Notes

1

COMPOSITION OF BLOOD AND NORMAL ERYTHROPOIESIS

1.1 INTRODUCTION

Blood consists of a fluid component- **plasma**, and a cellular component comprising of red cells, leucocytes and platelets, each of them with distinct morphology and a specific function. **Erythrocytes** or red cells are biconcave discs. They do not have a nucleus and are filled with hemoglobin which carries oxygen to tissues and carbon dioxide from the tissues to the lungs. **Platelets** are small cells. They also do not have a nucleus and are essential for clotting of blood. **Leucocytes** play an important role in fighting against infection. All these cells arise from a single cell called as the **Hematopoietic stem cell**. The process of formation of these cells is called **Hematopoiesis.** In this lesson we will learn the different stages in the development of red cells. The process of formation of red cells is called **erythropoiesis.**

OBJECTIVES

After reading this lesson, you will be able to:

- explain the composition of blood
- describe various stages in the formation of red cells
- explain the precautions in handling blood and blood products
- explain steps for preventing injury from sharp items.

1.2 SITES OF HEMATOPOIESIS

It begins in the early prenatal period, within the first two weeks, in the **yolk sac** in the form of blood islands and is known as **primitive hematopoiesis**. The red cells formed at this time are nucleated and contain embryonic type of hemoglobin which differs in the type of globin chains from the adult hemoglobin.

Definitive hematopoiesis begins a little later from the mesodermal tissue located in the **aorta-gonad-mesonephros** region and the cell giving rise to all haematopoietic elements is the **Hematopoietic Stem Cell (HSC)**.

In **fetal life (upto the 3rd month)** the major site of hematopoiesis is the **liver** with some contribution from the **spleen.**

From the **4th month of fetal life** hematopoiesis begins in the **bone marrow** throughout the skeleton, and at the time of birth it is the **bone marrow** which is hematopoietically active with only few foci of hepatic hematopoiesis. Till puberty hematopoiesis is actively seen in all bones but in **adults** it is confined to the **axial skeleton** mainly to the ends of long bones and the flat bones such as ribs, sternum and pelvis. After birth hematopoiesis is mainly confined to the bones.

1.3 HEMATOPOIETIC STEM CELL (HSC)

The hematopoietic stem cell (HSC) is a pluripotent cell that is the common progenitor of all cellular elements in the blood i.e. red cells, granulocytes, monocytes, platelets and lymphocytes. The cell has the capacity of **self renewal(form more cells of it's type), proliferation(multiply rapidly) and differentiation** along all cell lineages, and morphologically resembles a small lymphocyte. These HSCs are used for transplantation.

Erythropoiesis

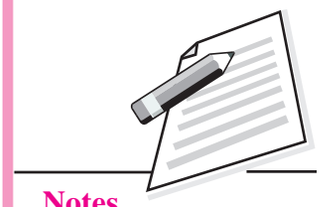
The erythroid progenitors develop from the HSC and give rise to **BFU- E (Burst Forming Units- Erythroid)and CFU –E (Colony Forming Units –Erythroid)** under the influence of a chemical **Erythropoietin (EPO)**.

Erythroblast is a term used for all forms of nucleated RBC. The least mature erythroid precursor cell is called a **proerythroblast.** It is a large cell with a rim of basophilic cytoplasm, a large nucleus which occupies most of the cell. Nuclear chromatin is coarse and prominent nucleoli are seen.

Basophilic/early normoblast This is smaller than the proerythroblast with a smaller nucleus but a more basophilic cytoplasm due to increased numbers of ribosomes in the cytoplasm. These ribosomes are involved in the production of hemoglobin. Nucleoli are not seen at this stage.

Polychromatic/intermediate normoblast This is the last precursor cell capable of mitosis and is smaller than the basophilic erythroblast. Its cytoplasm appears greyer due to the increased acidophilic staining caused by the presence of hemoglobin. Nuclear chromatin undergoes condensation.

Orthochromatic or late normoblast It is the smallest of the precursors and only slightly larger than a mature erythrocyte. Hemoglobinization is complete and so



the cytoplasm appears eosinophilic. The nucleus shrinks and the chromatin is greatly condensed giving the nucleus a homogeneous appearance.

As the proerythroblast matures through stages of basophilic, polychromatophilic and orthrochromatic erythroblasts, the cytoplasm becomes progressively more abundant and its colour changes from deep blue (due to high RNA content) to pink due to the increase in hemoglobin. The nucleus becomes smaller and dark staining(pyknotic).

Reticulocyte The cell is larger than a mature RBC, does not have a nucleus and has remnants of cytoplasmic ribosomal RNA which appear as a fine reticulum when stained with dyes such as new methylene blue and brilliant cresyl blue. On staining with Romansky stains it appears uniformly blue (polychromatophil). After its release from the marrow it remains in the spleen to undergo further maturation As the reticulocyte matures to an adult RBC it loses its ability to synthesize Hb.

The **mature RBC** is an anucleate biconcave disc lacking cytoplasmic organelles such as nucleus, mitochondria or ribosomes. Its major (90%) constituent is hemoglobin (Hb) which carries oxygen to tissues and carbon dioxide from the tissues. Besides Hb, it also contains enzymes required for energy production and for maintenance of Hb in a functional reduced state. The red cells are highly **deformable** under normal- physiological condition and this property allows them to pass easily through the sinusoids of the spleen.

The average **life span** of RBC is 100-120 days. After completion of their life span the RBC's are removed by the macrophages of liver and spleen(extravascular). A small fraction of RBCs may undergo destruction within the circulation that is intravascularly.

INTEXT QUESTIONS 1.1

1. Fluid component of blood is .....
2. ..... are filled with hemoglobin
3. ..... are essential for clotting of blood
4. ..... are essential for fighting against infections
5. Blood cells arise from ..... cells
6. The process of formation of blood cells is called .....
7. ..... cell is used for transplantation

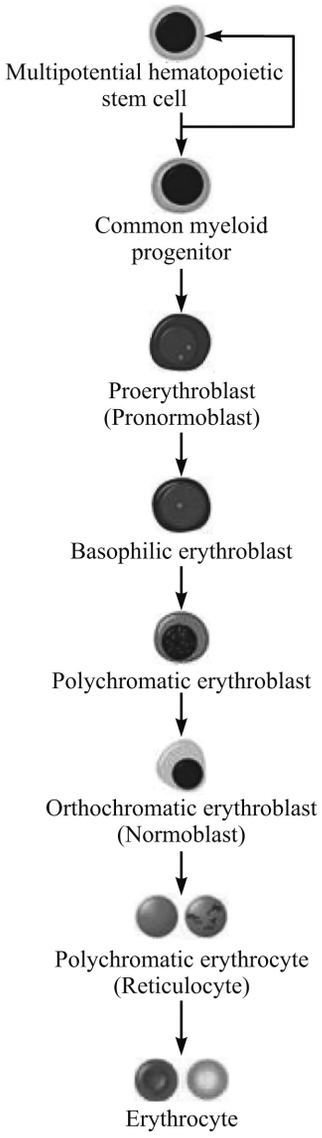
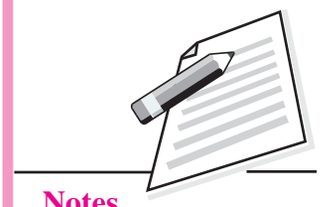


Fig. 1.1: Different stages in the formation of red cells. Note as the cell matures the size decreases, hemoglobinization occurs, the nucleus becomes small and dense

1.4 PRECAUTIONS IN HANDLING BLOOD AND BLOOD PRODUCTS

Human blood components, products made from **human** blood and certain other materials are treated and handled as if known to be potentially infectious and has a risk of transmitting infections such as HIV, HBV and others. Accordingly these are handled using precautions. This is called the **Universal precaution** (UP) approach.



Others which are handled using similar precautions are

- other body fluids containing visible blood, tissues, semen, vaginal secretions, cerebrospinal fluid, synovial fluid, pleural fluid, peritoneal fluid, pericardial fluid and amniotic fluid.
- any unfixed **tissue** or organ (other than intact skin) from a **human** (living or dead)
- HIV-containing cell or **tissue** cultures, organ cultures and HIV-containing culture medium or other solutions
- blood, organs or other tissues from experimental animals infected with HIV or HBV.

UP does not apply to feces, nasal secretions, sputum, sweat, tears, human breast milk, urine or vomitus unless they contain blood.

The **precautions** used are:

(a) Make full use of appropriate personal protective equipment (PPE).

(i) Use gloves when handling blood, blood products or other fluids for which UP are applicable (UP).

(ii) Use masks, safety goggles and/ or face shields for procedures that may involve splashing of blood or body fluids, creation of aerosols (or droplets of blood or body fluids) or exposure of mucous membranes or eyes, nose or mouth to same. (UP)

(iii) Use gowns, aprons, lab coats, coveralls and other protective body clothing needed to prevent exposure of body parts to blood, blood products or body fluids (UP). Note: Use protective caps, hoods and footwear in order to prevent exposure of head, hair or feet to blood or blood products (as may occur in autopsy) when required.

(iv) Use pocket masks or noncontact resuscitation bags or other regulation devices to resuscitate a patient to minimize exposure that may occur during emergency mouth-to-mouth resuscitation. (UP). Note: Remove contaminated PPE immediately or as soon as possible after completion of tasks or as soon as contaminated. Remove all PPE before leaving the work area.

Hand washing

Hands and other skin surfaces must be washed immediately after contact with blood.

Hands shall be washed each time gloves are removed.

Minimize splashing and splattering during hand washing.

To prevent injury from sharp items

- Use and dispose of needles, scalpels and other sharp items carefully.
- Use and clean instruments or devices after procedures cautiously.
- Do not bend, break or recap needles.
- Dispose off contaminated needles or sharps in a nearby specially designed puncture proof container immediately after use.

Biological Safety Cabinets (BSC)

BSC are required for procedures that may generate an aerosol (vortexing, grinding, blending, etc.).

Dermatitis

Employees who have exudative lesions or weeping dermatitis should refrain from handling blood until the condition resolves.

Exposure Occurrence

If an exposure incident to another person's blood occurs, immediately wash the exposed area with warm water and soap. If the exposed area is in the mouth, rinse mouth with water or mouthwash. If the exposure is in the eyes, flush with warm water or saline if available

Report the incident immediately with the following information:

- how, when and where the incident occurred and with whose blood/ secretions
- Your blood may then be tested for HIV but only with your consent.

The source individual's blood will also be tested if available and the results of the test will be made known to you.

INTEXT QUESTIONS 1.2

1. The average life span of the red cell is

   (a) 100 days (b) 105 days

   (c) 90 days (d) 120 days

2. Most of the red cells are removed after their life span in

   (a) Macrophages (b) The circulation

   (c) Both (d) Either

3. All of the statements are true of orthochromatic normoblast except

   (a) It is capable of mitosis (b) Hemoglobinization is complete

   (c) The nucleus is pyknotic (d) It is the smallest of the precursors

4. ..... is required for procedures that generate aerosol

5. ..... is used while handling blood and blood products

WHAT HAVE YOU LEARNT

- Erythroid cells develop from a hematopoietic stem cell in the marrow.
- The earliest identified erythroid precursor is the **Proerythroblast**.
- As the proerythroblast matures through stages of **basophilic, polychromatophilic and orthrochromatic erythroblasts**, the cytoplasm becomes progressively more abundant and its colour changes from deep blue (due to high RNA content) to pink due to the increase in hemoglobin. The nucleus becomes smaller and dark staining(pyknotic).
- **Reticulocyte** is the next stage. It is larger than the mature RBC, lacks a nucleus and has RNA remnants in the cytoplasm.
- The **mature RBC** is an anucleate biconcave disc which contains Hb, the oxygen carrier.
- The average **life span** of RBC is 100-120 days. After completion of their life span the RBC's are removed by the macrophages of liver and spleen(extravascular). A small fraction of RBCs may undergo destruction within the circulation that is intravascularly.

TERMINAL QUESTIONS

1. Define erythropoiesis
2. Explain the sites of hematopoiesis
3. Describe various stages of erythropoiesis

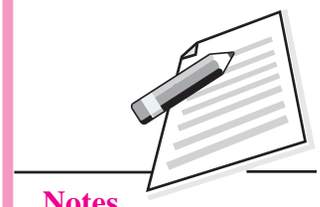
ANSWERS TO INTEXT QUESTIONS

1.1

1. Plasma
2. Erythrocytes
3. Platelets
4. Leucocytes
5. Hematopoietic
6. Hematopoiesis
7. Hematopoietic stem cell

1.2

1. 120 days
2. Macrophages
3. It is capable of mitosis
4. Biological safety cabinets
5. Personal protective equipements



Notes

2

TECHNIQUE OF BLOOD COLLECTION

2.1 INTRODUCTION

Blood is collected from the vein for various hematological investigations. In order to obtain accurate and precise results in the laboratory which will help the clinician to make a correct diagnosis of the patient's disease, it is of paramount importance to collect the blood sample in a correct manner.

Each sample is sent to the laboratory accompanied by a **laboratory requisition form** filled in by the clinician. Brief clinical details and any other relevant information must be mentioned on the form.

Prior to blood sample collection it is essential to check the patient's identity and make sure that it corresponds to the name and other details mentioned on the requisition form.

Blood can be withdrawn from the vein, usually the antecubital vein on the forearm (Venous blood) or from the finger or heel (Capillary blood). **Venous blood** is preferred. It can be collected using a **syringe and needle** or a **vacuum tube.** Both these methods will be described seperately.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the technique of collection of venous blood using a syringe and needle and a vacuum tube
- describe the method of collection of capillary blood
- explain the various anticoagulants used in hematology laboratory and their action.

2.2 COLLECTION OF VENOUS BLOOD

Blood is usually withdrawn from the antecubital vein or any other vein which is well identified on the forearm. The vein selected should be large, readily accessible, and sufficiently close to the surface to be seen and palpated

Preparation of venipuncture site

Clean the skin of the area around the identified vein with 70% isopropyl alcohol in a circular fashion beginning at the site and moving outward. Allow to dry spontaneously. Do not touch the venipuncture site after it has been cleaned.

Apply a tourniquet 3-4 inches above the venipuncture site. Ask the patient to make a fist a few times. Veins suitable for puncture will then become more apparent. Veins can become distended and easier to enter by allowing the arm to hang down for 2 or 3 minutes or by gently slapping the site of puncture.

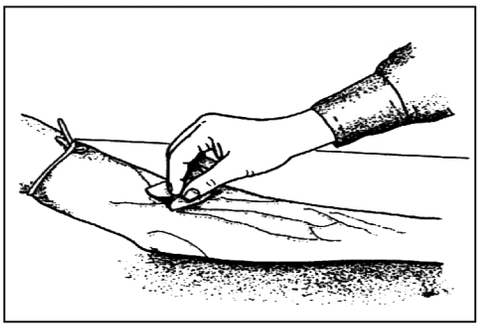


Fig. 2.1: Clean the area of the venipuncture site using circular anticlockwise motion

Collection of venous blood using a syringe

1. Clean hands thoroughly with soap and water.
2. Write the name and hospital number of the patient on the tube in which blood is to be collected. A printed label with these particulars can also be used for patient identification.
3. Place the needle into the syringe. Keep the cap over the needle capped till it is used. Check that the syringe works smoothly.
4. With the needle bevel up and parallel to the surface of the skin insert it into the vein. Appearance of blood in the hub of the needle indicates that the needle has successfully entered the vein. Release the tourniquet as soon as blood enters the syringe.

5. Withdraw the piston slowly to avoid frothing.
6. After obtaining the requisite amount of blood, place a sterile gauze pad over the point where the needle entered the skin and deftly withdraw the needle simultaneously while applying pressure over the site.
7. Deliver the blood gently into the specified receiver. Cap it firmly to prevent leakage.
8. Maintain light pressure on the gauze pad over venipuncture site till the bleeding stops and then cover the puncture site with a small adhesive dressing.
9. Destroy the needle in a special device (needle destroyer) immediately after use. DONOT break, bend or recap needles after use.
10. Place the used swab, syringe and any other contaminated material in a puncture resistant container for adequate disposal.

Collection of venous blood using a vacuum tube

The Vacutainer system consists of a double-pointed needle, a plastic holder or adapter, and several vacuum tubes with rubber stoppers of various colors depending on the sample to be collected. The color of the vacuum tube indicates the anticoagulant which it contains. The blood is directly collected into the tube from the vein.

Procedure

1. Place the identification details of the patient on each vacuum tube and ensure that they tally with the form.
2. Identify the vein and clean the area as described before. Apply a tourniquet 3-4 inches above the identified vein. Do not touch the venipuncture site after cleaning.
3. Place the vacuum tube in a reusable plastic holder and attach a disposable needle to it. Insert the tube into the holder till the top of the stopper is level with the marked guideline.
4. Place the patient's arm in a downward position to reduce the risk of backflow of any anticoagulant into the patient's circulation.
5. Insert the needle into the vein. Push the tube into the needle, puncturing the stopper/vacuum seal.
6. Remove the tourniquet as soon as the blood appears in the tube. Do not allow contents of the tube to come into contact with the stopper
7. Do not allow the contents of the tube to come into contact with the stopper during the procedure.

8. If more than one sample is required, successive tubes may be fitted into the holder after removing the previous tube and blood collected. The needle remains in the vein. While each successive tube is filling, the previous tube may be inverted gently to mix the sample well. Do not shake vigorously as this may lead to hemolysis of the blood sample.
9. After completion of blood collection, remove the holder and cover the site with a sterile swab and apply pressure till bleeding stops completely.
10. Destroy the needle in the destroyer without recapping it.

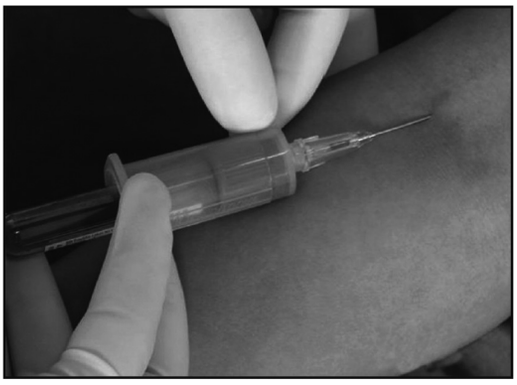


Fig. 2.2: Collection of venous blood using a vacuum tube

2.3 TYPES OF VACUUM TUBES

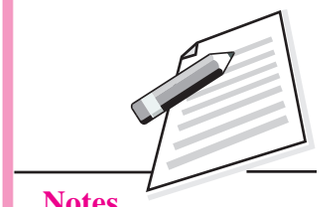
Vacuum tubes with different colored caps are available. Each contains a different anticoagulant and is used for various hematological tests as described below.

Color of cap	Anticoagulant	Test
Purple	EDTA	complete blood counts
Red	-	for tests which need serum
Blue	Sodium citrate	coagulation tests
Grey	fluoride	blood sugar

2.4 COLLECTION OF CAPILLARY BLOOD

This can be obtained by skin puncture with a needle or lancet and is specially used in small children or very obese adults in whom venepuncture fails. Samples can be used for making peripheral blood films, performing hematocrit/Hb and point of care testing.

In adults capillary blood sample can be obtained from the lateral side of tip of the 3rd or 4th finger while in infants the sample can be obtained by a deep puncture of the plantar surface of the heel.



Procedure

1. Clean the area with 70% alcohol and allow it to dry spontaneously. Puncture the skin to a depth of 2-3 mm with a sterile disposable lancet/needle.
2. Wipe the first drop of blood and squeeze gently to allow free flow of blood and collect the sample. In an adequate puncture, large drops of blood should exude spontaneously. Do not squeeze firmly as this gives unreliable results.

INTEXT QUESTIONS 2.1

Match the following

Test	Colour of cap
1. Complete blood count	(a) Grey
2. Coagulation test	(b) Purple
3. Blood sugar	(c) Red
4. Tests which need serum	(d) Blue

2.5 ANTICOAGULANTS USED IN HEMATOLOGY

The anticoagulants used commonly in the hematology laboratory are

EDTA: Ethylene Diamine Tetra Acetic acid

Sodium citrate

Heparin

EDTA: This is used for complete blood counts. The sodium and potassium salts can both be used but the dipotassium salt is preferred as it is more soluble. The dilithium salt can also be used but like the disodium salt is less soluble. The dipotassium salt is used in solid form. The International Council for Standardization in Hematology recommends use of the dipotassium salt at a concentration of 1.50±0.25mg/ml of blood. Coating the inside of a blood collection vial with a thin layer of EDTA improves the speed of uptake by blood. It exerts it's action by removing calcium which is essential for coagulation.

If EDTA is used in excess, it causes shrinkage of red cells and leucocytes. A significant decrease in hematocrit and increase in MCHC also occur. Platelets swell and disintegrate causing a false high count. It is thus important to add the correct amount of blood to the vial.

Trisodium citrate: This is used in coagulation studies in a ratio of 9 volumes of blood to one volume of anticoagulant (32g/L) (0.5ml citrate+4.5ml blood). It binds calcium thus preventing coagulation. It can also be used for estimation of ESR by Westergren method in a ratio of 4 volumes of blood to 1 volume of sodium citrate.

Heparin: The sodium or lithium salt of heparin is used at a concentration of 10-20IU/ml of blood for osmotic fragility and for red cell enzyme studies like glucose-6-phosphate dehydrogenase. It does not change red cell size and is the best anticoagulant for osmotic fragility. It can also be used for immunophenotyping. Heparin is not suitable for complete blood counts as it induces platelet and leucocyte clumping. It should also not be used for making peripheral smears as it gives a faint blue background color after staining smears by Romanowsky dyes.

Types of Samples Collected for Hematological Investigations

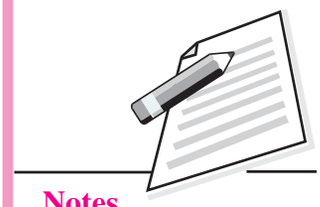
Whole blood: This is used for performing complete blood counts including reticulocyte count and for making peripheral blood films.

Serum: If blood is allowed to clot at room temperature, a straw colored fluid appears which is called serum. This may be admixed with red cells which can be removed by centrifugation at 1200g for 10 minutes. It is used for various biochemical tests and also serum protein electrophoresis to diagnose plasma cell disorders such as multiple myeloma. Serum lacks coagulation factors.

Plasma: This is obtained by centrifugation of anticoagulated blood and is used for coagulation studies.

INTEXT QUESTIONS 2.2

1. The anticoagulant used for complete blood counts is
   (a) EDTA
   (b) Heparin
   (c) Sodium citrate
   (d) Any of the above
2. For coagulation studies the ratio of blood to anticoagulant is
   (a) 9 : 1
   (b) 7 : 1
   (c) 8 : 1
   (d) Any of the above



3. Complete the sentence

   EDTA exerts it's action as an anticoagulant by .....

WHAT HAVE YOU LEARNT

- Blood is collected from the vein for various hematological investigations
- Blood can be withdrawn from the vein, usually the antecubital vein on the forearm or for capillary blood form the finger or heel
- Blood can be collected using a syringe and needle or a vacuum tube
- The vein should be large, readily accessible, and sufficiently close to the surface of the skin to be seen and palpated
- Write the patient name and hospital number on the tube
- The skin area around the identified vein should be cleaned with 70% isopropyl alcohol
- Apply a tourniquet 3-4 inches above the venipuncture site
- Insert the needle into the vein and withdrawn the required amount of blood slowly
- Deliver the blood in the specific container
- Maintain even pressure on the venipuncture site till bleeding stops
- Destroy the needle used immediately
- Vacuum tubes with different coloured caps are available and each contains different anticoagulant used for various hematological tests
- Capillary blood can be obtained from the lateral side of tip of 3^{rd} or 4^{th} finger and for infants from plantar surface of heel
- The anticoagulants used commonly in the hematology laboratory are Ethylene Diamine Tetra Acetic acid (EDTA), Sodium citrate, Heparin

TERMINAL QUESTIONS

1. Explain the different types of vacuum tubes
2. What the commonly used anticoagulants in hematology

Notes

ANSWERS TO INTEXT QUESTIONS

2.1

1. (b)
2. (d)
3. (a)
4. (c)

2.2

1. (a)
2. (a)
3. Removing the calcium

Notes

3

ESTIMATION OF HEMOGLOBIN

3.1 INTRODUCTION

Hemoglobin is the major constituent of the red cell cytoplasm, accounting for approximately 90% of the dry weight of the mature cell. It is comprised of **heme** and **globin**.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the structure of hemoglobin
- list the function of hemoglobin
- explain the various laboratory methods for estimation of hemoglobin
- enumerate the advantages and disadvantages of each method
- discuss the normal value and interpretation of abnormal results

3.2 STRUCTURE OF HEMOGLOBIN

The Hemoglobin molecule is a tetramer consisting of two pairs of similar polypeptide chains called **globin chains**. To each of the four chains is attached **heme** which is a **complex of iron in ferrous form and protoporphyrin.**

The major (96%) type of hemoglobin present in **adults** is called HbA and it has 2 alpha globin chains and 2 beta globin chains ($\alpha_2\beta_2$). The gene that codes for the formation of α globin chains is located on chromosome 16 and that which

codes for the formation of β globin chains is on chromosome 11. In adults, a minor amount of HbA2 ($\alpha_2\beta_2$) is also present and constitutes less than 3.5%.

During embryonic and fetal life other different types of hemoglobins predominate. These include **Gower-1** (present in early embryonic life), **Gower-2 and Hb Portland**. After the eighth week of development, embryonic hemoglobins are replaced by **Fetal hemoglobin** ($\alpha_2\beta_2$). This remains the predominant hemoglobin until after birth and constitutes 50-90% of the total hemoglobin. After birth, it's concentration decreases to less than 2% by 30 weeks of age. HbA is then the predominant hemoglobin.

The structure and time of occurrence of various hemoglobins is shown in the table below.

Name	Structure	Present in
Adult Hb (HbA)	$\alpha_2\beta 2$	Adults
Fetal Hb (HbF)	$\alpha_2\gamma_2$	Fetal life
Hb Portland	$\zeta_2\gamma_2$	Embryonic life
Gower-1	$\zeta_2\varepsilon_2$	,,
Gower-2	$\alpha_2\varepsilon_2$	,,

Function of Hemoglobin Heme has the ability to bind oxygen reversibly and carry it to tissues. It also facilitates the exchange of carbon dioxide between the lungs and tissues. Thus, **hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide.**

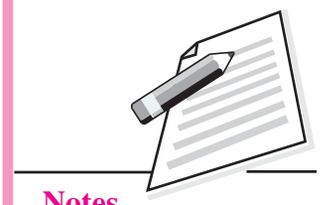
3.3 ESTIMATION OF HEMOGLOBIN IN THE LABORATORY

Various methods are available for estimation of hemoglobin in the laboratory.

I. Methods based on development of color. These are
   - Sahli's or acid hematin method
   - Cyanmethemoglobin method
   - Oxyhemoglobin method
   - Alkaline hematin method

II. Measurement of oxygen combining capacity

III. Measurement of iron content



3.4 METHODS BASED ON COLOR DEVELOPMENT

The **commonly used** methods are **Sahli's/ acid hematin** method and **Cyanmethemoglobin** method. The details of these methods are described below.

Sahli's/acid hematin Method

Principle: Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till it's color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Equipment required

Hemocytometer which consists of

- comparator box which has brown colored glass on either side
- Hb pipette which is marked upto $20mm^3$(0.02ml blood)
- Tube with markings of Hb on one side
- glass rod
- dropper

Reagents required

N/10 HCl

Distilled water

Sample: Venous blood collected in EDTA as described earlier

Procedure

1. Add N/10 HCl into the tube upto mark 2g%
2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.
3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.
4. Allow to stand undisturbed for 10min.
5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.

6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%.

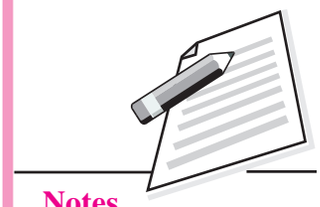
Fig. 3.1: Hb comparator box with brown glass on either side and tube with acid hematin solution in centre. The color of the solution is matched with the glass and the concentration of Hb is read directly

Advantages

- Easy to perform
- Quick
- Inexpensive
- Can be used as a bedside procedure
- Does not require technical expertise

Disadvantages

- Less accurate.
- All hemoglobins (oxyhemoglobin, sulphemoglobin) are not converted to acid hematin and hence the value of Hb obtained is less than the actual value.
- The color of acid hematin develops slowly.
- Color of acid hematin fades with time and dilution must be done exactly after 10 min when the color development is maximum
- Individual variation in matching of color is seen.



- If the matching point is passed, the whole procedure has to be repeated.
- Color of glass in the comparator box tends to fade with time .
- Lack of a true standard.

Cyanmethemoglobin method

This is the **internationally recommended method** for determining hemoglobin

Principle: Blood is diluted in a solution containing potassium cyanide and potassium ferricyanide. The latter converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540nm or in a colorimeter using a yellow green filter.

Equipment required

Hb pipette

Spectrophotometer

Reagents required

Drabkin's solution pH7.0-7.4 which contains

Potassium cyanide	50 mg
Potassium ferricyanide	200 mg
Potassium dihydrogen phosphate	140 mg
Nonionic detergent	1 ml
Distilled water	1 L

The solution should be clear and pale yellow in color. When measured against water as a blank in a spectrometer at a wavelength of 540 nm, the absorbance must be zero. **The solution is unstable if exposed to light and can be stored at room temperature in brown borosilicate bottles for several months.** However, if the room temperature is higher than 30°C, the solution should be stored in a refrigerator but brought to room temperature before use. The solution must **never be frozen.**

The pH of the solution must be checked every month.

Discard the solution if found to be turbid/if pH is outside range/ it's absorbance is not zero at 540 nm.

Do not pipette Drabkin's solution by mouth.

Sample: Venous blood collected in EDTA

Notes

Procedure

1. Take 5ml of Drabkin's solution in a test tube.
2. Mix the blood sample by gentle inversion and draw 0.02ml of blood into the Hb pipette. Wipe the outer surface of the pipette to remove excess blood.
3. Place the pipette into the tube containing Drabkin's solution and slowly expel the blood into the solution. Mix well and let it stand undisturbed for 5min.
4. Measure the absorbance of this solution at 540nm in a spectrophotometer after adjusting the OD at 0 by using Drabkin's solution as blank.
5. Calculate the hemoglobin concentration using a standard curve.

Advantages

- All forms of Hb except sulphemoglobin are converted to HiCN.
- Visual error is not there as no color matching is required.
- Cyanmethemoglobin solution is stable and it's color does not fade with time so readings may not be taken immediately.
- Absorbance may be measured soon after dilution.
- A reliable and stable reference standard is available from World Health Organisation for direct comparison.

Disadvantages

- Diluted blood has to stand for a period of time to ensure complete conversion of Hb.
- Potassium cyanide is a poisonous substance and that is why **Drabkin's solution must never be pipetted by mouth.**
- The rate of conversion of blood containing carboxyhemoglobin is slowed considerably. Prolonging the reaction time to 30min can overcome this problem.
- Abnormal plasma proteins cause turbidity when blood is diluted with Drabkin's solution.
- A high leucocyte count also causes turbidity on dilution of blood. Centrifuging the diluted blood can help overcome the turbidity.

Preparation of Standard curve for Hemoglobin estimation by Cyanmethemoglobin method

In a laboratory which tests several samples in a day, it is more convenient to prepare a standard curve for Hb.

A WHO International reference HiCN standard is available commercially as 10ml sealed ampoules. This solution is stable for years. The exact concentration of Hb present in the solution is indicated on the label.

Preparation of standard curve

1. Make serial dilutions of the reference solution eg 1in2, 1in4, 1in8 and so on with Drabkin's solution. As the Hb present in the solution is known, the Hb concentration of each dilution will also be known.
2. Take the OD of each dilution in the colorimeter against a blank of Drabkin's solution.
3. Plot the OD against the Hb concentration on a linear graph paper. The absorbance is plotted on the vertical axis and the hemoglobin concentration on the horizontal axis.The points should lie in a straight line that passes through the origin.
4. From this graph a table of readings and the corresponding Hb value can be prepared. This is more convenient than the graph specially when a large number of readings have to be taken. After OD of the sample is taken, the corresponding Hb value can be directly read from the table.

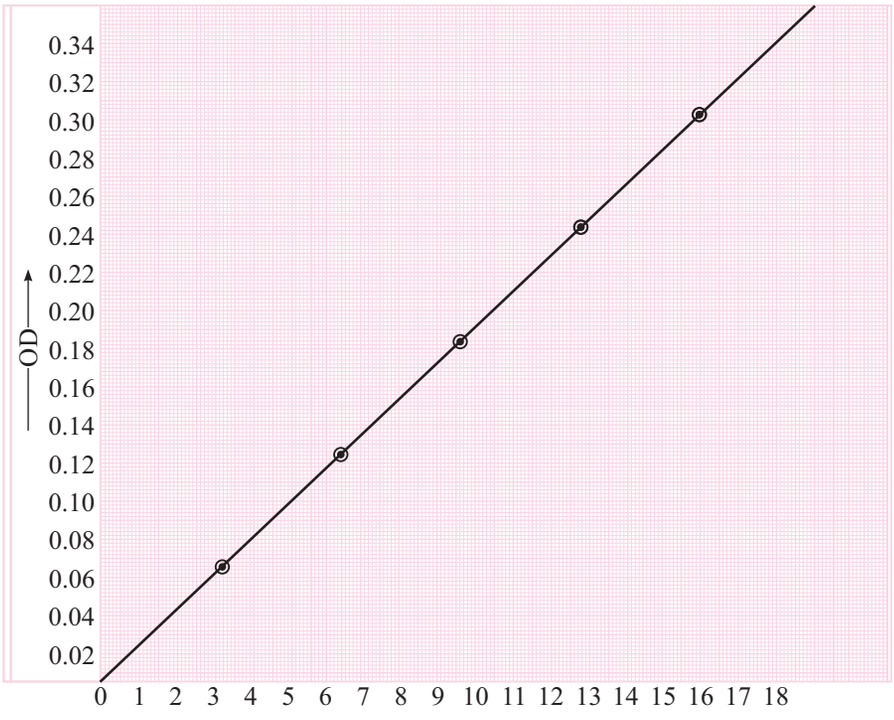


Fig. 3.2: Standard curve for Hb

3.5 OXYHEMOGLOBIN METHOD

Hb is converted to oxyhemoglobin by reaction with ammonia and the color of the solution is measured in a photocolorimeter**.**

The **advantages** of this method are that it is simple, quick and it's reliability is not affected by increased bilirubin level.

However, the **s**olution of oxyhemoglobin fades very quickly. It is not possible to prepare a stable standard.The method does not give satisfactory results in the presence of carboxyhemoglobin, methemoglobin and sulphemoglobin.

3.6 ALKALINE HEMATIN METHOD

Blood is converted to alkaline hematin by addition of alkali such as sodium hydroxide and the color measured in a colorimeter at 540 nm.

It gives a true estimate of Hb and is not affected by the presence of plasma proteins and lipids.

However, it is not used routinely as it is less accurate than the cyanmethemoglobin method and some forms of Hb such as HbF are resistant to alkali denaturation.

3.7 MEASUREMENT OF OXYGEN CARRYING CAPACITY

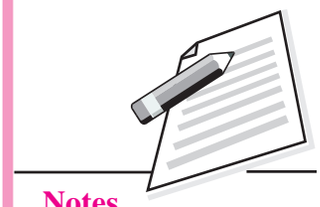
The oxygen combining capacity of blood is 1.34ml oxygen per gm of Hb. This is a measure of the function of Hb. It is not used routinely as it is not practical and gives results 2% lower than the other methods.

3.8 IRON CONTENT OF HEMOGLOBIN

Iron content can be measured and converted to Hb by using the formula 0.347 mg iron 100g Hb. It is however, impractical for routine use.

INTEXT QUESTIONS 3.1

1. Hemoglobin is comprised of ..... & .....
2. Hemoglobin molecule consists of two pairs of ..... chains
3. The predominant hemoglobin in adult is .....
4. Hemoglobin serves as primary medium of exchange of ..... & .....



3.9 DIRECT READING HEMOGLOBINOMETERS

Hemoglobinometers based on the cyanmethemoglobin/oxyhemoglobin method are available. The instrument has a built in filter and a scale and gives direct reading of Hb in g/dl. They are used in field surveys. It is imperative that the instrument is calibrated frequently.

Normal value of Hb

Males 15 ± 2 g/dl

Females 13.5 ± 1.5 g/dl

Significance of Hb estimation: Hemoglobin estimation is used as a screening test for detecting anemia. This is a frequently identified abnormality in our population. Anemia is not a diagnosis by itself and if detected, it's underlying cause must be ascertained. Hence, accurate Hb estimation is essential so that further tests can be done to ascertain it's cause and the patient treated accordingly.

WHO Cut Off Value below which Anemia is Present

Subject	Hb(g/dl)
Males	<13
Females	<12
Pregnant women	<11

INTEXT QUESTIONS 3.2

1. HbA is comprised of
   (a) $\alpha_2\beta_2$ (b) $\alpha_2\gamma_2$
   (c) $\alpha_2\delta_2$ (d) $\alpha_2\varepsilon_2$
2. In pregnant women anemia is present if Hb (g/dl)is less than
   (a) 12 (b) 10
   (c) 11 (d) 13
3. Cyanmethemoglobin method is preferred as
   (a) A reference standard is available for comparison
   (b) The color of the solution does not fade with time
   (c) No visual error is there
   (d) All of the above

WHAT HAVE YOU LEARNT

- The Hemoglobin molecule is a tetramer consisting of two pairs of similar polypeptide chains called **globin chains**. To each of the four chains is attached **heme** which is **a complex of iron in ferrous form and protoporphyrin**
- The major (96%) type of hemoglobin present in **adults** is called HbA ($\alpha_2\beta_2$).
- Hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide
- Various methods are available for estimation of hemoglobin in the laboratory. These are based on the estimation of hemoglobin by measurement of it's color
- The acid hematin or Sahli's method is easy to perform and gives quick results but is not recommended as it does not give accurate results
- The **Cyanmethemoglobin method** is the internationally recommended method for estimating hemoglobin which uses Drabkin's solution(a mixture of potassium cyanide and ferricyanide) to convert Hb to cyanmethemoglobin, the color of which is then measured in a spectrophotometer at 540nm and compared with a WHO reference standard
- Hb estimation is used as a screening test for detection of anemia

TERMINAL QUESTIONS

1. Describe the structure of Hemoglobin
2. List the functions of hemoglobin
3. Explain various laboratory methods for estimation of hemoglobin

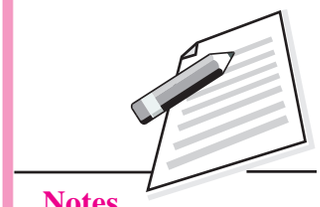
ANSWER TO INTEXT QUESTIONS

3.1

1. Heme & globin
2. Globin
3. HbA
4. Oxygen & carbon dioxide

3.2

1. (a) $\alpha_2\beta_2$
2. (c) 11
3. d.



4

HEMATOCRIT

4.1 INTRODUCTION

Hematocrit is a commonly performed investigation in hematological laboratories for evaluation of patients with anemia.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the methods of estimation of hematocrit
- describe the normal value and interpretation of result
- explain the calculation of red cell indices
- classify anemia.

4.2 HEMATOCRIT

Hematocrit or packed cell volume (Hct/PCV) is the volume occupied by red cells after blood is centrifuged at a high speed.

Methods for estimation

Hematocrit is measured by two methods:

- Microhematocrit method
- Automated method

The Macrohematocrit method which was used earlier is not used now.

Microhematocrit method

Sample: Blood collected in EDTA

Equipment required

1. Capillary tubes 75 mm in length with an internal diameter of 1mm.
2. Microhematocrit centrifuge
3. Reading device

Procedure

1. Place the capillary tube in the venous blood sample allowing blood to enter the tube by capillary action. Leave the last 15mm unfilled.
2. Seal the tube with modeling clay. Make sure there is no air trapped between the clay and the column of blood.
3. Place the tube in the microhematocrit centrifuge with the sealed end to the outer rim and centrifuge at 12000g for 5min.
4. Read the result using a microhematocrit reading device.

Fig. 4.1: Capillary tubes (right) and the microhematocrit centrifuge (left)

Fig. 4.2: Filled capillary tubes placed in the centrifuge

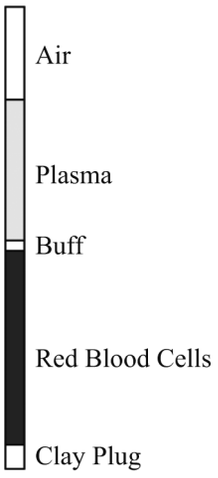


Fig. 4.3: The tube as it appears after centrifugation

Precautions

1. PCV increases with storage so it must be measured within 6 hrs of sample collection.
2. Capillary tubes must be of defined specifications.
3. Centrifuges must be checked at regular intervals for speed and accuracy.

Normal value

Men : 0.45±0.05L/L or 45±5%

Women : 0.41±0.05L/L or 41±5%

Notes

Significance of measuring hematocrit

- it is used as a screening test for anemia.
- along with red cell count and hemoglobin it is used for calculating red cell indices which are essential for classification of anemia.
- it can be used as a reference method for calibrating automated hematology analysers.
- it provides a rough guide to Hb measurement.

Precautions

1. PCV increases with storage so it must be measured within 6 hrs of sample collection.
2. Capillary tubes must be of defined specifications.
3. Centrifuges must be checked at regular intervals for speed and accuracy.

Automated method

This will be discussed in the chapter on automated cell analysers.

Normal value

Men : 0.45±0.05L/L or 45±5%

Women : 0.41±0.05L/L or 41±5%

Reduced Hematocrit is seen in anemia.

Increased hematocrit is seen in polycythemia.

Red cell indices

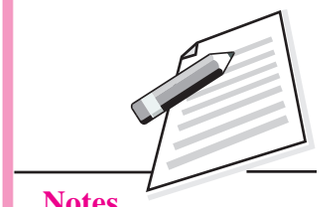
These indices are used in the morphological classification of anemia. They can be calculated manually. However, in most laboratories they are obtained by automated hematology analysers.

The **three indices** are

- Mean Corpuscular Volume (MCV)
- Mean Corpuscular Hemoglobin (MCH)
- Mean Corpuscular Hemoglobin Concentration (MCHC)

Mean corpuscular volume indicates the average volume of each red cell.

$$MCV = \frac{\text{Packed cell volume \%}}{\text{Red cell count (millions/mm}^3\text{)}} \times 10 \text{ femtolitres}$$



Notes

Hematocrit

Normal value 90±10fl

Interpretation: In the presence of anemia if

MCV < 80fl: microcytic anemia

MCV > 100fl: macrocytic anemia

MCV80-100 fl:normocytic anemia

Mean corpuscular hemoglobin reflects the average amount of Hb in each red cell.

$$MCH = \frac{\text{Hemoglobin (g/dl)}}{\text{Red cell count (millions/mm}^3\text{)}} \times 10 \text{ picogram}$$

Normal value: 29.5 ± 2.5pg

Interpretation: In the presence of anemia if

MCH < 27pg: hypochromic anemia

Mean corpuscular hemoglobin concentration is the average concentration of Hb in a given volume of packed red cells

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

Normal value: 33±1.5g/dl

Limitation of red cell indices: These are mean values and cannot express the variation that occurs within a population. They may be normal if the number of abnormal cells is small. If two populations of cells are present eg microcytic and macrocytic, the indices can again be normal.

Use of red cell indices: They are used in the classification of anemias.

Anemia: This is defined as a decrease in the red cell mass to adequately deliver oxygen to peripheral tissues. To establish the presence of anemia in the laboratory we can use Hb, hematocrit or packed cell volume and the red cell count. Because it is the easiest to measure, hemoglobin is the most frequently used parameter to detect anemia. Though the parameters can be measured manually it is best to measure them on an automated analyser.

Classification of anemia

I. **Morphological classification:** Anemia is classified on morphological grounds into three groups

Notes

- Microcytic hypochromic anemia MCV<80fl,MCH<27pg
- Macrocytic anemia MCV>100fl
- Normocytic,normochromic anemia MCV80-100fl, MCH27-32pg

Advantages of morphological classification

1. It is of practical, clinical value. Each subtype is associated with distinct causes and thus narrows the probable diagnosis.
2. It allows a comprehensive laboratory approach to be followed to reach a definitive diagnosis.
3. The initial step in making the classification depends on readily available laboratory tests.

II. Kinetic classification: Divides anemias into 2 groups:

(a) Impaired erythrocyte production

(b) Increased erythrocyte production

The causes of each are given below

Impaired production

Iron deficiency anemia

Anemia of chronic disease

Hypoplastic anemia

Anemia due to infiltrative disorders

Megaloblastic anemia

Thalassemia

Increased production

Hemolytic anemia

Treated nutritional anemia

INTEXT QUESTIONS 4.1

1. Calculate the MCV in this patient with Hct of 45% and red cell count of 5×10^{12}/L
2. If the Hb of this patient is 8g/dl, what will be the morphological subtype of anemia
3. The Hb of a male patient is 15g/dl and the red cell count is $5x10^{12}$/L. Calculate the MCH.

WHAT HAVE YOU LEARNT

- **Hematocrit** is a commonly performed investigation. It is used to **screen for anemia** and for **calculation of red cell indices**. Three red cell indices can be calculated from Hb, Hct and red cell count. These are **MCV, MCH and MCHC**. The red cell indices are helpful in the **classification of anemia on morphological grounds.**

TERMINAL QUESTIONS

1. Classify anemia
2. Describe the methods of Hematocrit measurement

ANSWERS TO INTEXT QUESTIONS

4.1

1. 90 fl
2. Normocytic
3. 30 pg

Notes

5

SELECTION AND REGISTRATION OF DONORS

5.1 INTRODUCTION

Blood Transfusion services form an essential part of the health care system. A well equipped transfusion centre ensures provision of efficient medical care.

The main aim of the Blood Transfusion centre is to provide safe blood and blood products free of pathogenic organisms to patients who need them.

To meet this aim, recruitment of healthy donors is of paramount importance. Infact, blood donation forms the cornerstone of a blood transfusion service. It is essential that in order to ensure an overall safety of the entire transfusion process, an effective programme for donor selection and screening is implemented.

OBJECTIVES

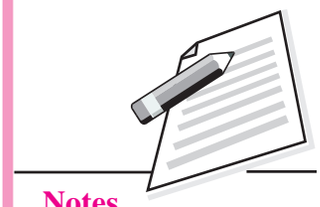
After reading this lesson, you will be able to:

- enlist the types of donors
- explain the criteria for selection of donors
- describe the conditions for deferring blood donors

5.2 TYPES OF BLOOD DONORS

There are four types of donors

- **Voluntary/unpaid** donors are those who donate blood of their own free will and do not receive any monetary benefit for the donation.



- **Paid/professional** donors receive payment for donation of blood.
- **Replacement** donors are friends and relatives of the patient who replace the unit of blood issued to the patient.
- **Autologous** donors are those who donate blood for their own use at a later time.

The World Health Organization recommends that the donor base should mostly be voluntary. Reliance on replacement and professional donors should be phased out.

Steps in selection of blood donors (Donor Screening)

All donors who come to the Transfusion centre are screened to ensure that they are in good health. This helps in avoiding transmission of infection/occurrence of any other untoward effect to the recipient and also protects the donor.

There are **four aspects of donor screening**

1. Donor registration
2. Medical history
3. Physical examination
4. Laboratory testing

1. **Donor registration:** The following information must be carefully recorded to enable the Transfusion centre to contact the donor, if required, at a later date.
    - Donation date and time.
    - Name of the donor
    - Father's /Husband's name
    - Age
    - Gender
    - Occupation
    - Address with Telephone numbers.
    - Blood group, if known

    Records of these details as filled in the form are maintained in the blood bank for 10years.

2. **Medical History:** A qualified and trained person must take a detailed medical history of each donor. If the donor is found to have any abnormal condition he must be referred to the Physician of the transfusion centre who will decide if blood is to be collected.

Notes

The donor may be provided with educational material informing them of high risk activities for transmission of HIV infection. They should be informed of the significance of not donating blood if they have indulged in any of these activities. Donors must also be ensured that all this information will be kept confidential. (See box below)

High risk activities for transmission of HIV infection

Homosexual male (male donors who have had sex with another male)

Males or females who have had sex with multiple partners

Commercial sex workers

Intravenous drug abusers

Professional paid donors

Symptoms suggestive of HIV infection experienced in last 6 months

Unexpected weight loss>10% of original weight

Night sweats

Unexplained fever > 99°C for more than10 days

Lymphadenopathy

Persistent diarrhea

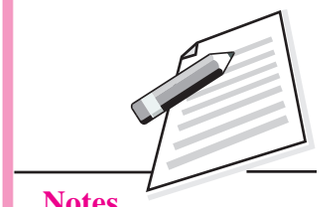
Persistent cough with expectoration

White patches in mouth

Certain aspects in the history may make a donor unfit for donation temporarily or permanently. These points must be enquired from the donor carefully. Causes of permanent and temporary deferral are given below.

Causes of permanent deferral of donors

- High risk group for HIV infection as given above
- HIV antibody positive
- Cardiovascular disease/heart disorders: myocardial infarction, angina, coronary artery disease on medication
- Patients who have undergone open heart surgery
- Patients with Hepatitis B virus/Hepatitis C virus infection
- Malignant diseases
- Abnormal bleeding tendency
- Endocrinal disorders



- Severe allergic disorder
- Polycythemia vera
- Chronic liver disease
- Chronic kidney disease
- Donors on drugs such as anticonvulsants, anticoagulants, antithyroid drugs, cytotoxic drugs, immunosuppressive drugs, vasodilators, drugs for Parkinson's disease, insulin.

Causes for temporary deferral of donors

Condition	Period of deferral
Major surgery	1 year
Minor surgery	6 months
Dental manipulation	3 days
Dental surgery	1 month
Transfusion with blood/components	1 year
Exposure to hepatitis by tattoo/acupuncture/contact with hepatitis patient	1 year
Travel to area endemic for malaria	1 yr after return
Malaria	3 months after treatment
Syphilis	1 yr after completion of therapy
Tuberculosis	5 years
Pregnancy	6 months
Abortion	6 months
Lactation	Till baby is weaned
Immunization: polio, measles, mumps, yellow fever	2 weeks from vaccination
Rubella, anti tetanus serum, anti venom Hepatitis B immune globulin, anti rabies and gamma globulin	4 weeks from vaccination 1 yr after vaccination
Drugs : Oral Antibiotics	3 days
Injectable antibiotics	4 days
Cortisone	7 days

Notes

Note

1. Do not defer for these vaccinations : Tetanus, Diptheria, Typhoid, Prophylactic Hepatitis B
2. Donors on salicylates(aspirin) should not be allowed to donate blood for platelet rich plasma/platelet concentrate if these drugs are used within the last 72 hours

Based on the history the donor may be

1. Accepted- donor continues on in the donation process
2. Deferred temporarily-donor is asked to come after a specified time
3 Deferred permanently - cannot be accepted as a blood donor under any circumstances

3. Physical examination: After the history all donors must be examined carefully to ensure their physical fitness. The following criteria must be met:

1. Age 18-65 years
2. Weight- If the donor weighs more than 45kg, he can donate 350ml of blood and donors weighing more than 60kg can donate 450ml blood.
3. Blood pressure must be normal.
4. Pulse: between 80-100/min, regular.
5. Afebrile i.e. body temperature not > 37. 5°C.
6. Venipuncture site must be free of any lesion.
7. Systemic examination: Heart, Lungs: normal.

   Per abdomen: no organomegaly detected.
8. Interval between donation must be 12 weeks.

Laboratory Tests

Certain laboratory tests are performed on donors prior to collection of blood.

1. **Hemoglobin :** Hb estimation is done prior to each donation by any of these methods:

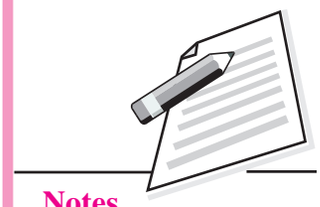
   Sahli's method

   Cyanmethemoglobin method

   Hemocue

To be fit for donation the Hb must be greater than 12.5g/dl.

If Hb estimation is not available hematocrit can be estimated and must be greater than 38%.



2. ABO and Rh grouping must be done on all donors.
3. Record all results on the donor form.

INTEXT QUESTIONS 5.1

1. An ideal donor is a
   (a) Voluntary donor (b) Replacement donor
   (c) Professional donor (d) Autologous donor
2. All are causes for permanent deferral of a donor except
   (a) Cardio-vascular disease (b) Malignancy
   (c) Abnormal bleeding tendency (d) Major surgery
3. A donor on antibiotics is deferred for
   (a) 3 months after last dose (b) 3 weeks after last dose
   (c) 3 days after last dose (d) 1 day after last dose
4. Minimum hemoglobin level accepted for blood donation is
   (a) 10 gm/dl (b) 12 gm/dl
   (c) 12.5 gm/dl (d) 11 gm/dl
5. The minimum interval between two whole blood donations should be
   (a) 6 months (b) 1 year
   (c) 3 months (d) 2 weeks

WHAT HAVE YOU LEARNT

Donors can be voluntary, replacement or professional. Voluntary donors are preferred. Prior to donation all donors must be screened carefully with a history, physical examination and Hb estimation to ensure their fitness for donation. Certain conditions make the donor unfit for donation temporarily or permanently.

TERMINAL QUESTIONS

1. Describe various aspects of donor screening
2. Explain the reasons for defering blood donors

ANSWERS TO INTEXT QUESTIONS

5.1

1. (a) Voluntary donor
2. (d) Major surgery
3. (c) 3 days after last dose
4. (c) 12.5 gm/dl
5. (c) 3 months

6

COLLECTION OF BLOOD FROM DONORS

6.1 INTRODUCTION

Collection of blood from donors is one of the most important functions of the blood bank.

Failure to follow approved procedures can lead to severe and potentially fatal consequences in the recipient and also the donor.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the procedure of collection of blood from donors as per recommended guidelines.
- discuss the care and storage of the collected blood
- explain the care of donors after donation of blood

6.2 BLOOD COLLECTION

The process of blood collection begins with donor information and counseling.

A. Pre-Donation Information

- Written or verbal information is given to blood donors before donation regarding their rights, the procedure of donation and tests which will be done on the collected unit of blood.

- The donor must sign the donor form indicating that the donation procedure has been understood and he agrees to donating blood and to testing for infectious agents that can be transmitted by transfusion.

B. Pre-Donation counseling

- Counselling is provided to potential donors in privacy before blood donation. The possible consequences of learning test results must also be explained to them.

Equipment required for collection of blood

1. **Blood collection bags**: PVC plastic bags which are closed system of single, double, triple, or quadruple bags for collection of 350 ml or 450 ml blood are available commercially. These bags contain an **anticoagulant solution, citrate phosphate dextrose –adenine (CPDA-1).** The volume of anticoagulant is 49ml for 350ml of blood and 63ml for 450ml of blood. The **shelf life** of blood collected in this is **35 days.**
2. Sphygmomanometer
3. Machine for mixing of blood
4. Weighing scale
5. Sterile cotton swabs, spirit and band aid
6. Plastic clips

Blood Collection Room: The room where blood is collected should be clean, well lit and preferably air-conditioned so that the donor is comfortable during the collection process.

Collection personnel: Blood should be collected only by a medical officer or adequately trained persons in the presence of the medical officer.

Donor identification: The name, age, sex of the donor should be entered prior to collection and a number assigned to each donor. Mention this number on the blood bag and pilot tubes.

Method of phelobotomy

1. Wash hands well with soap and water and wear sterile gloves.
2. Inspect the bag for any leak or damage. The anticoagulant solution must be clear and not have any particulate matter.
3. Make the donor lie down comfortably.

4. Identify the donor. The number on the bag must correspond with that on the form. Write the date of collection and expiry on the bag. Also mention the blood group of the donor, if known.
5. Place the bag on a balance.
6. Choose the site of venipuncture in the anticubital area of the arm after careful inspection of both arms. It should have no local infection/scars.
7. Apply a sphygmomanometer cuff 1 1/2 inches above the area and inflate to 50-60 mm Hg.This will help in identification of the vein. The vein should be large and firm but not superficial.
8. Deflate the cuff and clean 4-5cms of the area in a circular, centrifugal manner i.e. starting at the site of venipuncture and moving outward (Fig. 6.1). Clean with spirit first, then iodine and again with spirit. Allow to dry for 30 seconds. Do not touch the cleaned area after preparation.

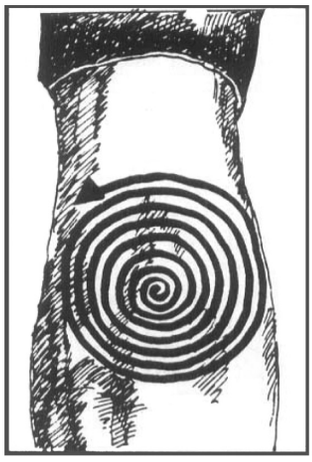


Fig. 6.1

9. Inflate blood pressure cuff to maintain a pressure of 50-60mm Hg. Remove the cap from the needle just before the venipuncture and insert into the vein.
10. Ask the donor to open and close fist during collection of blood.
11. Mix blood and anticoagulant at periodic interval during collection. This can be done manually or with an automated mixing equipment.
12. Do not leave the donor unattended during the entire procedure which takes 8-10 minutes
13. Monitor the volume of blood being drawn using a balance or blood collection monitor Scale. (One ml of blood weighs 1.05 g .Thus, 350 ml of blood weighs 367 g. and 450 ml weighs 472 g).
14. After the requisite amount of blood has been collected, clamp the tubing attached to the bag with a plastic clip.

15. Deflate the cuff, place a sterile swab at the puncture site and apply pressure over it and ask the donor to raise the arm
16. Let the donor remain on the couch for 5-10 min after the collection. Apply a band aid over the site after bleeding stops.
17. Provide light refreshment to the donor.

Care of the collected unit of blood

1. Take the bag to the processing table
2. Loosen the plastic clip and with light pressure on the bag transfer 4-5ml of blood to the pilot tubes for blood grouping and transfusion transmitted infections. Recheck that the number on the tube is the same as that on the bag.
3. Seal the tube with a sealer and separate the needle.
4. Strip the blood bag tubing, pushing the blood back into the bag. Mix the blood in collected bag by gentle inversion.

6.3 LABELLING OF BLOOD BAG AND IT'S STORAGE

Labeling of blood bag

A sticker is placed on the collected unit of blood. Each blood group is designated with a different color

Pink : Blood groupA

Yellow : Blood groupB

Blue : Blood group O

White : Blood group AB

The following information is written on the bag:

Donor number

Date of collection

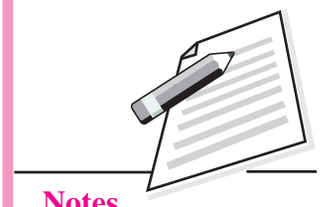
Date of expiry

Blood group

Negative for transfusion transmitted infections

Storage of collected blood

Keep the blood bag at 2-6^{o}C in the refrigerator immediately after collection. Blood is stored in a blood bank refrigerator in which the temperature is



controlled. An inbuilt alarm is also present to alert the laboratory staff in case the temperature rises above the range. Blood of the same group is stored in one shelf. The shelf life of blood is 35 days.

If platelets are to be harvested, blood bag should be kept at 20-24°C until platelets are separated.

Adequate storage ensures that the therapeutic efficacy of blood and it's components is maintained.

Donor reactions

Most donors tolerate the procedure of blood donation well. However, some donors may develop reactions during or after donation of blood. These can be mild such as **feeling of faintness or dizziness**. The donor may complain of weakness, profuse sweating and pallor. Sometimes these vasovagal attacks are accompanied with **loss of consciousness**. The skin feels cold, pulse rate is increased and the blood pressure may fall. The donor's head should be placed in a low position with the legs raised and fluids/light food may be given. Tight clothing must be loosened. Blood pressure, pulse and respiration must be monitored closely till the donor recovers. Cold compresses may be applied to the donor's head if required. Reassurance of the donor with sufficient rest before he leaves is essential.

A **hematoma** can form at the site of venipuncture. Application of firm pressure after donation helps prevent this. If required, ice may be applied to the site for 5 minutes.

Nausea and vomiting may also occur. The donor must be made comfortable and an emesis basin may be provided. The head must be turned to one side to prevent aspiration. Ask the donor to breathe slowly and deeply.

Muscular spasm or twitching may be seen in some donors.

Convulsions can be seen in some donors. This is an emergency and the blood bank physician must be informed immediately. Adequate help must be sought while providing necessary assistance so that the donor does not injure himself.

Sudden cardiac arrest is a very rare reaction.

All donor reactions must be recorded. Written instructions must be provided by the blood bank physician for managing these reactions.

Instruction to donors after donation

1. Increase intake of fluids for next 48 hours. No extra/special diet is needed.
2. Do not smoke or drive for next half an hour.

3. Do not drink alcohol for next 24 hours.
4. Do not go for a flight for next 24 hours if the donor is pilot by profession.
5. Avoid strenuous exercise/lifting heavy weights for 24hours.
6. If bleeding occurs from phlebotomy site raise the arm and apply pressure on the venipuncture site.
7. If feeling faint or dizzy lie down with legs slightly raised above the head level. If symptoms still persist consult nearest doctor, blood bank doctor or clinician.

Tests to be performed on donor blood

The following tests are done on blood collected in pilot tubes:

1. ABO grouping
2. Rh grouping
3. Screening for transfusion transmitted infections: Hepatitis B virus (HBsAg), hepatitis C virus (HCV), Human Immuno Deficiency virus (HIV-1,2), syphilis and malaria

INTEXT QUESTIONS 6.1

1. A 45 kg donor can donate
   (a) 350 ml of blood (b) 450 ml of blood
   (c) 400 ml of blood (d) 300 ml of blood
2. The shelf life of blood collected in CPDA-1 is
   (a) 35 days (b) 40 days
   (c) 36 days (d) 21 days
3. The collected blood is screened for all except
   (a) HIV (b) HBsAg
   (c) HepatitisA virus (d) Malaria
4. Anticoagulant used in blood is
   (a) EDTA (b) CPD
   (c) Heparin (d) CPDA-1
5. If platelets are to be prepared from the blood, it is stored at
   (a) 2-6°C (b) 20-22°C
   (c) 8-10°C (d) Any of the above

WHAT HAVE YOU LEARNT

- Blood collection entails that proper procedures from selection of the type of bag to be used to the post donation instructions to the donor is strictly implemented. Special attention to the labeling which allows donor identification must be given, as also maintenance of an aseptic and closed system for actual collection of blood to minimize any risk to the donor / recipient. The donor blood can be stored for 35 days at 2-6°C.The blood collected in pilot tubes must be screened for transfusion transmitted infections and blood grouping must also be done. Adverse reactions can occur in the donor during or after donation which must be managed.

TERMINAL QUESTIONS

1. Explain briefly the tests performed on blood donors.
2. Explain the care of donors after donation

ANSWERS TO INTEXT QUESTIONS

6.1

1. (a) 350 ml of blood
2. (a) 35 days
3. (c) Hepatitis A virus
4. (d) CPDA-1
5. (b) 20-22°C

Notes

7

ABO BLOOD GROUPING

7.1 INTRODUCTION

Several blood group systems have been described in humans. Of these, the ABO blood group system is most significant.

OBJECTIVE

After reading this lesson, you will be able to:

- explain commonly used terms in ABO grouping
- describe antigen antibody reactions
- describe the basis of ABO grouping.
- explain the techniques of ABO grouping.

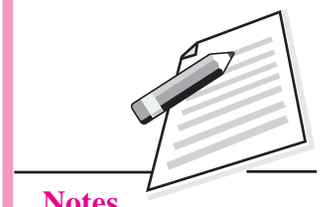
7.2 SOME COMMON ASPECTS OF IMMUNO-HEMATOLOGY

We will learn about some commonly used terms before beginning ABO grouping. This will help in better understanding of the subject.

Antigen: An antigen is a substance usually a protein which when introduced into an individual who recognizes it as foreign, leads to the production of antibody. This antibody specifically reacts with the antigen.

On the **red cell surface** there is presence of glycoproteins and glycolipids which act **as antigens**. They are called **blood group antigens**. These antigens can be on the surface, below or protrude from the red cell membrane. If introduced into the body of an individual who lacks the antigen, an immune reaction can occur.

Antibodies: These are immunoglobulins present in the serum and can be of 5 types: IgG, IgM, IgD, IgA and IgE.



If red cells carrying an antigen are introduced into the circulation of an individual who lacks that antigen, antibodies will form and cause destruction of the introduced red cells. These are **immune** or acquired antibodies and are **IgG** in nature. They react best at 37°C.

Certain antibodies occur without antigenic stimulus and are called **naturally occurring antibodies** e.g. **ABO antibodies**. They are IgM in nature and react at room temperature.

INTEXT QUESTIONS 7.1

1. Substance leading to production of antibody is .....
2. ..... & ..... acts as antigens.
3. Immunoglobulin of sera are .....
4. Antibodies are ..... present in sera.
5. Antibodies without antigenic stimulus is called as .....

7.3 ANTIGEN ANTIBODY REACTIONS

The antigen antibody reactions relevant to blood banking are:

- sensitization
- agglutination
- hemolysis
- neutralization

Sensitization is the combination of antigen and antibody. This is a reversible reaction.

Agglutination is the clumping of red cells. It occurs when sensitized cells come into contact with each other resulting in formation of bridges between them and formation of aggregates. It is the most common procedure in blood banking.

Hemolysis as the name suggests is destruction of red cells resulting in the release of hemoglobin from the cells due to the action of complement. This is used in antibody screening tests.

Neutralization: Blood group antigens when added to serum containing antibody can neutralize it. This is used in determining secretor status. If the strength of the antibody reduces, the antigen antibody reaction is assumed to have occurred.

Genotype: This refers to the genes present on the chromosome inherited from each parent irrespective of whether they produce any product which is detectable.

Phenotype: This is used to describe the observable effect of the inherited genes or expression of the genes i.e. the blood group.

INTEXT QUESTIONS 7.2

Match the following

1. Sensitization	(a) Observable effect of the inherited genes
2. Agglutination	(b) Destruction of red cells
3. Hemolysis	(c) Genes on chromosome
4. Genotype	(d) Combination of antigen & antibody
5. Phenotype	(e) Clumping of red cells

7.4 ABO BLOOD GROUP SYSTEM

The ABO grouping system is subdivided into 4 types based on the presence or absence of **antigens A and B** on the red cell surface as shown below. Red cells that only have antigen A are called group A. Those that only have **B antigen** are called **group B**. Cells that have **both A and B antigens** are group **AB**. Cells that **lack both antigens are O.**

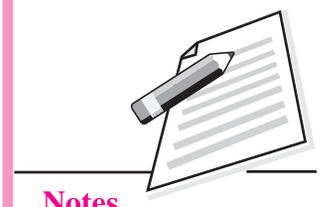
7.5 ANTIBODIES

The ABO antibodies ; anti-A and anti-B are **naturally occurring** antibodies and are present in the sera of individuals who lack the corresponding antigen. Cells with **A antigen** will have **anti-B in the serum**. Cells with **B antigen** will have **anti-A in the serum** and cells with **AB antigens** will **not have any antibody**. Group O individuals will have both anti-A and anti-B antibodies. These antibodies are IgM in nature.

The antigens and the corresponding antibodies in each blood group are shown below.

Table 7.1

Group	Antigen	Antibody
A	A	Anti-B
B	B	Anti-A
AB	A and B	None
O	None	Anti-A, Anti-B



Genetics: All features in humans are controlled by **genes** present on **chromosomes**. Each cell has 23 pairs of chromosomes. There is one locus on chromosome 9 occupied by one of the three alleles A, B, O. The genes of the ABO system are inherited as mendelian codominant. Each individual inherits one gene from each parent. The chromosome from the mother carries one of A,B or O gene. Similarly the chromosome from the father also has one of A, B or O gene. The gene on each chromosome determines the blood group as shown below. The A and B genes are dominant over the O gene.

Table 7.2

	Father	Mother	
	OO	AA	Genotype
Children	AO AO	AO AO	Genotype
Blood group of children	A A	A A	Phenotype

Mother group A, father group O and all children are group A.

INTEXT QUESTIONS 7.3

Match the following

Blood Group	Antigen
1. Group A	(a) Has both A & B antigen
2. Group B	(b) Antigen A
3. Group C	(c) Lack of A & B antigen
4. Group D	(d) Antigen B

Technique of ABO grouping: Various techniques are available for ABO grouping in the laboratory. These are

1. Slide technique
2. Tube technique
3. Microplate technique
4. Gel card technique

Slide technique

This can be performed in emergency or outdoor camps but **must not be performed as a routine test.**

Notes

Material required

1. Glass slides/white tile
2. Monoclonal Antisera A and Antisera B
3. Glass rod for mixing
4. Marker pen

Sample: Blood collected in a plain vial. Sample must be tested within 48hours. It should be kept in the refrigerator till processed. There should be no evidence of hemolysis in the sample.

Method

1. Mark one side of the glass slide as A and the other side as B.
2. Put one drop of antisera A on the side marked as A and one drop of antisera B on the side marked as B.
3. Add one drop of test blood sample/20% cell suspension to each antisera.
4. Mix the blood with the reagent using a clean stick. Spread the mixture over an area of 15mm diameter.
5. Gently rock the slide to and fro and look for agglutination.
6. Record the result.

Interpretation

Agglutination if present indicates a positive result

Advantages

1. Can be used in emergency and blood camps for preliminary grouping.
2. Easy to perform
3. Quick

Disadvantages

1. Not reliable for weak reactions as negative results cannot be checked microscopically.
2. Serum testing cannot be performed.
3. The test mixture tends to dry fast.
4. Drying causes aggregation of cells which can be interpreted as agglutination.
5. Less sensitive than tube technique.

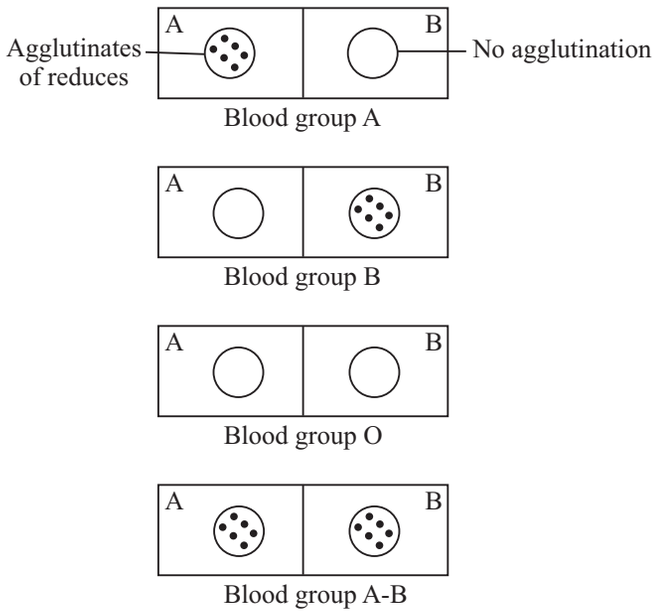


Fig. 7.1

Tube technique

This is the recommended method for grouping. It involves

- cell grouping or forward grouping: testing test red cells with known antisera.
- reverse or serum grouping: testing serum of donor/patient with known cells.

The procedure for cell and serum grouping is described separately but in all samples both the procedures should be done simultaneously and the results crosschecked.

Reagents required

1. Monoclonal Antisera-A and Antisera-B. Antisera-A, B is optional.
2. Normal saline
3. Known cells of group A, B, O

Equipment required

1. Centrifuge
2. Glass tubes 12 × 100 mm
3. Glass tubes 75 × 10 mm

Notes

Cell or forward grouping

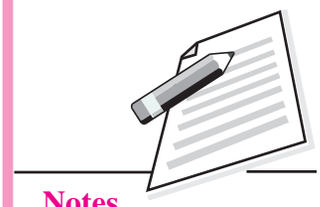
In this the donor/patient red cell are tested with known antisera.

Method

1. Check that the name and number of the donor/patient on the vial matches with the form. Write the same donor number on each tube in which grouping will be performed.
2. Centrifuge the sample to separate the cells and serum.
3. Prepare a 2-5% cell suspension of test red cells in normal saline as follows
   - Add the cells to a pre labeled tube (75 × 10mm) filled three fourth with normal saline.
   - Mix and centrifuge at 1000- 2000 rpm for 1-2 minutes. Decant the supernatant completely.
   - Add saline and repeat the procedure till the supernatant is absolutely clear.
   - After three washes, decant the supernatant and to the cell button add saline by counting the drops to make a 2-5% cell suspension.(10ml of normal saline and 0.2 ml/0.5 ml for 2 and 5% respectively).
   - Invert gently several times to make an even suspension.
4. Label three tubes as Anti-A, Anti-B and Anti-A,B.
5. Add one drop of Anti-A to tube marked A, one drop of Anti-B to tube marked B and one drop of Anti-A,B to tube marked A,B.
6. Add one drop of 2-5% red cell suspension of donor/patient to each tube and mix gently. Leave at room temperature for15- 30min or centrifuge at 1000rpm for 1 minute after 5-10 min incubation at room temperature.
7. Resuspend the cell button and check for agglutination. Also look for any evidence of hemolysis in the supernatant which is read as a positive result.
8. If no agglutination is seen, the contents of the tube must be examined microscopically.
9. Record the results.
10. An autocontrol (patient's serum and cells) can be set up in grouping. No agglutination should be seen in this tube.

Serum grouping

In this the serum of the donor/patient is tested with known cells. The Acells, Bcells and O cells are obtained by pooling fresh group A, B and O cells from



at least 3 individuals of these known groups and a 5% cell suspension (1ml of normal saline and 50µl of washed red cells)is prepared in a similar manner as the cell suspension in cell grouping by washing in saline. The cell suspensions must be prepared fresh everyday and may be tested using the corresponding antisera before use. The unit number from which the pooled red cells are prepared must be entered in the blood grouping register.

Method

1. Label three tubes as A cell, B cell and O cell.
2. Place two drops of the donor/patient serum in each tube.
3. Add one drop of A cells to tube marked A, one drop of B cells to tube marked B and one drop of O cells to tube marked as O.
4. Mix the contents by gentle shaking and leave undisturbed at room temperature for 30-60min or centrifuge at 1000rpm for 1minute.
5. Look for agglutination. Also look for any evidence of hemolysis in the supernatant which is read as a positive result.
6. If no agglutination is seen, the contents of the tube must be examined microscopically.
7. Record the results immediately.

Interpretation

Presence of agglutination or hemolysis is a positive result.

A smooth cell suspension after the button is resuspended is a negative result.

Grading agglutination reactions

The reaction result obtained in grouping is graded as follows:

H Hemolysis. This is a positive result

0 No agglutination, only a smooth suspension

1+ Many small clumps, supernatant has free cells

2+ Many small clumps with clear supernatant

3+ 2-3 clumps, no free cells

4+ One big clump, no free cells

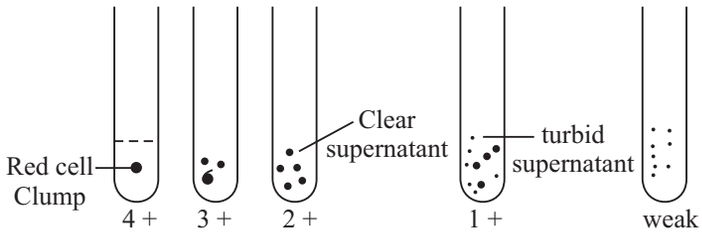


Fig. 7.2: Grading agglutination reactions for blood grouping by tube technique

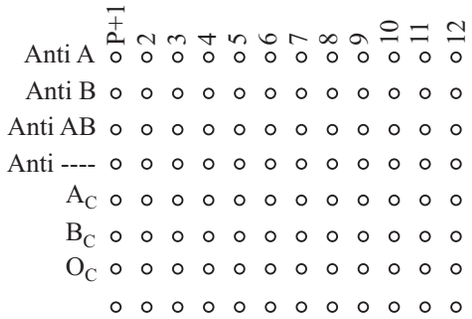


Fig. 7.3: Blood grouping by microplate technique

Advantages

1. Easy to perform
2. Accurate
3. The cell mixture can be incubated for a long time without drying.
4. The centrifugation used enhances the reaction and hence even weak antigens/antibodies can be detected.
5. Uses smaller quantity of reagents.

Interpretation of result

The results seen in different blood groups are shown below

Table 7.3

	Cell grouping			Serum grouping		
Group	**Anti-A**	**B**	**AB**	**Ac**	**Bc**	**Oc**
A	4+	-	4+	-	2+	-
B	-	4+	4+	2+	-	-
O	-	-	-	2+	2+	-
AB	4+	4+	4+	-	-	-

Recording results of ABO grouping

Each blood transfusion centre must have a record sheet in which blood grouping results are recorded.

All reactions must be graded.

Results must be recorded immediately after the test is performed.

Tallying results of forward and reverse grouping

The results of forward and reverse grouping must tally with each other. If a disparity is noted between the cell and serum grouping, inform the Blood Bank incharge and follow the necessary instructions on how to resolve it. Do not release the unit for transfusion till the discrepancy is resolved.

Microplate technique

The microplate consists of a plastic tray with 96 wells as shown in the fig below.

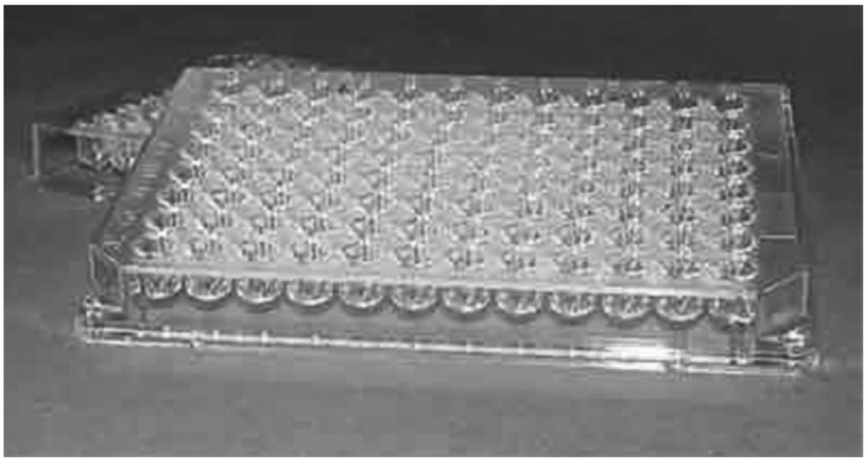


Fig. 7.4: A microplate with wells

Materials required

1. Microplate: U bottom
2. Plate centrifuge
3. Plate shaker

Reagents

1. Antisera A,B,AB. A working dilution of these antisera is prepared in saline.

Notes

Method

1. Arrange the blood samples in serial order.
2. Seperate the cells and serum. Make a 2-3% cell suspension of patient/donor cells as described in tube technique.
3. Each microplate has 8 wells vertically. In the first three wells, put one drop of diluted Anti-A, Anti-B and Anti-A,B respectively. In the next three wells put one drop of Ac, Bc and Oc. The remaining two wells can be used for Rh typing.
4. Add one drop of 2%cell suspension in the first three wells.
5. Put one drop of patient/donor serum in the next three wells.
6. Gently tap the plate and incubate at room temperature for 1hour. If a microplate centrifuge is available, centrifuge at 200g for 1min after 15min incubation.
7. Resuspend the red cells using a microplate shaker/manually briefly.
8. Record results.

Interpretation

Positive result : carpet of red cells which line the bottom of the well.

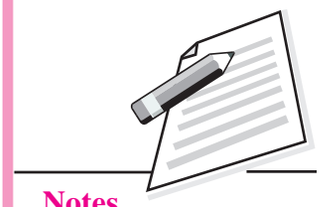
Negative result : compact button with smooth edges which streams when the plate is tilted.

Advantages

1. Uses small volume and low concentration of sera and red cells making it cost effective.
2. Easily handled microplate in place of 96 tubes
3. Large number of samples can be processed at the same time. There is economy of space and time.
4. The technique can be automated which helps in reduction in reading and transcription errors.

Gel card method for blood grouping

Microtubes containing sephadex gel prepared in a buffer such as LISS (low ionic strength saline) or saline are available. In the cards used for blood grouping, red cell specific antisera and a preservative are also added to the gel at the time of manufacture.each card contains six such microtubes.



Materials required

1. ID-Card "Diaclon ABO/D + reverse typing cards" containing monoclonal anti-A_1, anti-B and anti-D within the gel matrix. The microtube control is the negative control. Two microtubes with neutral gel serve for reverse grouping with A & B cells.
2. ID-Diluent 2(modified low ionic strength saline)
3. Test cell reagents- ID Diacell A_1 & B, O in a 0.8% ±0.1% suspension. This is available in 10ml vials which are ready to use.
4. 1D-Dispenser
5. ID-Pipetor and tips
6. ID-Working Table
7. ID-Centrifuge

Sample

A 5% red cell suspension is prepared by adding 0.5ml of Diluent 2 to 50µl of whole blood or 25 µl of packed cells and mixing gently.

Procedure

1. Identify the ID-Card with unique patient/donor no.
2. Remove the aluminum foil from as many microtubes as required by holding the ID card in upright position.
3. Pipette 50 µl ID Diacell A_1 to microtube 5(A1).
4. Pipette 50 µl ID Diacell B to microtube 6(B).
5. Pipette 50 µl of patient's serum to both microtubes 5&6.
6. Pipette 10 or 12.5 µl of patient's red cell suspension to microtubes 1-4 (A,B,D,ctrl)
7. Incubate at room temp for 10 min
8. Centrifuge the ID Cards for 10 mins in the ID Centrifuge.
9. Read and record the results.

Interpretation

Positive: agglutinated cells forming a red cell line on the surface of gel or agglutinates dispersed in gel

Negative: compact button of cells on the bottom of the microtube

Precautions in blood grouping

1. Perform the grouping at room temperature(20-24°C).
2. Include both cell and serum grouping as this serves as a check.
3. Use antisera as per manufacturer's instructions.
4. Store antisera in the refrigerator when not in use.
5. Check the antisera regularly with known cells.
6. All glassware must be dry and clean.
7. Label all tubes accurately.
8. Record results immediately.
9. Use correct speed and time of centrifugation.

WHAT HAVE YOU LEARNT

- Antigen is a substance which when introduced into an individual leads to the production of antibody
- On the red cell surface there is presence of glycoproteins and glycolipids which act as antigen, they are called group antigen
- Antibodies are Immunoglobulins present in the serum and are IgG, IgM,IgD,IgA & IgE
- Certain antibodies occur without antigenic stimulus and are called naturally occurring antibodies like ABO antibodies
- Antigen Antibody reaction may be sensitization, agglutination, hemolysis and neutralization
- ABO grouping system is subdivided into 4 types based on the presence or absence of Antigen A & B on the red cell surface
- Slide, Tube, Microplate & Gel card techniques are various techniques available for ABO grouping

TERMINAL QUESTIONS

1. Write the reaction seen with Anti-A and Anti-B with each blood group. Mark positive result as + and negative result as

Group	Anti-A	Anti-B
AB		
A		
O		
B		

2. Write the reaction with the known cells seen in the blood groups. Mark as in Q1.

Group	A cells	B cells	O cells
B			
AB			
A			
O			

ANSWERS TO INTEXT QUESTIONS

7.1

1. Antigen
2. Glycoproteins & glycolipids
3. Antibody
4. Immunoglobulin
5. Naturally occurring antibody

7.2

1. d 2. e 3. b 4. c 5. a

7.3

1. b 2. d 3. a 4. c

Notes

8

ERYTHROCYTE SEDIMENTATION RATE (ESR)

8.1 INTRODUCTION

Erythryocyte Sedimentation Rate (ESR) is the measurement after 1hour of the sedimentation of red cells when blood is allowed to stand in an open ended glass tube mounted vertically on a stand.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the methods used for measuring ESR
- enlist the factors influencing the sedimentation of red cells
- discuss the significance of measuring ESR
- describe the reticulocyte count and it's significance

8.2 METHODS OF MEASURING ESR

ESR can be measured in the laboratory by two methods

- Westergren method
- Automated ESR analyser

The International Council for Standardization in Hematology recommends the use of Westergren method as the standard method for measuring ESR.

Westergren method

The Westergren pipette is a glass pipette 30 cm in length and 2.5mm in diameter. The bore is uniform to within 5% throughout. A graduated scale in mm extends over the lower 20 cm.

Sample: Venous blood collected in EDTA. Four volumes of blood is diluted in 1 volume of citrate. Alternatively, 2ml blood is directly collected in 0.5ml of 3.8% trisodium citrate.

Equipment required

1. Westergren pipette
2. Stainless steel rack for holding pipette
3. Timer

Method

1. Mix the blood sample well and draw in the Westergren pipette to the 0mm mark with a rubber teat.
2. Place the tube exactly vertical in the rack which has a rubber cork at the bottom. Fix with adjustable screws after removing the teat.
3. Leave undisturbed for exactly 60min free from vibrations and not exposed to direct sunlight.
4. Read the column of clear plasma above the column of sedimented red cells to the nearest mm.

Normal value

Males 0-10mm1st hour

Females 10-20mm 1st hour

Fig. 8.1: Westergren pipette

Fig. 8.2: Westergren pipette filled with blood and placed vertically on the rubber cork in the rack

Precautions

1. Westergren pipette must be clean and dry.
2. Dilute the blood sample just before setting up the ESR.
3. Never use mouth suction to fill the pipette.
4. The Westergren pipette must be placed vertically.
5. The ESR must be set up at room temperature (18-25°C).

Automated method

Automated ESR analyser is a variation of the standard Westergren technique for determination of the erythrocyte sedimentation rate. The fundamental parameters are exactly the same as the Westergren method i.e. the same anticoagulant is used as the Westergren method and the ratio of blood to anticoagulant is also the same. Blood is collected in a pre-evacuated tube which is placed in the rack of the analyser. Several samples can be placed in different slots of the rack. The instrument uses a video camera and an electronic card. The video camera scans the tubes in the rack at regular intervals. These images are transferred to the microprocessing card which digitizes the images and analyses them. The system calculates the distance between the zero level of blood and plasma/cell interface as the sedimentation progresses. After multiple observations during the time of study the system mathematically calculates the ESR value which is equivalent to the value as by Westergren method at 1 hour.

Advantages

1. The tube in which blood is collected is pre-evacuated. No manual filling of blood is required as for Westergren method.
2. It is quick and results are available in 20 minutes.
3. Data of previous samples can be stored.
4. Multiple samples can be analysed simultaneously.

Fig. 8.3: Automated ESR analyser

Precautions

1. The instrument must be placed on a horizontal surface.
2. The surface on which the instrument is placed must be free of vibration.
3. Sample must be processed within 6hours of collection.
4. Blood must be collected to the mark in the tube as specified by the manufacturer.
5. To enable the camera to capture images, light must travel through the tube and hence no labels must be put on the tube. The tube can be identified by numbers.

Mechanism of sedimentation of red cells

Red cell sedimentation depends on the difference in specific gravity between red cells and plasma. As the density of red cells is more they settle down. The settling of red cells produces a settling force while the upward movement of plasma produces a retarding force. As both the forces are equal, very little settling occurs normally. When red cells form rouleaux, their mass increases and the rate of fall also increases thus increasing ESR. Rouleaux formation is limited normally by the negative charge on the red cells.

Stages in ESR

Sedimentation occurs in **three stages**. In the **first stage**, the red cells form rouleaux. In the **second stage**, sinking of the aggregates occurs at a constant speed. In the **final stage**, the rate of sedimentation slows as the aggregated cells pack at the bottom of the tube.

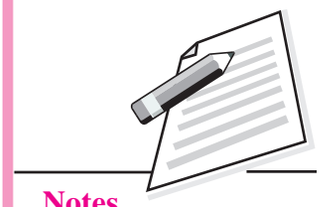
INTEXT QUESTIONS 8.1

1. Methods of measuring ESR are ..... & .....
2. Red cell sedimentation dependence on the difference in specific gravity between ..... & .....
3. ESR is influenced when red cells form .....
4. ..... facilitate rouleaux formation.

8.3 FACTORS AFFECTING RED CELL SEDIMENTATION

The rate of fall of red cells is influenced by a number of factors which interact with each other closely. These are

1. **Rate of rouleaux formation:** Sedimentaton of red cells is greatly influenced by the extent to which the red cells form rouleaux. More is the formation of rouleaux, higher will be the ESR. Rouleaux formation is in turn influenced by the concentration of fibrinogen and other acute phase proteins such as CRP, haptoglobin, ceruloplasmin and others. Rouleaux formation is also enhanced by immunoglobulins. All these proteins facilitate rouleaux formation and thus increase ESR. On the other hand, albumin retards rouleaux formation and ESR.
2. **Ratio of red cells to plasma** or the hematocrit also influences ESR. In **anemic patients**, the number of red cells is less and the proportion of plasma is more. The higher proportion of plasma increases rouleaux formation and hence increases ESR.
3. **Type of red cells** sickle cells and spherocytes do not form rouleaux and hence ESR is decreased in Hereditary spherocytosis and sickle cell anemia.
4. **Plasma viscosity ESR** increases as the plasma viscosity increases. Viscosity is dependent on the concentration of fibrinogen and other plasma proteins.
5. **Length of the tube:** Longer the tube, longer will be the duration of the second phase and higher will be the ESR.
6. **Verticality of the tube:** If the tube is not vertical, sedimentation occurs more quickly due to streaming of blood down the wall of the sloped tube.
7. **Temperature** sedimentation is accelerated as the temperature increases. ESR is hence always measured at room temperature(18-25°C).



INTEXT QUESTIONS 8.2

1. ESR increases when plasma viscosity .....
2. ESR is increased when plasma is .....
3. In sickle cell anemia, the ESR is .....
4. Sedimentation is accelerated when temperature .....

8.4 SIGNIFICANCE OF MEASURING ESR

ESR is a useful screening test for the presence of an underlying acute phase response. Most acute and chronic infections, neoplastic diseases are associated with alteration in plasma proteins and increased ESR. In patients with Tuberculosis, it provides an index of disease activity and of the response to therapy. Similarly in Rheumatoid arthritis, ESR is an index of activity. It is useful in the diagnosis of temporal arteritis and polymyalgia rheumatica.

Causes of elevated ESR

1. Tuberculosis
2. Rheumatoid arthritis
3. Myocardial infarction
4. Multiple myeloma and other plasma cell disorders
5. Pregnancy
6. Neoplastic diseases
7. Degenerative diseases

Causes of reduced ESR

1. Polycythaemia
2. Hypofibrinogenemia
3. Hereditary spherocytosis
4. Congestive heart failure
5. Sickle cell anemia

Reticulocyte count

Reticulocytes are immature red cells. They contain remnants of ribosomal RNA that was present in the cytoplasm of the nucleated precursors. Ribosomes have

the property of reacting with certain dyes such as new methylene blue and brilliant cresyl blue to form a blue precipitate of granules. This reaction takes place only in unfixed vitally stained preparations.

If a blood film is fixed in methanol, reticulocytes appear as diffusely basophilic cells and are called **polychromatophils**.

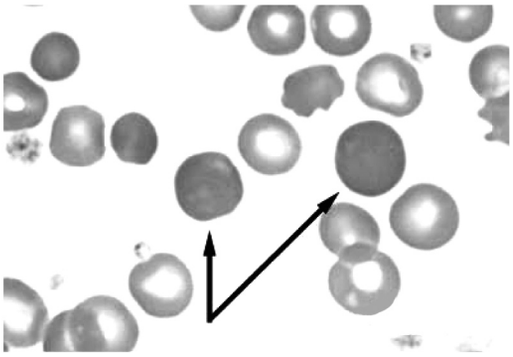


Fig. 8.4: Basophilic red cells or polychromatophils as seen on peripheral smear

Method

Reticulocytes are immature red cells. They contain remnants of ribosomal RNA that was present in the cytoplasm of the nucleated precursors. Ribosomes have the property of reacting with certain dyes such as new methylene blue and brilliant cresyl blue to form a blue precipitate of granules. This reaction takes place only in unfixed vitally stained preparations. New methylene blue stains the granules and filaments in reticulocytes more uniformly than brilliant cresyl blue and is preferred. Azure blue is a good substitute for new methylene blue.

Sample

Venous blood collected in EDTA

Reagents required

New methylene blue 1g dissolved in 100ml of phosphate buffer pH6.5.

Method

1. Place 2-3 drops of new methylene blue in a 75x10mm glass tube.
2. Add 2-3 drops of blood and mix.
3. Keep at 37°C for 15-20 min.
4. After resuspending the mixture, make films on glass slides as peripheral smears are made and allow to dry. The film has a greenish appearence

5. Examine under the microscope and choose an area of the film where the cells are spread and the staining is good. Red cells have a bluish hue while reticulocytes are identified by the presence of dark blue granules or filaments. Count the number of reticulocytes under oil immersion seen in 1000red cells and express as a percentage.

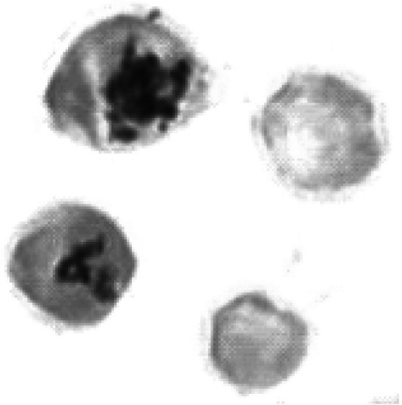


Fig. 8.5: Reticulocytes with granules

Normal value

Adults 0.5-2.5%

Infants 2-5%

Significance of reticulocyte count

Reticulocyte count is a reflection of erythropoietic activity or the rate of production of red cells. The count is **increased** in conditions with accelerated erythropoiesis as in hemolytic anemias. The counts correlate with the severity of hemolysis.

In nutritional anemias, the count is low. On treatment with hematinics the count rises. Hence, reticulocyte count is used to **assess response to hematinic therapy** in these patients.

The reticulocyte count is **low** in aplastic anemia and red cell aplasia. When aplastic crisis occurs in hemolytic anemia the reticulocyte count is low.

WHAT HAVE YOU LEARNT

- ESR is the measurement after 1hour of the sedimentation of red cells when blood is allowed to stand in an open ended glass tube mounted vertically

on a stand. It can be measured in the laboratory by Westergren method or by an automated analyser. Several factors influence ESR including concentration of fibrinogen and other acute phase proteins. ESR is a screening test for acute phase response and is elevated in acute and chronic infections, degenerative and neoplastic diseases.

- Reticulocytes are precursors of red cells which have remnants of ribosomal RNA. This stains with dyes such as new methylene blue to form blue precipitates or granules. On blood films fixed with methanol, reticulocytes appear as basophilic cells called polychromatophils. Reticulocyte count is a reflection of erythropoietic activity.

TERMINAL QUESTIONS

1. What is the standard method for measuring ESR
2. Name two conditions in which ESR is elevated.
3. Name two conditions in which ESR is reduced.
4. Name two dyes used for reticulocyte count.

ANSWER TO INTEXT QUESTIONS

8.1

1. Western method and automated ESR analyser
2. Red cells and plasma
3. Rouleaux formation
4. Proteins

8.2

1. Increases
2. High
3. Decreased
4. Increases

9

STAINING OF PBF AND INTERPRETATION OF NORMAL AND ABNORMAL RED CELL MORPHOLOGY

9.1 INTRODUCTION

A peripheral blood smear (peripheral blood film) is a glass microscope slide coated on one side with a thin layer of venous blood. The slide is stained under a microscope. Examination of a stained peripheral smear is an integral part of laboratory evaluation of patients. It provides information on red cells, leucocytes and platelets and is used to supplement the information provided by automated hematology analyzers. Any parasite if present, can be identified on the smear. Abnormal cells can also be detected in patients with hematological malignancies.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of staining
- explain red cell abnormalities seen on the smear and their interpretation

9.2 STAINING OF PERIPHERAL SMEAR

Peripheral blood smears can be prepared from fresh blood to which no anticoagulant is added or from EDTA blood. Clean glass slides should be used for making blood films. Each smear should be labelled with the patient number.

Notes

Smears are routinely stained with **Romanowsky stains** with very good results. These dyes have the property of making subtle distinction in shades of staining. They also stain granules differentially.

Commonly used Romanowsky stains

1. Giemsa
2. Wright
3. Leishman
4. Jenner's

Of these, Leishman and Wright are widely used in routine staining. However, the results are inferior to Giemsa and Jenner.

The International Council of Standardization in Hematology advocates a combination of pure **azure B and eosin Y** in the stain to be used.

Azure B being a basic dye binds to anionic molecules such as phosphate group of DNA and proteins of the cell nuclei. Eosin Y is an acidic dye and binds to basic molecules such as Hemoglobin and cationic sites on proteins. This difference in binding of the dye to various structures of the cell results in staining of that cell component with particular dyes. Thus red cells stain pink, the leucocyte cytoplasm is light pink, the nuclei are purplish black and the granules of the different leucocytes stain a different color.

The **advantages** of using this combination are that the procedure can be standardized and produces consistent results from batch to batch. The **disadvantage** is that the stain is expensive.

9.3 MAKING A PERIPHERAL BLOOD SMEAR

Examination of a stained peripheral smear is an integral part of laboratory evaluation of patients. It provides information on red cells, leucocytes and platelets and is used to supplement the information provided by automated hematology analyzers . Any parasite if present, can be identified on the smear. Abnormal cells can also be detected in patients with hematological malignancies.

This is possible only if the peripheral smear is well prepared.

Sample

Peripheral blood smears can be prepared from fresh blood to which no anticoagulant is added or from EDTA blood. Clean glass slides should be used for making blood films.

Method

1. Place a 1" × 3" glass microscope slide on a counter top of a laboratory bench. If frosted slides are used, the frosted end should face upward.
2. Write the laboratory number of the patient on the slide.
3. Place a 2 - 3 mm drop of blood approximately 1/4" from the edge, using a glass capillary tube.
4. Hold the slide by the narrow side between the thumb and forefinger of one hand at the end farthest from the frosted end.
5. Grasp a second slide ("spreader slide") between the thumb and forefinger of the other hand.
6. Place the edge of the spreader slide on the lower slide in front of the drop of blood (side farthest from the frosted end).
7. Pull the spreader slide toward the frosted end until it touches the drop of blood. Permit the blood to spread by capillary motion until it almost reaches the edges of the spreader slide.
8. Push the spreader slide forward at a 30^{o} angle with a rapid, even motion.

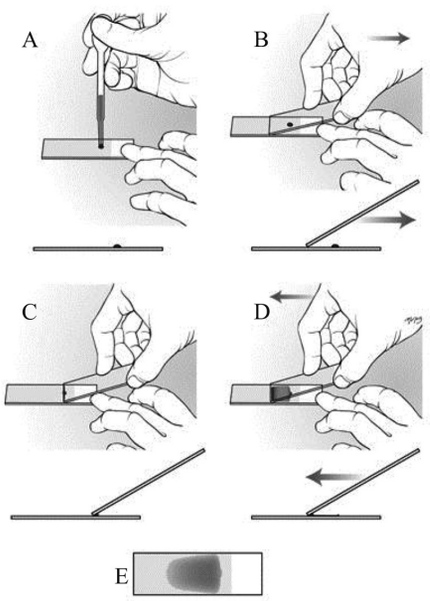


Fig. 9.1

A : Place a drop of blood on the slide

B : Hold the spreader as shown

C : Pull the spreader over the drop of blood so that it spreads

D : Push the spreader with a firm motion

E : This is how the prepared film appears

Notes

9.4 MAKING STAIN SOLUTION

Wright stain

This is available commercially in powder form. Take 2.5g of powder and add 2.5L of methanol to it. Shake well. Keep the stain for 4-5 days for maturation. Filter the stain prior to use.

Giemsa stain

Take 1g of Giemsa powder in a conical flask. Add 100ml of methanol and warm the mixture to 50°C. Keep for 15 min. Shake occasionally. Filter and keep for few hours before using.

9.5 STAINING PERIPHERAL BLOOD SMEARS

Peripheral blood smears are stained with Romanowsky dyes.

The method of **Wright staining** is described.

Reagents required

1. Wright stain
2. Buffer water pH 6.8 This has

Solution A Potassium dihydrogen phosphate 9.1g/L

Solution B Di sodium hydrogen phosphate 9.5g/L

Mix 50.8ml of solution A and 49.2ml of solution B.

Method

1. Make a smear and air dry it.
2. Place the smear on a staining rack. Flood it with Wright stain and leave for 2 minutes. This is the time required for fixation(methanol acts as a fixative).
3. Add twice the amount of buffered water, pH 7 from a plastic wash bottle.
4. Leave for 10 minutes.
5. Wash the stain with buffered water till the smear has a pinkish tinge.
6. Wipe the back of the smear and stand upright to dry.

Precautions

- stain films as soon as possible after they have been dried.

- do not leave the smears unfixed for more than a few hours. If smears are left unfixed for a long time, there is distortion of cellular morphology. Also, dried plasma stains the background of the smear a pale blue. If a delay is expected always fix the smear.
- the staining rack should be leveled.
- do not let the stain solution dry over the smear.

Giemsa staining

The slides are fixed in methanol first. The stain is poured over the slide and kept for 20 min. the smear is washed as above.

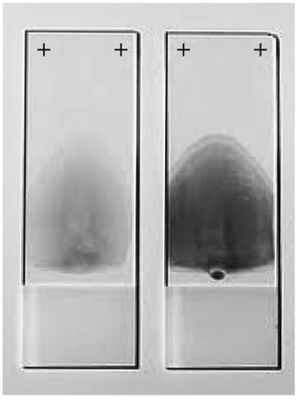


Fig. 9.2: Peripheral blood film before and after staining

INTEXT QUESTIONS 9.1

1. ..... & ..... can be detected by pheripheral blood smear.
2. ..... is routinely used in pheripheral smear.
3. ..... is a basic dye used in staining.
4. ..... is a acidic dye used in staining.

9.6 INTERPRETATION OF RED CELL MORPHOLOGY ON THE PERIPHERAL SMEAR

Normal

Normal red cells appear well spread. The inner one third of each cell is devoid of hemoglobin and appears clear. There is very little variation in size or shape of normal red cells.

Anisocytosis

This means that the red cells are showing variation in size. It can be due to larger red cells or macrocytes or smaller red cells (microcytes).

Poikilocytosis

This refers to variation in shape of red cells. It can be seen in nutritional anemias, thalassemia and other hemolytic anemias.

Macrocytes

As the name suggests, these are red cells which are larger in size. They are found in

- megaloblastic anemia
- aplastic anemia
- myelodysplastic syndromes
- chronic liver disease

INTEXT QUESTIONS 9.2

Match the following

1. Anisocytosis	(a) Cells are larger in size
2. Poikilocytosis	(b) Cells are smaller in size
3. Macrocytes	(c) Cell vary in shape
4. Microcytes	(d) Cell vary in size

Microcytes

These are red cells which are smaller in size. They are seen in

- iron deficiency anemia
- anemia of chronic disease
- thalassemia
- other hemoglobinopathies
- sideroblastic anemia

Hypochromia

It refers to the presence of red cells in which the area of central pallor is increased. It is seen in

- iron deficiency anemia
- anemia of chronic disease

- thalassemia
- other hemoglobinopathies
- sideroblastic anemia

Polychromasia

This appearance is of reticulocytes which appear pale blue on smears fixed and stained subsequently. So in patients with reticulocytes an increased nuber of polychromatophils are seen in the smear. They can be seen in

- hemolytic anemias
- nutritional anemias after treatment with hematinics

Target cells

These are cells in which there is a central round stained area and a peripheral rim of hemoglobin which are sepearted by non staining cytoplasm.

They are found in

- chronic liver disease
- iron deficiency anemia
- thalassemia
- other hemoglobinopathies

Spherocytes

These are spheroidal red cells with a regular outline. They are seen in

- hereditary spherocytosis
- immune hemolytic anemia
- hemolytic disease of the new born
- bacterial toxins

Basophilic stippling

This means the presence of numerous blue granules in the red cells. They are found in

- thalassemia
- lead poisonimg
- unstable hemoglobins
- megaloblastic anemia

Howell Jolly bodies

These are nuclear remnants and are present singly in a small number of red cells. They are basophilic and are seen in

- after splenectomy
- pernicious anemia

Agglutination

This refers to clumping together of red cells as seen in auto immune hemolytic anemia.

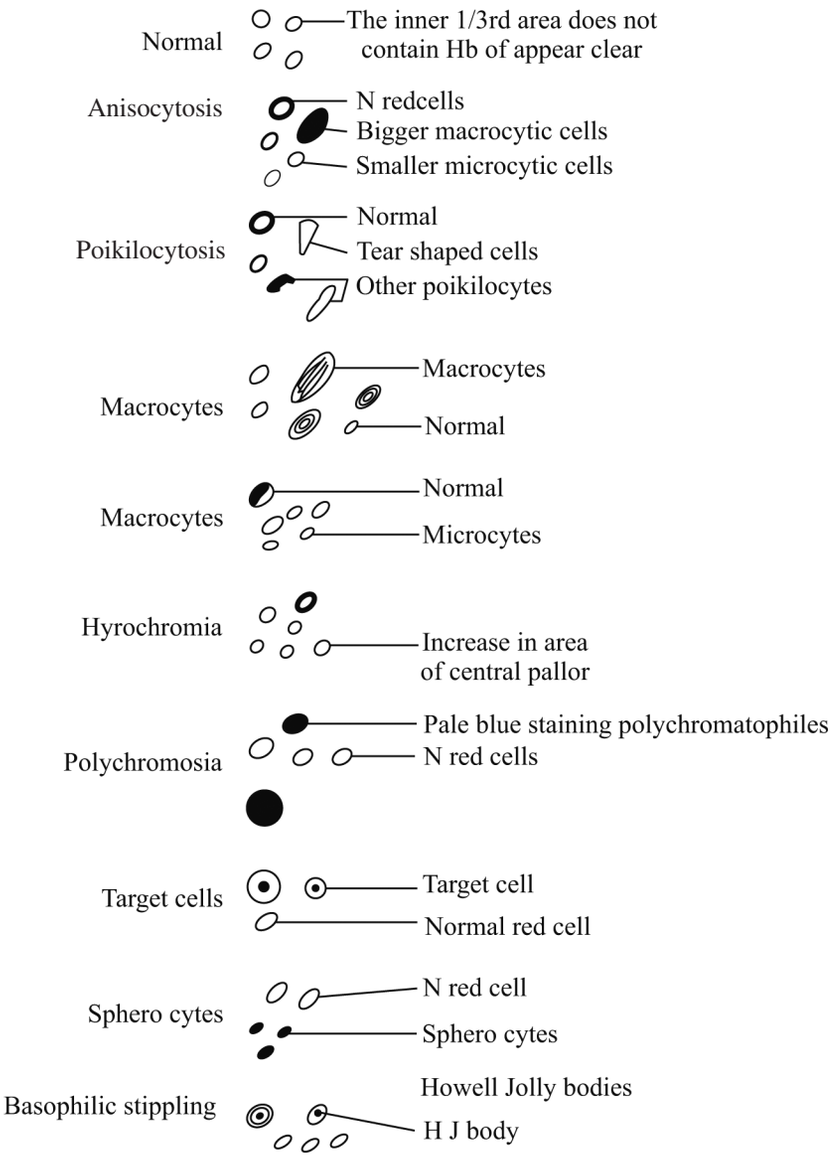


Fig. 9.3: Interpretation of red cell morphology

INTEXT QUESTIONS 9.3

1. Macrocytosis is seen in
   (a) Megaloblastic anemia (b) Iron deficiency anemia
   (c) Thalassemia (d) All of the above
2. Polychromasia represents
   (a) Nucleated red cells (b) Leucocytes
   (c) Reticulocytes (d) None of the above
3. The fixative present in Leishman stain is
   (a) Methanol (b) Ethanol
   (c) None of the above (d) Either of the above

WHAT HAVE YOU LEARNT

- Peripheral blood smears are stained with Romanowsk stains which include Leishman, Wright, Giemsa and Jenner's. They contain a mixture of azure B and Eosin Y. Of these, Leishman and Wright stains are commonly used. Several morphologic abnormalities can be seen in the red cells each of which are seen in specific conditions.

ANSWERS TO INTEXT QUESTIONS

9.1

1. Parasites & abnormal cells
2. Romanowsky stain
3. Azure B
4. Eosin Y

9.2

1. d 2. c 3. a 4. b

9.3

1. (a) Megaloblastic anemia
2. (c) Reticulocytes
3. (a) Methanol

Notes

10

MATURATION AND DEVELOPMENT OF LEUCOCYTES

10.1 INTRODUCTION

The leucocytes develop from the multipotent hematopoietic stem cell which then gives rise to a stem cell committed to formation of leucocytes. Both these cells cannot be identified morphologically by routine methods. The various types of leucocytes are granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes. The three cell types develop separately and accordingly these processes will be discussed separately.

OBJECTIVES

After reading this lesson, you will be able to:

- explain the various stages in the development of leucocytes.
- describe the different types of leucocytes seen normally in PBF.

10.2 MYELOPOIESIS

This is the process of formation of myeloid cells. It is restricted to the bone marrow after birth. The committed progenitor cell for granulocytes and monocytes is the **GM-CFU** which proliferates and differentiates to form myeloblast and monoblast.

The **myeloblast** is the earliest morphologically identifiable cell. It is 10-18µm in size. The cytoplasm is scant and basophilic, usually agranular and may contain a few azurophilic cytoplasmic granules in the blast transiting to the next stage

of promyelocyte. It has a large round to oval nucleus with a smooth nuclear membrane. The chromatin is fine, lacy and is evenly distributed throughout the nucleus. Two-five nucleoli can be identified in the nucleus.

The next stage of maturation is the **Promyelocyte.** It is larger than a myeloblast, 12-20 µm with more abundant cytoplasm which has abundant primary azurophilic granules . The nucleus is round to oval, has slightly more condensed chromatin and nucleoli are not prominent.

The next stage is the **Myelocyte** which is identified as being smaller than a promyelocyte, 12-18 µm. The cytoplasm is eosinophilic and an eccentric round to oval nucleus with coarse chromatin and no visible nucleoli can be seen. **Specific granules** appear in the cytoplasm at this stage and therefore, a myelocyte can be identified as a neutrophilic, eosinophilic or basophilic myelocyte based on the staining properties of the secondary granules. These granules are smaller than the azurophilic granules.

INTEXT QUESTIONS 10.1

1. Types of leucocytes are ....., ... & .....
2. Types of granulocytes are ....., ... & .....
3. Process of formation of myeloid cells are .....
4. Earliest morphologically identified cell is .....

Metamyelocyte is easily identified by its smaller size, dense and clumped chromatin and an indented, horse shoe shaped nucleus with no nucleoli. The cytoplasm is filled with primary, secondary and tertiary granules but the secondary granules predominate. The presence of granules and their staining properties determine whether it is neutrophilic, eosinophilic or basophilic metamyelocyte.

Neutrophilic metamyelocytes give rise to **band form** in which the nucleus becomes sausage shaped with further condensation of the chromatin.

Finally the **polymorphonuclear neutrophil** is formed which has coarsely clumped nuclear chromatin and 3-5 lobes in the nucleus. The lobes are connected by filamentous strands of chromatin.

Cell division is limited to myeloblast, promyelocyte and myelocyte. With the later stages undergoing differentiation but no further cell division. Hence myeloblasts, promyelocytes and myelocytes comprise the **proliferative or mitotic compartment**, whereas cells from metamylocyte stage onwards comprise the **maturation storage compartment** of the bone marrow.

The **half life of neutrophils** in circulation is 6-8 hrs. On completion of the life span, cells may undergo apoptosis or phagocytosis by macrophages, or undergo cell death through mechanisms dependent on reactive oxygen metabolite.

Eosinophils and basophils follow the same pattern of proliferation, differentiation, maturation and storage in the bone marrow. The **eosinophils** are recognized by their coarse, bright orange reddish granules and a bi or trilobed nucleus. The half life of eosinophils is approx 18 hrs.

Basophils are similar in size to a lymphocyte, have abundant coarse, purplish black granules overlying and obscuring the nucleus. The granules contain histamine, heparin, proteases.

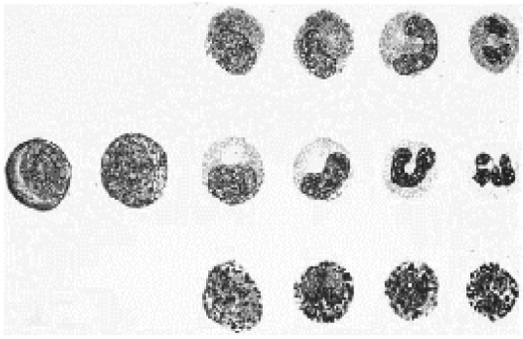


Fig. 10.1: centre row From left to right:myeloblast, promyelocyte, myelocyte, metamyelocyte, band form and mature neutrophil. The top row depicts development of eosinophils and the bottom row development of basophils

Formation of monocytes

The committed progenitor cell GM-CFU also differentiates to form monoblast under the influence of GM-CSF and M-CSF. **Monoblast** is morphologically similar to myeloblast. Monoblast gives rise to **promonocyte** which has basophilic cytoplasm, an indented nucleus, fine chromatin, and may contain a nucleolus. They mature into **Monocytes** which are 15-18 µm in size with a large centrally placed oval or indented nucleus, delicate chromatin and no nucleoli. The cytoplasm is abundant, pale blue in color and has a ground glass appearance due to numerous clear or lilac vacuoles. Monocytes have a short half life in blood of 4.5 to 10 hrs only and migrate from the blood to reside in various tissues as **tissue macrophages.**

Formation of lymphocytes : Lymphopoiesis

The mature lymphocytes are a heterogenous population of cells that differ from each other in terms of origin, lifespan and preferred sites of location within the lymphoid organ. The earliest identifiable precursor is a **Lymphoblast**. It is 14-18µm in size, with scant basophilic cytoplasm and high nuclear: cytoplasmic

(N/C) ratio. The nucleus shows coarse clumped chromatin with 1-2 prominent nucleoli. The lymphoblasts mature to **prolymphocytes** which are smaller cells, 10-12 µm in size with scant cytoplasm, more condensed chromatin and 0-1 nucleoli. The mature lymphocyte can be a **small lymphocyte** approximately 8-10µm in size with deep purplish blue round or slightly indented nucleus and dense chromatin. Nucleoli are not present. There is a very small rim of basophilic cytoplasm. In the **large granular lymphocyte** the cytoplasm is more abundant and contains several reddish granules. Both forms are seen in peripheral blood.

Around 60 - 80% of lymphocytes in the peripheral blood are T cells while B cells constitute about 10-15% of peripheral blood lymphocyte population.

For formation of B cells, the lymphoid progenitor gives rise to a **Precursor B cell** which forms the **immature B cell** and finally the **mature B cell**. B lymphocytes are not stored in the BM except for a brief period and are released into circulation to populate the secondary lymphoid organs The B cells occupy the lymphoid follicles.

Each of these stages can be identified by the expression of certain **cell surface antigens** which can be detected by immunophenotyping. Some of these antigens are described.

Table 10.1

Cell	Antigen
Progenitor B cell	TdT, CD34, CD19,Ig gene rearrangement
Pre B cell	cIg, CD 10, CD 19, CD20
Immature B cell	sIg , CD 19 ,CD20. TdT, CD 34 and CD 10 are not expressed
Mature B cell	coexpression of surface IgD (high) and IgM (low), CD 20, CD 22.

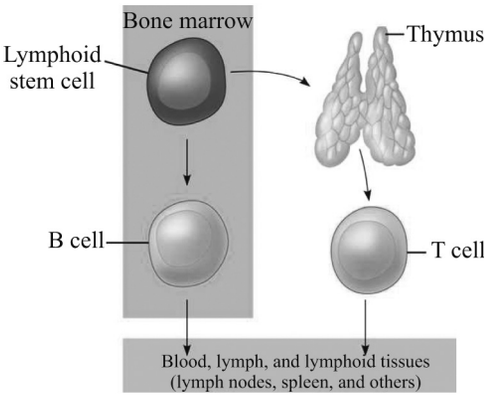
This expression of antigens is used in the classification of leukemias and lymphomas.

The **T cells** populate the perifollicular cortical areas in the lymph node. They are derived from **progenitors** which migrate to the **thymus** where they settle in the **corticomedullary junction**. **The stages in the development of T cells are similar to B cells.** The antigens expressed in each of the stages are described now

The earliest progenitor cells committed to the development of T cells express TdT and CD 34.

The immature cortical thymocytes express CD7, TdT, cCD3 and like B lymphocytes undergo TCR gene rearrangement. On further maturation

coexpression of CD 4 and CD8 occurs and later with further maturation the mature T cells express either CD4 or CD 8 antigen.



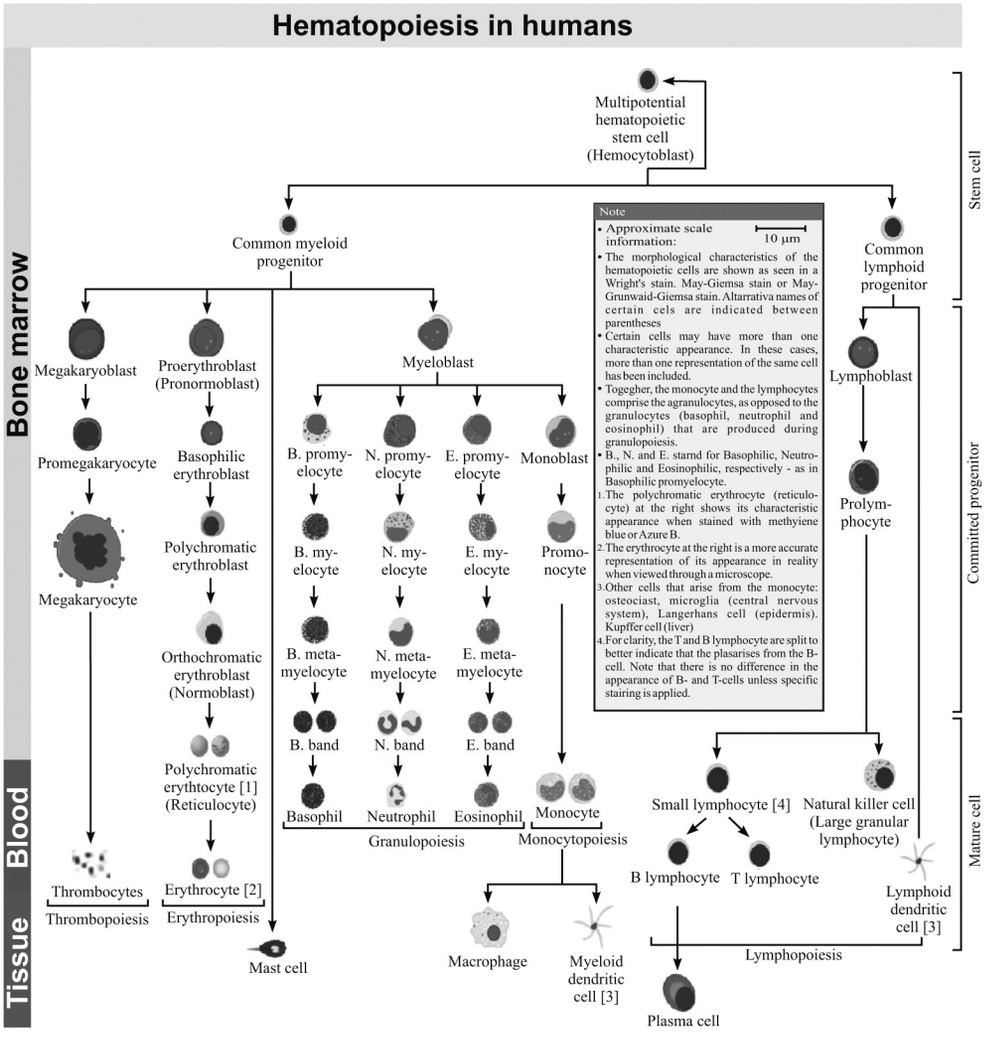
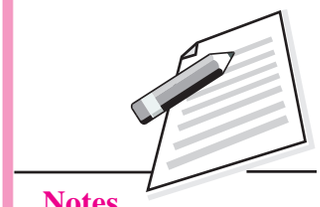


Fig. 10.2: Formation of monocytes, lymphocytes, neutrophil, eosinophil, basophil, red cells and platelets



INTEXT QUESTIONS 10.2

1. Band form arises from .....
2. Two compartments of bone marrow involved in myelopoiesis are ..... & ..... compartment.
3. Monoblast mature into .....
4. Tissue macrophages are .....
5. Precursor for lymphocytes are .....
6. The stages of cell development are by ..... and are detected by .....

10.3 FUNCTIONS OF WHITE CELLS

Neutrophils are phagocytic cells which migrate to the site of microbial infection, and kill the microorganisms.

Monocytes and tissue macrophages also have microbicidal activity although, less effective than that of neutrophils. They are also involved in **phagocytosis and degradation of necrotic material**.

Eosinophils are involved in **defence against parasites and in allergic reactions**.

Basophils along with mast cells are store houses of inflammatory mediators that are released during **IgE mediated hypersensitivity reactions** and are responsible for the associated inflammatory response and tissue injury.

Lymphocytes are non phagocytic cells. Both B and T lymphocytes with their ubsets are actively involved in **humoral and cell mediated immunity** through their interaction with monocyte –macrophage system.

Cells seen in peripheral smear

Polymorphonuclear neutrophil: This cell has an eosinophilic cytoplasm, coarsely clumped nuclear chromatin and a lobulated nucleus, in which the lobes are connected by filamentous strands of chromatin. Two types of granules are present in the mature neutrophils- **primary granules** or azurophilic granules which contain myeloperoxidase, lysozyme, acid phosphatase and acid hydrolase mainly and **secondary granules** or **specific granules** which contain lysozymes, alkaline phosphatase and lactoferrin mainly.

Eosinophil: These cells are about 8μ in diameter and are recognized by their coarse, bright orange reddish granules and a bilobed nucleus**.** The lobes are larger than those of the neutrophils.

Basophils are 10-15 µm in size, have abundant coarse, purplish black granules which fill the cytoplasm and obscure the nucleus.

Lymphocytes: Most lymphocytes in blood are small, about 10 µm in size though larger forms are also present. The nucleus stains deep purplish blue, is round or slightly indented and has dense chromatin. Nucleoli are not visible. There is a narrow rim of basophilic cytoplasm, which is more abundant in the large lymphocyte. Granules are present in the larger forms.

Monocytes are 15-18 µm in size with a large centrally placed oval or indented nucleus with delicate chromatin and no nucleoli. The cytoplasm is abundant pale blue gray in colour and has a ground glass appearance due to numerous clear or lilac vacuoles.

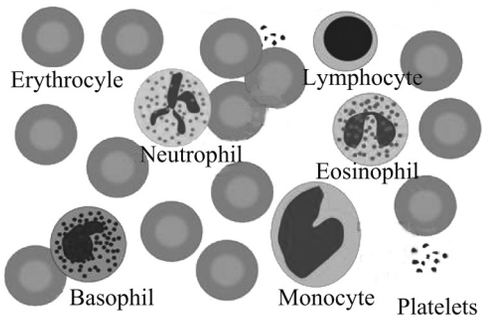


Fig. 10.3

INTEXT QUESTIONS 10.3

Math the following

1. Neutrophils	(a) Hypersensitivity reactions
2. Eosinophils	(b) Humoral and cell mediated immunity
3. Basophils	(c) Phagocytic cells
4. Lymphocytes	(d) Involved in allergic reactions

Normal white cell count

The normal value of total leucocyte count in different age groups is given below.

Table 10.2

Group	TLC × 10^9/L
Adults	4-11
Infants at birth	9-30
6 months-2 years	6-17.5
Children 4-7 years	5-15
Children 8-12 years	4.5-13.5

The counts are higher in infancy and childhood than in adults. The counts drop over the first seven days of life to about 14 × 109/L. there is an increase in TLC in pregnancy and following parturition. Counts also tend to be higher in the afternoon.

Absolute leucocyte count

The absolute count for a particular type of blood cell is the total white blood cell count multiplied by the differential percentage for that cell type.

Normal value of absolute count of leucocytes

Table 10.3

Cell	Absolute count $\times 10^9$/L
Neutrophil	1.8-7.7
Lymphocyte	1.0-4.8
Eosinophil	0.04-0.5
Monocyte	0.2-1.0
Basophil	0.01-0.1

Neutropenia

This implies a decrease in the number of neutrophils with an absolute count<1500cells/µl. based on the count neutropenia can be mild(1000-1500cells/ µl), moderate(500-1000cells/ µl) and severe(<500cells/µl).

Causes of neutropenia

- Bacterial infections such as typhoid, tuberculosis,gram- negative sepsis
- Viral infections such as hepatitis B, cytomegalovirus, Epstein-Barr virus, human immunodeficiency virus, hepatitis C virus
- Drugs
- Immune-mediated causes
- Transfusion reactions
- Neonatal alloimmune neutropenia
- Chronic autoimmune neutropenia
- Chronic idiopathic neutropenia
- Pure white cell aplasia
- Autoimmune disorders: systemic lupus erythematosus, rheumatoid arthritis, Wegener's granulomatosis

Nutritional causes: megaloblastic anaemia, copper deficiency, zinc excess

Bone marrow disorders: aplastic anaemia, replacement by lymphoma,leukaemia, myelofibrosis, multiple myeloma

Congenital or chronic neutropenias

Cyclic neutropenia

Inborn errors of metabolism

10.4 TOTAL LEUCOCYTE COUNT AND DIFFERENTIAL LEUCOCYTE COUNT

TLC

White blood cells or leucocytes can be counted manually in the laboratory using a Neubauer chamber.

Principle

Blood is diluted with Turk's fluid which lyses the red cells and colors the leucocytes which can then be counted.

Sample

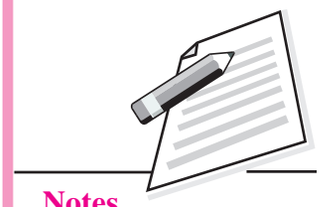
Venous blood collected in EDTA.

Equipment required

Hemocytometer which has

- WBC pipette which has a white bead in the bulb and the stem has markings of 0.5, 1 and 11.
- Improved Neubauer chamber

Fig. 10.4: WBC pipette: Note the white bead in the bulb and the markings



Reagents required

1. Turk's fluid which contains

   Glacial acetic acid 2ml

   1% gentian violet 5 drops

   Add water to make 100ml

Method

1. Mix the blood sample well gently.
2. Fill blood in the WBC pipette upto 0.5 mark. Wipe the excess blood.
3. Fill the pipette upto 11 mark with Turk's fluid.
4. Rotate the pipette in the palm of the hand to mix blood and diluting fluid.
5. Discard the first 2 drops and charge the Neubauer chamber after placing a cover slip over the area with the markings. Place the tip of the pipette close to the cover slip while charging the chamber. There should be no air bubbles in the area after charging.
6. Wait for 2-3 minutes.
7. Count the white cells under the microscope in the 4 corner squares each of which has 16 squares.
8. TLC = $\frac{N \times 20}{4 \times 0.1}$ i.e. $N \times 50$ (X109/L)

   N : number of cells counted in 4 squares

   20 is the dilution

   0.1 is the depth of the chamber

Alternative method of diluting blood for TLC

Take 3.8ml of Turk's fluid in a 75x10 mm glass tube and add 0.2 ml of blood to it. Charge the Neubauer chamber using a Pasteur pipette.

Table 10.4 Normal value of TLC in different age groups

Group	TLC $\times 10^9$/L
Adults	4-11
Infants at birth	9-30
6months-2 years	6-17.5
Children 4-7 years	5-15
Children 8-12 years	4.5-13.5

Precautions

1. The Neubauer chamber must be clean and free from dust.
2. There should be no air bubbles in the Neubauer chamber after charging.

Sources of error

1. Dust particles or clumped red cell debris may be counted as leucocytes.
2. Clumping of leucocytes may also cause error.

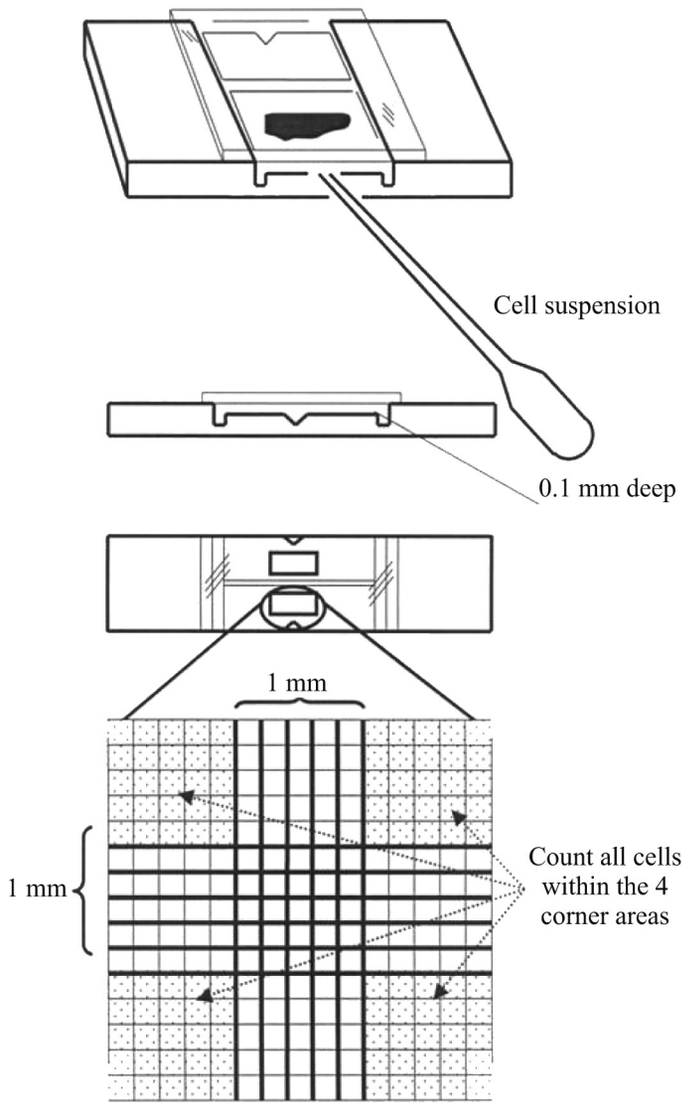


Fig. 10.5: Improved Neubauer chamber: Charging it and WBC counting is done in the 4 squares marked

Differential leucocyte count (DLC)

This refers to enumeration of different types of leucocytes on a stained peripheral smear. One hundred cells are counted and the number of each cell type is expressed as a percentage.

Apply a thin layer of oil over the stained smear and identify the area where DLC is to be done by first examining the smear under low power.

Count the cells using a40x objective in the identified area.

Move the slide in a zig zag manner till at least 100 cells are counted while avoiding the lateral edges of the smear.

Note the different types of leucocytes in the zone of morphology shown in the figure just next to the tail and express as a percentage.

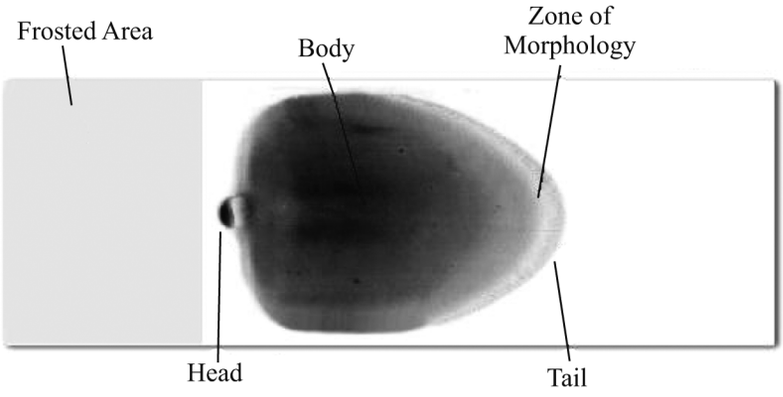


Fig. 10.6: Differential Leucocyte Count

Cells seen in peripheral smear

Polymorphonuclear neutrophil: This cell has an eosinophilic cytoplasm, coarsely clumped nuclear chromatin and a lobulated nucleus, in which the lobes are connected by filamentous strands of chromatin. Two types of granules are present in the mature neutrophils- primary granules or azurophilic granules which contain myeloperoxidase, lysozyme, acid phosphatase and acid hydrolase mainly and secondary granules or specific granules which contain lysozymes, alkaline phosphatase and lactoferrin mainly.

Eosinophil: These cells are about 8µ in diameter and are recognized by their coarse, bright orange reddish granules and a bilobed nucleus**.** The lobes are larger than those of the neutrophils.

Basophils are 10-15 µm in size, have abundant coarse, purplish black granules which fill the cytoplasm and obscure the nucleus.

Lymphocytes: Most lymphocytes in blood are small, about 10 µm in size though larger forms are also present. The nucleus stains deep purplish blue, is round or

slightly indented and has dense chromatin. Nucleoli are not visible. There is a narrow rim of basophilic cytoplasm, which is more abundant in the large lymphocyte. Granules are present in the larger forms.

Monocytes are 15-18 µm in size with a large centrally placed oval or indented nucleus with delicate chromatin and no nucleoli. The cytoplasm is abundant pale blue gray in colour and has a ground glass appearance due to numerous clear or lilac vacuoles.

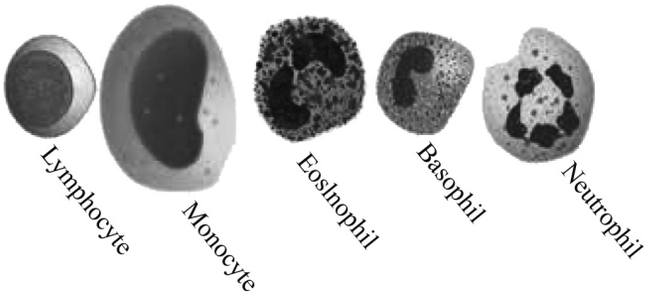


Fig. 10.7: Peripheral smear

Normal value of DLC

Table 10.5

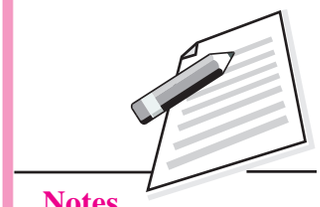
Cell	%
Neutrophils	40-75
Lymphocytes	20-40
Eosinophils	2-6
Monocytes	2-10
Basophils	0-1

INTEXT QUESTIONS 10.4

1. Normal TLC in adults (a) 1.8 – 7.7/L
2. TLC in infants at birth (b) 4 – 11/L
3. Normal neutrophil count (c) 0.04 – 0.5/L
4. Normal eosinophil count (d) 9 – 30/L

WHAT HAVE YOU LEARNT

- Leucocytes develop from multipotent hematopoietic stem cell
- Leucocytes are Granulocytes, Monocytes and Lymphocytes
- Granulocytes are Neutrophils, Eosinophils and Basophils



- Process of formation of myeloid cells is Myelopoiesis
- Neutrophils are phagocytic cells which migrate to the site of microbial infection
- Moncytes & Macrophages have microbicidal activity
- Eosinophils are involved in defense against parasites and in allergic reactions
- Basophils are released during IgE mediated hypersensitivity reactions
- Lymphocytes are non phagocytic cells

TERMINAL QUESTIONS

1. Explain Myelopoiesis
2. What are the functions of white cell

ANSWERS TO INTEXT QUESTIONS

10.1

1. Granulocytes, monocytes & lymphocytes
2. Neutrophils, eosinophils & basophils
3. Myelopoiesis
4. myeloblast

10.2

1. Neutrophilic metamyelocytes
2. Proliferative & maturation storage
3. Monocytes
4. Monocytes
5. Lymphoblast
6. Cell surface antigens, immunophenotyping

10.3

1. c 2. d 3. a 4. b

10.4

1. b 2. d 3. a 4. c

Notes

11

FORMATION OF PLATELETS AND THROMBOCYTOPENIA

11.1 INTRODUCTION

Platelets are small 2μm in diameter with a volume of 8fl. They are anucleate fragments which on Romanowsky stained smears are seen in clumps with occasional reddish granules. They show variation in size and shape. Platelets play an important role in coagulation.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the steps in formation of platelets
- explain the causes of thrombocytopenia

11.2 FORMATION OF PLATELETS

Platelet formation begins in the yolk sac and then shifts to the liver and finally the marrow. Platelets are formed from cells in the bone marrow called as **megakaryocytes** under the influence of thrombopoietin.

The first morphologically recognizable megakaryocyte is called a **megakaryoblast or stage 1 megakaryocyte** which is 6-24 μm in size with a relatively large nucleus, loose chromatin, multiple nucleoli and scant basophilic cytoplasm. It comprises of 20% of all megakaryocytes in the normal bone marrow.

This gives rise to the **stage II megakaryocyte** which is 14-30 μm in diameter,and has a lobulated nucleus and more abundant but less intensely basophilic cytoplasm. It comprises 25% of marrow megakaryocytes.

Mature megakaryocytes or stageIII/IV megakaryocytes are very large cells, 40-60 µm in size with eccentrically placed highly lobulated single nucleus. The cytoplasmic basophilia is further reduced. Stage 4 megakaryocytes comprise of 50% of marrow megakaryocytes and are wholly engaged in platelet formation.

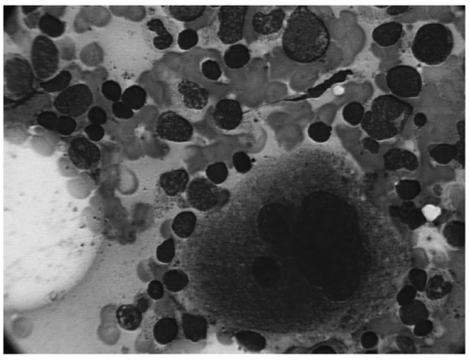


Fig. 11.1: A stage IV megakaryocyte in the bone marrow

Platelets are formed by fragmentation of the cytoplasm of megakaryocyte. It has been shown that each megakaryocyte can give rise to 1000 to 5000 platelets. Each day an adult human produces 1×10^{11} platelets. In times of increased demand platelet production can rise more than 10 times. The normal platelet count is $1.5\text{-}4\times10^{9}$/L. For any individual the count remains within a fairly narrow range.

Platelets are tiny anucleate structures 7.5 to 10.5 fl in size. On peripheral smear they appear as tiny blue gray structures with reddish granules. Platelets released from marrow under conditions of stress e.g. in thrombocytopenia are larger and often beaded in shape. These are termed as **stress platelets**, whereas the normal young platelets recently released from the marrow are called **reticulated platelets**. Platelets have a life span of 8-12 days after which they are removed by the spleen.

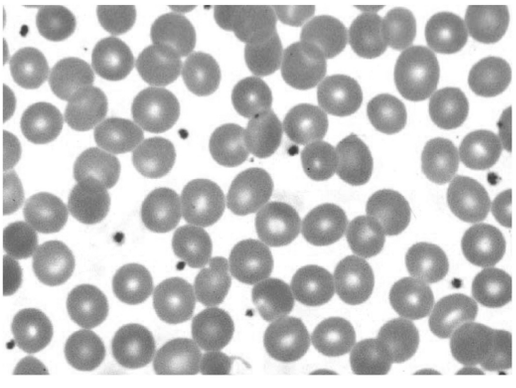


Fig. 11.2: Platelets as seen on peripheral smear

INTEXT QUESTIONS 11.1

1. Platelets are anucleate fragments which are ..... in size.
2. Platelets plays an important role in .....
3. Platelets which are formed from the bone marrow cells under the influence of thromboprotein are called as .....
4. Fragmentation of cytoplasm of megakaryocyte results in the formation of .....
5. ..... are nothing but the platelets released from marrow under stress.

11.3 STRUCTURE OF PLATELETS

The plasma membrane of platelets consists of phospholipids, glycolipids and several surface receptors such as glycoprotein IIb-IIIa which mediate interaction of platelets and other cells as required for normal function of platelets. Platelets possess secretory granules: α granules(PF4,vWF,PDGF,VEGF, β thromboglobulin) and dense bodies (ADP ,Ca, serotonin and ATP). The contents are released when the platelet is activated and they facilitate the coagulation process. Platelets exist in two distinct forms: resting and activated. The activated state results from stimulation by agonists such as thrombin.

Function of platelets

Platelets play a central role in normal hemostasis. They participate in primary hemostasis by adhering to sites of vascular injury and forming a primary hemostatic plug. After vascular injury, platelets come in contact with subendothelial collagen. On contact with the collagen, platelets undergo the following changes: they adhere to subendothelial collagen via vWF which is a bridge between platelet surface gpIb and the exposed collagen. This interaction stabilizes the platelet adhesion against the shear forces of flowing blood. It's importance is highlighted by the fact that deficiency of von Willebrand factor (von Willebrand disease) or gp Ib(Bernard Soulier syndrome) results in a bleeding tendency due to lack of primary hemostasis. The platelets undergo shape change and then there is release of the granule content. ADP is the most important content which causes platelet aggregation and augments release of ADP from other platelets.

After activation of platelets there is expression of a phospholipids complex on the platelet surface which provides a critical nucleation site for calcium and factor binding which then initiates the coagulation pathway leading to formation of thrombin. Platelets enhance contact activation and help in generation of tissue

factor thus further promoting coagulation and leading to fibrin formation. Thrombin results in further aggregation and with fibrin stabilizes the aggregated platelets creating a secondary hemostatic plug.

Causes of thrombocytopenia

A fall in platelet count below the normal level is called thrombocytopenia. It is the most common cause of abnormal bleeding. The causes are tabulated below.

A decreased platelet count (thrombocytopenia) can result from a marrow production problem or a peripheral platelet destructive process. Bleeding complications or even death can result in the presence of a severely decreased platelet count.

INTEXT QUESTIONS 11.2

Match the following

1. Decreased platelet content	(a) Von Willebrand disease
2. Two forms of platelet	(b) TTP, DIC
3. Hemostasis	(c) Thrombocytopenia
4. Lack of primary hemostasis	(d) Resting, Activated
5. Non immune cause of thrombocytopenia	(e) Platelet

Table 11.1 Causes of thrombocytopenia

I. Deficient platelet production

- Hypolasia of megakaryocytes due to drugs, irradiation and aplasia of bone marrow
- Defective thrombopoiesis

II. Accelerated platelet destruction(most common)

A. Immune causes

(a) Autoimmune

- idiopathic

Secondary infections, pregnancy, collagen

- infections, collagen vascular diseases, drugs, pregnancy, miscellaneous

(b) Alloimmune

- neonatal thrombocytopenia
- posttransfusion purpura

B. Non immune

- TTP, DIC
- platelet damage by abnormal vascular surfaces

III. Abnormal pooling of platelets

- disorders of spleen
- massive blood transfusion

Platelet count

The platelet count is one of several laboratory assays of importance in the functional evaluation of the hemostatic system.

Platelet count can be done manually. In most laboratories it is measured by automated hematology analysers which provide more accurate counts.

Equipment required

Neubauer chamber

Reagents required

1%ammonium oxalate

Sample

Venous blood collected in EDTA

Method

1. Make a 1 in 20 dilution of blood by mixing 0.2ml of blood with 3.8ml of diluent.Mix well.
2. Fill a Neubauer chamber with the mixture using a Pasteur pipette.
3. Place the chamber in a moist petri dish and leave undisturbed for 20min to allow the platelets to settle down.
4. Examine under the low power with the condenser kept down.The platelets appear as refractile particles.
5. Count the number of platelets in the RBC counting area in the four peripheral squares and the central square.

   Platelet count = N × 1000/mm^3

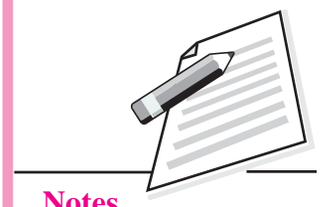
Normal platelet count is 150-400×10^9/L

INTEXT QUESTIONS 11.3

1. The commonest cause of thrombocytopenia is

   (a) increased pooling (b) decreased production

   (c) increased destruction (d) any of the above



2. The granule content which causes platelet aggregation is
   (a) ADP (b) ATP
   (c) Serotonin (d) All of the above
3. What is the normal platelet count

WHAT HAVE YOU LEARNT

- Platelets are tiny anucleate structures 7.5 to 10.5 fl in size. They are formed by cytoplasmic fragmentation of cells in the bone marrow called megakaryocytes. On peripheral smear they appear as tiny blue gray structures with reddish granules. They have a life span of 10 days after which they are removed by the spleen. Platelets play an important role in coagulation. They adhere to subendothelial collagen at sites of injury, release the contents of their granules and aggregate to form a primary hemostatic plug. They also provide a surface for interaction of coagulation factors.
- Thrombocytopenia refers to a decrease in the number of circulating platelets. It results from deficient production, increased destruction and abnormal pooling of platelets.

TERMINAL QUESTIONS

1. Explain the formation of platelets
2. What are the causes of thrombocytopenia

ANSWERS TO INTEXT QUESTIONS

11.1

1. 7.5 – 10.5 fl
2. Coagulation
3. Megakaryocytes
4. Platelets
5. Stress platelets

11.2

1. c 2. d 3. e 4. a 5. b

11.3

1. c 2. a 3. 1.5-4 × 10^9/L

Notes

12

RHESUS BLOOD GROUP SYSTEM

12.1 INTRODUCTION

The Rhesus blood group system is the second most important system in transfusion practice. Rh typing is routinely performed along with ABO grouping in all donors and recipients.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the nature of Rh antigens and antibodies
- explain the significance of Rh grouping
- discuss the method of Rh grouping

12.2 ANTIGENS OF THE RH SYSTEM

The most significant antigen of the Rh system is D because it is highly antigenic. An individual is labeled Rh positive or negative depending on the presence or absence of the D antigen on the surface of the red cells. The D antigen has greater immunogenicity than all other red cell antigens. In India, 95 percent of the population is Rh positive and 5 percent is Rh negative.

Besides antigen D, **four other antigens** of the Rh system have been described: ***C,c, E, e.*** Unlike the ABO antigens the Rh antigens are **only present on red cells**.

Antibodies

The Rh antibodies are always **immune** in nature. They are of IgG subtype and form after exposure to red cells possessing the D antigen. This exposure may occur through **transfusion** or **during pregnancy**.

Rh antibodies are the most important cause of **hemolytic disease of new born** and can cross the placenta to cause destruction of fetal red cells. Once formed their effect persists for many years. Even if their level in the circulation falls, subsequent exposure to the antigen results in a very rapid secondary response.

Rh genes

There are two homologous genes on the short arm of chromosome 1 which code for the nonglycosylated polypeptides that express Rh antigenic activity. One gene is designated **RHD** and it confers D activity on the red cell. It is **present in D-positive** individuals while D-negative individuals have no genetic material at this site. The D antigen has no allele. D- negative individuals lack RHD .

At the other adjacent locus, is a gene called **RHCE**. This determines the presence or absence of C, c, E and e antigens.

INTEXT QUESTIONS 12.1

1. ..... in all donors and recipients are important to perform Rh typing.
2. Rh system's most significant antigen is ..... because of its high .....
3. Rh antigens are only located on .....
4. ..... & ..... are the two homologous genes on the short arm of chromosome 1 which expresses Rh antigenic activity.
5. RHD determines the ..... on the red cells and RHCE concludes the presence or absence of .....

12.3 SIGNIFICANCE OF RH TYPING

Rh antibodies are immune in nature and form after exposure to red cells containing D antigen. Exposure can occur during pregnancy or after transfusion of an Rh negative individual with Rh positive blood. More than 80 percent of D negative individuals who receive a D positive blood transfusion develop Anti-D. To prevent this, the blood of all recipients and donors is routinely tested for D antigen to ensure that D negative individuals are identified and receive D

negative blood only. As Anti-D antibodies can cause hemolytic disease of new born, Rh negative pregnant women are screened during pregnancy for the development of unexpected antibodies.

D^u phenotype

D^u is a weaker variant of D antigen. D^u red cells are agglutinated by some anti-D antisera but not by others. Those which are not agglutinated by anti-D antisera can be detected by AHG technique.

Importance of detecting D^u

1. Though D^u is less antigenic than D, these red cells may be destroyed if transfused to a patient who has anti-D. Hence these donor units must be labeled as Rh positive and du testing must be done on donors.
2. D^u testing is not done routinely on recipients as they are labeled as Rh negative and transfused with Rh negative blood.
3. If a neonate is D^u positive, he can suffer from HDN if the mother has anti-D.
4. Rh negative mothers of a D^u positive neonate must receive RhIg.

Technique of Rh grouping

In Rh grouping, testing is done only for D antigen. Tests for other antibodies are performed only when specifically indicated.

The **methods** for Rh typing are

Slide

Tube

Microplate

Gel card

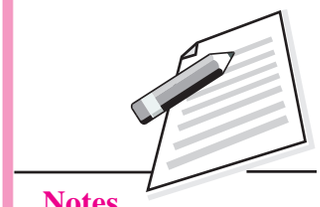
Types of Anti-D antisera

Different types of anti-D antisera are available commercially. These include

(a) Polyclonal human anti-D serum

(b) High protein antisera: This is used for slide grouping. It has macromolecular additives and gives rapid, reliable results.

(c) Saline reactive antisera: This is prepared from IgG antibodies which are modified so that they agglutinate antigen positive cells suspended in saline.



(d) Monoclonal antisera These are of three types: IgM anti-D monoclonal antisera, blend of IgM and IgG monoclonal antisera and blend of monoclonal IgM and polyclonal IgG. IgM anti-D antisera are highly specific and saline reacting. They react equally well at room temperature and at 37°C. They can be used for slide or tube test. For Du testing a blend of IgM and IgG monoclonal is used.

INTEXT QUESTIONS 12.2

1. Exposure of Rh antibodies on red cells containing D antigen may occur during ..... & .....
2. D^u red cells are agglutinated by ..... and not by others
3. Detection of non agglutinated D^u red cells by Anti –D antisera can be done by .....
4. ....., ..., ... and ..... are the four methods of Rh typing.
5. ..... is used for slide grouping.
6. For slide or tube test ..... and for D^u testing a blend of ..... are used.

12.4 SLIDE TECHNIQUE

This can be performed in emergency or outdoor camps but **must not be performed as a routine test.**

Material required

1. Glass slides/white tile
2. Monoclonal Antisera A and Antisera B
3. Glass rod for mixing
4. Marker pen

Sample: Blood collected in a plain vial. Sample must be tested within 48hours. It should be kept in the refrigerator till processed. There should be no evidence of hemolysis in the sample.

Method

1. Mark one side of the glass slide as A and the other side as B.
2. Put one drop of antisera A on the side marked as A and one drop of antisera B on the side marked as B.

3. Add one drop of test blood sample/20% cell suspension to each antisera.
4. Mix the blood with the reagent using a clean stick. Spread the mixture over an area of 15mm diameter.
5. Gently rock the slide to and fro and look for agglutination.
6. Record the result.

Interpretation

Agglutination if present indicates a positive result

Advantages

1. Can be used in emergency and blood camps for preliminary grouping.
2. Easy to perform
3. Quick

Disadvantages

1. Not reliable for weak reactions as negative results cannot be checked microscopically.
2. Serum testing cannot be performed.
3. The test mixture tends to dry fast.
4. Drying causes aggregation of cells which can be interpreted as agglutination.
5. Less sensitive than tube technique.

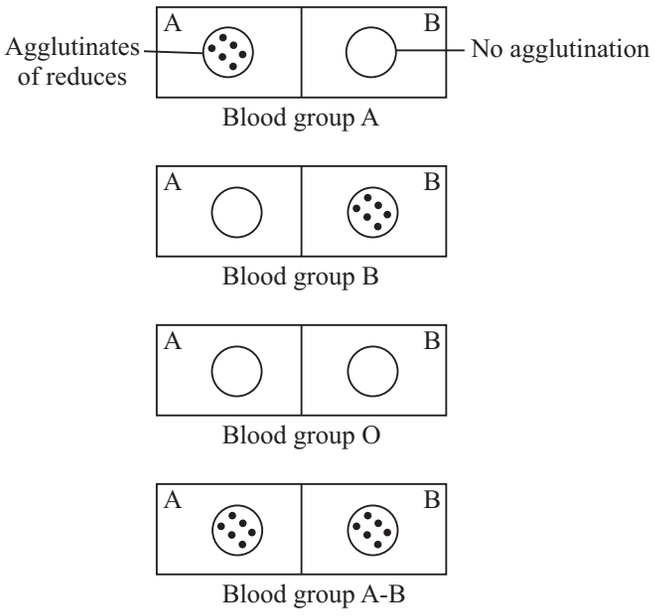
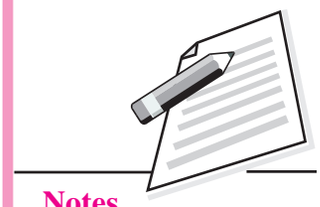


Fig. 12.1: ABO grouping by slide technique



Tube technique

This is the recommended method for grouping. It involves

- cell grouping or forward grouping: testing test red cells with known antisera.
- reverse or serum grouping: testing serum of donor/patient with known cells.

The procedure for cell and serum grouping is described separately but in all samples both the procedures should be done simultaneously and the results crosschecked.

Reagents required

1. Monoclonal Antisera-A and Antisera-B. Antisera-A,B is optional.
2. Normal saline
3. Known cells of group A, B, O

Equipment required

1. Centrifuge
2. Glass tubes 12 × 100mm
3. Glass tubes 75 × 10mm

Cell or forward grouping

In this the donor/patient red cell are tested with known antisera.

Method

1. Check that the name and number of the donor/patient on the vial matches with the form. Write the same donor number on each tube in which grouping will be performed.
2. Centrifuge the sample to separate the cells and serum.
3. Prepare a 2-5% cell suspension of test red cells in normal saline as follows
    - Add the cells to a pre labeled tube (75 × 10mm) filled three fourth with normal saline.
    - Mix and centrifuge at 1000- 2000 rpm for 1-2 minutes. Decant the supernatant completely.
    - Add saline and repeat the procedure till the supernatant is absolutely clear.
    - After three washes, decant the supernatant and to the cell button add saline by counting the drops to make a 2-5% cell suspension.(10ml of normal saline and 0.2ml/0.5ml for 2 and 5% respectively).
    - Invert gently several times to make an even suspension.

Notes

4. Label three tubes as Anti-A, Anti-B and Anti-A,B.
5. Add one drop of Anti-A to tube marked A, one drop of Anti-B to tube marked B and one drop of Anti-A,B to tube marked A,B.
6. Add one drop of 2-5%red cell suspension of donor/patient to each tube and mix gently. Leave at room temperature for15- 30min or centrifuge at 1000rpm for 1 minute after 5-10min incubation at room temperature.
7. Resuspend the cell button and check for agglutination. Also look for any evidence of hemolysis in the supernatant which is read as a positive result.
8. If no agglutination is seen, the contents of the tube must be examined microscopically.
9. Record the results.
10. An autocontrol (patient's serum and cells) can be set up in grouping. No agglutination should be seen in this tube.

Serum grouping

In this the serum of the donor/patient is tested with known cells. The Acells, Bcells and O cells are obtained by pooling fresh group A, B and O cells from at least 3 individuals of these known groups and a 5% cell suspension (1ml of normal saline and 50µl of washed red cells)is prepared in a similar manner as the cell suspension in cell grouping by washing in saline. The cell suspensions must be prepared fresh everyday and may be tested using the corresponding antisera before use. The unit number from which the pooled red cells are prepared must be entered in the blood grouping register.

Method

1. Label three tubes as A cell, B cell and O cell.
2. Place two drops of the donor/patient serum in each tube.
3. Add one drop of A cells to tube marked A, one drop of B cells to tube marked B and one drop of O cells to tube marked as O.
4. Mix the contents by gentle shaking and leave undisturbed at room temperature for 30-60min or centrifuge at 1000rpm for 1minute.
5. Look for agglutination. Also look for any evidence of hemolysis in the supernatant which is read as a positive result.
6. If no agglutination is seen, the contents of the tube must be examined microscopically.
7. Record the results immediately.

Interpretation

Presence of agglutination or hemolysis is a positive result.

A smooth cell suspension after the button is resuspended is a negative result.

Grading agglutination reactions

The reaction result obtained in grouping is graded as follows:

H Hemolysis .This is a positive result

0 No agglutination, only a smooth suspension

1+ Many small clumps, supernatant has free cells

2+ Many small clumps with clear supernatant

3+ 2-3 clumps, no free cells

4+ One big clump, no free cells

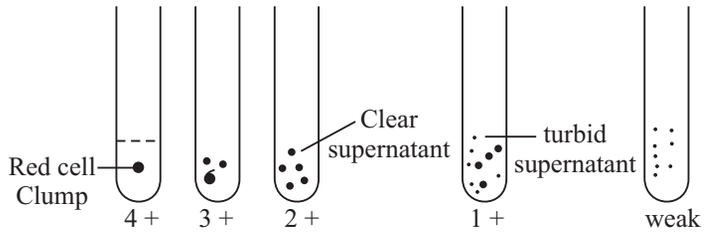


Fig. 12.2: Grading agglutination reactions for blood grouping by tube technique

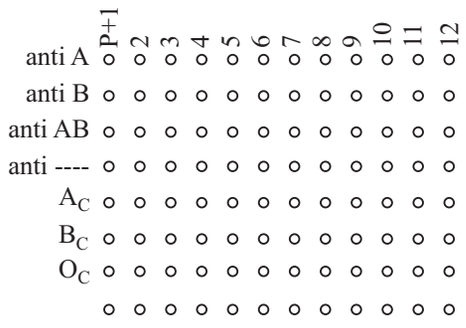


Fig. 12.3: Blood grouping by microplate technique

Advantages

1. Easy to perform
2. Accurate
3. The cell mixture can be incubated for a long time without drying.

4. The centrifugation used enhances the reaction and hence even weak antigens/antibodies can be detected.
5. Uses smaller quantity of reagents.

Interpretation of result

The results seen in different blood groups are shown below

Table 12.1

Group	Cell grouping			Serum grouping		
	Anti-A	B	AB	Ac	Bc	Oc
A	4+	-	4+	-	2+	-
B	-	4+	4+	2+	-	-
O	-	-	-	2+	2+	-
AB	4+	4+	4+	-	-	-

Recording results of ABO grouping

Each blood transfusion centre must have a record sheet in which blood grouping results are recorded.

All reactions must be graded.

Results must be recorded immediately after the test is performed.

Tallying results of forward and reverse grouping

The results of forward and reverse grouping must tally with each other. If a disparity is noted between the cell and serum grouping, inform the Blood Bank incharge and follow the necessary instructions on how to resolve it. Do not release the unit for transfusion till the discrepancy is resolved.

WHAT HAVE YOU LEARNT

- The most significant antigen of the Rh system is D because it is highly antigenic. An individual is labeled Rh positive or negative depending on the presence or absence of the D antigen on the surface of the red cells. The D antigen has greater immunogenicity than all other red cell antigens. In India, 95percent of the population is Rh positive and 5percent is Rh negative. Besides antigen D, four other antigens of the Rh system have been

described: ***C,c, E, e.*** Unlike the ABO antigens the Rh antigens are only present on red cells. The Rh antibodies are always immune in nature and form after exposure to red cells containing D antigen. Exposure can occur during pregnancy or after transfusion of an Rh negative individual with Rh positive blood. More than 80 percent of D negative individuals who receive a D positive blood transfusion develop Anti-D. To prevent this, the blood of all recipients and donors is routinely tested for D antigen to ensure that D negative individuals are identified and receive D negative blood only. As Anti-D antibodies can cause hemolytic disease of new born, Rh negative pregnant women are screened during pregnancy for the development of unexpected antibodies. Rh typing can be done by slide, tube, microplate or gel technique.

TERMINAL QUESTIONS

1. Explain the significance of Rh typing
2. Explain the different types of anti-D antisera

ANSWERS TO INTEXT QUESTIONS

12.1

1. ABO grouping
2. D & Antigenicity
3. Red cells
4. RHD & RHCE
5. D activity & C,c,E and e antigens

12.2

1. Pregnancy, transfusion
2. Anti –D antisera
3. AHG technique
4. Slide Tube Microplate & Gel card
5. High protein antisera
6. IgM anti-D antisera, IgM and IgG monocolonal

Notes

13

PRETRANSFUSION OR COMPATIBILITY TESTING

13.1 INTRODUCTION

Pretransfusion compatibility testing serves to select a compatible unit of blood for the recipient which when transfused does not cause any adverse effect. It ensures safe transfusion therapy.

OBJECTIVES

After reading this lesson, you will be able to:

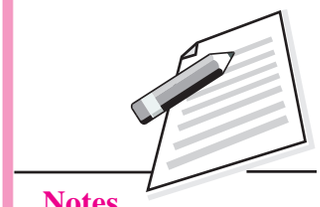
- describe the steps involved in pretransfusion testing
- explain the method of compatibility testing

13.2 STEPS INVOLVED IN COMPATIBILITY TESTING

I. Receiving the sample of the patient in the blood bank

The sample of blood should be collected after proper identification of the recipient patient. The sample is sent to the blood bank in a clean, dry plain tube. The sample is labeled with the following information: name, age, sex, ward, bed number and hospital number. The sample must be collected by the doctor and adequately labeled.

Each sample is accompanied with a **blood request form**. Details such as name, age sex, hospital number, ward and bed number as also clinical details are mentioned on this form. The requistion form should contain the signature of the doctor requesting the blood along with the date.



If the patient has received blood transfusion in the past the blood group, presence of any irregular antibodies and any adverse effect after transfusion must be mentioned.

The technician who receives the sample must ensure that the details on the form and the sample vial tally with each other.

II. ABO and Rh grouping

ABO and Rh grouping is performed on the recipient sample by standard techniques as described in the chapter on blood grouping. Tube technique or gel card method is used most often.

III. Antibody screening of the recipient's serum

The patient's serum is screened for the presence of commonly encountered irregular antibodies. These unexpected or irregular antibodies are produced after transfusion or after pregnancy. Antibody screening is done by the following methods in order to detect all clinically significant antibodies.

- saline technique at room temperature
- enzyme technique
- indirect antiglobulin technique at 37°C

Panel cells

For antibody screening the group O panel cells may be obtained commercially or by using pooled group O cells. These cells must carry the antigens of the common blood group systems like Rh, Kidd, Kell, Duffy, MNS, Lutheran and Lewis. This pooled group is tested against known antisera and if there is agglutination, these cells can be used for further antibody screening. The cell panel can be preserved for long periods by freezing in small aliquots after adding cryoprotective agents like glycerol. When needed, the aliquots can be thawed for screening.

While performing the test for screening antibodies, fresh serum of the patient is tested against panel cells (pooled or commercially obtained), patient's own cells (autocontrol) and cord cells (O group). A unit of blood whose red cells lack the corresponding antigen is then selected for transfusion in such a recipient.

If the specificity of the antibody cannot be ascertained and there is an urgent need for blood, the patient's serum should be cross matched with several units of the same ABO and Rh type as the patient to select compatible blood.

Notes

IV. Selection of blood

Donor blood on which ABO and Rh grouping and screening for various transfusion transmitted infections has been done is selected for transfusion.

Points to remember while selecting blood for transfusion:

- Blood of the same ABO group as the patient is preferred.
- If blood of the same ABO group is not available, alternate ABO group which are compatible may be transfused as shown below:

ABO group of patient	1st choice	Alternative choice
A	A	O
B	B	O
AB	AB	A (preferred) or B
O	O	None

- In patients with blood group AB, if AB group blood is not available, it is preferable to give group A blood rather than group B blood as Anti-B in group A individuals is weaker than Anti –A in group B donors. If the patient needs more than one unit of blood in sucession, it is advisable not to change from group A to group B blood or vice-versa.
- Bombay blood group patient should be given Oh blood only.

Selection based on Rh group

- Rh negative individuals must receive Rh negative blood only.
- For patients who already have Rh antibodies, select blood which lacks the corresponding Rh antigen.
- D^u donor blood is considered Rh positive.
- D^u recipient is treated as Rh negative.

V. Compatibility testing

Though used interchangeably, cross matching is only a part of compatibility testing. Compatibility testing comprises of the following:

- Review of patient's transfusion history and records
- ABO & Rh testing of donor as well as the recipient
- Antibody screening of the recepient's and donor's serum
- Cross matching

The purpose of cross match is to exclude the presence of any antibody (autoantibody or alloantibody) in the recepient's serum that will react with donor cells when transfused. This includes

(a) Major crossmatch is testing of recipient's serum with donor's red cells.

(b) Minor crossmatch is testing of donor's serum with recipient's red cells. If the donor's serum has been screened for irregular antibodies with a panel of cells and no antibody has been detected, minor crossmatch can be excluded.

Compatibility tests are done to ensure safe blood transfusion

Saline technique

This detects IgM antibodies in the recipient's serum that react at room temperature or lower against the donor red cells.

Method

1. Put 2 drops of recipient serum in a labeled test tube.
2. Add 1 drop of 2-5%donor red cells suspended in saline to the tube.
3. Mix and incubate for 5-10min at room temperature.
4. Centrifuge at1000rpm for 1min.
5. Observe for hemolysis or agglutination.
6. Confirm all negative results under the microscope.
7. Alternatively, the tube may be incubated for 30-60min at room temperature and the result read. Centrifugation is optional.

Interpretation

Agglutination or hemolysis indicates incompatibility of donor and recipient blood.

Limitation

The technique does not detect clinically significant IgG antibodies

Note

- Immediate spin technique can be used in emergency
- As the technique does not detect IgG antibodies, it is inadequate as a complete compatibility test.
- Incubation improves the sensitivity of the test.

Notes

Antiglobulin compatibility testing

Indirect antihuman globulin test is the most widely used technique to detect compatibility between donor red cells and recipient serum. It detects IgG antibodies in the recipient serum which may react with the donor red cells.

Method

1. Place 2 drops of patient's serum in a labeled test tube.
2. Add 1 drop of 2-5% donor red cells suspended in saline to the tube.
3. Incubate at 37°C for 30-60min.
4. Centrifuge at 1000rpm for1min.
5. Check for hemolysis or agglutination.
6. If there is no hemolysis or agglutination, wash the cells three times with normal saline decanting the supernatant after each wash.
7. Add 2 drops of polyspecific AHG serum.
8. Centrifuge at 1000rpm for 1minute.
9. Look for agglutination.
10. Add IgG coated red cells to negative AHG test.
11. Centrifuge and check for agglutination which must be present at this stage.
12. If there is no agglutination, the test is invalid.

Interpretation

Hemolysis or agglutination at any stage indicates incompatibility.

Note

- Saline and Antiglobulin crossmatch can be done in separate tubes.
- Alternatively, after centrifuging in saline technique (step5), the tube is incubated at 37°C for 30min and IAT is then done.
- Donor red cells can be pretreated with **enzyme such as papain** and then crossmatching done. It enhances agglutination.
- **Bovine albumin 22%** can be added to the serum cell mixture to increase sensitivity. It enhances agglutination.
- Suspension of donor red cells **in low ionic strength saline (LISS)** increases sensitivity. On reducing the ionic strength of saline, the number of ions which can form clouds around the antigen antibody molecules is reduced. This increases the binding of antigen and antibody to each other.

VI. Labeling of blood bag and Issue of blood

On the unit of blood which has been crossmatched, a label containing the following information is attached

- Donor's identification number
- ABO and Rh group of donor
- Patient's name and hospital number
- ABO and Rh group of patient

Crossmatch report

A crossmatch report is sent with the blood which is being issued which includes

- Donor's identification number
- ABO and Rh group of donor
- Expiry date
- Patient's name and hospital number
- ABO and Rh group of patient
- Interpretation of crossmatching results
- Date of issue of blood

INTEXT QUESTIONS 13.1

1. In a patient with group AB, if AB blood is not available, it is preferable to use blood of group

   (a) A (b) B

   (c) O (d) Any of the above

2. A D^u donor blood is considered

   (a) Rh negative (b) Rh positive

   (c) Either

3. If group O blood is transfused to a patient with group A it is preferable to give

   (a) Plasma (b) Whole blood

   (c) Packed red cells (d) Any of the above

WHAT HAVE YOU LEARNT

- Compatibility testing is done to select a unit of blood for the recipient which does not cause any adverse effects. An adequately labeled sample is received in the blood bank along with a blood request from. The details on the form and the vial must tally with each other. ABO and Rh grouping and antibody screening is done on the recipient sample. A unit of blood is then selected and major cross matching (recipient serum tested with donor red cells) done. The unit is then issued along with a cross matching report.

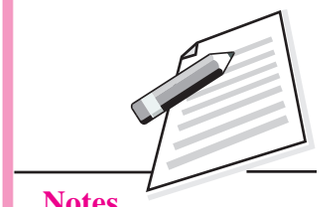
TERMINAL QUESTIONS

1. What are the steps involved in compatibility testing
2. Describe the methods of compatibility testing

ANSWERS TO INTEXT QUESTIONS

13.1

1. (a) A group
2. (b) Rh Positive
3. (c) Packed red cells



14

TRANSFUSION REACTIONS

14.1 INTRODUCTION

Transfusion of blood and blood products are reported to cause reactions during or after procedure specially in patients who receive multiple transfusions. These are referred to as **transfusion reactions (TR)** and may vary in severity from mild to life threatening and fatal reactions. The transfusion centre must maintain a record of all reported transfusion reactions.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the types of transfusion reactions
- explain the investigations of these reactions

14.2 TRANSFUSION REACTION

Transfusion reactions are **classified** into immunological and non-immunological, each of which being further subclassified as acute (occur within24hours) and delayed (occur in days-months) (Table 14.1).

Table 14.1 Classification of transfusion reactions

Type	Immediate	Delayed
Immunological reactions	Hemolytic Febrile non haemolytic	Hemolytic Alloimmunization to red cell, platelet, leucocyte antigens

Notes

Non immunological	Anaphylactic	Post transfusion purpura
	Allergic	GVHD
	Transfusion related acute lung injury	Immunomodulation
	Septicemia	Transfusion transmitted infections
	Circulatory overload	Transfusion hemosiderosis
	Hyperkalemia	Pulmonary microembolisation
	Hypocalcemia	

Immunological reactions: Immediate

1. Acute Hemolytic transfusion reaction (AHTR)

Hemolytic transfusion reactions are of two types:

A. Intravascular (acute hemolytic) transfusion reaction

B. Extravascular (delayed hemolytic) transfusion reaction

Intravascular transfusion reactions occur soon after transfusion of blood and are caused by red cell antibodies in the recipient which destroy the donor red cells. Usually associated with transfusion of ABO incompatible blood, this type of reaction (also known as acute hemolytic TR) is very severe and life threatening. It occurs as a result of activation of the complement system which mediates IgM induced red cell destruction within the circulation.

Causes of hemolytic transfusion reaction

Acute hemolytic reactions can result from clerical errors or technical errors.

I. Clerical errors

These include the following

1. Incorrect identification of patient at the time of sample collection.
2. Incorrect labeling of recipient sample.
3. Incorrect labeling of blood bag.
4. Confusion in the identity of patient at the time of transfusion.
5. Issue of a wrong unit of blood.

II. Technical errors

These may result from

1. Error in blood grouping

2. Error in cross matching due to which incompatibility is not detected
3. Presence of weak antibodies which are not detected by routine tests.
4. Incorrect interpretation of test results.

The following investigations are done in patients with AHTR:

(a) Pre-transfusion and post-transfusion plasma of the patient is examined for evidence of free hemoglobin or bilirubin

(b) Measuring plasma haptoglobin and free hemoglobin levels in patient's urine

(c) Direct antiglobulin test is performed on pre-transfusion and post-transfusion sample

(d) Repeating the compatibility test of the patient's serum (Pre-transfusion and post-transfusion) against donor red cells from the bag

(e) Using indirect Coombs test checking the donor plasma against patient's red cells

INTEXT QUESTION 14.1

1. Reactions to blood transfusion during or after is referred as .....
2. Transfusion reactions are classified as ..... & .....
3. Commonest causes of hemolytic transfusion reaction is .....
4. Acute hemolytic reactions can result from ..... or ..... errors

2. Febrile non hemolytic transfusion reaction (FNHTR)

This is defined as an increase in temperature of 1°C or more above the baseline temperature of the patient during or within 24 hours of transfusion which cannot be ascribed to any other causes. It is associated with transfusion of cellular components and is more common in patients receiving multiple transfusions and in multiparous women.

FNHTR result from **Alloimmunization to antigens on leucocytes and platelets**. The antibodies (HLA antibodies and platelet specific antibodies) in the recipient react with the antigens on donor leucocytes and platelets with activation of complement and release of cytokines which release pyrogens.

The **symptoms** are **mild** and include **fever** with or without chills and rigor.

The reaction can be **prevented** by Leucoreduction of blood products and use of apheresis platelets

Notes

3. Allergic reactions

These reactions are mild and result from the reaction of IgE attached to mast cells/basophils of recipient with proteins or other soluble substances in donor plasma. There is release of histamine which causes itching and urticaria. The reactions are more frequent after transfusion of whole blood and plasma. The patient complains of redness, pruritis, skin rash and urticaria. The reactions can be **prevented** by transfusion of washed red cells, reducing the amount of plasma in the transfused blood by centrifugation and washing the cellular components to remove residual plasma.

4. Anaphylactic reactions

This is a immediate hypersensitivity reaction which may begin after infusion of only a few ml of plasma or plasma containing product. While some of these reactions are immediate others occur after as long as one hour after transfusion. The reaction is seen in patients who are IgA deficient and develop IgA antibodies after blood transfusion/pregnancy.

5. Transfusion Related Acute Lung Injury (TRALI)

This refers to the occurrence of acute lung injury within 6 hours of transfusion. It results from granulocyte specific and anti-HLA antibodies in the donor, which reacts with recipient leucocytes in the pulmonary microvasculature resulting in agglutination of granulocytes in the capillaries of the lung. There is activation of complement in the pulmonary vascular bed which leads to capillary endothelial damage and accumulation of fluid in the alveoli. Donors of plasma which causes TRALI should be examined for the presence of granulocyte specific antibodies and anti-HLA antibodies. The reaction can be **prevented** by use of leucocyte depleted blood components.

INTEXT QUESTIONS 14.2

1. Febrile nonhemolytic transfusion reactions occurs ..... or ..... hours of transfusion
2. Febrile hemolytic transfusion reactions results from ..... to antigen on ..... & .....
3. Febrile hemolytic transfusion reaction can be prevented by ..... of blood products & use of ..... platelets
4. Allergic reactions results from reaction of ..... attached to .....

5. In allergic reactions itching & urticaria is caused by release of .....
6. Anaphylactic reactions are seen in patients who are ..... deficient
7. Transfusion related acute lung injury results from ..... specific & ..... antibodies
8. Transfusion related acute lung injury can be prevented by use of ..... depleted blood component

14.3 NON IMMUNOLOGICAL REACTIONS: IMMEDIATE

1. Septicemia

This is an important cause of fatality associated with blood transfusion. The organisms implicated are Yersinia, Pseudomonas, Enterobacter, and Serratia. These enter and contaminate blood through blood bags, from a donor with asymptomatic bacteremia, unclean skin and skin core which enters from the collection needle. This type of transfusion reaction is uncommon as blood itself has bactericidal activity and storage of collected blood bags at low temperatures inhibits the growth of the microorganisms.

2. Circulatory overload

This results from transfusion of whole blood in excessive volume or high speed. Transfusion of blood to severely anemic patients with congestive heart failure and elderly patients with limited cardiac reserve may result in pulmonary edema.

To prevent these reactions, slow blood transfusion is given and the patient is closely monitored. Infusion of red cell concentrate in place of whole blood and volume reducing apheresis platelets can also be helpful in preventing these reactions.

Immunological reactions: Delayed

1. Delayed Hemolytic Transfusion Reaction (DHTR)

In DHTR there is destruction of the transfused red cells 2-10 days after blood transfusion. It results from an anamnestic antibody response to antigens of the Rh, Kidd, Kell and Duffy blood groups. These patients are immunized by a previous transfusion or pregnancy following which a primary antibody response occurs. This is delayed in onset, the peak is reached slowly and the antibody level gradually declines. The antibody is hence not detected during antibody screening and compatibility testing. On re-exposure to the immunogenic red cell antigen, a brisk immune response occurs and high IgG titers occur within few days. The donor red cells are coated with the antibody and they are removed by

Notes

macrophages of the reticuloendothelial system (mainly liver and spleen). The red cell destruction is hence also known as extravascular transfusion reaction**.** The patient may be asymptomatic. If symptoms are present they are mild and include fever, fall in hemoglobin and jaundice 5-7 days after transfusion. Investigations include screening for red cell antibodies. The direct antibody test is often positive. The antibody should be eluted from the red cells and identified.

2. Post transfusion thrombocytopenia

Severe thrombocytopenia can develop 7-10 days after platelet transfusion due to platelet specific antibodies. This is seen in patients who have been immunized earlier by platelet transfusion and in multiparous women..The patient complains of fever with chills and generalized purpura a week after the transfusion. The thrombocytopenia is self limiting.

3. Graft versus host disease (GVHD)

Cellular concentrates of red cells, platelets and granulocytes contain immunocompetent T lymphocytes. When transfused into immunodeficient recipients, these donor T lymphocytes recognize the host HLA antigens as foreign and mount an immune response thus causing GVHD. Irradiation of blood products containing lymphocytes prior to transfusion to patients at risk helps prevent GVHD.

Non immunological complications: Delayed

1. Iron overload

Each unit of red cells contains 0.25g of iron. Patients who receive repeated blood transfusion such as thalassemia major, other hemolytic anemias progressively accumulate iron. The iron gets deposited initially in the reticuloendothelial system and then in the parenchymal cells of various organs causing growth retardation, sexual dysfunction, hepatic and cardiac dysfunction and other features. The accumulated iron is removed by the administration of iron chelators.

2. Transfusion transmitted infections

Various infections can be transmitted by transfusion of blood. These are HIV, Hepatitis B, Hepatitis C, syphilis and malaria. It is thus mandatory to screen all collected units of blood for all these infections. The organisms, their component screened and the method of screening are given in Table 14.2.

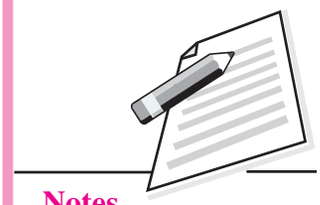


Table 14.2 Infectious agents transmitted through blood and their screening method

Agent	Component screened	Method
Hepatitis B	HBsAg	ELISA
Hepatitis C	Anti-HCV	ELISA
HIV	Anti-HIV1,2	ELISA, particle agglutination assays and spot test
Syphilis	Treponemal antigen	VDRL, TPHA
Malaria	Malaria antigen	Malaria antigen assay

INTEXT QUESTIONS 14.3

1. Each unit of blood is screened for the following infections except
   (a) HIV (b) HBsAg
   (c) Hepatitis A (d) Malaria
2. Hemolytic transfusion reaction is most often due to
   (a) Rh incompatibility (b) ABO incompatibility
   (c) Minor blood groups (d) Any of the above
3. The test preferably done for HIV screening is
   (a) ELISA (b) Rapid test
   (c) Both (d) Neither

WHAT HAVE YOU LEANT

- Reactions to blood transfusion during or after transfusion of blood and blood components are referred as transfusion reactions
- Transfusion reaction may be classified as Immunological & Non-immunological reactions
- Acute reactions occurs within 24 hours and delayed reactions occurs in days to months
- Immediate immune reactions are hemolytic transfusion reaction, febrile nonhemolytic transfusion reaction, allergic reactions, anaphylactic reactions, transfusion related acute lung injury

Notes

- Hemolytic transfusion reaction occurs commonly because of ABO incompatibility and result from clerical or technical errors
- Febrile nonhemolytic transfusion reaction result from Alloimmunization to antigens on leucocytes and platelets
- Febrile hemolytic transfusion reaction can be prevented by leucoreduction of blood products and use of apheresis platelets
- Allergic reactions are caused by IgE attached to mast cells/basophils
- Anaphylactic reactions is seen in patient with IgA deficiency
- Transfusion related Acute lung injury results from granulocyte specific and anti HLA antibodies and can be prevented by use of leucocyte depleted blood components
- Immediate nonimmunological reactions are bacterial contamination, circulatory overload
- Circulatory overload can be prevented by slow blood transfusion
- Delayed immunological reactions are Delayed hemolytic transfusion reaction, post transfusion thrombocytopenia, Graft versus host disease
- Delayed non-immunological reactions are Iron overload, transfusion transmitted infections

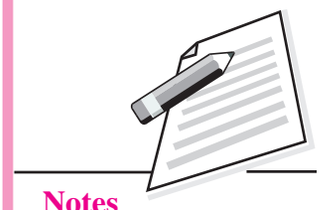
TERMINAL QUESTIONS

1. Classify transfusion reactions
2. Explain how you will investigate transfusion reactions
3. Describe immediate immunological reactions
4. Explain hemolytic transfusion reaction

ANSWERS TO INTEXT QUESTIONS

14.1

1. Transfusion reaction
2. Immune & Non-Immune
3. ABO incompatibility
4. Clerical, technical



14.2

1. During, within 24 hours
2. Alloimmunization, Leucocytes and Platelets
3. Leucoreduction, apheresis
4. IgE, mast cells/Basophils
5. Histamine
6. IgA
7. Granulocytes, Anti HLA
8. Leucocyte

14.3

1. Hepatitis A
2. ABO incompatibility
3. ELISA

Notes

15

INTRODUCTION TO ANEMIA

15.1 INTRODUCTION

Anemia is a state of decreased red cell mass of blood leading to decreased oxygen carrying capacity of body. Red cell indices are useful in classifying anemia. Anemias are classified etiologically and morphologically. In this chapter we will discuss about red cell indices and also about classification of anemia.

OBJECTIVES

After reading this lesson, you will be able to:

- define anemia
- explain red cell indices
- describe Normal red cell parameters
- classify anemia

15.2 DEFINITION

Anemia is defined as the state in which the red cell mass of blood is decreased below the normal level for the age and sex of the patient. As a result of this the oxygen carrying capacity of blood is decreased. It is characterized by a decrease in hemoglobin, packed red cell volume (PCV) and red blood cell (RBC) count.

15.3 RED CELL INDICES

Red cell indices play a vital role in classification of anemia. In well equipped laboratories, these parameters are provided by the automated analyzers. Manual

counting of cells and further calculation of the indices based on them is not accurate and is obsolete. The important red cell indices are as follows:

15.3.1 Mean Corpuscular Volume or MCV

MCV is defined as the volume of the average red blood cell expressed in femtoliters.

It is calculated by the formula:

MCV (fL) = PCV (L/L) ÷ RBC (x 10^{12}/L)

The normal MCV for men and women is 92 ± 9 fL

In electronic counters the MCV is determined by pulse height analysis.

15.3.2 Mean Corpuscular Haemoglobin or MCH

MCH is the average mass of hemoglobin per red cell expressed in picograms. It is calculated by the formula:

MCH (pg) = Hb (g/L) ÷ RBC (x 10^{12}/L)

The normal MCH for men and women is 29.5 ± 2.5 pg

In electronic counters the MCH is a derived value based on the hemoglobin and the RBC count.

15.3.3 Mean Corpuscular Haemoglobin Concentration or MCHC

MCHC is the measure of the concentration of hemoglobin in a given volume of packed red cells and is expressed as g/L

MCHC (g/L) = Hb (g/L) ÷ PCV (L/L) ×1000

The normal MCHC for men and women is 330 ± 15 g/L

In electronic counters this is a derived value from Hb and PCV (or MCV and RBC).

15.3.4 Red Cell Distribution Width or RDW

The RDW is a measure of variation of red cell size or anisocytosis. In electronic counters it is derived from the pulse height analysis and can be expressed either as a coefficient of variation (CV) (%) of the RBC volume or as the standard deviation (in fL).

The normal RDW as coefficient of variation (CV) is 12.8 ± 1.2 % and as standard deviation SD is 42.5 ± 3.5 fl.

The normal red cell parameters are given in Table 1

Table 15.1: The normal red cell parameters

	PARAMETER						
	Hb (g/dL)	PCV (L/L)	RBC ($x10^{12}$/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)
Male	14.5±1.5	0.45±0.05	5±0.5	87±5	29±2	330±15	12.8±1.2
Female	13.5±1.5	0.41±0.05	4.5±0.5	87±5	29±2	330±15	12.8±1.2
Neonate	18.0±4.0	0.6±0.15	6±1.0	100±5	34±3	330±40	12.8±1.2
Child	12.5±1.5	0.35±0.05	4.6±0.6	85±5	27±3	330±15	12.8±1.2

Table 1 – Hb – haemoglobin, PCV – packed cell volume, RBC – red blood cell, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin. MCHC – mean corpuscular hemoglobin concentration, RDW – red cell distribution width, fL – femtoliter, pg – picogram.

15.4 CLASSIFICATION OF ANEMIA

15.4.1 Based on Morphology of Red Cells and the Red Cell indices

15.4.1.1 Normocytic Normochromic Anemia

There is a decrease in hemoglobin, PCV and RBC count.

The MCV, MCH, MCHC and RDW are normal

The blood film shows decreased RBCs which appear normal in size and colour

Examples are anemia due to acute blood loss, hemodilution, decreased erythropoietin secretion and anemia associated with impaired marrow response.

15.4.2 Microcytic Hypochromic Anemia

There is decrease in hemoglobin, PCV and RBC count

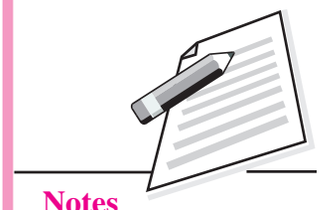
The MCV, MCH and MCHC are decreased. RDW may or may not be increased

The blood film shows small RBCs (microcytes) with increase in central pallor (hypochromic).

Examples are iron deficiency anemia, anemia of chronic disorders, disorders of globin synthesis (beta thalassemia minor), sideroblastic anemia and lead intoxication.

15.4.3 Macrocytic Anemia

There is decrease in hemoglobin, PCV and the RBC count.



The MCV and MCH are increased, MCHC is normal and the RDW is increased.

The blood film shows large number of macrocytes which are well hemoglobinised.

Macrocytic anemia is further divided into megaloblastic and nonmegaloblastic anemia.

Examples of megaloblastic anemia are folic acid or vitamin B12 deficiency, inherited disorders of DNA synthesis and drug induced disorders of DNA synthesis.

Nonmegaloblastic anemia can be due to hypothyroidism, liver disease, alcoholism and aplastic anemia.

15.4.5 Etiological Classification Based on the Cause of Anemia

15.4.5.1 Deficiency of building materials essential for the production of blood

(a) Iron deficiency anemia – red cells are unable to make normal amount of hemoglobin

(b) Vitamin B12 and folic acid deficiency –results in abnormal DNA synthesis leading to megaloblastic anemia.

(c) Anemia of protein calorie malnutrition – red cells are unable to make globin chains

15.4.5.2 Disease of the bone marrow interfering with normal haematopoiesis

(a) Aplastic and hypoplastic anemia

(b) Leukemia/lymphoma – abnormal proliferating cells infiltrating marrow

(c) Fibrosis of the marrow – primary or secondary

(d) Inflammatory conditions – tuberculosis, granuloma formation

15.4.5.3 Excessive blood loss

(a) Acute blood loss – accidents, trauma, surgery, hematemesis

(b) Chronic blood loss – gastrointestinal bleeding due to ulcers, cancer, piles, hookworm infestation, genitourinary due to repeated pregnancies, excessive periods.

15.4.5.4 Increased red cell destruction – Haemolytic anemias

These may be due to:

A. Defects inside the RBC (Intra corpuscular defects)

(a) Red cell membrane defects - example Hereditary spherocytosis, elliptocytosis

(b) Abnormalties of hemoglobin synthesis

1. Decreased globin synthesis – quantitative defect (Example Thalassemias)
2. Abnormal globin synthesis – qualitative defects (Example Sickle cell anemia)

(c) Abnormalities of red cell enzymes – G6PD deficiency

B. Defects outside the RBC (Extra corpuscular defects)

(a) Immune haemolytic anemias- alloimmune, auto immune, drug induced

(b) Parasites – eg malaria

(c) Bacterial – eg Clostridia

(d) Venoms – eg snake venoms

(e) Red cell fragmentation seen in disseminated intravascular coagulation, haemolytic uremic syndrome, march haemoglobinuria, prosthetic heart valves etc.

(f) Drug induced

INTEXT QUESTION 15.1

1. Define Anemia
2. Classify anemia according to the morphology of red cells.

   (a) ..... (b) .....

   (c) .....
3. Name the four red cell indices used to classify anemias and give their normal values.

   (a) ..... (b) .....

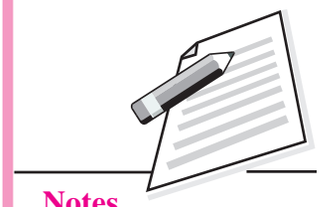
   (c) ..... (d) .....
4. Given the following red cell parameters describe the type of anemia and give one example.

   (a) Hb 10.0g/dl, PCV 32%, RBC 4.5×10^{12}/L, MCV 71fL, MCH 22.2pg, MCHC31.2%, RDW 19%.

   Type of anemia ..... Example .....

   (b) Hb 7.5g.dl, PCV 25%, RBC 2.2×10^{12}/L, MCV 114fL,MCH 34pg. MCHC 30%, RDW 26%

   Type of anemia ..... Example .....



(c) Hb 10g/dl PCV 29% RBC 5.5 × 10^{12}/L MCV 69fL MCH 20pg MCHC 31%, RDW 13%

Type of anemia ..... Example .....

WHAT HAVE YOU LEARNT

- Anemia is a state of decreased red cell mass leading to decreased oxygen carrying capacity of body.
- Red cell indices play a vital role in classification of anemia
- Red cell indices are
  - MCV – Mean Corpuscular Volume
  - MCH – Mean Corpusculat Hemoglobin
  - MCHC – Mean Corpuscular Hemoglobin Concentration
  - RDW – Red Cell Distribution Width which are useful in classifying anemia.
- MCV is defined as the volume of the average red blood cell expressed in femtoliters.
- MCH is the mass of hemoglobin red cells expressed in pictograms.
- MCHC is the measure of the concentration of hemoglobin in a given volume of PRC expressed as g/L.
- The RDW is a measure of variation of red cell size or anisocytosis.
- Morphologically Anemias are classified as
  - Normocytic Normochromic Anemia
  - Microcytic Hypochromic Anemia
  - Macrocytic Anemia
- Etiologically Anemias are classified as
  - Deficiency of building materials essential for the production of blood like Iron deficiency anemia, Vitamin B12 and folic acid deficiency, Anemia of protein calorie malnutrition.
  - Disease of the bone marrow interfering with normal haematopoiesis like Aplastic and hypoplastic anemia, Leukemia/lymphoma, Fibrosis of the marrow, Inflammatory conditionss.

- Excessive blood loss – Haemorrhagic anemias like Acute blood loss, Chronic blood loss.
- Increased red cell destruction – Haemolytic anemias like Defects inside the RBC (Intra corpuscular defects) Defects outside the RBC (Extra corpuscular defects).

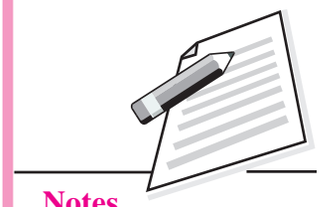
TERMINAL QUESTIONS

1. List the Red Cell Indices with their normal values
2. Classify anemias Morphologically
3. Classify anemia etiologically with examples

ANSWERS TO INTEXT QUESTIONS

15.1

1. Anemia is defined as the state in which the hemoglobin in blood is decreased below the normal level for the age and sex of the patient
2. (a) Normochromic Normocytic anemia
   (b) Hypochromic Microcytic anemia
   (c) Normochromic Macrocytic anemia
3. (a) Mean Corpuscular Volume, 82 – 92 fL
   (b) Mean corpusculat Hemoglobin, 27 – 32 pg
   (c) Mean Corpuscular Hemoglobin concentration, 32 – 36%
   (d) Red cell distribution Width, 11 – 13.5%
4. (a) Normochromic Microcytic Anemia, Anemia of chronic infection
   (b) Normochromic Microcytic Anemia, Megaloblastic Anemia
   (c) Normochromic Microcytic Anemia, Anemia of chronic infection



Notes

16

MICROCYTIC HYPOCHROMIC ANEMIA

16.1 INTRODUCTION

Microcytic hypochromic anemia is characterized by decreased haemoglobin, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and normal to increased Red Cell Distribution Width (RDW). RBC count is normal to increased depending upon the cause of anemia. The peripheral blood smear shows red cells which are smaller in size (microcytes) containing less haemoglobin (hypochromic).

OBJECTIVES

After reading this lesson, you will be able to:

- describe the basics of normal iron metabolism
- explain the biochemical parameters to measure iron in the body
- describe the causes of iron deficiency anemia
- discuss the laboratory diagnosis of iron deficiency anemia
- explain the differential diagnosis of hypochromic microcytic anemia

16.2 DEFINITION

Microcytic hypochromic anemia is characterized by decreased hemoglobin, PCV, MCV, MCH, MCHC and normal to increased RDW. RBC count is normal to increased depending upon the cause of anemia. The peripheral blood smear shows red cells which are smaller in size (microcytes) containing less hemoglobin (hypochromic).

Examples

Iron deficiency anemia

Anemia of chronic disorders

Disorders of globin synthesis

(eg. Thalassemia minor)

Sideroblastic anemias

Lead intoxication

16.3 IRON DEFICIENCY ANEMIA

16.3.1 Incidence

This is the most common type of anemia found worldwide and in India. It is seen in all age groups but is more common in women of the child bearing age and in children.

16.3.2 Cause

The anemia is caused by the deficiency of iron which is essential for the formation of normal hemoglobin.

16.3.3 Iron Metabolism

A basic understanding of iron metabolism is essential to understand the pathogenesis of iron deficiency anemia

16.3.3.1 Distribution of Iron in the Body

A. Hemoglobin – the major component of iron (60%) in the body is in the erythron (RBC + developing erythroid cells in bone marrow) 1 ml of RBC contains approximately 1 mg of iron

B. Myoglobin – Iron is also present in muscle tissue.

C. Enzymes – trace amounts of iron are present in certain enzymes in cells like cytochromes, catalases, peroxidases

D. Storage iron. Iron is recycled when RBC break down and is utilized for new hemoglobin synthesis. Iron is stored as

(a) Ferritin. The iron in macrophages is bound to a protein called apoferritin and is stored as ferritin.

(b) Haemosiderin – is another form of storage iron in macrophages.

16.3.3.2 Iron Intake

Iron is present in food as haeme iron and as non haeme iron. An average diet contains 10 – 15mg/day. Only 5 – 10% (~3.5mg/day) is absorbed from the food. Foods containing iron are meat, fish, eggs, liver, dhals, dried fruits, spinach.

16.3.3.3 Iron Requirements

Males 1mg/day Females 1.5mg/day Pregnancy and lactation 2mg/day

16.3.3.4 Iron Absorption

Iron absorption is an active process and requires ion transporters and enzymes which control the amount of iron absorbed. It is absorbed in the proximal small intestine at the brush border of the lining epithelium, close to the gastric outlet. In the stomach the gastric juices free the heme from its bound form and provide an acidic pH. The pathways for absorption are different for heme and non heme iron (Fe3+). The ferric (Fe3+) nonheme iron must first be converted into ferrous (Fe2+) form so that it can be transported across the membrane via a transporter (divalent metal ion transporter; DMT1). Heme iron is directly moved across the apical membranes through other transporters. Once inside the cell both types of iron have similar fate. Depending upon the body requirements, the absorbed iron is either retained in the mucosal cell or is transferred into plasma.

16.3.3.5 Iron Transport

Iron combines with a plasma protein called transferrin for transport in plasma. It is usually 30% saturated with iron so the serum iron is 100 µg/dL in females and 120 µg/dL in males. The normal percentage saturation of transferrin is 35 ±15%. The transferrin iron complex is attached to the transferrin receptors on the surface of macrophages and internalized. The iron is stored in macrophages in the marrow until required by the developing RBC to make haemoglobin. The transferrin returns to plasma

16.3.3.6 Iron Loss

Iron is lost from the body through shedding of skin and mucosal epithelial cells. Women lose iron during menstruation, in pregnancy and lactation

Iron Deficiency Anemia

16.3.4 Causes of iron deficiency

Iron deficiency occurs due to (a) decreased iron intake, (b) decreased absorption, (c) chronic blood loss or (d) increased requirement.

Notes

(a) **Decreased iron intake** – infants, elderly, poor dietary intake, decreased stores in babies born to iron deficient mothers.

(b) **Decreased absorption** – diseases of the stomach and duodenum and malabsorption.

(c) **Increased loss** – chronic blood loss due to gastro intestinal bleeding, hook worm infestation, excessive periods, multiple and frequent pregnancy etc.

(d) **Increased requirements** – Growing infants, children, adolescents and pregnant women.

16.3.4.1 Clinical presentation

Patients may be asymptomatic or present with fatigue, poor performance status, have difficulty in swallowing and in severe cases may have breathlessness. Some patients especially children may eat mud (pica). Patients have pallor, thin hair, ulcers in the angle of the mouth (angular stomatitis), bald tongue, flat and sometimes spoon shaped nails (koilonychia). There may be pedal edema and cardiac enlargement in severe anemia.

Stages in development of iron deficiency

Iron deficiency develops in a sequential manner and may be classified into 3 stages:

(a) Prelatent: Decreased storage iron without decrease in serum iron levels

(b) Latent: Exhausted iron stores with limited decrease in serum iron levels, the patients are still not anemic

(c) Anemia: Hemoglobin concentration falls and iron deficiency anemia is symptomatic.

16.3.4.2 Laboratory diagnosis

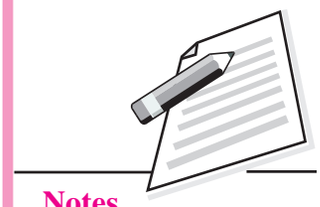
16.3.4.2.1 Complete blood counts

(a) Hemoglobin, PCV and RBC count are decreased. Anemia is commonly around 6 0 -7g/dl.

(b) MCV, MCH and MCHC are all decreased. The RDW is increased ~17 – 23%

(c) WBC count is normal.

(d) Platelet count may be normal or may show thrombocytosis.



16.3.4.2 Peripheral blood smear (PBS)

The peripheral blood smear (Fig. 16.2) shows variable morphology depending on the degree of iron deficiency. RBCs display variable degrees of anisocytosis (variation in size of RBC), microcytosis (RBC size <6.0µm diameter), hypochromia (increase in central pallor) and poikilocytosis (variation in shape). WBC morphology is normal Eosinophilia may be present in hook worm infestation. Thrombocytosis is frequently encountered in iron deficiency anemia.

16.3.4.3 Biochemical tests

The following iron profile is seen in patients with iron deficiency anemia:

- Serum iron is decreased (normal: 60-170µg/dL)
- Total iron binding capacity (TIBC) is increased (normal: 250-400 µg/dL)
- Transferrin saturation is decreased (normal: 16-50%)
- Serum ferritin is decreased (normal: 15-300 µg/L in males and 15-200 µg/L in females)

16.3.4.4 Bone marrow examination

There is minimal utility of bone marrow examination in cases of iron deficiency anemia as the diagnosis is based in CBC, PBS and biochemical findings. The most characteristic feature is absent or severely reduced macrophage iron (storage iron) as evidenced by Prussian blue staining. There is mild to moderate erythroid hyperplasia. The normoblasts appear small, have scant cytoplasm and irregular ragged cytoplasmic borders. The myeloid precursors and megakaryocytes are normal.

16.3.5 Differential Diagnosis

Other microcytic hypochromic anemias – Anemia of chronic disease, disorders of globin synthesis (beta thalassemia minor), sideroblastic anemia and lead intoxication.

INTEXT QUESTIONS 16.1

1. In Hypochromic microcytic anemia, red cells are ..... in size and contain ..... amount of Haemoglobulin
2. 1ml of RBC contain ..... mg of Iron
3. Iron is stored as ..... & .....
4. Iron in macrophages is bound to a protein called as .....
5. Iron requirement in pregnancy & Lactation is ..... mg.day

6. Iron combines with plasma protein called .....
7. Spoon shaped nails is described as .....
8. Children eating mud is described as .....

16.4 ANEMIA OF CHRONIC DISEASE

16.4.1 Definition

Anemia of chronic disorders is a type of anemia associated with a variety of chronic inflammatory, infectious or neoplastic disorders. The anemia usually develops when the above mentioned diseases persist for more than 1 or 2 months. It is secondary to inability of developing erythroid cells to utilize the available iron.

16.4.2 Associated Conditions

This type of anemia is commonly seen in the general population as it is associated with many chronic disorders. It may be associated with infections like tuberculosis, osteomyelitis, endocarditis, chronic abscesses; connective tissue disorders like rheumatoid arthritis, systemic lupus erythematosus; carcinoma, lymphomas and alcoholic liver diseases.

16.4.3 Pathogenesis

The most important abnormalities which occur in anemia of chronic disorder are: decreased red cells survival, inadequate marrow response, impaired iron mobilization and iron uptake by erythroid cells. These abnormalities are thought to be the effect of cytokines that are released during inflammation.

16.4.4 Laboratory Diagnosis

(a) Hemoglobin is ~9.0 -10.0g/dl, PCV and RBC count are mildly decreased.

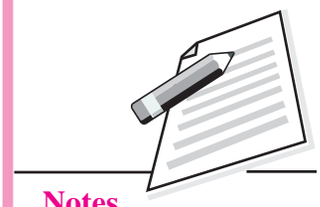
(b) The MCH is decreased, MCV may be normal or mildly decreased (MCV <72fl is rare), MCHC is normal, RDW is mildly increased.

(c) The peripheral blood smear show normocytic normochromic or microcytic hypochromic red cells. Changes in WBC and platelets will reflect the underlying disorder

(d) The reticulocyte count is decreased.

(e) The bone marrow examination shows normocellular marrow with normoblastic erythroid hyperplasia and normal myeloid and megakaryocytic maturation. The amount of hemosiderin within macrophages is increased.

(f) The Biochemical tests show decreased serum iron, decreased TIBC, decreased transferring saturation and increased or normal serum ferritin.



INTEXT QUESTIONS 16.2

1. Anemia of chronic disorders are due to the inability of cells to utilize .....
2. Infections which cause anemia are ....., ... & .....
3. Connective disorder which cause anemia are ..... & .....
4. ..... released during inflammation blocking the reuse of iron causing anemia

16.5 β THALASSEMIA MINOR

16.5.1 Definition

This is an inherited defect in haemoglobin synthesis where there is a decrease in globin chain synthesis. When the patient inherits two thalassemia genes, one from each parent, he/she develops a severe form of anemia called thalassemia major. A person who has one thalassemia gene has a mild hypochromic microcytic anemia which must be differentiated from iron deficiency anemia.

16.5.2 Laboratory Diagnosis

(a) Hemoglobin, PCV are mildly decreased. **RBC count is increased.**

(b) MCV, MCH are decreased but MCHC is normal or mildly decreased. RDW is only mildly increased ~14 – 15%

(c) Peripheral blood smear shows mild anemia, uniformly microcytic hypochromic red cells with no polychromasia. Target cells may be present. WBC and platelets are normal.

(d) Reticulocyte count is increased.

(e) Biochemical tests show normal serum iron, serum TIBC, transferrin saturation and ferritin.

(f) The diagnosis is made by performing hemoglobin analysis by HPLC (high performance liquid chromatography). Patients with beta thalassemia trait show the presence of raised HbA_2 in the range of 3.5 to 7.0%, HbF 1 to 3%; the rest being constituted by HbA.

Important

In the Indian population where the incidence of the beta thalassemia gene is 3-15% depending on the region, it is important to detect the thalassemia carrier

and offer genetic counseling and screening of the spouse to prevent the birth of children with beta thalassemia major.

16.6 SIDEROBLASTIC ANEMIA

16.6.1 Defintion

This is a refractory anemia with microcytic hypochromic red cells due to a defect in heme synthesis and the inability of mitochondria of developing red cells to incorporate iron into the heme molecule. It is characterized by the presence of ringed sideroblasts (erythroblast with perinuclear distribution of Prussian blue positive granules) in the bone marrow.

16.6.2 Types of Sideroblastic Anemia

A. Hereditary – usually seen in males, X linked inheritance, rarely autosomal.

B. Acquired

(a) Clonal: Associated with myelodysplastic syndrome

(b) Reversible: Drug induced (anti TB drugs, chloramphenicol), alcoholism

16.6.3 Pathogenesis

There is a defect in heme synthesis and iron is not incorporated into heme in the mitochondria. The iron transported to the mitochondria gets accumulated and can be demonstrated by the presence of stainable iron in the form of a ring around the nuclei of erythroblasts in the marrow. These granules contain iron inside mitochondria. The insufficient heme synthesis may also be secondary to enzyme defects in heme biosynthetic pathways. Since heme synthesis is affected, the red cells contain less haemoglobin and a microcytic hypochromic anemia results.

16.6.4 Laboratory Diagnosis

(a) The degree of anemia is highly variable

(b) MCV may be markedly reduced in severe cases. MCH and MCHC are decreased. RDW is increased.

(c) WBC and platelets are usually normal

(d) Peripheral blood smear shows marked anisocytosis and poikilocytosis with appearance of microcytes, normocytic normochromic and target cells

(e) Bone marrow shows erythroid hyperplasia increased iron stores with more than 15% ringed sideroblasts. The marrow also will have features of the underlying associated disorders if any.

(f) Serum iron is increased, TIBC decreased, Transferrin saturation is increased, serum ferritin is increased.

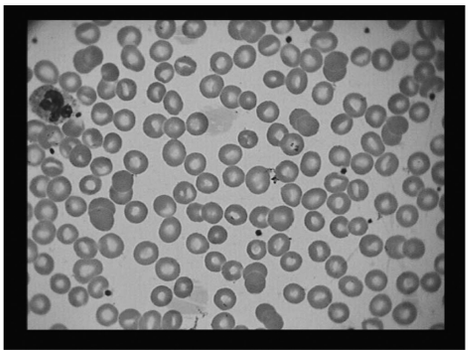


Fig. 16.1: Normal blood film

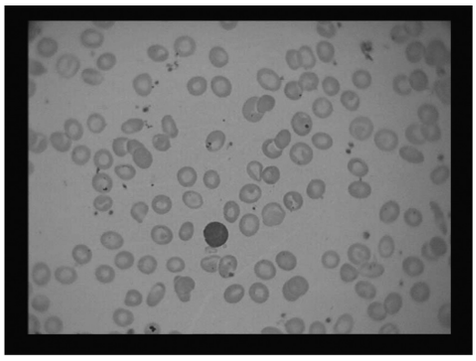


Fig. 16.2: Iron Deficiency anemia

INTEXT QUESTIONS 16.3

1. Thalasemia is caused because of decreased ..... synthesis
2. Diagnosis of Thalasemia is made by ..... test
3. Inability of red cells to incorporate iron into haeme molecule causes ..... anemia
4. In Sideroblastic anemia, RBC's may be ..... or .....

WHAT HAVE YOU LEARNT

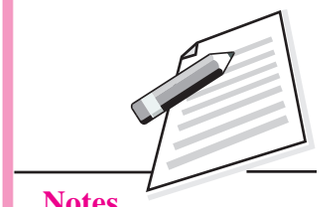
- Hypochromic microcytic anemias are characterized by decreased Hb, PCV, RBC, MCV, MCH, MCHC and increased RDW
- Blood film shows red cells smaller in size (microcytes), containing less hemoglobin (hypochromic)

Notes

- Iron deficiency anemia is caused by the deficiency of iron
- Iron is stored as Ferritin & Haemosiderin
- Iron requirement increases during pregnancy
- Iron combines with a plasma protein called Transferrin for transport in plasma
- In bone marrow absent or severly reduced macrophage iron (storage iron) is evidenced as by Prussian blu staining
- Serum iron is decreased with increased TIBC
- Transferrin saturation is reduced and serum Ferritin is reduced
- Chronic diseases like chronic inflammatory disorders and connective tissue diseases causes the erythroid cells unable to utilize the available irons
- Cytokines released during inflammation block the re use of iron
- Blood film shows anisocytosis, microcytosis & sometimes hypochromia
- Bone marrow shows normocellular marrow with normoblastic erythroid hyperplasia, delay in haemoglobinisation, normal iron store, and normal myeloid and megakaryocyte maturation
- Thalassemia minor is an inherited defect in haemoglobulin synthesis where there is globin chain synthesis
- Diagnosis of thalassemia is by High Performance Liquid Chromatography (HPLC)
- Defect in haeme synthesizes and inability of developing red cells to incorporate iron into haeme molecule causes sideroblastic anemia
- Sideroblastic anemia is X linked inheritance usually seen in males.

TERMINAL QUESTIONS

1. Name the cause of iron deficiency anemia
2. Describe the laboratory diagnosis of iron deficiency anemia.
3. Give the normal values for the following
   (a) Serum iron .....
   (b) Total iron binding capacity .....
   (c) Serum ferritin .....
   (d) Transferrin saturation .....



4. Define the following terms
   (a) Microcytosis .....
   (b) Hypochromia .....
   (c) Sideroblasts .....
   (d) Thalassemia minor .....

ANSWERS TO INTEXT QUESTIONS

16.1

1. Smaller & Less
2. 1 mg
3. Ferretin & Haemosiderin
4. Apoferritin
5. 2 mg
6. Transferrin
7. Koilonychia
8. Pica

16.2

1. Iron
2. Tuberculosis, Chronic abcesses & Osteomyelitis
3. Rheumatoid arthritis & Systemic Lupus Erythematosus
4. Cytokines

16.3

1. Globin chain
2. High Performance Liquid Chromatography (HPLC)
3. Sideroblastic
4. Normal or decreased

Notes

17

MACROCYTIC ANEMIAS

17.1 INTRODUCTION

Macrocytic anemias are characterized by the presence of macrocytes (RBC >9µm in diameter) with normal haemoglobin content. Examples of Macrocytic anemias are Megaloblastic anemia due to deficiency of cobalamin or folic acid, Aplastic anemia, Anemia in liver disease and hypothyroidism, Anemia in myelodysplastic syndrome

OBJECTIVES

After reading this lesson, you will be able to:

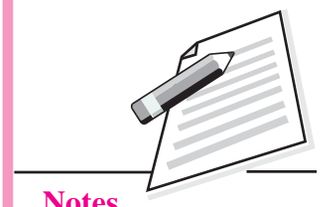
- define macrocytic anemias
- describe megaloblastic anemia
- explain laboratory diagnosis of megaloblastic anemias
- discuss the other causes of macrocytosis in brief

17.2 DEFINITION

Macrocytic anemias are characterized by the presence of macrocytes (RBC >9µm in diameter) with normal haemoglobin content. The MCV and MCH are increased, and MCHC is normal.

Examples

- Macrocytic anemia is further divided into megaloblastic and nonmegaloblastic anemia
- Examples of megaloblastic anemia are folic acid or vitamin B12 deficiency, inherited disorders of DNA synthesis and drug induced disorders of DNA synthesis.



- Nonmegaloblastic anemia can be due to hypothyroidism, liver disease, alcoholism and aplastic anemia

17.3 MEGALOBLASTIC ANEMIA

17.3.1 Definition

These are a group of disorders due to defective DNA synthesis and nuclear cytoplasmic asynchrony resulting in anemia with abnormally large red cells.

17.3.2 Cause of Megaloblastic Anemia

For normal DNA synthesis vitamin B_{12} or cobalamin and folic acid are essential. Megaloblastic anemia occurs due to the deficiency of either or both of these vitamins.

17.3.3 Pathogenesis

In megaloblastic anemia there is a defect in DNA synthesis, RNA synthesis remains unimpaired. There is impaired cell division due to which hemoglobin is synthesized in excess during the delay. As a result of this enlarged erythroid precursors are produced. These changes affect all proliferating cells.

17.4 VITAMIN B_{12} OR CYANOCOBALAMIN

17.4.1 Source

This vitamin is synthesized only by micro organisms and is stored in animal tissue especially the liver. A strict vegetarian diet is deficient in this vitamin.

17.4.2 Absorption

Vitamin B_{12} is called the EXTRINSIC FACTOR. It is present in food bound to proteins. The acidic pH of the gastric juices is needed to release it from protein. At this pH, it binds to the salivary R PROTEIN (haptocorrin). The parietal cells of the stomach mucosa secrete another protein called the INTRINSIC FACTOR (IF). The R PROTEIN- cobalamin complex and IF pass into the duodenum. In the duodenum, pancreatic secretions neutralize the pH and provide the enzymes that degrade R PROTEIN. The cobalamin binds to IF. The complex passes through the rest of the duodenum and jejunum. In the ileum there are receptors in the mucosa for IF. The cobalamin is absorbed by endocytosis and it then binds to a transport protein called TRANSCOBALAMIN II. (TCII)

17.4.3 Transport in plasma

The cobalamin is transported in plasma by the TC II – cobalamin complex.

17.4.4 Entry into cells

Cells have receptors for TC II. The TCII binds to the receptor and the cobalamin is internalized.

17.4.5 Enterohepatic Circulation of Cobalamin

0.5 - 9 µg of cobalamin bound to TC II is taken up by hepatocytes which have receptors for TCII and the cobalamin is secreted into bile. Most of this cobalamin is reabsorbed from the intestinal tract.

17.4.6 Excretion

The daily excretion of cobalamin is ~1 µg.

17.4.7 Requirements

The daily intake of cobalamin is 2 – 5 µg/day. The normal serum concentration is 180 – 640 ng/L

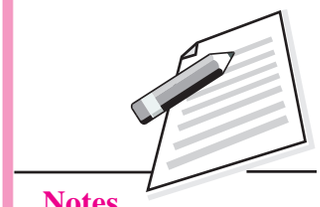
17.4.8 Causes of Vitamin B_{12} Deficiency

1. Dietary insufficiency. The pure vegetarian Indian diet contains very little cobalamin. Deficiency is also seen in old and bed ridden patients.
2. Increased requirements – pregnancy, lactation, growing children, hemolytic anemia.
3. Inability to absorb the vitamin – following gastric surgery, lack of hydrochloric acid in gastric juice, drugs suppressing acid production, lack of intrinsic factor due to auto antibodies to parietal cells or intrinsic factor, ileal resection, chronic diarrhea.
4. Competitive absorption of the vitamin by fish tape worm and bacteria in blind loop syndrome.

17.5 FOLIC ACID OR PTEROYL MONOGLUTAMIC ACID

17.5.1 Sources of Folic Acid

This vitamin if present in animal sources like milk, eggs, liver and in vegetable sources like fresh green vegetables, fruits and yeast. It is destroyed by prolonged cooking.



17.5.2 Absorption

Polyglutamates are broken down to monoglutamates and folic acid is absorbed in the small intestine, mainly the jejunum with the help of folate binding proteins.

17.5.3 Transport

There is no specific serum transport protein. It is bound loosely to serum albumin.

17.5.4 Entry into Cells

Cells have receptors for folate binding proteins. The folic acid is transported into the cell in vesicles.

17.5.5 Enterohepatic Circulation of Folic Acid

There is an enterohepatic circulation of folic acid similar to cobalamin.

17.5.6 Excretion

Approximately 200 µg of folate is excreted focally every day.

17.5.7 Requirements

The recommended dietary folate equivalent intake is 400 µg/day for adults, 600 µg/day in pregnant women and 500 µg/day in lactating women.

The normal serum folate is 3 - 20 µg/L

The normal red cell folate is 160 - 640 µg/L.

Dietary folate which enters the blood stream is rapidly cleared to the tissues. The serum folate is labile and is sensitive to short term changes in folic acid levels. In these situations it is better to assay the red cell folate level which will give a better assessment of tissue folate stores.

17.5.8 Causes of Folic Acid Deficiency

1. Dietary insufficiency: Deficiency is seen in old and bed ridden patients, ICU patients and by eating over cooked food.
2. Increased requirements – pregnancy, lactation, growing children, hemolytic anemia
3. Liver disease and chronic alcoholism
4. Inability to absorb the vitamin – following gastric surgery, sprue, chronic diarrhea, malabsorption.
5. Drugs – several chemotherapy drugs which are folic acid antagonists.

17.6 CLINICAL FEATURES OF MEGALOBLASTIC ANEMIA

There is moderate to severe anemia, pallor, breathlessness, mild jaundice, premature graying of hair, bald tongue, glossitis, angular stomatitis, and pigmentation of knuckles. Patient complains of tingling and numbness of hands and feet and poor memory. In severe megaloblastic anemia neurological symptoms may appear. Folic acid deficiency in early pregnancy leads to defective development of the nervous system resulting in congenital defects in the baby called neural tube defects.

17.7 LABORATORY DIAGNOSIS OF MEGALOBLASTIC ANEMIA

The features of megaloblastic anemia in both cobalamin and folate deficiency are similar and the two can only be differentiated by assay of the vitamins in blood.

17.7.1 Complete Blood Count

1. Hemoglobin, PCV and RBC count are all decreased. There is a greater reduction of RBC count than hemoglobin or PCV.
2. MCV and MCH are increased, MCHC is normal. RDW is markedly increased.
3. Peripheral blood smear (figure 3) shows moderate to severe anemia with marked anisocytosis, poikilocytosis and macrocytosis (RBC are >9μm in diameter). The red cells are normochromic. Nucleated RBC with megaloblastic change, Howell Jolly bodies, Cabot rings and basophilic stippling are seen. Neutrophils appear larger than normal (macropolycytes) and nuclei have 5 or more lobes (hypersegmentation). There may be variable thrombocytopenia.
4. WBC may be normal in number or may show leucopenia.
5. Platelet count may be normal or decreased.
6. Reticulocyte count is decreased.

17.7.2 Bone Marrow

Bone marrow is hypercellular and shows erythroid hyperplasia. The red cell precursors are large with nuclei showing opened up chromatin which appears like a fine lattice or sieve like. The cytoplasm of the late erythroid precursors appears mature but the nucleus appears immature. This is called **Megaloblastic Maturation**. The myeloid precursors show giant metamyelocytes and band

forms. Megakaryocytes are normal or decreased in number. The nuclei however show hyperlobulation and fragmentation.

17.7.3 Biochemical Parameters

(a) Serum folic acid and red cell folate levels are decreased.

(b) Serum cobalamin levels are decreased.

(c) Two metabolites of the metabolic pathway of these vitamins namely serum homocysteine and methyl malonic acid levels may be increased.

(d) Schilling test: To confirm that the malabsorption of cobalamin is secondary to lack of intrinsic factor.

17.8 TREATMENT

The treatment consists of replacing the deficient vitamin and dietary advice. Response to treatment is seen as an increase in the reticulocyte count which starts about the second or third day. Increase in hemoglobin is seen by the end of a week of treatment. Treatment should be continued for at least six months to build up the stores in the body.

Anemia in Chronic Liver Disease

In chronic liver disease there is anemia often associated with a mild degree of macrocytosis and raised MCV (usually between 100 to 110 fl). Other features that are present in the blood film are target cells and acanthocytes. These abnormally shaped red cells occur due to the deranged lipids in the RBC membrane. In liver disease due to chronic alcoholism there may be true megaloblastic anemia secondary to folic acid deficiency.

INTEXT QUESTIONS 17.1

1. Macrocytic anemias are characterized by ..... with normal ..... content
2. Megaloblastic anemia occurs because of deficiency of ..... and .....
3. Vitamin B12 is also called as .....
4. Minimum daily intake of folic acid is ...../day
5. Folic acid deficiency in pregnancy causes ..... in newborn baby

Notes

17.9 MACROCYTIC ANEMIA IN MYELODYSPLASTIC SYNDROME (MDS)

MDS is a preleukemic condition where the normal hematopoiesis in bone marrow is disturbed leading to morphologic and proliferative changes in the precursor cells. The ineffective hematopoesis affects all three cell lines. The red cells are macrocytic and have a raised MCV. It is important to differentiate between MDS and megaloblastic anemia because MDS may progresses into acute leukemia.

17.10 APLASTIC ANEMIA

17.10.1 Definition

Aplastic anemia is a condition where there is a failure of the bone marrow to produce red cells, white cells and platelets resulting in a PANCYTOPENIA.

17.10.2 Etiology

The bone marrow failure may be:

(a) Congenital or inherited – called Fanconi anemia

(b) Secondary to drugs, toxins, chemical, radiation exposure, or post certain viral infections

(c) Idiopathic where the cause is unknown.

17.10.3 Clinical Features

The patient presents with symptoms of anemia (tiredness, breathlessness, pallor) because marrow is not producing red cells. He/she may also have repeated and severe infections because normal WBC production is impaired. The patient may also have bleeding symptoms because of thrombocytopenia.

17.10.4 Laboratory Diagnosis

17.10.4.1 Complete Blood Counts

1. Hemoglobin, PCV, RBC count all reduced
2. MCV normal or increased, MCH normal, MCHC normal or mildly increased, RDW normal
3. WBC count show leukopenia with neutropenia.
4. Platelet count is decreased

5. Reticulocyte count is markedly decreased
6. Peripheral blood smear shows pancytopenia. The red cells are few in number, normochromic normocytic or macrocytic. There is no polychromasia. nRBC are not seen. There is leukopenia with neutropenia. The majority of WBC seen are mature lymphocytes. The platelets are reduced in number but have normal morphology.

17.10.4.2 Bone Marrow Examination

Both bone marrow aspirate and trephine biopsy should be done because it is easier to assess bone marrow cellularity in the biopsy. The bone marrow is markedly hypocellular (<25% for age). The particles are made up of fat and underlying supporting fibrous tissue. The aspirate smears are poorly cellular and show marked decrease in erythroid and myeloid precursors and absent or occasional megakaryocytes. Most of the nucleated cells seen are lymphocytes and plasma cells. Tissue basophils and mast cells are increased. Iron stain shows adequate iron stores.

17.10.4.3 Treatment

Supportive treatment with blood transfusions, antibiotics and platelet transfusion is given Definitive treatment is immunosuppression or bone marrow transplantation.

Macrocytosis is also seen in neonatal blood, hypothyroidism and when there is polychromasia

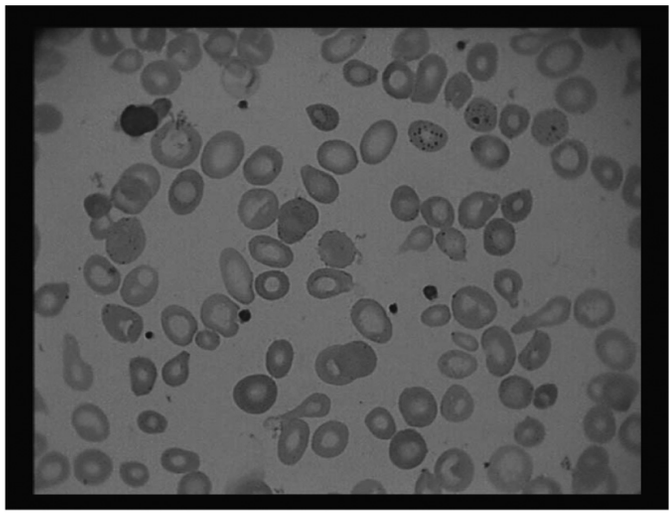


Fig. 17.1: Megaloblastic anemia

INTEXT QUESTIONS 17.2

Match the following

1. Vitamin B12	(a) Neural tube defects
2. Parietal cells	(b) RBC diameter > 9 μm
3. Folic acid deficiency	(c) Extrinsic Factor
4. Macrocytes	(d) Pancytopenia
5. Aplastic anemia	(e) Intrinsic Factor

WHAT HAVE YOU LEARNT

- Macrocytic anemias are characterized by presence of microcytes (RBC >9 μm in diameter) with normal haemoglobin content
- In macrocytic anemia, MCV and MCH is increased and MCHC is normal
- Megaloblastic anemia occurs due to deficiency of vitamin B12 or cobalamin and folic acid
- Vitamin B12 is synthesized only by microorganisms and a strict vegetarian diet is deficient in this vitamin
- Vitamin B12 is called Extrinsic factor and Parietal cells is called Intrinsic factor
- Cobalamin is transported in plasma by TC II – cobalamin complex
- Dietary insufficiency because of pure vegetarian diet, lactation & inability to absorb vitamin following gastric surgery, lack of hydrochloric acid caused vitamin B12 deficiency
- Folic acid is absorbed in small intestine mainly the jejunum
- Bone marrow in folic acid deficiency is hypercellular with erythroid hyperplasic
- The cytoplasm of the late erythroid precusors appears mature but nucleus appear immature and is called Megaloblastic maturation and Myeloid precursors show giant metamyelocytes and band forms
- Serum folic acid, red cell folate and Serum cobalamin levels are decreased
- Aplastic anemia results in pancytopenia
- In bone marrow examination, the smears are hypocellular and show marked decrease in erythroid and myeloid precursors and absent megakaryocytes.

TERMINAL QUESTIONS

1. Define megaloblastic anemia
2. Describe the blood and bone marrow findings in megaloblastic anemia.
3. Give the normal values for the following:-
   (a) serum cobalamin .....
   (b) serum folate .....
   (c) red cell folate .....
   (d) MCV .....
4. Name 4 conditions where red cells are macrocytic.
   (a) .....
   (b) .....
   (c) .....
   (d) .....

ANSWERS TO INTEXT QUESTIONS

17.1

1. Macrocytes, haemoglobin
2. Vitamin B 12 & Folic acid
3. Extrinsic factor
4. 50 µg
5. Neural tube defects

17.2

1. (c)
2. (e)
3. (a)
4. (b)
5. (d)

Notes

18

HEMOLYTIC ANEMIA

18.1 INTRODUCTION

Hemolytic anaemias are anaemias that result from increased destruction of red cells or due to a shortened life span of red cells

OBJECTIVES

After reading this lesson, you will be able to:

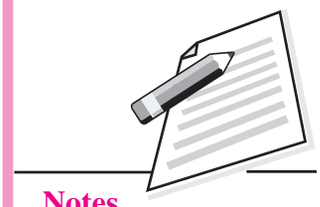
- define hemolytic anaemia, extravascular hemolysis & Intravascular hemolysis
- describe the haematological findings in hemolysis
- explain the biochemical findings in hemolysis
- classify hemolytic anaemia.

18.2 DEFINITION

Hemolytic anemia results from increased destruction of red cells or due to a shortened life span of red cells. They are characterized by increased red cell destruction together with compensatory regeneration.

18.3 PATHOGENESIS

The normal life span of the red cell is about 120 days. When there is hemolysis the bone marrow compensates by increasing erythropoiesis by 6 to 8 times the normal number of RBC produced. Anemia results when the bone marrow is no longer able to compensate the degree of hemolysis.



18.4 CLASSIFICATION OF HEMOLYTIC ANEMIA

Based on the site of red cell destruction, hemolytic anemia is classified as:

(a) Intravascular: when the destruction is predominantly within the circulation

(b) Extravascular: when the destruction is predominantly within tissue macrophages. The macrophages in the spleen, liver bone marrow and lymph nodes phagocytose and destroy red cells.

Hemolytic anemia can also be classified as due to intrinsic (defect in the red cell) or extrinsic (red cell is normal, anemia is secondary to exposure to drugs, infection or other factors) abnormalities.

18.4.1 Defects inside the RBC (Intracorpuscular defects)

These conditions are usually hereditary except paroxysmal nocturnal hemoglobinuria (PNH) which is an acquired condition.

(a) Red cell membrane defects - eg. Hereditary spherocytosis, elliptocytosis

(b) Abnormalities of hemoglobin synthesis

Decreased globin synthesis – quantitative defect eg. Thalassemias

Abnormal globin synthesis – qualitative defect eg. Sickle cell anemia

(c) Abnormalities of red cell enzymes – eg. G6PD deficiency

18.4.2 Defects outside the RBC (Extracorpuscular defects)

The red cells are normal but the life span is shortened because of external factors.

(a) Immune hemolytic anemia- alloimmune, autoimmune, drug induced

(b) Parasites – eg. Malaria

(c) Bacterial – eg. Clostridia

(d) Venoms – eg. Snake venoms

(e) Red cell fragmentation seen in disseminated intravascular coagulation, hemolytic uremic syndrome, march hemoglobinuria, prosthetic heart valves etc.

(f) Drug induced

(g) Burns

18.5 BIOCHEMICAL CHANGES DURING HAEMOLYSIS

When the red cells are destroyed, the hemoglobin is broken down to globin and heme.

(a) Globin chains are broken down to amino acids which are re-used for protein synthesis.

(b) The heme ring is opened up and the iron is released, bound to transferrin, recirculated and used for new RBC formation.

(c) The protoporphyrin ring is opened up and during this process carbon monoxide is released and excreted through expired air.

(d) Biliverdin is formed from the protoporphyrin**.** Biliverdin is converted to un-conjugated (indirect) free bilirubin by biliverdin reductase**.** The unconjugated bilirubin is bound loosely to serum albumin and is conjugated to bilirubin glucuronide (direct or conjugated bilirubin) in the liver.

(e) The conjugated bilirubin is excreted in bile giving it a yellow green colour

(f) Most of the bile secreted in the small intestine is reabsorbed and transported back to the liver. This is called the enterohepatic circulation of bilirubin.

(g) In the intestine the remaining bilirubin is converted to stercobilinogen

(h) Some of the stercobilinogen is reabsorbed and excreted as urobilinogen in urine.

Thus in hemolytic anemia, the serum total bilirubin is mildly increased, most of it is indirect bilirubin, urine urobilinogen and fecal stercobilinogen are increased and there is increased storage iron.

When red cells get destroyed in the blood stream releasing hemoglobin into plasma it is called **intravascular hemolysis**. The hemoglobin binds to a plasma protein called haptoglobin and the Hb-Haptoglobin complex is taken up by tissue macrophages. The hemoglobin is then degraded to biliverdin. When all the haptoglobin is saturated the free hemoglobin is increased resulting in **hemoglobinemia** and is excreted by the kidney as **hemoglobinuria**. Some of this hemoglobin in the glomerular filtrate is reabsorbed by the tubular epithelium and stored as hemosiderin. Demonstration of hemosiderin in urine **(hemosiderinuria)** indicates recent intravascular hemolysis.

INTEXT QUESTIONS 18.1

1. Haemolytic anaemias occurs because of ..... or ..... of red cells
2. Normal life span of red cells is .....

3. Normal mechanism of red cell destruction is .....
4. During hemolysis haemoglobin is broken down as ..... & .....
5. Bilirubin is excreted as ..... in urine
6. Match the following

1. Red cell membrane defect	(a) Thalassemias
2. Abnormal globin synthesis	(b) G6PD deficiency
3. Decreased globin synthesis	(c) Hereditary Sperocytosis
4. Abnormal red cell enzymes	(d) Sickle cell anaemia

18.6 GENERAL LABORATORY FEATURES OF HEMOLYTIC ANEMIA

A. Hemoglobin, PCV and RBC count are reduced.

B. Peripheral blood smear shows variable anemia with anisocytosis, poikilocytosis, polychromasia, fragmented red cells, nucleated RBC and basophilic stippling. These findings are seen in most of the hemolytic anemias. Depending on the underlying cause of hemolysis the more specific findings like sickle cells, spherocytes or target cells may be present.

C. Reticulocyte count is increased.

D. Bone marrow examination has limited role in the diagnosis of hemolytic anemia. The bone marrow shows cellular marrow particles and markedly hypercellular smears. There is marked erythroid hyperplasia with normoblastic maturation resulting in reduction of myeloid to erythroid ratio. Myeloid maturation and thrombopoiesis are normal.

E. Biochemical changes are as described above. Serum LDH is elevated.

F. Red cell life span is decreased.

G. Special investigations have to be done to confirm the diagnosis. These investigations will depend on the underlying cause of hemolysis.

18.7 HEREDITARY SPHEROCYTOSIS

18.7.1 Definition

This is an anemia characterized by the presence of many small red cells or spherocytes in the blood. The abnormal shape of the red cells is due to the lack of one or more of the cytoskeletal proteins (mainly spectrin and ankyrin) of the red cell membrane. The abnormal shape results in lack of flexibility of the cell membrane while passing through small capillaries. The red cells are trapped in the microcirculation and have a decreased life span.

18.7.2 Etiology

This red cell membrane defect is usually an inherited disorder. It is inherited as an autosomal dominant trait and affects both sexes equally.

18.7.3 Clinical Presentation

Young males and females present with history of repeated attacks on mild jaundice, fever, gall stones, leg ulcers and splenomegaly. Rarely when the defect involves more than one protein, there may be neonatal hyperbilirubinemia.

18.7.4 Laboratory Diagnosis

1. Hemoglobin, PCV, RBC count are mildly decreased
2. MCV and MCH are normal, MCHC is increased
3. Peripheral blood smear (figure 4) characteristically shows spherocytes which are well hemoglobinized red cells with a low mean cell diameter. Spherocytes lack the central pallor which is encountered in normal red cells. The number of spherocytes per high power field varies from patient to patient. Poikilocytes and polychromatophils may be seen. Spherocytosis can also be seen in autoimmune hemolytic anemia, ABO hemolytic disease of the newborn and bacterial toxins like Clostridium.
4. WBC and platelet counts are normal
5. **Osmotic fragility test**

   This test demonstrates that the surface area to volume ratio is reduced in the presence of spherocytosis. Normal red cells can withstand hypotonicity and can increase in size by about 70% (because of the biconcave shape) before their membrane is stretched. Spherocytes on the other hand when placed in progressively more hypotonic solutions, because of their round shape, are unable to swell further as water enters the cells and they rupture sooner than normal red cells. Thus spherocytes, whatever the reason for their formation will show increased osmotic fragility. Osmotic fragility is also increased in hereditary elliptocytosis and hereditary stomatocytosis. Iron deficient microcytic hypochromic red cells and thalassemic red cells show decreased osmotic fragility.
6. Direct Coomb's test must be performed to rule immune cause for the formation of spherocytes.
7. Biochemical tests show indirect bilirubinemia, increased excretion of urobilinogen and stercobilinogen and increased serum LDH.

8. Analysis of cytoskeletal proteins to demonstrate the protein responsible may be done by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)
9. Flow cytometric (dye binding) test

18.8 HEREDITARY ELLIPTOCYTOSIS

Hereditary elliptocytosis syndrome are a group of genetic disorders which are characterized by elliptocytes on peripheral blood smear (figure 5). While most are well compensated hemolytic anemias which are accidently identified during routine peripheral blood examination, some present with moderate to severe hemolytic anemia.

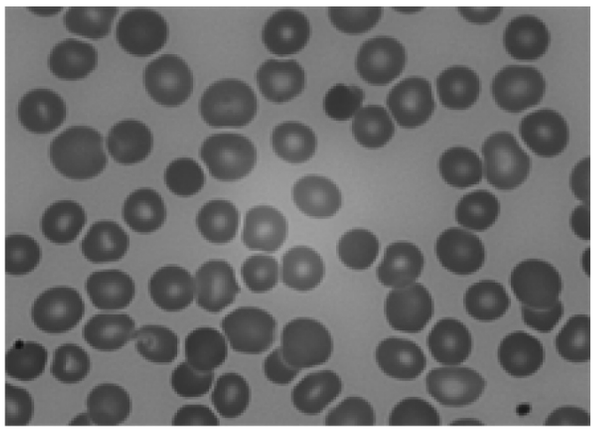


Fig. 18.1: Spherocytes

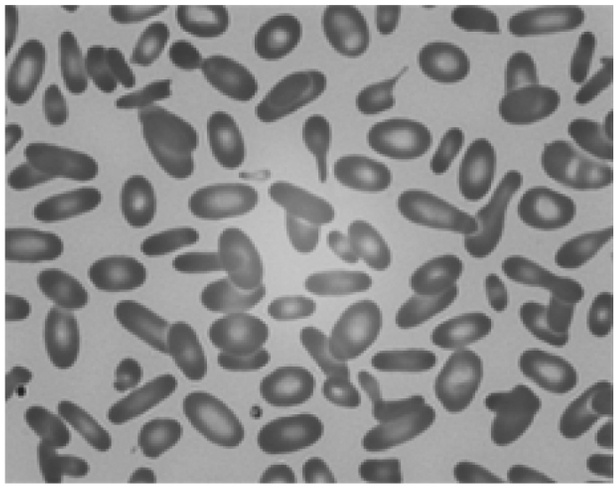


Fig. 18.2: Elliptocytosis

INTEXT QUESTIONS 18.2

1. Hereditary spherocytosis is characterized by presence of ..... in the blood
2. Hereditary sperocytosis is inherited as ..... trait
3. Autosomal dominant inherited disorder of red cell is .....
4. Absence of red cell membrane protein causes .....

18.9 PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

It is an acquired non-malignant clonal disorder where there is expansion of one or more hematopoietic stem cells. It occurs due to mutation in the PIGA gene which leads to production of stem cells which are deficient in glycosylphosphatidylinositol-anchored proteins (GPI-AP). These include complement defense proteins like CD55 (DAF) and CD59; RBC membrane proteins like acetyl choline esterase, etc.

PNH red cells are abnormally sensitive to hemolysis in the presence of complement and a low pH. Hemolysis may vary from mild to massive requiring transfusions and the patient may develop acute tubular necrosis of the kidneys. Classically, the patients have episodic nocturnal intravascular hemolysis and hemoglobinuria. In other patients there may be low grade intravascular hemolysis associated with infection or stress. Associated thrombocytopenia, leucopenia and thrombosis at unusual sites are frequently noted. PNH is often associated with aplastic anemia

Laboratory Diagnosis

1. Mild to severe degree of anemia may be seen.
2. Leucopenia and thrombocytopenia is frequent
3. Reticulocyte count is increased
4. Urine is positive for hemoglobinuria and hemosiderinuria.
5. Biochemical changes seen in other causes of intravascular hemolysis are present.
6. Ham (acidified serum lysis) test: Patient's red cells show abnormal tendency to hemolysis in the presence of a mild acid pH and fresh complement.
7. Direct Coombs test is negative.
8. Flow cytotometry analysis of GPI linked proteins.

WHAT HAVE YOU LEARNT

- Haemolytic anaemia result from increased destruction of red cells or due to shortened life span of red cells
- Normal life span of red cell is 120 days
- Normal mechanism of red cell destruction is called Extravascular haemolysis
- When red cells are destroyed they are broken down to globin and haeme
- Bilirubin is excreted as urobilinogen in urine
- Red cells when destroyed in blood stream, releasing Hb into plasma is called Intravascular haemolysis
- Haemolytic anaemia is classified based on causes as Defects inside RBCs and defects outside RBC
- Lack of cytoskeletal proteins of red cell membrane causes abnormal shape of red cell causing Hereditary Spherocytosis and is characterized by presence of Spherocytes
- Hereditary Spherocytosis is inherited as autosomal dominant inherited disorder
- Hereditary Ellipatocytosis is an autosomal dominant inherited disorder
- Absence of two or more red cell membrane protein causes Hereditary Pyropoikilocytosis

TERMINAL QUESTIONS

Write short notes on

1. Spherocytosis
2. Osmotic fragility test
3. Biochemical findings in Hereditary spherocytosis
4. PNH

ANSWERS TO INTEXT QUESTIONS

18.1

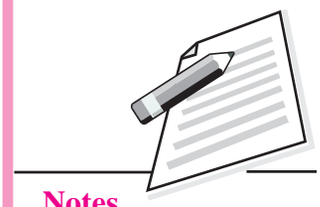
1. Increased destruction, shortened life span

Notes

2. 120 days
3. Extravascular hemolysis
4. Globin & Haeme
5. Urobilinogen
6. 1. (c)
   2. (d)
   3. (a)
   4. (b)

18.2

1. Spherocytes
2. Autosomal dominant
3. Hereditary Elliprocytosis
4. Hereditary Pyropoikilcytosis



19

HEMOLYTIC ANEMIA DUE TO ABNORMAL HEMOGLOBIN SYNTHESIS

19.1 INTRODUCTION

There are two main mechanisms by which anaemia is produced

(a) **Thalassemia:** A group of disorders characterized by quantitative defect in the synthesis of one or more globin chains. HbA or adult hemoglobin constitutes the maximum amount of red cell hemoglobin and is composed of two alpha and two beta chains.

When alpha (α) globin chains are not produced in normal numbers the condition is alpha (α) thalassemia.

When beta (β) globin chains are not produced in normal numbers the condition is beta (β) thalassemia

(b) **Hemoglobinopathies:** In these conditions there are structural abnormalities in the globin chains leading to qualitative defects. The globin chain is abnormal in structure due to one or more amino acid substitution or deletions or additions. The globin chain so formed is functionally abnormal and the red cell life span is shortened

OBJECTIVES

After reading this lesson, you will be able to:

- describe pathogenesis, lab diagnosis of thalassemia major and minor
- describe pathogenesis of haemoglobinopathies.

19.2 β THALASSEMIA

19.2.1 Inheritance and Pathogenesis

This is an inherited autosomal recessive disorder where there is a decrease in the number of beta globin chains being produced. In the heterozygous condition where the patient has one normal gene and the other thalassemia gene, the globin chain imbalance is compensated by an increase in delta chain production and an increase in HbA2. Thus patients who have one beta thalassemia gene are referred to as beta thalassemia trait and have a mild well compensated microcytic hypochromic anemia. This condition is called beta thalassemia minor.

When a person inherits one beta thalassemia gene from each parent he/she has homozygous beta thalassemia and is unable to produce normal HbA. The chain imbalance is so great as to produce moderate to severe anemia. This condition is called beta thalassemia Major or Cooley's anemia. The patient develops a life long transfusion dependent anemia.

19.2.2 Incidence in India

The thalassemia gene is present in the Indian population. It is more common in certain communities like Punjabis, Gujratis, Marwaris and Sindhis.

19.3 β THALASSEMIA MAJOR

19.3.1 Clinical Presentation

The patient usually presents by the age of 5 – 6 months after birth with pallor, frequent attacks of infections and hepatosplenomegaly. The bone marrow tries to compensate for the anemia by increased production of red cells. However these red cells are defective and undergo lysis – a term used for this situation is "ineffective erythropoiesis". The marrow cavity enlarges, the cortex of the bone thins and many skeletal abnormalities result due to this. Iron overload results in deposition and damage of organs like heart, liver and pancreas. The patient ultimately dies from frequent infections, resultant anemia and iron overload from repeated transfusions.

19.3.2 Laboratory Diagnosis

1. Hemoglobin, PCV, RBC count all decreased. Hb may be 3.0 – 4.0 g/dL
2. MCV, MCH and MCHC decreased. RDW is markedly increased.
3. WBC – Leukocytosis may be present. Many nucleated RBC are present, falsely elevating the WBC count.

4. Platelet count is normal. May be decreased if there is pooling in the spleen.
5. Peripheral blood smear (Fig. 19.1) shows moderate to severe anemia with marked anisocytosis, poikilocytosis, microcytosis, fragmented red cells and polychromasia. Many nRBC, red cell inclusions and basophilic stippling and target cells are also seen.

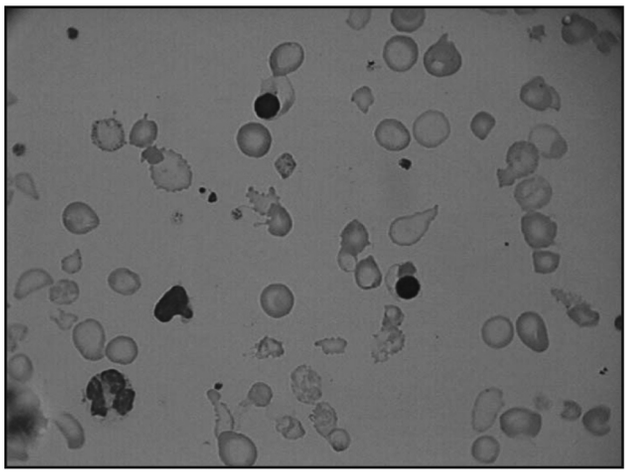


Fig. 19.1: Beta Thalassemia Major

6. Reticulocyte count is increased.
7. Biochemical tests show mild indirect bilirubinemia, increased excretion of urobilinogen and stercobilinogen, increased serum LDH and serum ferritin.
8. Hemoglobin electrophoresis using agarose gel at a pH of 8.6 or citrate agar electrophoresis at pH6.0 can be used to see the electrophoretic pattern of the hemoglobin and detects the common clinically significant hemoglobin variants. Isoelectric focusing is an alternative method.
9. The HbF may be quantified by a test called the Alkali Denaturation test
10. Hemoglobin analysis using High Performance Liquid Chromatography (HPLC) has largely replaced the more time consuming techniques described above. The various hemoglobins may be quantified using the HPLC method. The patient's hemoglobin in beta thalassemia major is mainly HbF with normal amounts of HbA2. In severe β^0 thalassemia there will be no detectable HbA, in β^+ thalassemia small amounts of HbA may be present.
11. Globin chain gene analysis can be done to identify various DNA mutations.
12. Detailed family studies, DNA analysis and screening of all blood relatives of the index case should be undertaken to identify carriers.
13. Genetic counseling should be given to prevent the birth of children with thalassemia.

19.3.3 Treatment

1. Blood transfusions to maintain Hb 10.5 to 11.0g/dL
2. Chelation of iron
3. Bone marrow transplantation

19.4 β THALASSEMIA MINOR

19.4.1 Definition

This is an inherited defect in haemoglobin synthesis where there is a decrease in globin chain synthesis. When the patient inherits two thalassemia genes, one from each parent, he/she develops a severe form of anaemia called thalassemia major. A person who has one thalassemia gene has a mild hypochromic microcytic anaemia which must be differentiated from iron deficiency anaemia.

19.4.2 Laboratory Diagnosis

(a) Haemoglobin, PCV, mildly decreased. **RBC count is increased.**

(b) MCV, MCH are decreased but MCHC is normal. RDW is mildly increased ~14 – 15%

(c) Blood film shows mild anaemia, uniformly microcytic hypochromic red cells with no polychromasia. Target cells may be present. WBC and platelets are normal. Fig. 19.2

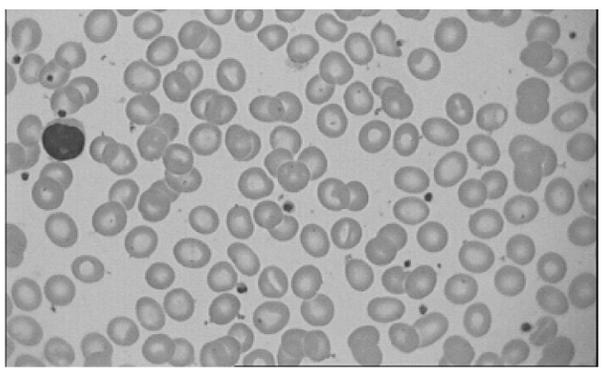


Fig. 19.2: Beta Thalassemia Minor

(d) Reticulocyte count is normal.

(e) Bone marrow is normal with normal iron stores

(f) Biochemical tests show normal serum iron, normal serum TIBC and ferritin

(g) The diagnosis is made by performing a haemoglobin analysis by HPLC (high performance liquid chromatography). Patients with beta thalassemia trait show the presence of raised HbA_2 in the range of 3.7- 7.0% with 80′ – 85% HbA and < 1.0%HbF

IMPORTANT

In the Indian population where the incidence of the beta thalassemia gene is 3- 15% depending on the region, it is important to detect the thalassemia carrier and offer genetic counseling and screening of the spouse to prevent the birth of children with beta thalassemia major.

19.5 HAEMOGLOBINOPATHIES

19.5.1 Definition

In these conditions there are structural abnormalities in the globin chains leading to qualitative defects in the hemoglobin. The globin chain is abnormal in structure due to one or more amino acid substitution, deletions or additions. The globin chain so formed is functionally abnormal and the red cell life span is shortened. There are numerous hemo-globinopathies described but most of them do not produce clinical disease.

19.5.2 Important Haemoglobinopathies

19.5.2.1 Sickle Haemoglobin

Sickle cell hemoglobin (HbS) is caused by a single amino acid substitution in the β globin chain. The sixth amino acid glutamic acid is replaced by valine (HbS $\alpha 2\beta 2^{6glu\text{-}val}$). The abnormal hemoglobin so formed tends to polarize under decreased oxygen tension and deforms the red cells into sickle shaped cells. These cells do not flow freely in the microcirculation. The result is stasis, hypoxic damage to distal tissues and hemolysis.

19.5.2.2 Geographic Distribution

Sickle cell anemia is found in Central and West Africa, the Middle East and along the old slave routes. In India HbS is found in the tribal belts around central India, Maharashtra, Madhya Pradesh, Bihar, Orissa, West Bengal, along the Western Ghats, Tamil Nadu and Kerala.

19.5.2.3 Inheritance

The condition is inherited as an autosomal recessive trait. When a child inherits two HbS genes, one from each parent he/she develops HbS anemia. When a child

inherits one HbS gene from either parent, he/she is called a HbS trait and is asymptomatic. A child may also inherit the HbS gene from one parent and another β globin gene abnormality from the other parent – the so called double heterozygous state eg HbS/bthal or HbS/HbD or HbS/ Hb C etc.

19.5.2.4 Clinical Presentation

The patient with HbS anemia (HbSS) suffers from jaundice, anemia, splenomegaly and frequent infections. Acute events like vaso-occlusive events, bone pains and CNS effects are common. Sequestration of irreversibly sickled cells in lungs, liver, spleen etc can lead to death at a young age.

19.5.2.5 Laboratory Diagnosis

1. Hemoglobin, PCV, RBC count are decreased.
2. MCV, MCH may be normal or decreased, MCHC is normal, RDW is increased.
3 Reticulocyte count is increased.
4. WBC count and platelets are usually increased.
5. Peripheral blood smear (Fig. 19.3) show variable anemia with anisocytosis, poikilocytosis, sickle shaped cells, target cells, polychromasia, nRBC and basophilic stippling. Howell Jolly bodies may be numerous in post splenectomy smears.

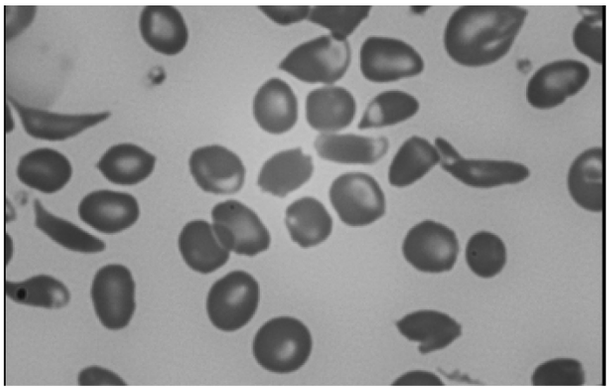


Fig. 19.3: Sickle cells

6. **Sickle cell preparation**

Principle: This is a simple test to demonstrate the decrease solubility and hence the ability of the cells containing HbS to form sickle cells in the absence of oxygen.

Procedure: Make a working solution of disodium hydrogen phosphate and sodium dithionite. Add 5 drops of this freshly prepared reagent to a drop of blood sample anticoagulated in EDTA on a clean glass slide. Place a cover slip over the diluted blood. Seal the edges of the coverslip with paraffin or nail polish to prevent air entry under the coverslip.

Result: Sickling takes place almost immediately in sickle cell anemia and within one hour in sickle cell trait (Fig. 19.4). It is observed at 40x with the condenser of the microscope lowered down.

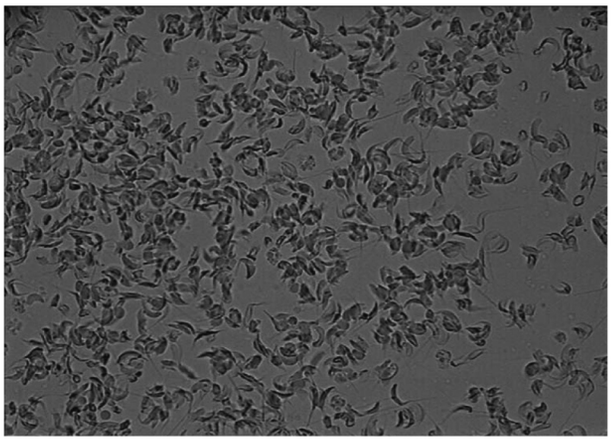


Fig. 19.4: Sickle cell preparation

7. **Solubility test for HbS**

Principle: Hb S is precipitated by high molarity phosphate buffer which increase the turbidity of the solution.

Procedure: The reagent contains KH_2PO_4, K2HPO_4, saponin and sodium dithionite in distilled water. Take 2mL of reagent in a test tube and add 10 µL of packed cells of the patient to it, mix and leave for 5 minutes at room temperature. The solution is now light pink or red in colour. Centrifuge at 1200 rpm for 5 minutes. Result: Observe the contents of the tube by holding the tube 2.5 cm in front of a white card with narrow black lines. The results are compared with appropriately put positive and negative controls.

(a) HbS/S – clear supernatant + curdy precipitate

(b) HbS/A – red supernatant + curdy precipitate

(c) HbA – clear solution, no precipitate

8. Hemoglobin electrophoresis at pH 8.6 on agarose gel or cellulose acetate (Fig. 19.5).

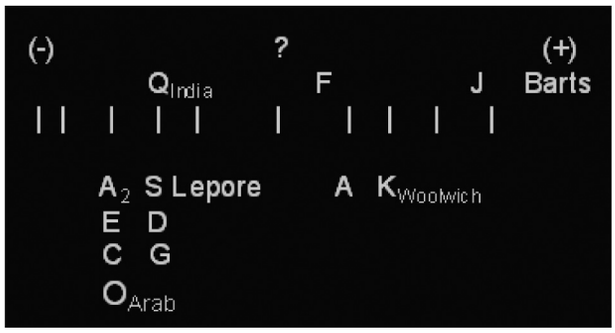


Fig. 19.5: Diagrammatic representation of separation of haemoglobins by electrophoresis

9. Hb S may be quantified using HPLC to separate the abnormal hemoglobin.
10. Biochemical tests show mild indirect bilirubinemia, increased excretion of urobilinogen and stercobilinogen and increased serum LDH.
11. Detailed family studies, DNA analysis and screening of all blood relatives of the index case should be undertaken to identify carriers.
12. Genetic counseling should be given to prevent the birth of children with sickle cell disease.

19.6 OTHER HAEMOGLOBINOPATHIES SEEN IN INDIA

19.6.1 Haemoglobin E

This abnormal hemoglobin is commonly seen in the North Eastern states of India. The abnormal hemoglobin is HbE $\alpha 2\beta 2^{26glu\text{-}lysine}$. It may present as homozygous HbE (HbE/E) or HbE trait (HbE/A) or HbE/β thal. The homogygous HbE and HbE trait are usually asymptomatic but HbE/β thal presents as a moderately severe hemolytic anemia.

19.6.2 Haemoglobin D

This hemoglobin is commonly seen in the Punjabi population. Hb D is $\alpha 2\beta 2^{26glu\text{-}lysine.}$ May present as homozygous HbD (HbD/D) or HbD trait (HbD/A) or HbD/β thal which is a moderately severe hemolytic anemia.

19.6.3 Haemoglobin C

This abnormal hemoglobin is found mainly in West Africa. HbC is $\alpha 2\beta 2^{26glu\text{-}lysine.}$ The heterozygotes are asymptomatic whereas the homozygous individuals develop a mid degree of hemolytic anemia. Significant numbers of target cells are seen in the peripheral blood smear (Fig. 19.6).

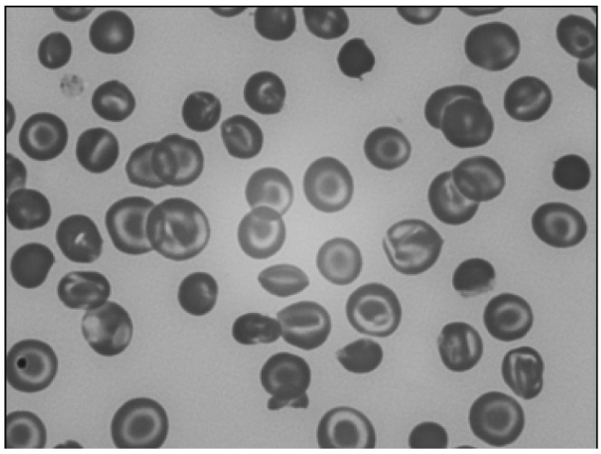


Fig. 19.6: Haemoglobin C

INTEXT QUESTIONS 19.1

1. β thalassemia is inherited as ..... disorder
2. Thalassemia minor causes ..... anaemia
3. Cooley's anaemia is also called as .....
4. Defective red cells undergoing lysis is described as .....
5. Single amino acid substitution in ß globin chain causes ..... anaemia
6. Sickle cell anaemia is inherited as ..... disorder

WHAT HAVE YOU LEARNT

- Decreased production of one or more normal globin chains causes thalassemia. Reduced Alpha (α) globin chains causes Alpha thalassemia and reduced beta (β) globin causes beta thalassemia
- Structural abnormalities in globin chain causes haemoglobinopathies
- β thalassemia is inherited as autosomal recessive disorder
- Heterozygous gene causes mild form of disorder called thalassemia minor
- Homozygous gene causes severe form of disorder called thalassemia major which is also known as cooley's anaemia
- Defective red cells undergoing lysis is described as ineffective erythropoiesis
- Haemoglobin electrophoresis using agarose gel shows mainly of HbF with normal amount of HbA2. In sever thalassemia there will be not detectable

Hb, in β+ thalassemia small amount of HbA may be present. No abnormal Hb are detectable

- HbF may be quantified by Alkali Denaturation test
- Structural abnormalities by substitution or deletion or addition of amino acid in globin chain leading to qualitiative defects
- Sickle Hb is caused by single amino acid substitution in β globin chain. The sixth amino acid glutamic acid is replaced by valine
- Solubility test is used for identifying sickle Hb.

TERMINAL QUESTIONS

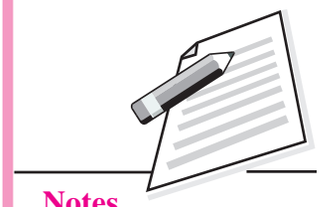
Write short notes on

1. Tests to demonstrate sickle haemoglobin
2. Thalassemia minor
3. Laboratory diagnosis of Thalassemia major

ANSWERS TO INTEXT QUESTIONS

19.1

1. Autosomal recessive
2. Hypochromic microcytic anaemia
3. Thalassemia major
4. Ineffective erythropoiesis
5. Sickle cell
6. Autosomal recessive trait



20

HEMOLYTIC ANEMIAS DUE TO ABNORMAL RED CELL ENZYMES

20.1 INTRODUCTION

The main metabolic substrate for the RBCs is glucose. It is metabolized by two pathways: approximately 90% of the glucose is metabolized through the Embden Meyerhoff (glycolytic) pathway and the rest by the hexose monophosphate (HMP) pathway.

In the Embden Meyerhoff (glycolytic) pathway glucose is metabolized to lactate through a series of enzymatic steps. Each molecule of glucose gives rise to 2 molecules of ATP. The ATP provides energy to maintain red cell volume, shape and flexibility. An ATP dependent pump in the red cell membrane actively keeps sodium out of the cell and potassium inside. The red cell has the enzymes that are needed for the glycolytic pathway. These enzymes help break down glucose to generate ATP which is the source of energy.

About 10% of the glucose is diverted to the Hexose Monophosphate shunt pathway and this is essential for protection of red cells from oxidative stress. This pathway is necessary for the generation of NADPH which then reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). GSH prevents the accumulation of H_2O_2 and the oxidation of hemoglobin to methemoglobin. When the level of GSH falls, H_2O_2 accumulates in the cell and oxidizes the hemoglobin to methemoglobin which becomes denatured and precipitates as Heinz bodies. These inclusions are rigid and attached to the red cell membrane and make the red cell susceptible to hemolysis. The NADPH required in this pathway is generated by the enzyme Glucose 6 phosphate dehydrogenase (G6PD).

Any of the enzymes in these pathways may be deficient or dysfunctional and result in hemolysis. The most common enzyme deficiencies are G6PD deficiency and pyruvate kinase deficiency.

OJECTIVES

After reading this lesson, you will be able to:

- describe G6PD deficiency disorder, its clinical presentation and Laboratory diagnosis
- describe extra-corpuscular causes of hemolysis
- explain haemolytic anemia due to red cell fragmentation

20.2 G6PD DEFICIENCY

Glucose 6 phosphate dehydrogenase (G6PD) is responsible for sending glucose 6 phosphate (G6PO4) into the HMP shunt and the generation of NADPH. Deficiency of G6PD enzyme results in red cells which are unable to degrade oxidant drugs and chemicals because the GSH is not being generated in these cells. On exposure to oxidant stress the deficient red cells form numerous Heinz bodies and undergo hemolysis. The disorder is a X linked recessive disorder**.** The patients with G6PD deficiency are further classified into variants (Class I to V) based on the magnitude of enzyme deficiency and severity of hemolysis.

20.2.1 Clinical Presentation

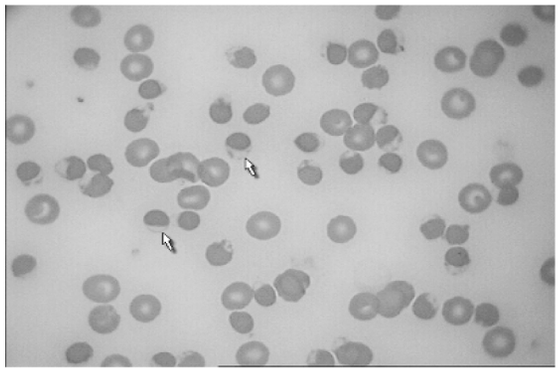
The clinical presentation depends on the variant of the G6PD deficiency. Most of the patients are asymptomatic under normal circumstances. However, these patients when exposed to oxidant stress (drugs, chemicals, infections, fever, fava beans) develop acute hemolytic anemia with passage of cola coloured urine, jaundice and in severe cases may develop renal shutdown.

20.2.2 Laboratory Diagnosis

1. Anemia develops secondary to the exposure of oxidative stress. The hematological features are those of hemolytic anemia and are not specific for G6PD deficiency.
2. Peripheral blood smear shows moderate to severe anemia with anisocytosis, poikilocytosis, polychromasia, irregularly contracted red cells, bite cells, spherocytes and nRBC. Bit cells or blister cells (Fig. 20.1) are red cells in

which the hemoglobin has been pushed to one side leaving an empty bleb or blisters.

3. Heinz body can be demonstrated by brilliant cresyl blue supravital staining.
4. Reticulocyte count is increased
5. Methemoglobin reduction test is a screening test for G6PD enzyme deficiency. It is based on the ability of the test red cells to generate NADPH and convert methemoglobin to hemoglobin. It must be remembered the reticulocytes contain trace amounts of the enzyme even in deficient cells and may give a false positive qualitative result during acute hemolysis
6. G6PD enzyme assays are available for quantifying the enzyme



Arrows indicate "blister cells"

Fig. 20.1: Oxidant haemolysis in G6PD deficiency

INTEXT QUESTIONS 20.1

1. Enzyme deficiencies like ..... & ..... cause haemolytic anemias
2. Cola coloured urine occurs in ..... deficiency
3. G6PD deficiency affects ..... & ..... are carriers
4. ..... test is used for quantifying the enzymes

20.3 EXTRACORPUSCULAR CAUSES OF HAEMOLYSIS

These anemias are caused by factors external to the red cell which is often an innocent by stander. The most important conditions are considered below

Notes

20.3.1 Immune Hemolytic Anemias

1. Pathogenesis

Immune hemolytic anemias are due to antibody production against the red cells. The antigen(s) are present on the red cell membrane and the antibody in the serum binds to the antigen to form an antigen – antibody complex on the cell surface. This complex is removed by macrophages in the liver and spleen (Extravascular hemolysis). During this process the red cell membrane is lost and microspherocyte form.

2. Types of Immune Hemolytic Anemia.

A. Alloimmune Hemolytic Anemia

This type of anemia occurs in two situations:

(a) Mismatched blood transfusion when blood of the wrong ABO group is transfused to a patient. The patient's serum contains naturally occurring IgM isoantibodies (anti A and/or anti B). The antibody binds to the transfused cells and produces intravascular hemolysis of the transfused red cells.

(b) Hemolytic disease of the new born which occurs during pregnancy when Rh negative mother has a Rh positive baby.

B. Autoimmune Hemolytic Anemia

In this condition the antibody is produced by the body against its own red cells. Based on the temperature at which these antibodies act, they can be classified as warm acting or cold active or a mixture.

Cold active antibodies donot attach to RBCs at room temperature, but their affinity increases as the temperature approaches 0°C. They are usually IgM type and fix complement. In contrast to this warm active antibodies act at 37°C, are IgG type and do not fix complement.

Autoantibodies may be idiopathic or secondary to other autoimmune disorders like systemic lupus erythematosus, lymphoproliferative disorders, infections like syphilis and drugs. Hemolysis in AIHA can be either intravascular or extravascular.

Drug Induced Immune Haemolytic Anemia

Drugs may cause an antibody mediated haemolytic anemia by different mechanisms. The anemia disappears when the drug is withdrawn.

3. Laboratory Diagnosis of Immune Hemolytic Anemias

A. Haemoglobin, PCV, RBC count is decreased.

B. In cold active AIHA, MCV may be artifactually elevated, producing a falsely elevate MCHC (due to red cell agglutination)

C. Reticulocyte count is elevated

D. Pheripheral blood smear shows variable anemia. Agglutination of red cells may be present when cold antibodies are present. Spherocytes are seen in warm type AIHA

E. Biochemical test show raised indirect bilirubin and LDH. Intravascular hemolysis presents with hemoglobinuria, hemosiderinuria and decreased serum haptoglobin

F. Serological tests: The diagnosis of AIHA is established by detection of antibodies and or complement on the surface of the patient's red cells. In Direct Coomb's test patient's red cells are mixed with sera containing antibodies. In Indirect Coomb's test, the patient's serum is tested against reagent red cells.

INTEXT QUESTIONS 20.2

1. Iso immune haemolytic anemia occurs because of .....
2. Alloimmune haemolytic anemia in new born due to .....
3. In autoimmune haemolysis anemia ..... test is positive
4. In alloimmune haemolytic anemia ..... test is positive

20.4 HAEMOLYTIC ANEMIA DUE TO RED CELL FRAGMENTATION

Occasionally an intravascular hemolytic process occurs when red cells are broken secondary to excessive physical trauma. This may occur inside blood vessels which are blocked because of narrowing or fibrin deposition. RBC may also be broken if there is mechanical obstruction like abnormal or synthetic heart valves. Turbulence in blood flow may also cause red cell fragmentation:

Causes of red cell fragmentation

A. Abnormalities of heart and large vessels

(a) Mechanical heart valves

(b) Renal artery stenosis

(c) Large hemangioma

B. Small vessel disease (microangiopathic hemolytic anemia)

(a) Hemolytic uremic syndrome (HUS)

(b) Thrombotic thrombocytopenic purpura (TTP)

C. Disseminated intravascular coagulation (DIC)

D. Lupus erythematosus

Laboratory Diagnosis

1. The blood findings depend on the degree of hemolysis. If the hemolysis is compensated, the Hb, red cell count and indices are near normal
2. Peripheral blood smear (Fig. 20.2) shows variable anemia, anisocytosis, poikilocytosis, fragmented red cells, polychromasia, nRBC and red cell inclusions.
3. Biochemical test show raised indirect bilirubin and LDH. Serum haptoglobin is reduced.
4. Urine may show hemoglobinuria or hemosiderinuria.

The term Microangiopathic Haemolytic Anemia may also be used for red cell fragmentation ocuring in association with small vessel disease.

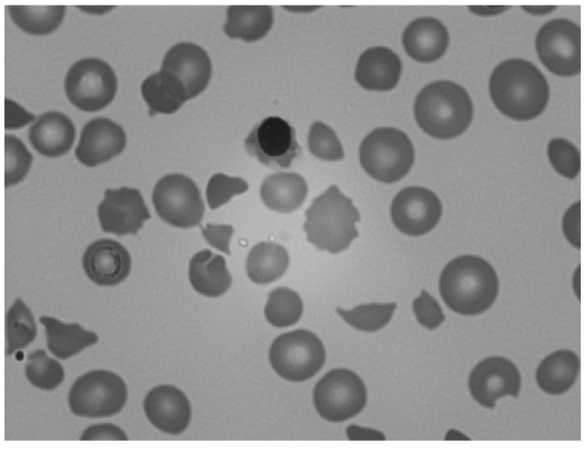


Fig. 20.2: Red cell Fragmentation in DIC

WHAT HAVE YOU LEANT

- Red cells has many enzymes that are needed for glycolytic pathway

- These enzymes help breakdown glucose to generate ATP which is the source of energy
- Any of the enzymes in the pathway may be deficient of dysfunctional and result in hemolysis
- Common enzyme deficiency are G6PD deficiency and Pyruvate kinase deficiency
- G6PD is responsible for sending G6PO4 into HMP shunt and generation of NADPH
- Deficiency of G6PD enzyme makes red cells unable to degrade oxidant drugs and chemicals as GSH is not generated in cells
- On exposure to oxidant stress, the red cells form Heinz bodies and undergo vascular hemolysis
- Immune hemolytic anemias are due to antibody production against red cells
- During this process red cell membrane is lost and destroyed by hemolysis
- Isoimmune hemolysis occurs because of mismatch blood transfusion causing intra vascular hemolysis
- Alloimmune hemolysis occurs as hemolytic disease of newborn due to anti D
- Autoimmune hemolytic anemia occurs because of idiopathic causes or because of other auto immune disorder like Systemic Lupus Erythemoatosus
- Hemolytic process occurs when red cells are broken inside small blood vessels because of narrowing or fibrin deposition

TERMINAL QUESTIONS

Write short notes on the following

1. Oxidant haemolysis in G6PD deficiency
2. Haemoglobinuria and haemosiderinuria
3. Autoimmune Hemolytic anemia
4. Microangiopathic hemolysis
5. Coomb's tests – Direct and Indirect
6. Types of Immune hemolytic anemia

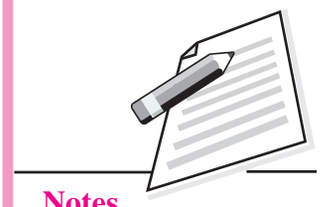
ANSWERS TO INTEXT QUESTIONS

20.1

1. G6PD & Pyruvate kinase
2. G6PD deficiency
3. Males & Females
4. G6PD enzyme assays

20.2

1. Mismatched blood transfusion
2. Anti D
3. Direct coomb's test
4. Indirect coomb's test



21

SCREENING FOR BLOOD TRANSFUSION TRANSMITTED DISEASES

21.1 INTRODUCTION

The microbial agents of importance to blood transfusion services are those that are transmissible by blood transfusion and can cause morbidity and mortality in recipients. Screening of all blood donations should be mandatory for infections namely HIV-1, HIV-2, HBSAG, HCV, Syphilis and Malaria.

OBJECTIVES

After reading this lesson, you will be able to

- explain about the importance of screening blood donations
- describe the infectious agents causing infections
- explain the laboratory diagnosis of transfusion transmitted infections
- perform the laboratory diagnosis of transfusion transmitted infections

21.2 SCREENING OF BLOOD DONATIONS

Screening of all blood donations should be mandatory for the following infections using following markers

- HIV-1 and HIV-2: screening for either a combination of HIV antigen-antibody or HIV antibodies.
- Hepatitis B: screening for hepatitis B surface antigen (HBsAg).

Notes

- Hepatitis C: screening for either a combination of HCV antigen antibody or HCV antibodies.
- Syphilis (Treponema pallidum): screening for specific treponemal antibodies.
- Screening of donations for other infections, such as those causing malaria, should be based on local epidemiological evidence

21.3 INFECTIOUS AGENTS

There are four main groups of micro-organisms known to cause infections namely viruses, bacteria, protozoa and fungi

Only first three groups of microbes – viruses, bacteria and protozoa – have been reported to be transmitted by blood transfusion. Individuals with fungal infections are usually too sick to be accepted as blood donors. Viruses are most commonly transmitted by transfusion.

21.4 TRANSMISSION OF INFECTIOUS AGENTS BY BLOOD TRANSFUSION

In order to be transmitted by blood transfusion, an infectious agent must be present in the donated blood. Each blood transfusion service or blood bank or laboratory should, therefore, screen for evidence of the microbes that are known to cause infections with this route of transmission.

21.4.1 Human Immunodeficiency Virus (HIV)

HIV causes AIDS. This syndrome was recognized in 1981, well before the discovery of the causative virus.

A. Laboratory Diagnosis of HIV Infection

Shortly after exposure, the core protein, p24, has been found in some individuals. Within few weeks antibodies to both envelope (gp41) and core (p24) proteins appear in almost all infected individuals. During the early phase of infection a non-specific acute “viral illness” may occur.

Once antibodies appear, they increase in titer even though the host is asymptomatic. During this phase of infection viral cultures of isolated lymphocytes demonstrate the presence of virus. As infection progress, changes in the ratio of T-lymphocytes with specific surface markers, CD4 (helper) to CD8 (suppressor) cell, are observed. The ratio of CD4 to CD8 in healthy and immune competent individual is about 2:1. In acquired immune deficiency syndrome (AIDS), the

virus destroys CD4 cells and their number in the body decline and there is decrease in CD4:CD8 ratio.

HIV positive persons with fewer than 200CD4+ T cells per µl are considered as having AIDS in the absence of symptoms and / or opportunistic infection.

The presence of anti-HIV in an asymptomatic individual means that the individual has been exposed to the virus. It is accepted that, in almost all cases, the virus will be present in the individual. Seroconversion is sequential samples means that the infection has been recent.

Antibodies are present as early as 14 days after infection, while others indicate that they may not be present until 28 days or more after infection. The antibodies produced are directed against both core and envelope proteins. The most important antibodies are specifically anti-p24 (core) and anti-gp41 (envelope). Although antibodies to other proteins are produced, the presence of these two antibodies has been found to provide the best confirmation to infection. It has also been found to be the best means of monitoring the progress of infection.

Following infection and prior to the production of antibodies, there is 'window period' of varying length during which the infection establishes. During this period when no antibodies are detected, viral antigen (p24, gp41) could be detected. The length of time that antigen can be detected is very short, often no more than 1-2 weeks.

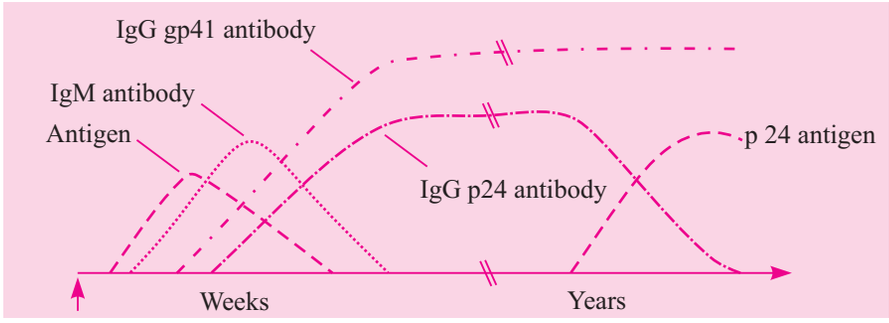


Fig. 21.1: Serological events following HIV infection

B. Testing for HIV Viral Markers

The detection of anti-HIV is the most suitable approach for identifying HIV-infected blood donations. The three main kinds of screening assays to detect anti-HIV available are:

(i) Enzyme Linked ImmunoSorbent Assays (ELISA/EIA)

(ii) Particle agglutination assays

(iii) Specialized rapid assays

Notes

While selecting assays for testing anti-HIV following features may be taken into account:

- High specificity
- High sensitivity
- Simplicity of test
- Incubation time
- Cost

(i) Enzyme Linked ImmunoSorbent Assay (ELISA)

It is the most commonly used assay and is based on the use of immobilized viral antigen which captures anti-HIV antibodies present in the test sample.

Manufacturers often use the terms first generation, second generation and third generation assay.

- First generation assays are purified or unpurified native virus or virus infected cells lysates prepared from cell structure.
- Second generation assays use recombinant antigen produced by cloning fragments of viral nucleic acid into yeast, growing large amount of the engineered yeast in bulk culture and purifying the viral proteins produced.
- Third generation assays are synthetic viral polypeptides artificially produced by chemical synthesis.

Assays are based on the same principles but differ in the way the viral antigen is immobilized:

- On the sides of wells of a polystyrene micro plate
- On the small polystyrene breads. This method needs specialized equipment.

There are three types of ELISA

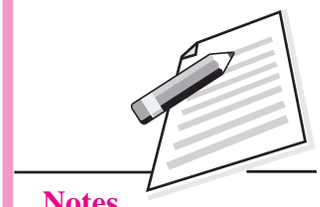
(a) Antiglobulin type ELISA:

Viral antibody present in test sample is bound to immobilized viral antigen and is detected by enzyme labeled anti-human antibody.

(b) Competitive ELISA:

It is widely used assay, in which antibody present in test sample competes with enzyme linked specific antibody for binding sites on immobilized antigen.

(c) Sandwich ELISA:



This is highly specific type of ELISA in which viral antibody in test sample is bound to immobilized antigen and then detected by free enzyme-labeled viral antigen.

(a) Antiglobulin Type ELISA method (or EIA)

It is solid phase enzyme immunoassay utilizing polystyrene wells of microplates or breads coated with HIV specific proteins representing HIV core and envelope antigens.

1. Serum or diluted serum is added to the well coated with HIV specific proteins (p24 & gp 41). Positive and negative controls are added to a number of wells on each plate run.
2. They are incubated for the defined period of time and at the correct temperature.
3. During the incubation, any specific antibody present in the test serum binds to the viral antigen.
4. At the end of incubation, the wells are washed at least three times with washing fluid to remove unbound serum. (Manual washing is done using multi-channel washer to fill and then empty the wells with wash fluid or by mechanical washing using an automated plate washer).
5. After final wash, the wash fluid is removed. It is very important that well are as drier as possible. The plate can be turned upside down and gently tapped dry on some absorbent tissue if the wells are still wet.
6. Conjugate solution is added to all wells and they are incubated at the defined period of time and at correct temperature.

   Conjugate solution contains anti-human globulin antibody which has been chemically linked to an enzyme usually horse radish peroxidase or alkaline phosphate. Conjugate binds to only human antibodies that are bound to the antigen immobilized on the wells. Conjugate does not bound in those wells that did not contain anti-HIV bound to antigen.
7. At the end of incubation, the wells are again washed three times to remove excess, unbound conjugate and are prepared for the next stage of the assay as described earlier in step 4 & 5.
8. Substrate solution is added immediately to all wells and incubated in dark for the defined period of time at correct temperature.

   When the substrate solution is added, color develops in the wells containing bound conjugate due the activation of substrate by enzyme. Wells having no bound conjugate do not change the color of the substrate. Thus reactive wells having anti-HIV positive sera are colored, and the wells

containing no anti-HIV sera are colorless. The controls show appropriate color changes.

9. At the end of incubation, diluted acid (1 N H2SO4) solution is added to all wells to stop reaction. The acid inactivates the enzyme and fixes the color. The intensity of color change is directly proportional to the antibody concentrate present in the samples/controls.

   The color change can be read visually.

10. The optical densities (OD values) of the solutions in the microwells are measured by ELISA reader at the specific wave length after determining the cut-off value and the results are determined.

(b) Competitive ELISA Method

The principles of the competitive Elisa are the same as that of antiglobulin assay but it differs slightly in the way in which anti-HIV is detected.

- The conjugate and the test sera are added at the same time in wells and incubated together.
- The conjugate is enzyme-labeled anti-HIV antibody in competitive ELISA, rather, than labeled non-specific antibody as in antiglobulin-type ELISA.
- The conjugate anti-HIV competes with anti-HIV in test sera for the antigen binding site.

  A test sample containing anti-HIV will block the binding of conjugate to antigen while sample not containing anti-HIV will allow binding of the conjugate to antigen.

Method

1. Undiluted sera and conjugate are added in wells at the same time. Positive and negative controls are also put.
2. The wells are incubated for the defined period and at correct temperature. During this period anti-HIV present in the test serum competes with the conjugated anti-HIV for the binding sites on the viral antigen.
3. At end of the incubation period, the wells are washed with washing fluid to remove excess sera and conjugate and prepared for the next step as described in the antiglobulin type assay.
4. Substrate solution is immediately added to all wells and incubated in dark for the defined period and at correct temperature.
5. At the end of the incubation period, dilute acid (1 N H2SO4) solution is added to all wells to stop reaction.

6. An intense color signifies a non-reactive sample, while lack of color signifies a reactive specimen.
7. The optical densities (OD values) of the solutions in the microwells are read by the ELISA reader after determining the cut-off value and the results are determined.

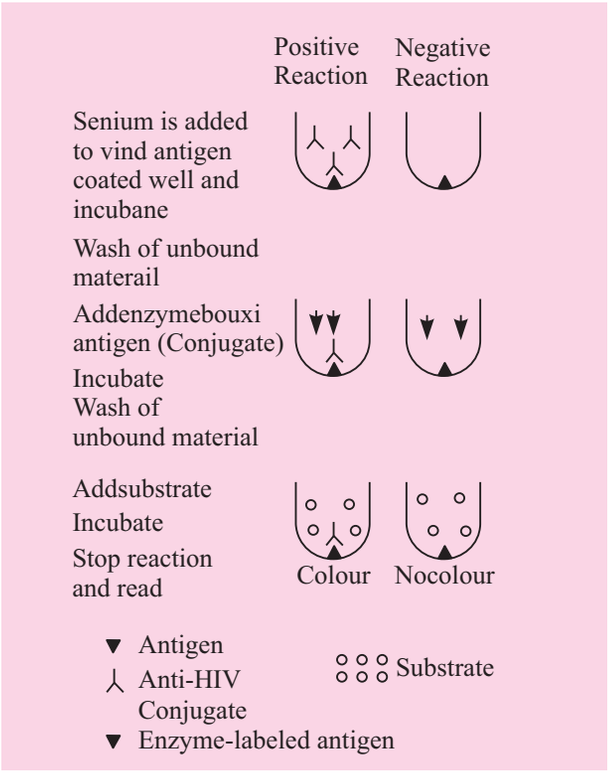


Fig. 21.2: Sandwich Elisa Method

(c) Sandwich ELISA Method

The basic principle of the sandwich ELISA is again the same as that of antiglobulin-type ELISA, but it differs in the way in which the anti-HIV is detected.

- Antigen, usually synthetic peptides are attached to the surface of wells in microplates.
- The conjugate is enzyme-labeled synthetic antigen in sandwich ELISA, rather than enzyme-linked anti-human immunoglobulin in the antiglobulin-type assay.
- During the incubation period, the conjugated antigen binds anti-HIV antibody bound to the antigen immobilized on the microwells.
- A Sandwich is built of antigen-antibody-antigen.

Notes

- At the end of incubation, diluted acid (1 N H_2SO_4) solution is added to all wells to stop reaction.
- An intense color signifies a reactive sample having HIV, while lack of color signifies a non – reactive specimen having no HIV.
- The optical densities (OD values) of the solution in the microwells are read by the ELISA reader after determining the cut-off value, and the results are determined.

Method

1. Sera or diluted sera are added to antigen bound wells. The positive and negative controls are also put. The wells are incubated for the defined period of time and at the correct temperature. During this period anti-HIV present in the sera bind to the antigen.
2. At the end of incubation period. The wells are washed with washing solution to remove the excess sera and the wells are prepared for the next step as described earlier.
3. The conjugate is added to the wells. The wells are incubated for the defined period of time and at the correct temperature. During this period, the conjugate binds to antibody bound to the immobilized antigen. A sandwich is built up of antigen-antibody-antigen.
4. At the end of incubation period. The wells are washed with washing fluid to remove unbound conjugate and the wells are prepared for the next step, as described earlier.
5. Substrate solution is immediately added to all wells and they are incubated at the defined period of time and at correct temperature.
6. At the end of incubation period, dilute acid (1 N H2SO4) solution is added to all wells to stop reaction.
7. Color develops in the wells having sera containing anti-HIV and no color will develops in the wells having sera with no anti-HIV.
8. The optical densities (OD values) of the solutions are read by the ELISA reader after calculating cut-off value, and the results are recorded.

Precautions in ELISA

- Hemolysed, lipemic or contaminated sample sera should not be used.
- Correct volumes of test samples, conjugate and substrate are added.
- Instructions given by the manufacturers of kits should be strictly followed.

(ii) Particle Agglutination Assays

Particle agglutination assay detects the presence of anti-HIV by the agglutination of particles coated with HIV antigens. Particles are made of gelatin or latex. The assay is performed in microplates.

Method

1. HIV antigen is immobilized on particles made out of gelatin or latex.
2. Test samples and controls are diluted in microwells with test dilutent which is provided with assays.

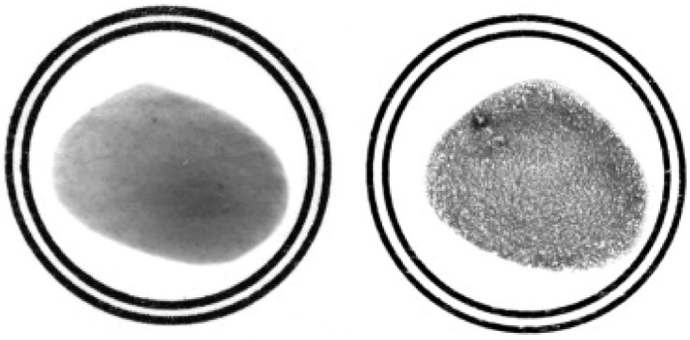


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Fig. 21.3: Particle agglutination assays

3. The HIV coated particles are added to dilute samples and controls. Then they are incubated, usually at room temperature (20-24ˆ C) for the defined period. During incubation, the particles are agglutinated by anti-HIV present in the serum.
4. At the end of the incubation period, the results of tests can be read with naked eyes. If gelatin particles are used, they appear in bluish color. If latex particles are used, they appear white in color which can be seen against a black background.
5. A reactive result appears as an event mat of agglutinated particles across the bottom of wells. A non-reactive result appears as a button or ring of non-agglutinated particles, that settle in the center of well.

Advantages of particle agglutination assay

- Expensive equipment is not needed.
- Do not have different stages of reactions.
- Do not need washing equipment.
- Results can be read visually

Notes

Disadvantages of particle agglutination assay:

- Subjective error in weak reaction
- Test serum having non-specific agglutinins, may agglutinate both sensitized and nonsensitized particles.

(iii) Specialized Rapid Spot Test

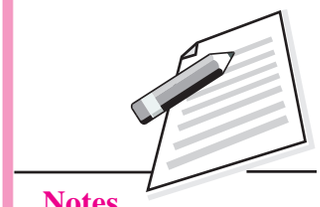
It detects anti-HIV. It is rapid and simple and based on the ELISA/ EIA technique. The HIV antigen, usually recombinant or synthetic peptide, is immobilized on either porous or semi-porous membrane usually set in a well, in a plastic cassette absorbent pad. Most of the specialized rapid assays are in the form of a kit having everything required for the test.

Method

1. The test sample and buffer solution (provided with the kit) are put on the porous membrane and allowed to soak in. Pre-dilution of the test sample may be required. This is achieved by the addition of drops of diluents and sample in a suitable vial. The diluted sample is directed put on the porous membrane.
2. It is incubated for 10-15 minutes. During this period the sample passes through the membrane and if it contains anti-HIV antibodies, it will bind to the HIV antigen on the membrane.
3. The membrane is rinsed with the buffer solution to remove unbound antibodies.
4. Then conjugate is added. The composition of the conjugate varies between assays use an enzyme conjugate anti-human immunoglobulin as in antiglobulin-type ELISA. When such conjugate is used, a further wash step and addition of chromogen is required to visualize the results. Some assays use proteins A labeled with colloidal gold as the conjugate. It will bind to anti-HIV present and gives a red/purple color.
5. The final result is read visually and compared with the expected results described by the manufacturer.
6. Although control is not required, it is good practice to set up a negative control and a weak positive control.

C. HIV Antigen Detection Assays

These are sensitive and specific tests targeting viral antigens or nucleic acids and their availability has led to proposals for using these assays in screening of donated blood to detect HIV infected blood earlier than with current antibody assays.



The detection of HIV antigen has very limited value in blood transfusion service; however, it may be useful in the following conditions:

- Detection of HIV infection during window period before seroconversion.
- Confirmation of HIV infection in infants born to HIV infected mothers.
- May help to resolve the indetermine Western Blot test results.
- To monitor HIV infected patients on antiviral therapy.

Methods for HIV antigen testing.

- ELISA for testing HIV p24 antigen
- Polymerase Chain Reaction (PCR)
- Viral isolation

Screening for HIV Antigen (ELISA)

Screening for HIV antigen (usually p 24) is performed using sandwich ELISA. The difference between HIV-antigen and anti-HIV antibody screening is that HIV-antigen test uses a sandwich of antibody-antigen-antibody, unlike HIV antibody screening which comprises a sandwich of antigen-antibody-antigen.

- Antibody (usually mono-clonal) is bound to the surface microwells.
- Test serum and positive and negative controls are added to the microwells and incubated. At the end of incubation period, the excess serum is washed off.
- Conjugate is added and incubated. The conjugate is an enzyme-labeled specific antibody (usually monoclonal).
- During the incubation, the conjugate binds HIV-antigen bound to the anti-HIV immobilized on the microwell. A sandwich is formed of antibody-antigen-antibody.
- The excess conjugate is washed away and substrate (chromogen) is added in the same way as in the antiglobulin assay and incubated.
- At the end of the incubation period, diluted acid (1H2SO4) is added to all wells to stop the reaction.
- An intense color signifies a reactive sample having HIV, while lack of color signifies a non – reactive specimen having no HIV.
- The optical densities (OD values) of the solution in the microwells are read by the ELISA reader after determining the cut-off value, and the results are determined (Fig. 21.4).

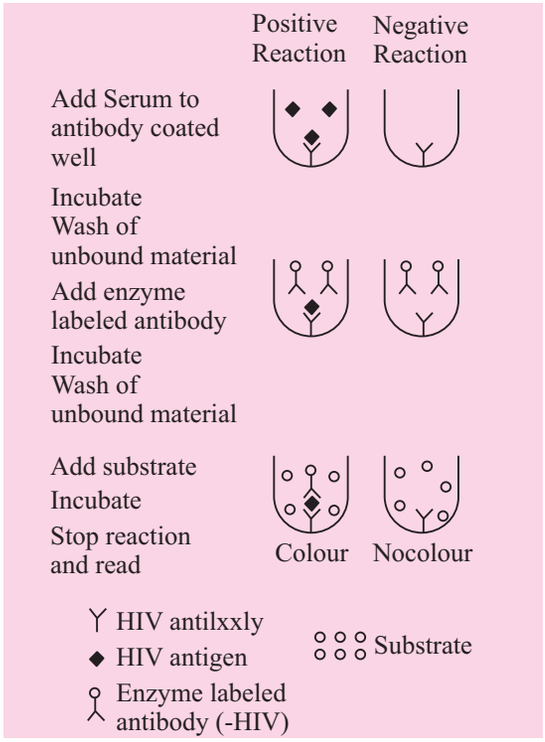


Fig. 21.4: HIV Antigen ELISA

21.4.2 Transfusion Associated Hepatitis

At least four viruses have been associated post-transfusion hepatitis:

1. Hepatitis A virus (HAV)
2. Hepatitis B virus (HBV)
3. Hepatitis C virus (HCV)
4. Hepatitis D virus (HDV)

Several other viruses causing hepatitis have been reported. Hepatitis E virus has been reported to cause epidemic hepatitis associated with contaminated water. The majority of transfusion associated hepatitis is due to HBV or HCV.

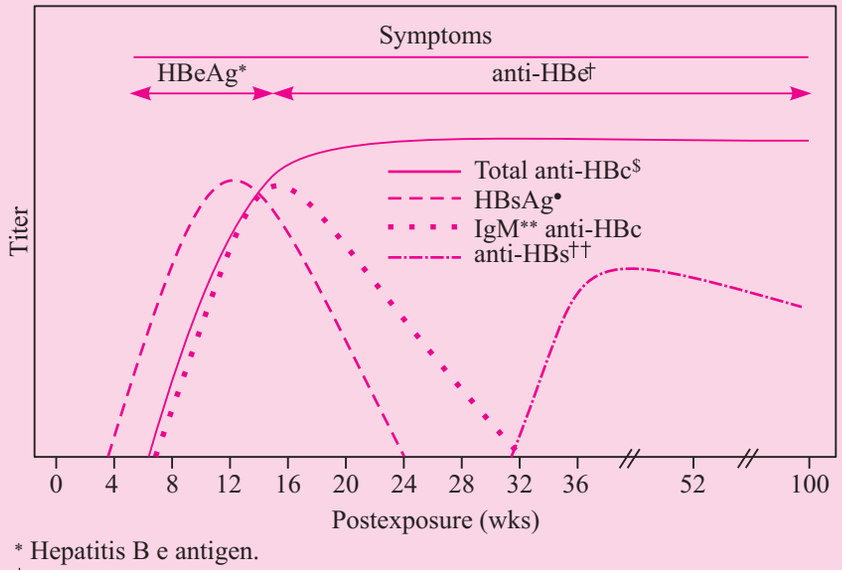
Serological Findings

The incubation period of HBV infection is about 30 to 150 days during which the patient has no sign and symptom but the virus may be detected.

- When an individual is infected by HBV several of antigens and antibodies can be detected by serological tests. Usually the first marker of HBV to appear is HBsAg. It remains detectable from a few a days to several months. This marker is also found in some (5% - 10%) infected persons who become chronic carriers of HBV.

- Anti-HBs becomes detectable after HBsAg disappear which indicates recovery from acute illness. Sometimes appearance of anti-HBs is delayed for weeks and months after HBsAg becomes undetectable. During this period, called 'core antibody window', anti-HBc may be the only detectable marker of recent HBV infection.

Figure 21.5 typical serologic course of acute hepatitis B virus infection with recovery



* Hepatitis B e antigen.
† Antibody to HBeAg.
$ Antibody to hepatitis B core antigen.
• Hepatitis B surface antigen.
** Immunoglobulin M.
†† Antibody to HBsAg.

Fig. 21.5: Markers in HBV infection

- Shortly after infection and before clinical signs and symptoms or biochemical changes in liver functions occur, two other markers – HbeAg and anti-HBC are detectable in the serum of infected person.
- Initially the anti-HBc is IgM, however as the infection progress IgG anti-HBc appears and the later persists in persons who recover from the infection.
- HbeAg usually disappears when the patient enters the convalescent phase.
- In others with chronic infection (persistence of HBsAg for longer than 6 months), HbeAg is cleared, and anti-HBe is found.
- In persons who do not develop immunity to HBV, HBsAg, HBeAg, and anti-HBc can be present.

- Individuals, who recover from infection, will have anti-HBs and/or anti-HBc in their sera.

Serological markers of hepatitis B infection and their diagnostic significance.

Marker	Significance
HBsAg	Active infection, acute or chronic
Anti-HBs	Clinical recovery, infection resolved, immunity develop
Anti HBC (IgM)	Early acute infection
Anti-HBc (IgG)	Active or past infection (carrier state)
HBeAg	Acute or serious chronic infection
Anti-HBe	Resolution of acute infection, may signal late sequelae

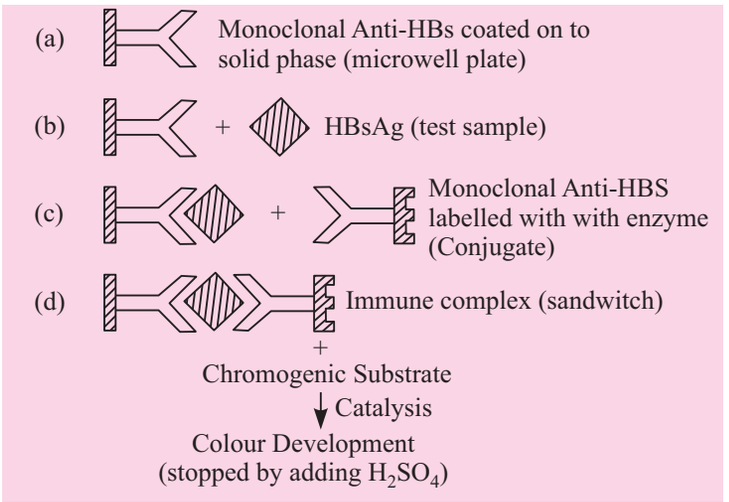


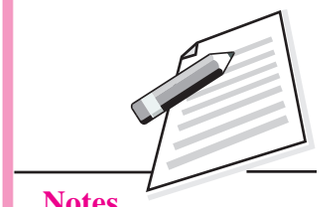
Fig. 21.6: Diagrammatic sequence for detection of HBsAg by ELISA

Enzyme Immunoassay (ELISA/EIA)

It is based on a one step 'sandwich' principle. It involves the use of solid support (wells of microplate or breads) coated with unlabeled anti-HBs antibody. Number of commercial kits is available and the recommended procedure by the manufacturers should be followed. The general steps of the technique of the test in microplate are given below:

See figure 21.6.

1. Put appropriate volume of the test sample and equal volume of positive and negative controls in the wells of microplate coated with anti HBs.



2. Incubate for the correct period of time at defined temperature.
3. At the end of incubation period, the wells are washed to remove excess of serum and dry the wells as much as possible before the next stage of assay.
4. Then and appropriate volume of conjugate (enzyme-linked anti-HBs antibody) to each well. Enzyme label is usually horseradish peroxidase.
5. Incubate for the correct period of time at defined temperature.
6. After incubation, the wells are washed and are prepared for the next step.
7. Add appropriate volume of chromogen substrate into wells.
8. Incubate the plate in dark for correct time, development of colour suggests the presence of HBsAG, and no or low color suggests absence of HBsAG.
9. The results can be read visually or by ELISA reader.

21.4.3 Syphilis

Etiological agent for syphilis is Treponema pallidum. Blood and its components may transit syphilis. The incubation period for transfusion transmitted syohilis is 1 to 4 months, the blood recipient exhibiting the signs and symptoms of secondary syphilis.

Transfusion-transmitted syphilis is not a major hazard of modern transfusion therapy, its chief reasons are:

- The spirochetes do not survive in citrated blood stored 4ˆC for 72 hours.
- Now the blood is mostly collected from the voluntary donors and sexually promiscuous persons are excluded from blood donation.
- Most patients who need transfusion of blood or its components receive antibodies therapy because of their clinical condition.
- Widespread use of penicillin and other antibiotics to treat syphilis and its carrier might account for the rarity of infected cases.

Role of serological tests for syphilis in prevention of posttransfusion syphilis:

Spirochetemia usually is common in the early stages during the invasion of lymphnodes. The screening tests used for blood donors often are negative in early syphilis when spirochetes could be transmitted by blood transfusion. Only 25% of patients with primary syphilis have a reactive serological test for syphilis (STS), and the rest do not become positive until the 4^{th} week after the onset of primary syphilis. By the time the person develops a positive STS, the spirochetemia has typically cleared.

Notes

In addition, a high proportion of healthy people have biological false positive reactions even they do not have circulating spirochetes. It may be due to viral infections, immunization, lupus, erythematosus and dysproteinemias.

The prevention of spirochete transmission is not well accomplished by STS test. Anyhow it is considered safe to test blood donation for syphilis due to the following reasons:

- There is possibility of transmitting syphilis.
- Demand of fresher blood for exchange transfusion, and platelets.
- Screening for spirochetes helps to exclude donors who are in high risk group for HIV and HBV infection.
- Cost for STS is low.

Tests

The serological tests are of two types:

- Non-specific tests
- Specific test

Non-specific tests

The most commonly used test in blood donor screening are non-specific tests because they are simple, rapid and economical, they are:

- Venereal Disease Research Laboratory (VDRL) test
- Rapid plasma regain (RPR) test

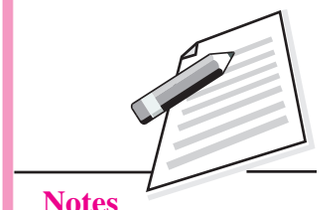
Specific tests

These tests are mainly confirmatory tests and have lengthy procedures. They are not suitable for routine screening of donor's blood. Tests are:

- Treponema pallidum hemagglutination test (TPHA)
- Fluroscent treponemal antibody absorption test (FTA-ABS)
- Ttreponema pallidum immobilization test (TPI)
- Enzyme Immuno Assay

21.4.4 Malaria

Malaria is caused by intra-erythrocytic protozoan parasites, Plasodium vivax, P. falciparum, P. ovale or P. malariae. The usual mode of transmission is via the bite of anopheles mosquoti.



Screening tests for the malaria

1. Microscopic examination of thick and thin blood smears.

   It is difficult to find parasites in blood film in short time especially if the density of parasites is less than 100 per microlitre of blood. However it is best practical method for testing malarial parasites

2. Tests for antibody to malarial parasites are:
   - Indirect fluorescent antibody test (IFA)
   - ELISA
3. Immuno-diagnostic testing, including EIA methodology
4. Nucleic acid probe methodology, including polymerase chain reaction (PCR)
5. Rapid card test – malaria pLDH test
6. Clean the area to be lanced with an alcohol swab.
7. Squeeze the end of the fingertip and pierce with a sterile lancet provided.
8. Wipe away the first drop of blood with sterile gauze or cotton.
9. Take a sample pipette provided, and while gently squeezing the tube, immerse the open end in the blood drop and then gently release the pressure to draw blood into the sample pipette up to the black line.
10. Add 5µl of whole blood into Sample Well (sample well)
11. Add two drops (60µl) of assay buffer into buffer well.
12. Read the test result in 20 min.
13. Negative reaction

    The presence of only one band within the result window indicates a negative result.

14. P. vivax or other plasmodium sp. Positive reaction

    The presence of two color bands indicates a positive result for P. vivax or other plasmodium sp. The pLDH present in the sample reacts with the pan anti-pLDH conjugate and move through the test strip where the pLDh is captured by pan specific anti-pLDH.

15. Invalid

The test is invalid if the line in C area does not appear. If this occurs, the test should be repeated using a new strip.

Notes

None of the above methods except microscopic examination of blood smear is practical and possible.

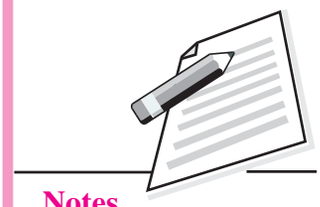
Since there are no appropriate methods for screening malarial parasites in blood donation, it has been suggested that chemoprophylaxis therapy for malaria should be given to all recipients of blood in highly endemic areas.

INTEXT QUESTIONS 21.1

1. The ratio of CD4 to CD8 in healthy and immune competent individual is .....
2. In Acquired Immune Deficiency Syndrome (AIDS), the virus destroys ..... cells and the CD4:CD8 ratio is .....
3. HIV positive patients with ..... number of CD4 cells per µl are considered as having AIDS
4. The three main kinds of screening assays to detect anti-HIV are ....., ... & .....
5. The most commonly used assay is .....
6. Types of ELISA are ....., ... & .....
7. The most commonly used ELISA assay is .....
8. The highly specific type of ELISA is .....
9. Methods for HIV antigen testing .....
10. Example of Non-specific test for screening of syphilis is .....
11. Example of Confirmatory test used for screening of syphilis is .....

WHAT HAVE YOU LEARNT

- The microbial agents of importance to blood transfusion services are those that are transmissible by blood transfusion and can cause morbidity and mortality in recipients.
- Screening of all blood donations should be mandatory for infections namely HIV-1, HIV-2, HBSAG, HCV, Syphilis and Malaria.
- There are four main groups of micro-organisms known to cause infections namely viruses, bacteria, protozoa and fungi



- Only first three groups of microbes – viruses, bacteria and protozoa – have been reported to be transmitted by blood transfusion. Viruses are most commonly transmitted by transfusion.
- The ratio of CD4 to CD8 in healthy and immune competent individual is about 2:1.
- In Acquired Immune Deficiency Syndrome (AIDS), the virus destroys CD4 cells and their number in the body decline and there is decrease in CD4:CD8 ratio.
- HIV positive persons with less than 200CD4+ T cells per µl are considered as having AIDS in the absence of symptoms and / or opportunistic infection
- The presence of anti-HIV in an asymptomatic individual means that the individual has been exposed to the virus. It is accepted that, in almost all cases, the virus will be present in the individual.
- Seroconversion is sequential samples means that the infection has been recent.

  The three main kinds of screening assays to detect anti-HIV available are:

  d. Enzyme Linked ImmunoSorbent Assays (ELISA/EIA)

  e. Particle agglutination assays

  f. Specialized rapid assays

- Enzyme Linked ImmunoSorbent Assay (ELISA) is the most commonly used assay and is based on the use of immobilized viral antigen which captures anti-HIV antibodies present in the test sample.
- There are three types of ELISA namely Antiglobulin type ELISA, Competitive ELISA, Sandwich ELISA
- Precautions in ELISA
  - Hemolysed, lipemic or contaminated sample sera should not be used.
  - Correct volumes of test samples, conjugate and substrate are added.
  - Instructions given by the manufacturers of kits should be strictly followed.
- Particle agglutination assay detects the presence of anti-HIV by the agglutination of particles coated with HIV antigens.
- It detects anti-HIV. It is rapid and simple and based on the ELISA/ EIA technique.

Notes

- The detection of HIV antigen has very limited value in blood transfusion service; however, it may be useful in the following conditions:
  - Detection of HIV infection during window period before seroconversion.
  - Confirmation of HIV infection in infants born to HIV infected mothers.
  - May help to resolve the indetermine Western Blot test results.
  - To monitor HIV infected patients on antiviral therapy.
- Methods for HIV antigen testing are ELISA for testing HIV p24 antigen, Polymerase Chain Reaction (PCR), Viral isolation
- At least four viruses have been associated post-transfusion hepatitis namely Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV)
- Etiological agent for syphilis is Treponema pallidum. Blood and its components may transit syphilis.
- The incubation period for transfusion transmitted syphilis is 1 to 4 months, the blood recipient exhibiting the signs and symptoms of secondary syphilis.
- The serological tests are Non-specific tests and Specific test
- Non-specific tests are most commonly used test in blood donor screening are non-specific tests because they are simple, rapid and economical, they are Venereal Disease Research Laboratory (VDRL) test and Rapid plasma regain (RPR) test
- Specific tests are mainly confirmatory tests and have lengthy procedures. They are not suitable for routine screening of donor's blood, they are Treponema pallidum hemagglutination test (TPHA), Fluroscent treponemal antibody absorption test (FTA-ABS), Ttreponema pallidum immobilization test (TPI), Enzyme Immuno Assay
- Screening tests for the malaria are Microscopic examination of thick and thin blood smears
- Tests for antibody to malarial parasites are Indirect fluorescent antibody test (IFA) and ELISA
- Immuno-diagnostic testing, including EIA methodology, Nucleic acid probe methodology, including polymerase chain reaction (PCR), Rapid card test – malaria pLDH test

TERMINAL QUESTIONS

1. Write a short note on screening of blood donations
2. What are the screening assays to detect anti-HIV
3. Describe briefly the types of ELISA
4. Write a short note on methods of HIV antigen testing
5. Explain the non specific test of diagnosis of syphilis
6. Explain the specific tests of diagnosis of syphilis
7. Write a short note on screening test for malaria

ANSWERS TO INTEXT QUESTIONS

21.1

1. 2 : 1
2. CD4
3. Reduced
4. < 200 cells
5. Enzyme Linked ImmunoSorbent Assay (ELISA)
6. Antiglobulin type ELISA, Competitive ELISA & Sandwich ELISA
7. Competitive ELISA
8. Sandwich ELISA
9. ELISA for testing HIV p24 antigen, Polymerase Chain Reaction (PCR), Viral isolation
10. Venereal Disease Research Laboratory (VDRL) test
11. Treponema pallidum hemagglutination test (TPHA)

Notes

22

ANTI GLOBULIN TEST

22.1 INTRODUCTION

The anti globulin test (Coombs test) was introduced by Coombs, Mourant and Race in 1945. The test was developed to detect antibodies which can bind the antigen on the surface of RBCs but cannot agglutinate them (incomplete antibodies).

OBJECITVE

After reading this lesson, you will be able to:

- describe the principle of Anti-Globulin test
- explain the procedure of Direct and Indirect Anti Globulin test
- describe the uses of Direct and Indirect Anti globulin test

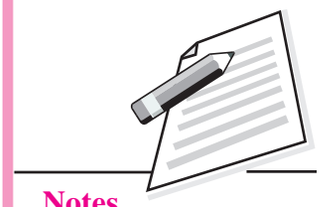
22.2 PRINCIPLE

The test is based on the principle that antihuman globulin (AHG) antibodies combine with RBCs coated with human immunoglobulin or complement (in vivo or in vitro). The AHG acts as a bridge and causes agglutination of the RBCs.

There are two type of Anti Globulin Test

Direct Anti globulin Test (DAT)
It reflects in vivo antibody sensitization of RBCs
Patient's RBC s are tested against reagent serum

Indirect Anti globulin Test (IAT)
It detects presence of IgG antibody in serum (in vitro sensitization)
Patient's serum is tested against reagent RBCs



22.3 DIRECT ANTI GLOBULIN TEST (DAT)

To identify if a patient's RBCs are coated with immunoglobulin, complement or both, AHG with reactivity to human immunoglobulin and/or compliment is added to the patient's RBCs. If cross linking and subsequent agglutination is present, direct Coomb's test is positive.

Specimen: EDTA blood

Materials

1. Test tubes
2. AHG reagent
3. Positive control cells (IgG coated)
4. Centrifuge and microscope

Procedure

1. Make 5% cell suspension of patient blood by washing 3 times with normal saline.
2. After last washing decant the supernatant completely.
3. Take 1 drop of patient's cell suspension in a test tube.
4. Add 2 drops of A.H.G reagent with the patient's cell suspension in the test tube.
5. Mix well and centrifuge the mixture for at 1500 RPM for 1 minute.
6. Gently shake the tube and examine with naked eye and under microscope to see the agglutination.
7. If the test result is negative, add a drop of control cells.
8. Mix well and centrifuge the mixture for at 1500 RPM for 1 minute and look for agglutination. If no agglutination is seen, the result is invalid.

Result

Presence of agglutination means a positive DAT. This indicates the presence of human immune globulin or complement bound to RBCs.

Absence of agglutination means a negative DAT.

Uses of DAT

1. Presence of autoantibodies against RBC as in the case of warm autoimmune hemolytic anemia (AHA).
2. To detect hemolytic transfusion reactions when incompatible blood is transfused, the donor cells get coated with recipient's antibodies and the DAT is positive.

3. To detect hemolytic disease of Newborn: detects the presence of maternal antibodies attached to fetal RBCs.
4. Antibodies induced by drugs.

22.4 INDIRECT ANTI GLOBULIN TEST (IAT)

Reagent RBCs (Coomb's control cells) are incubated in the presence of patient's serum. On adding AHG reagent, IgG coated red cells agglutinate

Specimen: Patient's Serum

Materials

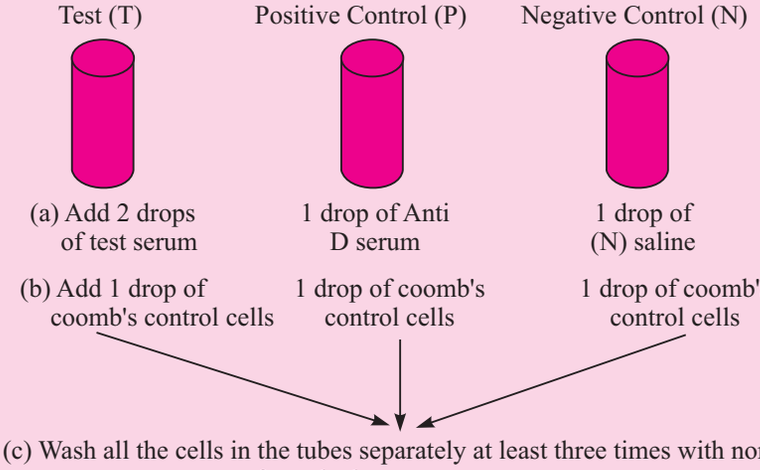
In addition to the material used in DAT;

1. O positive red blood cells (Coomb's control cells)
2. Anti D serum
3. Normal saline

O positive red blood cells are prepared as follows:

1. A pool of 'O' positive blood group cells is taken from 3 separate donors.
2. These cells are saline washed at least 3 times & a 5% saline suspension of the washed cells is made.

Procedure : Take three test tubes labeled as test, positive control & negative control.



(c) Wash all the cells in the tubes separately at least three times with normal saline to remove any traces of attached serum.

+

(d) Add 2 drops of anti human globulin to each test tube.
(e) Incubate for 5-10 min. at room temperature
(f) Centrifuge at 1500 RMP for 1 min.
(g) Re-suspend the cells and observe all the tubes for agglutination with naked eye & microscopically.

Anti Globulin Test

Test	Positve Control	Neg. Control	Result
Agglutination	Agglutination	No Agglutination	Positive
No Agglutination	Agglutination	No Agglutination	Negative

If positive control shows no agglutination or the negative control shows agglutination it means the test has not been performed correctly and needs to be repeated.

Interpretation

A positive test means patient's serum contains Anti D antibodies.

A negative test means patients serum does not contain anti D antibodies

Uses of IAT

(a) Cross matching

To detect the presence of recipient antibodies bound to donor RBCs.

(b) Typing of erythrocyte antigen

(c) Detecting presence of Anti D antibodies in a Rh negative woman married to an Rh positive man.

Sensitivity of IAT can be increased by

1. Optimizing the temperature at 37ˆC
2. Increasing the ratio of serum to red cells
3. Addition of LISS (low ionic strength saline), albumin and enzymes

INTEXT QUESTIONS 22.1

1. Antiglobulin test is commonly known as .....
2. ..... test detects antibodies bound to RBCs
3. ..... test detects unbound antibodies
4. Common used of Direct Antiglobulin tests are ..... & .....
5. Common used of Indirect Antiglobulin tests are ..... & .....

WHAT HAVE YOU LEARNT

- Antiglobulin test is commonly known as coomb's test
- Antiglobulin test is used to detect incomplete RH antibodies
- It is based on the principle that antihuman globulin antibodies combine with RBCs coated with human immunoglobulin or complement
- Direct and Indirect antiglobulin tests are the two types of antiglobulin tests
- Direct antiglobulin tests detects antibodies bound to RBCs
- Indirect antiglobulin test detects the presence of unbound antibodies in serum
- DAT is used to detect hemolytic transfusion reactions and also to detect hemolytic diseases of newborn
- Indirect anitglobulin test is used in cross matchin and to detect the presence of Anti D antibodies in Rh negative woman married to Rh positive man.

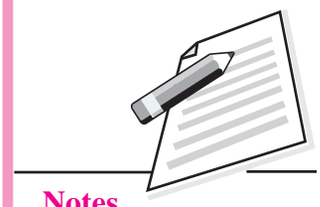
TERMINAL QUESTIONS

1. List the types of Antiglobulin test
2. Enlist the uses of Direct Antiglobulin test
3. Enlist the uses of Indirect Antiglobulin test

ANSWERS TO INTEXT QUESTIONS

22.1

1. Coomb's test
2. Direct Antiglobulin
3. Indirect Antiglobulin
4. Hemolytic transfusion reaction & Hemolytic diseases of Newborn
5. Cross matching & Detecting presence of Anti D antibodies



Notes

23

LEUKEMIA

23.1 INTRODUCTION

Leukemia is a disease of unknown etiology and fatal termination characterized by uncontrolled, abnormal and widespread proliferation of leukocytes and their precursors in bone marrow and blood.

OBJECTIVES

After reading this lesson, you will be able to:

- classify Acute Lymphoblastic leukemia
- describe FAB classification of AML
- explain Cytochemistry stains in diagnosis of leukemia
- explain Immunophenotyping technique in diagnosis of Leukemia
- describe Chronic myeloid leukemia, Chronic lymphocyte leukemia, Chronic myeloproliferative disorders, Myelodysplastic syndromes

23.2 LEUKEMIA

23.2.1 Definition

Leukemia is a disease of unknown etiology and fatal termination characterized by uncontrolled, abnormal and widespread proliferation of leukocytes and their precursors in bone marrow and blood.

23.2.2 Classification

This is based on the clinical course of the disease and the type of cell line that is involved.

Notes

In the early 1990s the **French – American – British** group introduced the FAB classification of acute leukemia based mainly on the morphological appearance of blasts in Romanowsky stained smears of peripheral blood and bone marrow.

More recently (2002) the WHO classification of leukemia included in addition to morphology, immunophenotyping of the blasts and cytogenetic studies.

ACUTE LEUKEMIA is of sudden onset and rapid progression unless treated and is characterized by the **presence of blast cells** in peripheral blood and bone marrow.

CHRONIC LEUKEMIA is of gradual onset and slow progression and is characterized by the **presence of more mature cells** in the peripheral blood and bone marrow.

23.2.3 Diagnostic Tools in the Diagnosis of Leukemia

A. Morphology of the blasts in peripheral blood and bone marrow.

B. Cytochemistry of blasts in the various leukemias

Leukocyte cytochemistry uses techniques to identify enzymes or other cytoplasmic products in cells. Cytochemistry is useful for

1. Identification of myeloid blasts from lymphoid blasts
2. Differentiation of granulocytic and monocytic components of acute myeloid leukemia
3. Detection of unusual lineages eg basophils.
4. Detection of the absence of certain enzymes in a malignant clone eg Leukocyte alkaline phosphatase in Chronic Myeloid Leukemia.

The common Cytochemical stains used are

(a) Myeloperoxidase (MPO) This enzyme is present in primary and secondary granules of granulocytes. MPO splits H_2O_2 and in the presence of a chromogenic electron donor forms an insoluble reaction product. Substrates are benzidine substitutes. The reaction product is stable. MPO is not inhibited by heparin, oxalate or EDTA. The substrate is 3,3 diaminobenzidine with a phosphate buffer at pH 7.3. A brown granular deposit is seen in blasts of myeloid origin.

(b) Sudan Black B (SBB). This lipophilic dye binds with granule components in granulocytes and monocytes irreversibly. Staining reaction is comparable to MPO.

(c) Periodic Acid Schiff reaction (PAS). Periodic acid oxidizes 1-2 glycol groups to produce dialdehydes. Dialdehydes give a red reaction when exposed to the Schiff reagent which is leucobasic fuchsin. A positive

reaction is seen with carbohydrates especially glycogen, polysaccharides, mucoproteins, glycoproteins etc. Blood smears are fixed with formalin vapour, exposed to Periodic acid and then Schiff reagent and counter stained with Haematoxylin. WBC show diffuse, confluent red colour. ALL blasts show "block" positivity and the method can be used to differentiate ALL from AML.

(d) Acid phosphatase. This enzyme is present in haemopoietic cells. It is useful for the diagnosis of T cell ALL and Hairy cell leukemia. Air dried smears fixed in methanol, acetone, citric acid buffer are exposed to naptholAS-BI phosphate substrate at an acid pH of 5.0. The reaction is detected with the use of hexazotised pararosanaline dye. Nuclei are counter stained with haematoxylin. T cells show strong localized polar positivity. Other WBC also show variable positivity. In hairy cell leukemia the cells react equally positive in the presence and absence of tartaric acid.

(e) Esterase stains. WBC granules contain several types of esterases that hydrolyse acyl or chloroacyl esters of α naphthol or naphthol AS. **Specific esterase** stain with naphthol AD chloroacetate ester (CAE) and is positive in the myeloid series and strong positive in promyelocytes including Auer rods. **Non specific esterase** (NSE) stains with α naphthyl acetate ester (ANAE) or α naphthyl butyrate ester or (ANBE) and is positive in 80% of the monocyte cell line. A stain combining the two substrates sequentially or in combination in one single step may be used to differentiate myeloblasts from monoblasts.

(f) Neutrophils contain alkaline phosphatase enzyme in their granules. The reaction between leukocyte alkaline phosphatase in cells, naphthol AS phosphate substrate, alkaline buffer at pH 9.0 is detected using a coupling azo dye (Fast Blue BB salt or Fast Garnet GBC) A counter stain is used for nuclei. The azo dye product is blue or brown granules in neutrophil cytoplasm. The neutrophils are scored as 0 = negative, 1+ = occasional granules in cytoplasm,2+ = moderate granulation, 3+ = heavy granulation and 4+ = heavy granulation overlapping the nucleus.

A positive control that is used is usually from a pregnant female. The abnormal neutrophils in CML have markedly decreased levels of this enzyme. In CML neutrophils stain negative for the enzyme

(g) Toludine Blue stain is used to stain the metachromatic granules in basophils and mast cells.

C. Immunophenotyping is done to determine whether the cells are of myeloid or lymphoid origin and if lymphoid, whether they are of T or B cell origin. The cells at various stages of development express antigens on the surface.

Monoclonal antibodies are available commercially to detect these antigens. The antibodies are tagged with a fluorescent label. The test cells are incubated with a series of specific antibodies and by observing the degree of binding and the specificity of the antibody using a flow cytometer it is possible to identify and quantify the cells for diagnosis and for follow up of therapy. Flow cytometry is also used to detect the presence of cytoplasmic or surface immunoglobulins and the presence of certain cytoplasmic enzymes eg myeloperoxidase.

D. Cytogenetics. Many of the blasts show specific cytogenetic markers which help identify them as well as predict their behavior to chemotherapeutic drugs. Standard cytogenetics, RT-PCT, FISH are some techniques that are used.

E. Cytospin of Cerobrospinal Fluid is used to detect the presence of small numbers of lymphoblasts in CSF in ALL. The cytospin is a special centrifuge which is gentle on the cells and produces minimum distortion of the blast cells.

INTEXT QUESTIONS 23.1

1. Acute leukemia is characterized by ..... in pheripheral blood and bone marrow
2. Chronic leukemia is characterized by ..... in pheripheral blood and bone marrow
3. Common cytochemical stains used in diagnosis of leukemia are ....., ..., ..., ..., ..., ..., ...
4. ..... reaction method is used to diefferentiate ALL from AML
5. ..... test is used in diagnosis of T cell & Hairy cell Leukemia
6. ..... is used to differentiate myeloblasts from monoblasts
7. ..... is used for identifying the origin of cells
8. Cytospin of CSF is used to detect ..... in ALL

23.3 ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

23.3.1 Clinical Presentation

ALL affects children in the 2 – 10 years age group commonly but is also seen in adults. The patient presents with fever, weakness, pallor, infections bleeding and enlarged lymph nodes and spleen. Childhood ALL is associated with a good prognosis.

23.3.2 Laboratory Diagnosis

1. Haemoglobin, PCV, RBC count are decreased.
2. MCV, MCH and MCHC are normal RDW is normal. There is normochromic normocytic anaemia.
3. WBC count is elevated (20.0 – 100.0x10^9/L). Rarely the count may be low in subleukemic leukemia.
4. Platelet count shows moderate to severe thrombocytopenia
5. The blood film shows normochromic normocytic anaemia, leukocytosis with many blast cells and thrombocytopenia.
6. The differential cells show many blast cells There is neutropenia. The FAB classification describes 3 types of ALL depending on the morphology of the blasts. They are

A. ALL L1

The blast is small, 14 – 16µm in diameter, the cytoplasm is barely visible, the nucleus occupies the whole cell, has condensed chromatin and nucleoli are not seen. Figure 23.1.

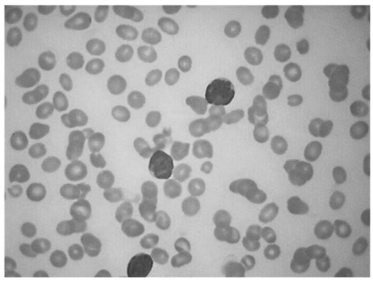


Fig. 23.1: ALL L1

B. ALL L2

The blast is 15 - 18µm in diameter, nucleus occupies most of the cell. The nuclear membrane is thick, chromatin is coarse, 1 – 2 nucleoli are seen. The chromatin around the nucleolus is condensed. The cytoplasm is pale blue, forms a rim around the nucleus and does not contain any inclusions. Figure 23.2.

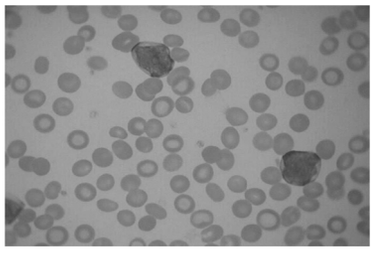


Fig. 23.2: ALL L2

C. ALL L3

The blast is 18 – 20 µm in diameter, nucleus has reticular chromatin, 1-2 prominent nucleoli, cytoplasm is moderate in amount blue in colour and there are punched out vacuoles in the cytoplasm and overlying the nucleus. Figure 23.3

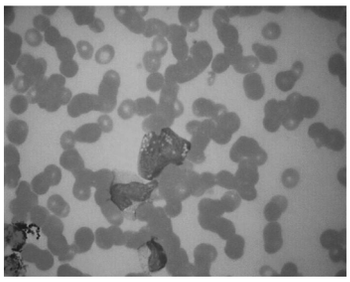


Fig. 23.3: ALL L3

7. Cytochemistry. Special stains are done to differentiate ALL from AML. These are
   A. Myeloperoxidaes stain and Sudan Black B stains are negative in ALL.
   B. Periodic Acid Schiff stain may show block positive staining in the cytoplasm of ALL blasts with no differentiation between T and B cells.
8. Immunophenotyping differentiates the blasts into T and B cells. B cells are CD19+, CD10+ and may show cytoplasmic CD22. T cells are CD2+, CD5+, CD7+
9. Bone marrow examination shows solidly cellular marrow with suppression of normal erythropoiesis, myelopoiesis and megakaryocytes. The marrow is replaced by proliferating blasts. In the FAB classification there must be >30% blasts in the marrow and in the WHO classification there must be >20% blasts in marrow.
10. Biochemical tests show raised serum uric acid levels, raised serum LDH and serum creatinine may be elevated

23.4 ACUTE MYELOID LEUKEMIA (AML)

23.4.1 Clinical Presentation

AML affects young adults who present with fever, weakness, pallor, infections, bleeding, bone pains and splenomegaly.

23.4.2 Laboratory Diagnosis

1. Haemoglobin, PCV, RBC count are decreased.
2. MCV, MCH and MCHC are normal RDW is normal. There is normochromic normocytic anaemia.

3. WBC count is elevated (20.0 – 100.0x10^9/L). Rarely the count may be low in subleukemic leukemia.
4. Platelet count shows moderate to severe thrombocytopenia
5. The blood film shows normochromic normocytic anaemia, leukocytosis with many blast cells and thrombocytopenia.
6. The differential cell count shows many blast cells There is neutropenia. The FAB classification describes 8 types of AML depending on the morphology of the blasts. They are

A. AML M0

The blast is 15 - 18µm in diameter, nucleus occupies most of the cell, nuclear membrane is fine, chromatin is fine, 2 – 3 nucleoli are seen. The cytoplasm is pale blue, forms a rim around the nucleus and does not contain any inclusions. These cells are CD 13+,CD 33+ and CD 117+ by flow cytometry <3% of cells show MPO/SBB positivity

B. AML M1

The blast is 15 - 18µm in diameter, nucleus occupies most of the cell, nuclear membrane is fine, chromatin is fine, 2 – 3 nucleoli are seen. The cytoplasm is pale blue, forms a rim around the nucleus and may contain thin rod like pink structures called Auer rods. This is formed by the condensation of primary granules and when present are diagnostic of AML. Figure 23.4 Myeloperoxidase and Sudan Black B stains show early positivity in 30 – 100% of cells

C. AML M2

The blast is 15 - 18µm in diameter, nucleus occupies most of the cell, nuclear membrane is fine, chromatin is fine, 2 – 3 nucleoli are seen. The cytoplasm is pale blue, forms a rim around the nucleus and contains fine early granulation and thin rod like pink structures called Auer rods. Myeloperoxidase and Sudan Black B stains show positivity in 30 – 100% of cells.

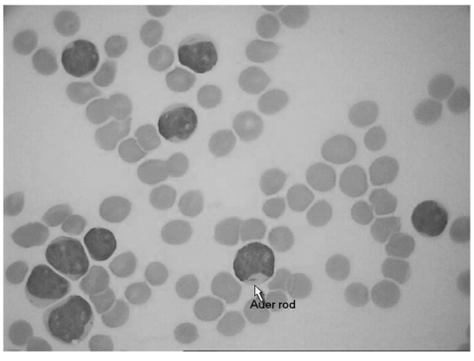


Fig. 23.4: Acute Myeloid Leukemia (AML M2)

D. AML M3 (Promyelocytic leukemia)

The blast is 18 – 20 µm in diameter, nucleus has reticular chromatin, 1-2 prominent nucleoli. The nucleus appears folded on itself or may be bilobed. The cytoplasm is moderate in amount and filled with fine pink azurophilic granules. Many Auer rods are seen and these cells are called faggot cells. Figure 23.5. Sometimes the granulation is not prominent and the leukemia is called AML M3 Hypogranular variant .The myeloperoxidase stain and SBB stains are strong positive. 90% of the cells show specific esterase positivity. AML M3 is associated with a cytogenetic abnormality namely T(15:17)

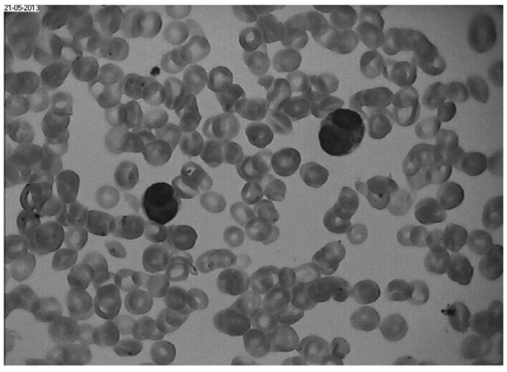


Fig. 23.5: Acute Myeloid Leukemia (AML M3)

E. AML M4 (Myelomonocytic leukemia)

In this variant two types of blasts are present – myeloblasts as described above and up to 20% monocytes and monoblasts. The monoblaste are 20 – 22 µm in diameter, nucleus has reticular chromatin, 2-3 prominent nucleoli and the nucleus may be indented. The cytoplasm is abundant, vacuolated and may contain few azurophilic granules and sometimes Auer rods. MPO, SBB and non specific esterase are positive A variant of this leukemia with prominent eosinophilia and a cytogenetic abnormality inversion16 is known. Figure 23.6.

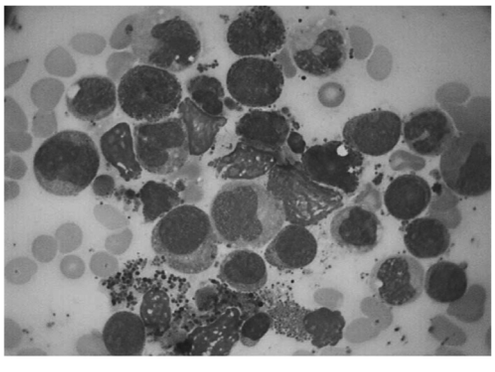
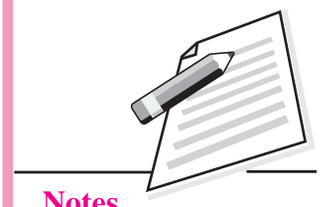


Fig. 23.6: Acute Myelomonocytic Leukemia (AML M4) Eosinophilic



F. AML M5 (Monocytic leukemia)

In this variant the predominant cell line is monocytoid and is a mixture of monocytes and monoblasts which make up >80% of the cells.MPO, SBB and combined esterase are positive. In addition to cytochemistry there is also increase in serum and urinary lysozyme excretion

G. AML M6 (Erythroleukemia)

In this variant there are two cell lines involved erythroid which must be greater than 50% in marrow and myeloblasts which makes up greater than 20% of the non erythroid cells in marrow. Auer rods and dysplasia may be seen. PAS positivity is seen in erythroblasts.

H AML M7 (Megakaryoblastic leukemia)

In this variant the cell line involved is megakaryoblastic. The blasts superficially resemble lymphoblasts. Flow cytometry is needed to identify these blasts which show positivity for CD 41 and CD 61 which are platelet markers.

7. Cytochemistry. Special stains are done to differentiate ALL from AML. These are
   A. Myeloperoxidaes stain and Sudan Black B stains are negative in ALL.
   B. Periodic Acid Schiff stain may show block positive staining in the cytoplasm of ALL blasts with no differentiation between T and B cells.
   C. Esterase stains are done to differentiate the monocyte cell line
8. Immunophenotyping is done to identify the myeloid, monocytoid, erythroid and megakaryocyte cell lines.
9. Bone marrow examination shows solidly cellular marrow with suppression of normal erythropoiesis, myelopoiesis and megakaryocytes. The marrow is replaced by proliferating blasts.
10. Biochemical tests show raised serum uric acid levels, raised serum LDH and serum creatinine may be elevated
11. Cytogenetic studies are performed to demonstrate typical abnormalities in certain leukemias and to monitor response to treatment.

23.5 CHRONIC MYELOID LEUKEMIA (CML)

23.5.1 Clinical Presentation

This condition affects all age groups mostly young adults. There is anaemia, fever, weight loss, sweating, bone pain and enlarged spleen. The leukemia runs a chronic course of 2 – 5 years unless treated and transforms into acute leukemia, myeloblastic or lymphoblastic as a terminal event.

23.5.2 Laboratory Diagnosis

1. Haemoglobin, PCV, RBC count are decreased.
2. MCV, MCH and MCHC are normal RDW is normal. There is normochromic normocytic anaemia.
3. WBC count is elevated (100.0 – 800.0x10^9/L).
4. Platelet count is normal or increased.
5. The blood film looks like a bone marrow preparation because of the marked leukocytosis. The red cells show normochromic normocytic anaemia. The entire series of myeloid cells are seen. Myeloblast make up 2 -5% of the cells , myelocytes ~ 20%, neutrophils ~20%. There is an increase in basophils which make up 5 – 10% of the cells. Eosinophils may also be increased. Lymphocytes are normal. Platelets may be normal or increased. Figure 23.7. A falling haemoglobin, increasing blast percentage and increase in basophils indicate blast transformation.

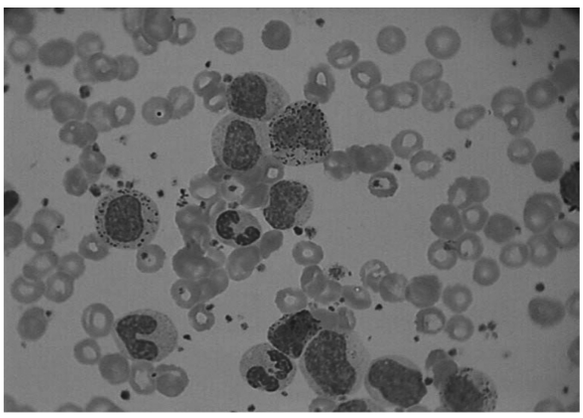


Fig. 23.7: Chronic Myeloid Leukemia

6. The bone marrow is solidly cellular with no fat spaces, There is marked myeloid hyperplasia with all stages of maturation, basophilia and increased megakaryocytes. The erythroid series is normal
7. Cytochemistry Leukocyte Alkaline phosphatase (LAP)

   Neutrophils contain alkaline phosphatase enzyme in their granules. The abnormal neutrophils in CML have markedly decreased levels of this enzyme. Blood smears made from finger prick are stained using a phosphate substrate, alkaline buffer at pH 9.0, a coupling azo dye and a counter stain. In the presence of the enzyme the reaction product is seen as blue or brown (depending on azo dye used) granular deposit in neutrophil cytoplasm. In CML neutrophils stain negative for the enzyme.

8. Cytogenetics

   A typical translocation between 19 :23 called the Philadelphia chromosome is demonstrated in CML.

9. Biochemistry

   Increased serum LDH, serum uric acid and serum vitamin B_{12} are seen

23.6 CHRONIC LYMPHOCYTIC LEUKEMIA

23.6.1 Clinical Presentation

This condition affects older age groups and is common in Caucasians. There is anaemia, fever, weight loss, sweating, enlarged lymph nodes and spleen. The leukemia runs a chronic course of 2 – 5 years unless treated and may be associated with frequent infections because of depressed immunity.

23.6.2 Laboratory Diagnosis

1. Haemoglobin, PCV, RBC count are mildly decreased or normal
2. MCV, MCH and MCHC are normal RDW is normal.
3. WBC count is elevated (50.0 – 250.0x10^9/L).
4. Platelet count is normal. Rarely there may be immune thrombocytopenia.
5. The blood film shows leukocytosis. The red cells are normochromic normocytic. 70 – 90 % of the WBC are mature lymphocytes. Many cells are smear cells. There is a persistent absolute lymphocytosis. Platelets are normal. Figure 23.8.

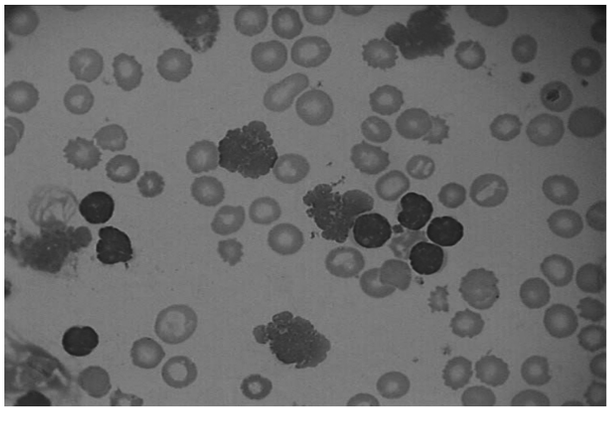


Fig. 23.8: Chronic Lymphocytic Leukemia

6. The bone marrow is solidly cellular with no fat spaces. 90% of the cells in the marrow are mature lymphocytes and a relative decrease in erythroid and myeloid series. Megakaryocytes are normal.

Notes

7. Flow cytometry shows the presence of B cell markers with one aberrant T cell marker, namely CD5
8. Biochemistry

   Increased serum LDH, serum uric acid may be seen

23.7 CHRONIC MYELOPROLIFERATIVE DISORDERS

This is a group of disorders where there is proliferation of the various components of the marrow leading to a chronic disease. These include:-

(a) Chronic myeloid leukemia – as described above

(b) Polycythemia rubra vera – erythrocytosis, leukocytosis and thrombocytosis

(c) Myelofibrosis - abnormal megakaryocyte proliferation leads to deposition of fibrous tissue in marrow. The normal haematopoiesis takes place in other organs like liver and spleen (extramedullary haematopoiesis) resulting in a leukoerythroblastic blood picture.

(d) Essential thrombocythemia – proliferation of abnormal megakaryocytes giving rise to thrombocytosis with abnormal platelets which also have platelet dysfunction

(e) Chronic myelomonocytic anaemia

23.8 MYELODYSPLASTIC SYNDROMES OR PRELEUKEMIA

In this group of disorders the maturation of cells is abnormal and dysplastic and may involve one or more cell line in the marrow. These conditions usually progress to acute leukemia There are several types of MDS which include

(a) Refractory anaemia – persistent unexplained anaemia in older age group patients. Bone marrow shows evidence of erythroid hyperplasia with dysplastic maturation.

(b) Sideroblastic anaemia – persistent unexplained hypochromic microcytic anaemia in older patients with the presence of increased iron stores and ringed sideroblasts in marrow.

(c) Refractory anaemia with excess blasts (RAEB) Marrow shows 5 - 20 % myeloblasts and the cell lines show dysplasia

(d) RAEB –T refractory anaemia with excess blasts in transformation. This condition is reclassified as AML with dysplasia in the WHO classification

INTEXT QUESTIONS 23.2

1. Basophils are increased in .....
2. Inclusions diagnostic of Acute Myeloid Leukemia are called .....
3. A special cytochemical stain done in Chronic Myeloid Leukemia is .....
4. A Chromosomal abnormality seen in Chronic Myeloid Leukemia is .....
5. A Special stain used to differentiate myeloblast from lymphoblast is .....
6. FAB classification stands for .....
7. Promyelocytes contain ..... granules.
8. The characteristics of Chronic Lymphocytic Leukemia in peripheral blood are ..... and .....

WHAT HAVE YOU LEARNT

- Leukemia is characterized by uncontrolled, abnormal & widespread proliferation of leukocytes and their precursors in bone marrow and blood
- French – American – British classification is based on morphological appearance of blasts in pheripheral blood & bone marrow
- WHO classification of leukemia includes morphology, immunophenotyping of blasts & cytogenic studies
- Acute leukemia is of sudden onset & slow progression and is characterised by presence of more mature cells in pheripheral blood and bone marrow
- Leukocyte cytochemistry uses techniques to identify enzymes or other cytoplasmic products in cells
- Common cytochemical stains used are Myeloperoxidase, Sudan Black B (SBB), Periodic Acid Schiff reaction (PAS), Acid Phosphatase, Esterase stain, Azo dye, Toludine Blue stain
- Immunophenotyping is done to determine whether the cells are of myeloid or lymphoid origin
- Cytospin of Cerebrospinal fluid is used to detect the presence of small numbers of lymphoblasts in CSF in ALL

Notes

- Acute Lymphoblastic Leukemia(ALL) affects children but also seen in adults
- In ALL the blood film shows normochromic normocytic anaemia, leukocytosis and megakaryocytes
- In FAB classification there must be >30% blasts and in WHO classification there must be >20% blasts in marrow
- Acute Myeloid Leukemia (AML) affects young adults, blood film shows normocromic, normocytic anaemia, leukocytosis with many blast cells & thrombocytopenia
- Chronic myeloid leukemia affects all groups mostly young adults
- Blood film shows marked leukocytosis, the red cells show normochromic normocytic anaemia
- Cytochemical stain of Leukocyte Alkaline Phosphatase (LAP) is useful in the diagnosis
- Chronic Lymphocytic Leukemia affects older age group
- In CLL, the blood film shows, the red cells are nomochromic normocytic and a persistent absolute lymphocytosis

TERMINAL QUESTIONS

1. Classify Acute Lymphoblastic Leukemia
2. Describe FAB classification of Acute Myeloid Leukemia
3. Describe Diagnostic tools in Leukemia
4. Write briefly on Chronic Myeloid Leukemia
5. Write briefly on Chronic lymphoblastic leukemia
6. Write briefly on Myelodysplastic syndromes
7. Write briefly on chronic myeloproliferative disorders.

ANSWERS TO INTEXT QUESTIONS

23.1

1. Presence of blast cells
2. Presence of more mature cells

3. Myeloperoxidase, sudan Black B, Periodic Acid Schiff reaction, Acid Phosphatase, Esterase, Azo dye, Toludine Blue stain
4. Periodic Acid Stiff (PAS)
5. Acid phosphatase
6. Esterase stain
7. Immunophenotyping
8. Lymphoblasts

23.2

1. Chronic Myeloid Leukemia
2. Auer rods
3. Leukocyte Alkaline Phosphatase
4. Phildelphia
5. Sudan Black B stain
6. French-American-British
7. Azurophilic
8. Leukocytosis and absolute Lymphocytosis

Notes

24

HAEMOSTASIS

24.1 INTRODUCTION

Haemostasis means "arrest of bleeding". During haemostasis several mechanisms interact to slow blood flow, block the vessel wall defect with a platelet plug (primary haemostasis), convert fibrinogen to a jelly like fibrin clot (coagulation of blood) and later re-establish the flow of blood through a mechanism of slow clot lysis (fibrinolysis).

OBJECTIVES

After reading this lesson, you will be able to:

- describe the 4 phases of Haemostasis
- describe the coagulation of blood
- explain Von Willebrand's Disease
- explain the causes of thrombocytopenia
- discuss bleeding Time test
- interpret PT/APTT/TT
- explain INR
- describe D – dimmer

24.2 HAEMOSTASIS

Haemostasis means "arrest of bleeding". During haemostasis several mechanisms interact to slow blood flow, block the vessel wall defect with a platelet plug (primary haemostasis), convert fibrinogen to a jelly like fibrin clot (coagulation of blood) and later re-establish the flow of blood through a mechanism of slow clot lysis (fibrinolysis). These complex physiological processes may be divided into phases:

A. Vascular Phase.

B. Platelet Phase.

C. Coagulation Phase and

D. Fibrinolytic Phase.

During haemostasis all the phases interact. It is convenient to consider them under these headings when investigating a patient with haemostatic problems.

A. The Vascular phase

The blood vessel plays a major role in maintaining the blood in a liquid state. The entire surface of the vessel wall is lined by a single layer of endothelial cells (EC) which rest on a basement membrane. The blood inside the blood vessel is not in touch with the sub-endothelial tissues. The EC secrete a major protein called the von Willebrand factor (VWF) into the plasma and sub-endothelium. External to the basement membrane lies the sub-endothelium rich in collagen, elastin, fibronectin, tissue factor (TF), VWF etc. External to the sub-endothelium lies the smooth muscle layer of variable thickness depending on the type of vessel. External to the smooth muscle layer is the adventitial layer. Figure 24.1.

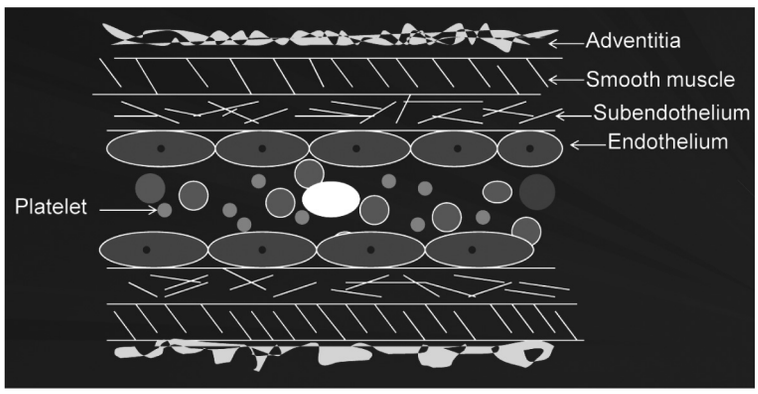


Fig. 24.1: The Blood Vessel Structure

Table I summarizes the role of the Vascular Phase.

Table I. Role of Vascular Phase in Haemostasis.

Blood vessel	Property	Function
Endothelial cell	Non reactive surface	Smooth blood flow Platelets are repelled
	VWF	1. Binds to platelet GPIb-IX and GPIIb-IIIa and mediates platelet adhesion to vessel wall. 2. Carries FVIII and prevents its early degradation.
	Antiplatelet activity	Negative charge on EC repels platelets, prostacyclin production and local degradation of ADP

	Anticoagulant property	1. Heparan sulphate on EC + antithrombin III inactivate serine proteases ie, thrombin, FXIa, FIXa etc. 2. Thrombin + Thrombo-modulin on EC activate Protein C and S which inactivates FVa and FVIIIa
	Fibrinolytic activity	EC releases plasminogen activator
Sub endothelial tissues	Collagen, elastin, VWF etc	Activate platelets
	Tissue factor	Complexes with FVII to form tissue thromboplastin for extrinsic pathway
Smooth muscle	Vasoconstriction and dilatation	Regulates blood flow

Table I:- EC – endothelial cell, VWF – von Willebrand factor, GP – glycoprotein

B. The Platelet Phase

Platelets are formed from megakaryocytes in bone marrow. The normal platelet count is 150- 450 x10^9/l. The life span of the platelet is 8 - 9 days. On a stained blood smear platelets are anucleate, round or discoid bodies, 1 – 2 mm in size with fine purple pink granules. The platelet has a phospholipid cell membrane into which are inserted glycoproteins (GP) which act as major cell receptors and antigens of the platelets. The role of platelets in haemostasis may be summarized as follows:

1. **Adhesion**. When EC is damaged platelets adhere to sub-endothelial tissues. Normal platelet number, presence of the receptor GPIb-IX complex on the platelet membrane (receptor for VWF), VWF in plasma and normal structure of collagen are necessary for adhesion. Figure 24.2

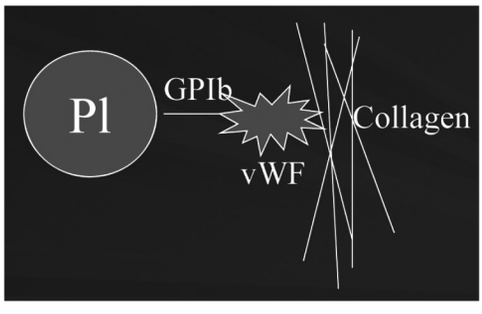
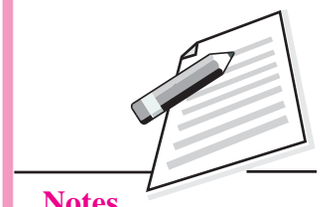


Fig. 24.2: Platelet Adhesion

2. **Aggregation**. Platelets then stick to one another to form the primary platelet plug. Normal platelet number, presence of GPIIb-IIIa complex on the



platelet surface (receptor for fibrinogen), plasma fibrinogen and calcium ions are necessary for platelet aggregation. Figure 24.3.

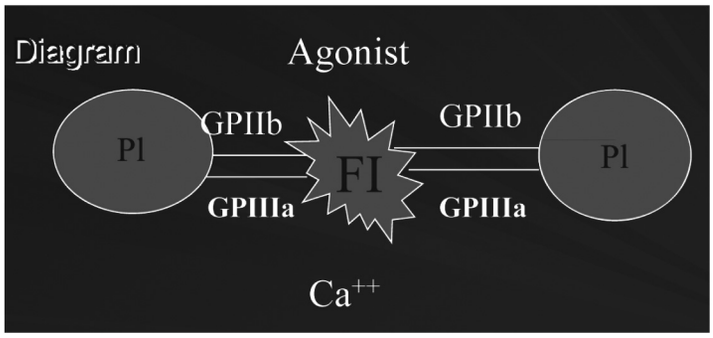


Fig. 24.3: Platelet Aggregation

3. **Platelet Release reaction**. As platelets aggregate they release their a and d granule contents which further sustain the aggregation response.
4. **The phospholipid surface** for the formation of coagulation complexes during blood coagulation is provided by the plasma membrane of the platelets.
5. **Clot retraction.** The GPIIb-IIIa complex on platelets anchor fibrin strands and pull them together to ensure a strong fibrin clot.
6. **Wound healing.** Platelet derived growth factor (PDGF) released from a granules promotes fibroblast proliferation and healing.

The platelet plug that is formed provides primary haemostasis. This must be reinforced with fibrin deposition to sustain haemostasis.

Table II lists some of the **abnormal platelet - vessel wall interactions** which result in bleeding.

Table II Abnormalties in Primary Haemostasis

Defect	Condition
Abnormal collagen (adhesion defect)	Ehlers Danlos syndrome, Marfans syndrome, Senile purpura
Absence of GPIb-IX (adhesion defect)	Bernard Soulier syndrome
Absence of VWF (adhesion and coagulation defect)	Von Willebrand's disease
Absence of GPIIb-IIIa (aggregation defect)	Glanzmann,s thrombasthenia, drug induced.
Absence of FI (aggregation + coagulation defect)	Afibrinogenemia

Absence of a or d granules or both (release defect)	Gray platelet syndrome and d storage pool disorder
Absence of receptors for ADP, collagen epinephrine, arachidonic acid etc.	Aggregation and release defects, drug induced
Absence of phospholipid release	Scott syndrome
Absence of enzyme systems	"aspirin" like defects

INTEXT QUESTIONS 24.1

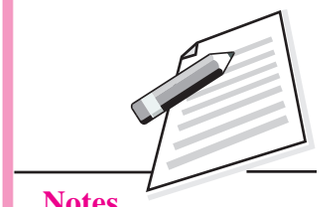
1. Haemostasis means .....
2. The endothelial cells of blood vessel secrete a protein called ..... factor
3. Platelets are formed from ..... in bone marrow
4. Normal platelet count is .....
5. When endothelial cell is damaged ..... adheres to sub-endothelial tissues
6. ..... ions is necessary for platelet aggregation
7. ..... factor promotes fibroblast proliferation and healing
8. Absence of phospholipid release causes ..... syndrome

C. The Coagulation Phase

In this phase liquid blood coagulates into a stable jelly like clot. Table III lists the coagulation proteins.

Table III Coagulation Factors

Factor	Synonym	Production site	Plasma half life	Replacement
FI	Fibrinogen	Liver	3 - 5 days	CRYPPT.
FII*	Prothrombin	Liver	2.5 – 3 days	FFP, PCC
FIII	Tissue factor	Subendothelium	-	-
FIV	Calcium ions	-	-	-
FV**	Labile factor Proaccelerin	Liver, mega-karyocyte	0.5 day	FFP



FVII*	Stable factor, Proconvertin	Liver	3 – 4 hours	FFP, Plasma, rFVII
FVIII**	Anti haemo-philic globulin	Liver	8 – 12 hours	CRYPPT. FFP, FVIII conc.
FIX*	Christmas factor	Liver	1 day	FFP, Plasma, PCC, FIX conc.
FX*	Stuart Prower factor	Liver	1.5 days	FFP, Plasma, PCC
FXI	Plasma thromboplastin antecedent	Liver	3 days	FFP
FXII	Hageman factor	Liver	2 days	FFP
FXIII	Fibrin stablising factor	Liver	9 – 10 days	CRYPPT, FFP
	Prekalleikrein**			FFP
	High molecular weight kinnin-ogen**			FFP

Table III: *Vitamin K dependent factors, ** cofactors. FFP – fresh frozen plasma, CRYPPT – cryoprecipitate, PCC – prothrombin complex

Blood coagulation takes place in a series of enzyme reactions, each successive step being catalysed by the active enzyme formed in the previous step. The process gains speed and force like a cascade. During the process the activated enzymes form **complexes** on phospholipid membranes in the presence of calcium ions. This localizes the clotting process to the site of injury. The complexes are formed by the vitamin K dependent factors (F II, VII, IX and X). These factors are produced in the liver in their precursor form. They bear g glutamic (Gla) residues at the amino terminal end of the molecule. A post ribosomal modification in the hepatocyte involves carboxylation of the Gla residues in the presence of a carboxylase, vitamin K, carbon dioxide and oxygen. The g carboxy glutamic acid residues of these proteins bind calcium ions which then serve as a bridge to bind the proteins to phospholipid surfaces.

For ease of understanding the Coagulation Phase has been divided into (a) Intrinsic Pathway (b) Extrinsic Pathway and (c) Common Pathway as shown in Figure 24.4

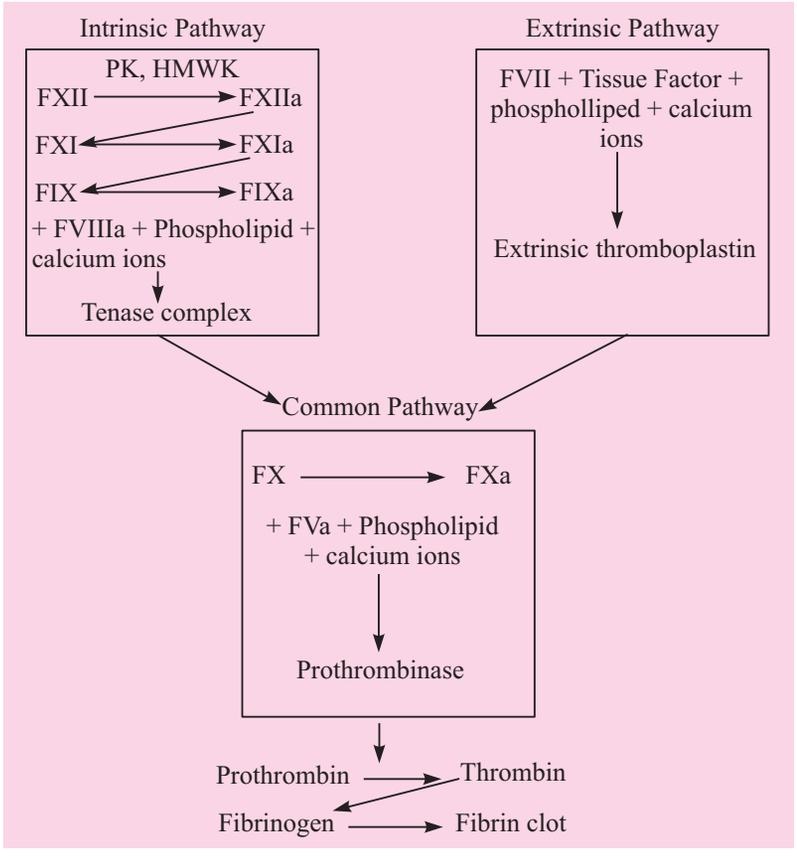


Fig. 24.4: Schematic representation of the coagulation pathways

In the Intrinsic pathway, when blood comes into contact with a non endothelial surface, FXII is activated to FXIIa. Prekallikrein and High Molecular Weight Kinninogen are cofactors. FXIIa activates FXI to FXIa which in turn activates FIX. FIXa complexes with phospholipid (PL) in the presence of calcium ions and activated FVIII to form a complex called tenase or intrinsic thromboplastin.

In the Extrinsic Pathway, when blood comes in contact with tissue factor present in the subendothelium, FVII is activated and forms a complex with TF, PL and calcium ions to form extrinsic thromboplastin.

In the Common Pathway, both intrinsic and extrinsic thromboplastins activate FX. FXa forms a complex, Prothrombinase, with FVa, PL and calcium ions. Prothrombinase converts prothrombin to thrombin. Thrombin is a potent serine protease which splits off fibrinopeptides A and B from fibrinogen to produce fibrin monomers. Fibrin monomers polymerize to form fibrin clot.

Thrombin also activates FV, FVIII, FXI and FXIII. In the body the activation of FVII through TF and the generation of small amounts of thrombin is the

dominant pathway. Once thrombin is generated the process is sustained through the intrinsic pathway.

FXIIIa acts on the fibrin clot in the presence of calcium ions to stabilize it.

The extrinsic and common pathways are tested "in vitro" by the Prothrombin time (PT) and the intrinsic and common pathways are tested by the activated partial thromboplastin time (APTT). The final conversion of FI to fibrin is tested by the Thrombin Time (TT). Since FXIIIa acts only after fibrin formation, its activity is not evaluated by PT or APTT.

Disorders of the coagulation pathway due to a **single factor** deficiency are usually inherited disorders. Haemophilia A or FVIII deficiency is the most common example. Multifactor deficiencies are usually acquired as seen in severe liver disease, disseminated intravascular coagulation or massive transfusion of bank blood

D. The Fibrinolytic Phase.

Fibrinolysis achieves recanalization of a blood vessel blocked by fibrin deposition. Plasminogen is a protein made in the liver and circulates in blood. Plasminogen is converted to the active enzyme, plasmin, by the action of tissue plasminogen activator (tPA) released from damaged EC. Plasminogen activators are also present in milk, semen, prostatic bed etc. Plasmin that is formed is immediately inactivated by circulating antiplasmin. Small amounts of plasmin adsorbed onto fibrin strands digest fibrin from within the clot. Fibrin breaks down to form fibrin degradation products (FDP) which are cleared by macrophages. Plasmin can also digest fibrinogen.

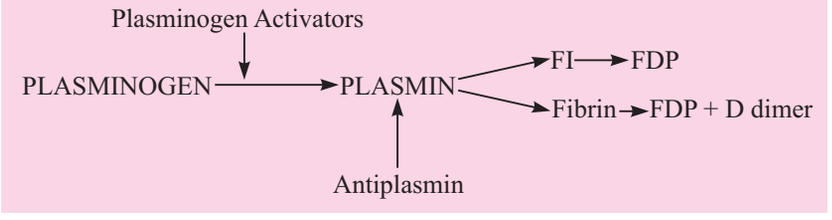


Fig. 24.5: The Fibrinolytic Pathway

Localization of the haemostatic process.

Uncontrolled and disseminated fibrin deposition is prevented by several mechanisms:-

1. Formation of membrane bound complexes localizing clot formation to site of injury.
2. Blood flow removes and dilutes activated coagulation factors.

Notes

3. Presence in plasma of normal inhibitor proteins which inactivate the activated coagulation factors. These are
   (a) Antithrombin III formed by the liver and complexed to heparan sulfate and inactivates the serine proteases – Thrombin, FXIa, FXa, FIXa, FVIIa.
   (b) Proteins C and S which are vitamin K dependent factors which inactivate FVa and FVIIIa.
   (c) Antiplasmin inactivates plasmin.
   (d) Tissue factor pathway inhibitor inhibits tissue factor.

Abnormalties of Haemostasis may result in **Bleeding** (Congenital or Acquired) and **Thrombosis** (Arterial or Venous)

INTEXT QUESTIONS 24.2

1. The extrinsic and common pathways are tested by ..... test
2. The intrinsic and common pathways are tested by ..... test
3. Final conversion of F1 to fibrin is tested by ..... test
4. Abnormalities of Haemostasis may result in ..... and .....

24.3 BLEEDING DISORDERS

All patients who present with bleeding need to be investigated. **It is also good practice to screen patients for a bleeding tendency prior to subjecting a patient to any form of surgery.** Preoperative evaluation of haemostasis occurs at two levels – an adequate history and screening tests.

History. Specific questions should be directed to

(a) Is there a history of a bleeding tendency? If so, what was the **age of onset** of the problem? Onset in childhood is associated with congenital causes. Recent onset suggests an acquired disorder eg. drug induced or liver disease.

(b) What is the **type of bleeding**? Bleeding into the skin and from mucosal surfaces – bruising, epistaxis, gum bleeding, menorrhagia suggest a vascular or platelet related problem. Bleeding into subcutaneous tissues, haematoma formation and haemarthrosis suggest a coagulation factor deficiency.

(c) **Delayed wound healing**, bleeding from umbilical stump, intracranial bleeds and frequent abortions are associated with FI and FXIII defects.

(d) Assess **severity of bleeding tendency** from frequency of episodes, days of hospitalization, transfusion requirements.

(e) Detailed history of **drug intake** especially of antiplatelet agents should be taken.

(f) **Family history**. In general haemophilia A and B are sex linked recessive disorders, von Willebrand's disease is autosomal dominant and all other coagulation factor deficiencies and platelet disorders are autosomal recessive in nature.

24.4 SCREENING TESTS FOR HAEMOSTASIS

These tests detect most abnormalities in haemostasis. If found to be abnormal the patient is referred for definitive tests which may be available only in specialized laboratories.

24.4.1 Blood Samples to be taken for screening tests are:

(a) 1 – 2 ml blood in EDTA anticoagulant for complete blood count. Platelet count done from a finger prick is not reliable.

(b) 4.5 ml venous blood mixed with 0.5ml of 3.2% sodium citrate for plasma clotting tests.

Blood must be taken from a clean venepuncture. If sampling is to be done from a central line, it must be flushed with normal saline, the first 4 – 5 ml of blood discarded and then the sample for tests must be drawn. The sample must be processed within 1 – 2 hours.

24.4.2 Tests to be done are:-

24.4.2.1 Platelet count. All platelet counts must be verified by a blood smear.

24.4.2.2 Bleeding Time. (BT) This measures the platelet vessel wall interaction. Two methods are available – the Template method (normal 2-9minutes) and the Ivy method (normal 2 – 6minutes). The BT is very operator dependent. It is accepted now that the BT is not indicated as a routine screening test and that a history taken well is just as reliable. **The BT is a poor predictor of abnormal surgical bleeding** and need not be done prior to surgery. Indications for doing BT are assessment of platelet function 4 –5 days after stopping asprin, assessing platelet function when patient has mild thrombocytopenia (platelet count > $50x10^9/l$), diagnosis of VWD and platelet dysfunction and to evaluate response to FFP, cryoprecipitate or desmopressin in VWD prior to surgery.

24.4.2.3 Prothrombin time (PT) is the time taken for citrated plasma to clot on the addition of tissue thromboplastin and calcium chloride. It measures

the extrinsic and common pathways and will detect abnormalities of FI, II, V, VII and X. A control plasma is always run with the test. PT is considered prolonged if the test and control values show a greater than 3 second difference. The normal PT by the manual technique is 12 - 14 seconds. A mild elevation of the PT is an early indication of deranged hepatocelluar dysfunction. For patients on oral anticoagulants the PT is reported as **the INR or International Normalised Ratio** to avoid problems that arise from the use of thromboplastins of varying sensitivities. Commercial thromboplastins are assigned an ISI (International Sensitivity Index) value after comparisons with a WHO standard. The ISI is used to calculate the INR. The INR is calculated as:

INR = (Patient's PT ÷ Mean normal PT)^{ISI} The normal INR is 0.9 – 1.2.

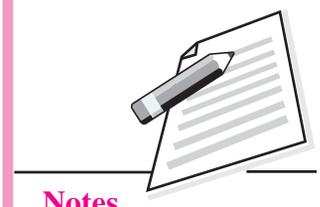
24.4.2.4 Activated partial thromboplastin time (APTT) has replaced the old and insensitive Clotting Time (CT) test. The APTT measures all the coagulation factors except FVII and FXIII. The normal manual APTT is 30 – 35". A greater than 5 second difference from the control is significant. A persistently short APTT (<25") indicates a hypercoagulable state. The APTT is used to monitor heparin therapy.

24.4.2.5 Thrombin time (TT) measures clottable fibrinogen. The normal TT is 12 –14" and a greater than 2 seconds difference from control is significant. TT is increased in deficiency of FI, dysfibrinogenemia, in the presence of heparin and with elevated levels of FDP.

24.4.2.6 Correction studies. When the PT/APTT/or TT are prolonged a correction study is performed by mixing equal parts of the test plasma and control plasma and repeating the test with the mixture. If the prolonged time is corrected, the study indicates a factor deficiency. Lack of correction indicates the presence of an inhibitor in the patient's plasma. Common inhibitors are heparin, lupus anticoagulant or an antibody to one of the coagulation factors.

24.4.2.7 Tests of fibrinolysis. For screening purposes it is sufficient to detect increased fibrinolysis in a patient using latex agglutination kits or ELISA techniques which demonstrate the presence of fibrin degradation products (FDP) and D-dimers.

24.4.2.8 Screening test for FXIII activity. Since FXIII acts after clot formation, the plasma clotting tests do not measure FXIII activity. A screening test called clot solubility in 5M urea or 1% acetic acid is performed to screen for FXIII activity.



INTEXT QUESTIONS 20.3

1. INR stands for .....
2. Normal INR is .....
3. Normal prothrombin tine is .....
4. Activated partial thromboplastin time measures are coagulation factors except ..... & .....
5. Normal Activated partial thromboplastin time is .....
6. Normal thrombin time is .....

24.5 COMMON BLEEDING PROBLEMS

24.5.1 Vascular Disorders

Many disorders of abnormal blood vessel structure are present as inherited or acquired conditions. Many of these disorders are present as syndromes and recognized because of typical signs. They present with easy bruising and purpura.

Von Willebrand's Disease

This is an inherited disorder of vascular dysfunction. The VWF is normally produced by the endothelial cells and secreted into plasma. The VWF has two functions (1) to mediate platelet adhesion to subendothelial collagen and (2) to carry FVIII and prevent it from being destroyed. VWD is an autosomal dominant disorder and occurs in both males and females.

Laboratory Diagnosis

1. Haemoglobin, PCV, RBC count are normal unless there is blood loss.
2. Platelet count is normal, morphology is normal.
3. Bleeding time is increased
4. Prothrombin time is normal, Thrombin time is normal
5. APTT is increased
6. FVIII activity is decreased
7. FXIII is normal

Treatment is infusion of FFP or cryoprecipitate both of which contain VWF.

24.5.2 Platelet Disorders

Thrombocytopenia is the most commonly encountered haemostatic problem. The availability of platelet concentrates for replacement therapy has made surgery possible for such patients.

Causes of Thrombocytopenia

A. Failure of Platelet Production in bone marrow.

Aplastic anaemia, Leukemia, Radiation, chemotherapy, Alcholism, Megaloblastic anaemia, marrow infiltration by tumour, lymphoma.

B. Increased destruction of platelets

(a) Immune causes – ITP (immune thrombocytopenic purpura), SLE, drug induced, post transfusion, malaria, viral infections, neonatal.

(b) DIC

(c) Hemolytic uremic syndrome, TTP

(d) Sepsis

C. Abnormal distribution of platelets – splenomegaly

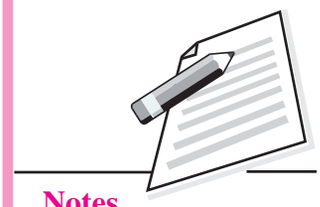
D. Dilutional – massive transfusion

Laboratory diagnosis

1. Complete blood counts shows thrombocytopenia
2. Platelet morphology may be abnormal depending on the cause for low platelets
3. Bleeding Time variably prolonged
4. Plasma clotting tests are normal unless there is factor consumption
5. Bone marrow examination to determine cause of thrombocytopenia

Disorders of Platelet Function are given in Table II

Coagulation Factor Deficiency: The deficiency of a single factor is usually due to an inherited deficiency. The commonest factor deficiency is Haemophilia A which is FVIII deficiency. Haemophilia B or deficiency of FIX is less common. Both haemophilias present with haematoma formation and haemarthrosis and are inherited as sex linked recessive disorders. Clinically severe form of the disorder has less than 1% factor activity and is associated with spontaneous bleeding by one year of age. Moderate deficiency has 2 – 5% factor activity and is associated with bleeding following trauma and haemarthrosis. Mild form of the disease has 5 – 15% factor activity and is asymptomatic unless the patient is subjected to trauma or surgery. The APTT will detect these patients whereas the CT will definitely miss them.



Laboratory Diagnosis

1. Haemoglobin, PCV, RBC normal or variably deranged.
2. MCV, MCH,MCHC,RDW normal
3. WBC count normal
4. Platelet count normal
5. Bleeding Time normal
6. PT normal
7. APTT prolonged
8. TT normal
9. FVIII/FIX assay.

Rare disorders of other coagulation factors are autosomal recessive and may involve any of the coagulation factors.

Acquired Disorders

(a) Liver disease – Most of the coagulation factors are made in the liver and hence chronic liver disease is associated with multifactor deficiency characterized by prolonged PT, APTT, low fI and increased D-dimers.

(b) Vitamin K deficiency.

Vitamin is a fat soluble vitamin which is needed for the normal formation of the Vitamin K dependent factor – FII,FVII, FIX and FX. Vitamin K needs bile for absorption. Deficiency is seen in

(a) Newborns: This is called haemorrhagic disease of the new born. It occurs because the liver of the newborn is immature and mother's milk lacks vitamin K. PT and APTT are markedly prolonged and TT is normal

(b) Obstructive jaundice

(c) Liver disease.

(d) Oral anticoagulants used to prevent thrombosis are vitamin antagonists

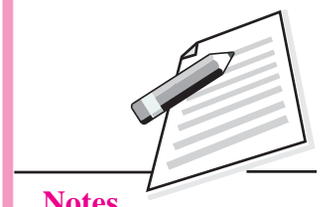
(c) Thrombotic Disorders

Thrombosis in blood vessels may be arterial (eg heart attack, stroke) or venous (eg Deep vein thrombosis). The conditions that are associated with thrombosis are usually acquired. They may rarely be inherited due to the decreased levels of naturally occurring inhibitors in blood. Thrombosis occurs as a result of abnormalities in blood vessels like atherosclerosis, stasis or pooling of blood, inflammation of blood vessels and abnormalities in blood flow due to viscosity, increased levels of fibrinogen, FVIII etc.

Notes

WHAT HAVE YOU LEARNT

- Haemostasis means arrest of bleeding
- Phases of Haemostasis are vascular phase, platelet phase, coagulation phase and fibrinolytic phase
- Blood vessel plays a major role in maintaining the blood in the liquid state
- The entire surface of the vessel is lined by Endothelial cells (EC)
- Endothelial cells secrete a major protein called Von Willebrand factor into plasma and sub-endothelium
- Platelets are formed from megakaryocytes in bone marrow and normal count is 150 – 450 X 10^{9}/L
- When endothelial cells is damaged platelets adhere to sub endothelial cells
- Platelet derived growth factor released from á granules promotes fibroblast proliferation and healing
- Blood coagulation takes place in a series of enzyme reactions each successive step being catalysed by active enzyme formed in previous step
- Coagulation phase has been divided into Intrinsic pathway, Extrinsic pathway and common pathway
- Extrinsic and common pathway is tested by Prothrombin Time (PT)
- Intrinsic and common pathway is tested by Activated Thromboplastin Time (APTT)
- Final conversion of FI to fibrin is tested by Thrombin time (TT)
- Hemophilia A or FVIII deficiency is a disorder of coagulation pathway due to single factor
- Abnormalities of haemostasis may result in Bleeding & Thrombosis
- Bleeding time measures platelet vessel wall interaction and is a poor predictor of abnormal surgical bleeding
- Prothrombin time (PT) is the time taken for citrated plasma to clot on the addition of tissue thromboplastin and calcium citrate
- Normal PT is 12-14 seconds and for patients on oral anticoagulants, PT is reported as INR or International Normalised Ratio and normal INR is 0.9 – 1.2
- Activated partial thromboplastin time (APTT) measures all coagulation factors except FVII and FXIII. Normal APTT is 30-35 seconds
- Thrombin time (TT) measures clottable fibrinogen and normal TT is 12 – 14 seconds



TERMINAL QUESTIONS

1. Draw and describe the coagulation pathway.
2. Short notes on:
   (a) Platelet function
   (b) Haemophilia
   (c) Prothrombin time

ANSWERS TO INTEXT QUESTIONS

24.1

1. Arrest of bleeding
2. Von Willebrand
3. Megakaryocytes
4. 150-450x10^9/l
5. Platelets
6. Calcium
7. Platelet derived growth
8. Scott

24.2

1. Prothrombin time
2. Activated thrombopastin time
3. Thrombin time
4. Bleeding and thrombosis

24.3

1. International Normalised Ratio
2. 0.9 – 1.2
3. 12-14 seconds
4. FVII & FXIII
5. 30 – 35 seconds
6. 12 – 14 seconds

Notes

25

AUTOIMMUNE HEMOLYTIC ANEMIA (AHA)

25.1 INTRODUCTION

AHA is a group of anemia in which an increased hemolysis is caused by antibodies directed against the patient's own RBCs. These auto antibodies are usually of Ig Type.

OBJECTIVES

After reading this lesson, you will be able to:

- define autoimmune hemolytic anaemia
- classify autoimmune hemolytic anaemia
- explain the diagnosis of autoimmune hemolytic anaemia

25.2 CLASSIFICATION

Autoimmune hemolytic anaemia is classified based on the etiology and also on the of temperature at which antibody reacts with RBC.

Etiological Classification

Etiologically autoimmune hemolytic anemia is classified as idiopathic and secondary. Secondary causes may be because of drugs like methyl dopa, mefenamic acid and L-dopa and diseases like malignancy of lymphoreticular system, systemic lupus erythematosus (SLE), rheumatoid arthritis, ulcerative colitis, paroxysmal cold hemoglobinuria (PCH), infections like Mycoplasma pneumonia and Cytomegalovirus (CMV).

Basis of temperature of reaction with RBC

On the basis of the temperature at which the antibody reacts with RBC, autoimmune hemolytic anemia may be classified as that caused by warm antibodies (which combines with RBC at a temperature of 37°C) and by cold antibodies (which combines at cooler peripheral circulation at temperature between 30 – 32°C to 24°C). Cold active antibodies are usually IgM, fix complement and lead to intravascular red cell destruction. Warm active antibodies are typically IgG, may or may not fix complement and destruction of RBCs occurs in the spleen.

Etiological Classification of AHA

- Idiopathic (50%)
- Secondary (50%)
  - DRUGS
    - e.g: methyl dopa mefenamic acid
    - L-dopa procainamide
  - DISEASES
    - Malignancy of lympho reticular system, SLE, RA, ulcerative colitis, paroxysmal cold hemoglobinuria (PCH) Infections: Mycoplasma pneumonia, CMV

AHA can also be classified according to the temperature at which antibody reacts with RBC.

TYPES OF AUTO ANTIBODIES

- **Warm Antibodies**
  They combine with RBCs at a temperature of 37° C
  - Idiopathic
  - **Secondary**
    Lymphomas, SLE
    Infections Drugs: methyldopa
- **Cold antibodies**
  They have little/no activity at 37°C
  Affinity increases as temperature decreases
  - Idiopathic
    Cold agglutinin disease
  - Secondary
    Mycoplasma pneumonia
    Infectious mononucleosis
    Lymphomas, PCH

25.3 DIAGNOSIS OF AUTOIMMUNE HEMOLYTIC ANAEMIA

1. Tests for hemolysis:

   Complete blood count with peripheral blood smear, serum bilirubin, lactate dehydrogenase, serum haptoglobin and urine hemoglobin

2. Serological test:

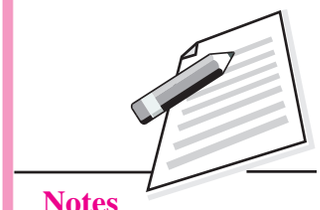
   The diagnosis of AHA depends on demonstrating the auto antibodies on surface of RBCs. This is done using the antiglobulin test wherein adding Antihuman Globulin (AHG) serum causes red cells to agglutinate.

INTEXT QUESTIONS 25.1

1. Autoimmune hemolytic anemia is caused by ..... type of antibodies
2. Autoimmune hemolytic anemia may be classified based on ..... and also on the basis of .....
3. Secondary etiological causes of autoimmune hemolytic anemia are ..... & .....
4. Warm antibodies combines with RBC at a temperature of .....
5. Cold antibodies combines with RBC at a temperature between ..... to .....
6. Autoimmune hemolytic anemia can be detected by ..... test
7. Secondary causes of cold antibodies of autoimmune antibodies are ..... & .....

WHAT HAVE YOU LEARNT

- Autoimmune hemolytic anemia are caused by antibodies directed against the patient's own RBCs which are usually Ig type
- Autoimmune hemolytic anemia are classified based on etiology and on the temperature at which antibody reacts with RBCs
- Etiological causes are drugs like methyl dopa, mefanamic acid, L-dopa and diseases like malignancy of lymphoreticular system, SLE, RA, ulcerative colitis
- Based on the temperature at which antibody reacts with RBCs they are classified as warm & cold antibodies



- Warm antibodies combine with RBCs at a temperature of 37°C
- Cold antibodies combine at temperature between 30 – 32°C and 24°C
- For diagnosis, tests for hemolysis and anti globulin test are done

TERMINAL QUESTIONS

1. Classify autoimmune hemolytic anaemia
2. Explain the diagnosis of autoimmune hemolytic anaemia

ANSWERS TO INTEXT QUESTIONS

25.1

1. Ig
2. Etiology & temperature at which antibody reacts with RBC
3. Drugs & Diseases
4. 37°C
5. 30 – 32°C to 24°C
6. Antiglobulin test
7. Infections & lymphoma

Notes

26

HEMOLYTIC DISEASE OF THE NEW BORN (HDNB)

26.1 INTRODUCTION

Hemolytic disease of the newborn is a condition in which IgG antibodies from maternal blood cross the placenta into the fetal circulation where they react with fetal red cells and break them.

OBJECTIVES

After reading this lesson, you will be able to:

- explain the pathology of Hemolytic disease of the newborn
- discuss the antenatal assessment of Hemolytic Disease of Newborn
- describe the tests on maternal and cord blood at delivery
- explain the prophylaxis for hemolytic disease of new born

26.2 PATHOPHYSIOLOGY

Hemolytic disease of the newborn is a condition which occurs due to destruction of fetal red cells by IgG antibodies from maternal blood. The antibodies cross the placenta during pregnancy and reach into the circulation of the fetus where they react with the red blood cells and break them. These antibodies are commonly directed against Rh or ABO blood group antigens on fetal red cells.

The risk of maternal immunization to blood group antigens depends on the following factors:

(a) Volume of incompatible fetal red cells that cross the placenta and reach the maternal circulation (fetomaternal hemorrhage)

(b) Immunogenicity of the red cell antigen and maternal immune response

Rh incompatibility reactions occur where an Rh negative mother becomes pregnant with an Rh positive fetus. A small number of RBCs from fetus cross the placenta to the mother's blood and the mother gets immunized. The first baby of these mothers is unaffected. However, during the second pregnancy with an Rh positive fetus, the passage of fetal blood cells to maternal blood causes a severe immune response. Large number of IgG antibodies are produced in the mother that cross the placenta, reach the fetal blood and destroy its RBCs. This fetomaternal hemorrhage can occur after caesarean section, vaginal delivery, abortion, invasive procedures or clinical maneuvers. Similar condition can occur in Rh negative females who receive incompatible blood transfusion.

The ABO incompatibility is much more common than Rh but is usually of less severity. The antibodies are usually of the IgM type (which cannot cross the placenta). IgG antibodies are more likely to occur when mother of blood group "O" carries fetus of either group A or B. The ABO incompatibility may be seen even in first pregnancy, but it is very less severe than Rh incompatibility because:

1. The fetal red cells express A&B blood group antigens weakly.
2. There is widespread distribution of carbohydrate antigens in fetal fluids & tissues which mimic A and B red cell antigens and neutralize large part of maternal anti-A and anti-B antibody.

26.2.1 Clinical Features

About 50% of the infants are asymptomatic or have mild disease, 30% have moderate anemia and hyperbilirubinemia and approximate 20% are severely affected and may develop hydrops fetalis

26.2.2 Laboratory Evaluation

All pregnant women should have their blood group (ABO and Rh) and antibody screening (indirect Coomb's test) done at the first visit to obstetrician. This will help to identify women who will require Rh immunoglobulin and monitor the increasing titer of anti D in maternal serum.

Tests on maternal and cord blood at delivery

The following tests should be carried out:

Notes

Tests on cord blood:

(a) ABO & Rh Group

(b) Direct Coomb's test

(c) Hemoglobin

(d) Bilirubin

Tests on maternal blood

(a) ABO & Rh Blood group

(b) Maternal red cell antibody (anti D) titre using indirect Coombs test

(c) Kleihauer- Betke acid dilution test for quantitative estimation of fetomaternal hemorrhage, this determines the prophylactic dose of anti D immunoglobulin to be given to mother.

(d) Amniotic fluid analysis by spectrophotometer; based on the fact that optical density of the amniotic fluid increases with its bilirubin concentration. Liley curve is plotted and management of the patients is based on the optical density at 450 nm.

(e) Noninvasive fetal monitoring using Doppler

Anti D Prophylaxis

Anti D is administered to Rh negative women during pregnancy and after delivery or abortion to minimize the risk of HDNB.

INTEXT QUESTIONS 26.1

1. Hemolytic disease of Newborn is due to ..... or ..... incompatibility
2. Rh incompatibility occurs when ..... mother becomes pregnant with ..... fetus
3. In ABO incompatibility the mother is of blood group ..... & the fetus is of group .....
4. Increased ..... in the serum is useful in identifying the risk of Hemolytic disease of Newborn
5. ..... test determines the feto maternal red cell damage

WHAT HAVE YOU LEARNT

- In HDNB IgG antibodies from maternal blood cross the placenta into the circulation of the fetus
- Majority of hemolytic disease of newborn is due to ABO incompatibility but cases of Rh incompatibility are clinically more severe
- Rh incompatibility occurs when Rh negative mother becomes pregnant with Rh positive fetus and the mother gets immunized when the fetal blood cells cross the placenta into maternal blood and produces antibodies
- During the second pregnancy when the fetal blood cells reach the maternal blood, large number of IgG antibodies are produced which cross the placenta and destroy fetal RBCs
- An increasing titre of Anti D in maternal serum is useful in identifying pregnancies at risk
- Anti D may be administered to Rh negative women during pregnancy and after delivery to minimize the risk of HDNB.

TERMINAL QUESTIONS

1. Explain the tests on maternal and cord blood at delivery
2. Explain briefly the pathogenesis of Hemolytic disease of Newborn

ANSWERS TO INTEXT QUESTIONS

26.1

1. ABO & Rh
2. Negative, Positive
3. O, A or B
4. Anti D titre
5. Kleihaurer- Betke test

Notes

27

BLOOD COMPONENTS

27.1 INTRODUCTION

In many clinical conditions a patient needs to be transfused blood taken from another person. This is the process of blood transfusion.

OBJECTIVES

After reading this lesson, you will be able to:

- describe blood components
- discuss the production of blood components
- explain the selection of blood components
- describe Hema pheresis

27.2 BLOOD COMPONENTS

Once blood has been taken from the donor it can either be kept as such to be used as whole blood or can be separated into its various components. Separating blood into its components has many advantages like:-

(a) Maximize the yield of products form a single donation.

(b) Ability to use optimal product for specific disease.

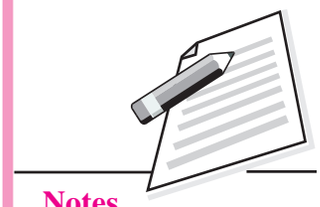
(c) Reducing the exposure of foreign material to the donor to minimum.

The most commonly used blood components are:-

(a) Whole blood.

(b) Packed cells.

(c) Platelet concentrate.



(d) Fresh frozen plasma (FFP)

(e) Cryoprecipitate

Others include:

(a) Leukodepleted packed cells

(b) Platelet rich plasma

(c) Platelet poor plasma

(d) Fresh plasma

27.3 PRODUCTION OF BLOOD COMPONENTS

Blood which is to be used for component production is collected into special collection system in which 2 or 3 smaller satellite bags are attached to the main collection bag.

Blood components are prepared from whole blood in large centrifuges which are refrigerated. The blood can be subjected to a light spin or a heavy spin depending upon components to be produced.

For preparation of platelet concentrate, centrifugation is performed at room temperature (20° C to 24° C); for all other blood components, centrifugation is carried out between 1°C and 6°C.

27.4 SELECTION OF BLOOD COMPONENTS

Whole Blood

Whole blood is the unmodified blood collected from the donor. 450 ml blood is collected from the donor which is added to 63ml of CPD (Citrate phosphate dextrose) or CPDA-1 anticoagulant.

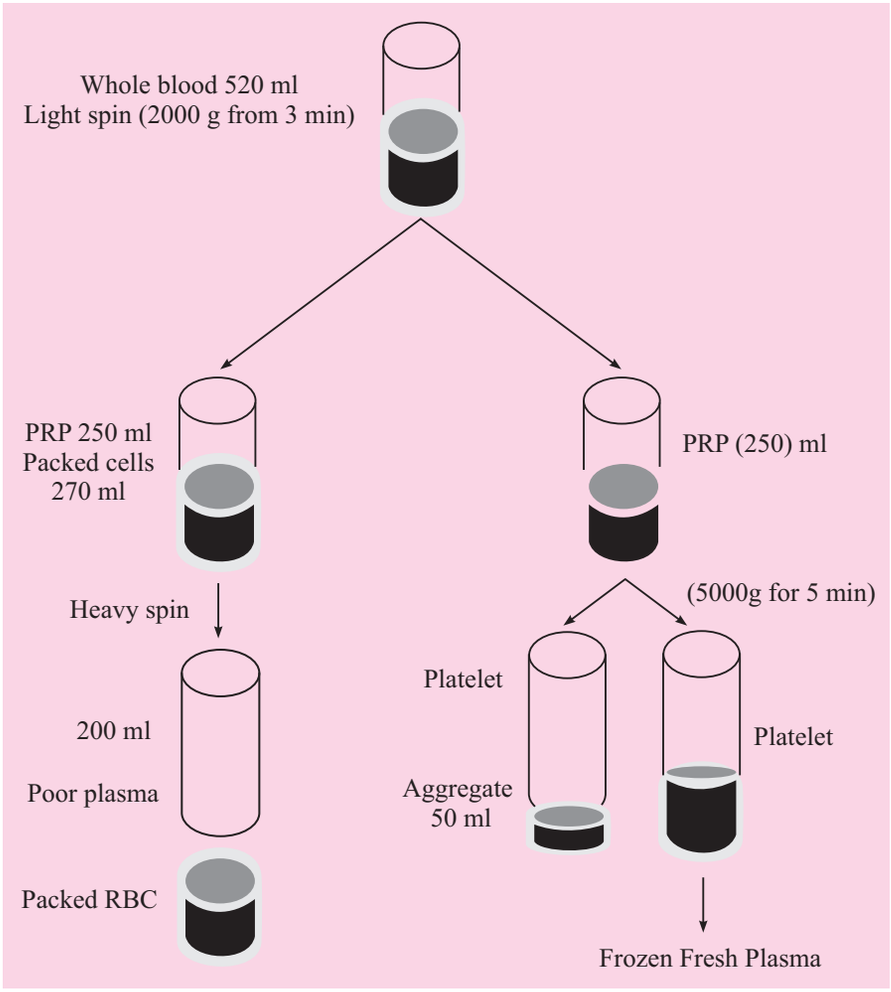
It can be stored at 1°-6°C for 21-35 days.

It is most commonly used in blood loss anemia e.g. Trauma

Packed Red Cells

The separation of RBCs from plasma concentrates the blood, the haematocrit is increased to 70%-80%. After removing plasma 100ml of an additional solution is added for long term storage, most common being CPD. This extends storage time to 42 days.

The major indication for use of packed red cells is chronic anemia when there is no loss of blood volume but of hemoglobin only.



Platelet Concentrates

Platelets are produced from the whole blood within eight hours of collection and are stable at room temperature (20° C-24° C) for up to 5 days. It is important that the pH is maintained at 6 or above for the platelets to remain functional. Each concentrate has a minimum of 5.5×10^{10} platelets.

Platelet concentrates are used to prevent bleeding from thrombocytopenia.

Fresh Frozen Plasma

Fresh frozen plasma is obtained by subjecting whole blood to a light spin and separating 200-260ml of upper part. It is then immediately frozen to -18° C or less within 8 hour of collection.

FFP is a rich source of plasma protein & coagulation factors. It is used in coagulation factor deficiencies & in disseminated intravascular coagulation (DIC).

27.5 HEMA PHERESIS

It is a procedure in which blood is withdrawn from the donor, anti coagulated and the desired component is separated from it. The remaining blood is returned back to the donor. Most commonly platelet aphaeresis is made in which platelets are separated from the donor blood and rest of the blood is transfused back.

INTEXT QUESTIONS 27.1

1. For preparation of platelet concentrate, centrifugation is performed at ..... temperature
2. ..... is used in blood loss caused from trauma
3. Packed red cells are prepared by separating ..... from plasma concentrates
4. Platelets concentrates are prepared from the whole blood within ..... hours of collection
5. Fresh frozen plasma is a rich source of ..... & .....

WHAT HAVE YOU LEARNT

- Blood taken from donor, can either be used as whole blood or can be separated into various components
- Commonly used blood components are whole blood, packed cells, platelet concentrate, fresh frozen plasma and cryoprecipitate
- Preparation of platelet concentrate, centrifugation is performed at room temperature at 20^{o}c to 24^{o}c.
- All other blood components are prepared by centrifugation carried out between 1^{o}c to 2^{o}c
- Whole blood is the unmodified blood collected from donor. 450ml blood is collected from the donor to which 63ml of CPD(Citrate Phosphate Dextrose) or CPDA-1 anticoagulant
- Packed red cells are prepared by separating RBCs from plasma concentration
- Platelets are produced from the whole blood within eight hours of collection

ANSWERS TO INTEXT QUESTIONS

27.1

1. 20°C – 24°C
2. Whole blood
3. RBC's
4. Eight hours
5. Plasma Protein & Coagulation factors