CHAPTER

12

Differential Leucocyte Count

Learning Objectives

After learning this practical the students should be able to:

1. Enlist the content of the Leishman stain and discuss their functions.
2. Make an ideal smear and describe the criterion for grading a smear as ideal smear.
3. Stain the smear for differential leucocyte count.
4. Evaluate the quality of smear.
5. Identify the neutrophil, eosinophil, basophil, monocytes and lymphocyte in smear slide.
6. Determine the number of each type of white blood cell present in the blood and expressed them as a percentage of each type among the total hundred cell counted.
7. Enlist the precautions while preparing the smear for differential leucocyte count.

Aim: To stain and study the different types of white blood cells in the smear. Determine the number of each type of white blood cell presents in the blood.

Principle: The drop of blood is used to make a peripheral smear which is fixed and stained with Leishman's stain and examined under oil immersion lens of microscope to conduct the differential leucocyte count.

Material and chemicals: Lancet, clean dry glass slides, cedarwood oil, Leishman's stain and microscope.

The constituent of Leishman stain is:

1. Eosin—it is a negatively charged acidic dye staining positively charged basic particles which include eosinophil granules and red blood cells.
2. Methylene blue—it is a positively charged basic dye staining negatively charged acidic particles such as basophil granules, cytoplasm and nuclei of the white blood cells.
3. Acetone free methyl alcohol which acts as a fixative and helps in fixing the blood smear over the slide and so also preserves the morphological characteristic of the white blood cells.

Note: Leishman stain is readily available in crystal form, 0.15 gm of Leishman stain powder is dissolved in 100 ml of acetone free methyl alcohol.

Procedure

I. Preparation of an Ideal Blood Smear

1. Make a sterile finger prick under aseptic precaution. Discard the first drop of blood and allow the blood to free flow.
2. Place a drop of blood at one of the ends of each of the glass slides.
3. Place the spreader slide at an angle of 45° just in front of the blood drop. (Fig. 12.1A)

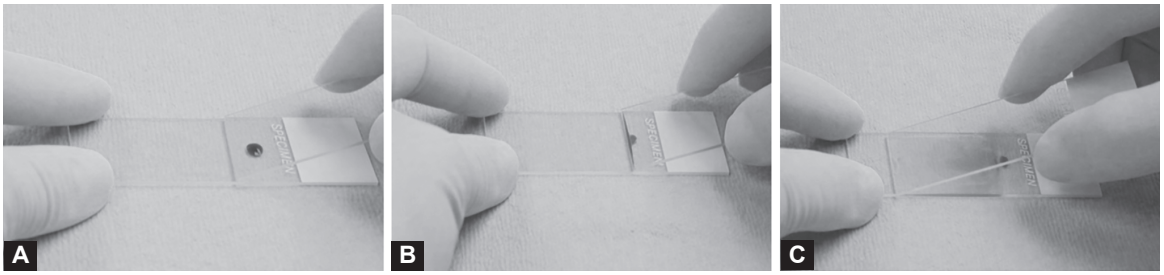


Fig. 12.1A to C: Technique of making the peripheral blood smear; (A) The spreader slide placed at an angle of 45 degrees just in front of the blood drop; (B) The drop spreads out along the line of contact of the spreader slide; (C) Move the spreader slide smoothly and evenly in forward direction maintaining an angle of 45 degrees; to make the blood smear.

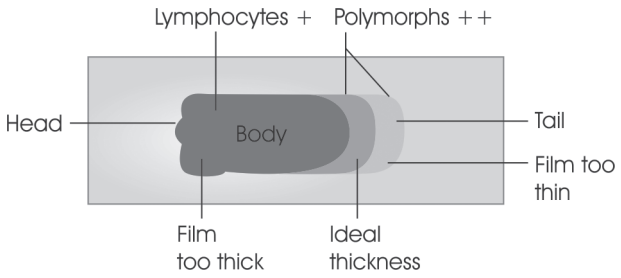


Fig. 12.2: Ideal peripheral blood smear should be ideally thick

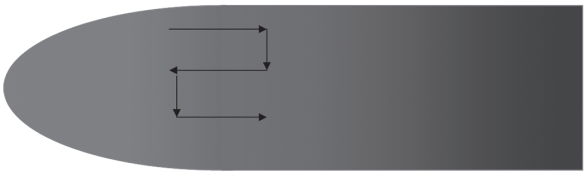


Fig. 12.3: Counting of cells in blood smear

N	E	N	N	N					

Fig. 12.4: Check box for cell count reporting

4. Move the spreader slide backwards to touch the blood drop.
5. The drop must spread out along the line of contact of the spreader slide (Fig. 12.1B)
6. Move the spreader slide smoothly and evenly in forward direction maintaining an angle of 45 degrees; to make the blood smear. (Fig. 12.1C)
7. Let the smear dry in air. Repeat the procedure and make three more blood smears.

A smear is described as having a head, body and tail.

Note: An ideal smear is uniform without any striations or vacuoles and neither too thin nor too thick.

Precautions for preparation of a blood smear:

1. Ensure that the complete drop of blood is uniformly spread along the edge of the spreader slide.
2. The gentle pressure should be constantly maintained when drawing the slide forward.
3. The spreader slide should be moved smoothly and evenly in forward direction maintaining an angle of 45 degrees; to make the ideal blood smear.
4. If spreader slide is moved slowly, the leucocytes will be unevenly distributed.
5. Place the slide on glass rods and let it dry.

Note: There should be no delay in making the smear after placing the drop of blood on the slide because delay will result in non-uniform distribution of the white blood cells, and most of the white cells will be visible at the thin edge of the smear.

Causes of Poor Blood Smear

1. If small drop of blood is taken it will produce thin slide; while large drop of blood will make the slide too thick.
2. If the spreader slide is not pushed smoothly and gently across the slide.
3. Failure to maintain an angle of 45 degrees with the slide while moving the spreader slide at a 30° angle.
4. The spreader slide is incompletely moved across the slide.
5. Use of contaminated greasy slide.

II. Fixing and Staining of Blood Smear

1. Place the slide on the staining stand rack in the washing sink.
2. Pour 10–12 drops of Leishman stain on the slide so as to cover it under the stain and wait for two minutes and this is the fixation time.
3. Add adequate amount of distilled water so that it spreads over the smear uniformly. Mix the water and stain by holding slide in hand and making a rocking movement or blowing over it gently. Ensure that the stain is completely covered with distilled water so as to clear any overstained portion.
4. Wait for another 8–10 minutes allowing time for proper staining.
5. After interval of 10 minutes wash the slide, holding it horizontally with tap water. Ensure water does not hit the smear directly, otherwise the stained portion may get removed.
6. Allow slide to dry placing it over the staining stand rack.

Note: The characteristic features of ideal blood smear.

1. An ideal smear covers around ¾ the entire slide area.
2. Ideal smear is tongue shaped, slightly rounded or curved at the feathery edge tail region. It is thick at head end, and thin at tail end.
3. The upper and lower edges of the smear are clear and visible.
4. The smear is smooth without any streaks or vacuoles.
5. It is single cell layer thick.

Examination of a blood film

1. The dry and stained film is examined without a coverslip under oil immersion objective.

2. Place a drop of cedarwood oil over the chosen area and move the oil immersion objective into position; making the lower end of the objective touch the drop of oil.
3. Focus the cell using fine adjustment knob.
4. The differential cell count is done by moving the slide along the central and periphery of the smear. A total of 100 cells are to be counted in which every white cell seen must be recorded as neutrophil, basophil, eosinophil, monocytes and lymphocyte appropriately.
5. White blood cells are found more in numbers along the upper edge, lower edge and tail but are poorly stained. The lymphocytes are more along body area while monocytes and neutrophils along the edges and tail of the smear.

Precaution while counting WBC under smear: Make your observation in one field and record the number of WBC according to its type; then move to another field in the snake-liked direction as shown in Fig. 12.3.

PRECAUTIONS

1. Clean and dry the slides before use.
2. The glass-spreader must have a smooth and clean edge for uniform spreading of the blood drop.
3. Prepare 2–3 slides at a time for practice and obtain an ideal smear.
4. Well-stained slide should be examined under the oil-immersion lens for appropriate results.

Then Find the Percentage of Each Type

Results

N = ... %, E = ... %, B = ... %,

L = ... %, M = ... %

Observe the cells and note them successively in the squares below.

Key Notes

Avoid delay while making blood smear after drop of blood is placed on slide. The delay may lead to abnormal distribution of the white blood cells, and a few large white cells may accumulate at the thin edge of the smear. Pressure to the spreader slide—the fingers should be positioned on the spreader slide as far towards edge as possible and then apply moderate and even pressure along the spreader slide.

CHARACTERISTICS OF DIFFERENTIAL LEUCOCYTES

Granulocytes

Neutrophils (Fig. 12.5)

1. They are also known as polymorphonuclear leukocytes.
2. They are ten to fourteen micron in diameter.
3. The nucleus is multilobed (1–6 lobes), cytoplasm is bluish pink in colour with plenty of red brown or purple colour granules.
4. Their main function is phagocytosis of bacteria. They are the first cells to reach an injured tissue and their count increases in the acute phase of any infections.
5. The increase in neutrophil is called neutrophilia. It is commonly seen in acute bacterial infections, lung abscesses, burns, acute haemorrhage and in physiological conditions such as after muscular exercise, after meals, pregnancy, lactation, etc.
6. The decrease in neutrophil is called neutropenia. It is seen in typhoid, paratyphoid fever, viral infections, malaria, aplastic anaemia and in bone marrow destruction due to irradiation.

Eosinophil (Fig. 12.5)

1. Eosinophil is 10–14 micron in diameter.
2. The nucleus is purple in colour and has bilobed and spectacle-shaped or horseshoe-shaped nucleus.

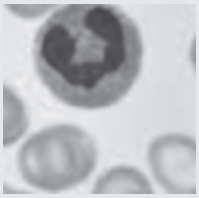
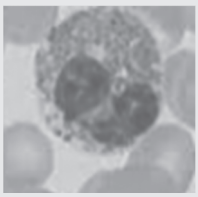
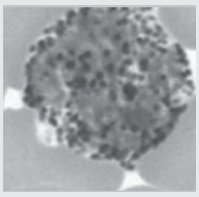
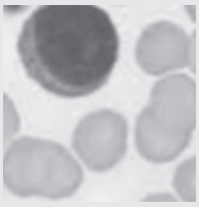
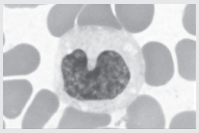
Revise the characteristic features of WBC			
Differential leucocytes	*Percentage of white blood cells*	*Morphology*	*Function*
Neutrophils 	50–70%	10–14 µm in diameter, multilobed nucleus (2–6 lobes) purple in colour, cytoplasm is blue in colour and fine sand like red brown coloured granules are present	Phagocytosis—engulf and destroy bacterial pathogens and also act as mediators of pyrexia response
Eosinophils 	1–4%	10–14 µm in diameter, nucleus purple in colour and has bilobed nuclei; eosinophilic pink coloured cytoplasm with large, coarse brick red coloured granules	Play a role in limiting allergic reactions. Mild phagocytic action and provides local mucosal immunity
Basophils 	< 1%	10–14 µm in diameter, purple coloured bilobed nuclei, basophilic blue coloured cytoplasm, coarse, large purplish blue granules overlying the nucleus	Function as mediators of inflammation, mild phagocytic action and contains hepari which is an anticoagulant
Lymphocytes 	20–40%	Small lymphocyte: 7–10 µm in diameter, large lymphocyte: 10–14 µm in diameter, dense, purple staining, oval or round nucleus; little cytoplasm	The most important cells of the immune system; effective in fighting infectious organisms; act against a specific foreign molecule (antigen)
Monocytes 	2–8%	10–18 µm in diameter indented kidney-shaped nucleus, pale in colour, cytoplasm abundant pale blue in colour and cell is agranular	Transform into macrophages; phagocytic cells and detoxify killing tumor cells

Fig. 12.5: Characteristic features WBC (neutrophil, eosinophils, basophils, lymphocytes, monocytes)

3. The cytoplasm is pink in colour having large coarse brick red coloured granules.
4. The eosinophil helps in phagocytosis and they release toxins from the granules and kill the pathogens especially parasites and worms.
5. The eosinophil count is increased in allergic condition and then by degrading mediators of inflammation such as bradykinin and histamine limit allergic reactions. The increase in eosinophil count is known as eosinophilia. It occurs

in allergic conditions like urticaria and bronchial asthma, parasitic infestations (round worm, dermatitis and tropical eosinophilia).

6. The decrease in eosinophil count is known as eosinopenia. It is seen in Cushing syndrome, ACTH or steroid treatment, acute infections, etc.

Basophil (Fig. 12.5)

1. The basophil is 10–14 micron in diameter.
2. The nucleus is bilobed and purplish blue in colour.
3. The cytoplasm is bluish, granular and basophilic in nature and has large coarse purple or blue coloured granules overlying the nucleus.
4. Basophils have mild phagocytic action. They are functional against hypersensitivity reactions. They liberate eosinophil chemotactic factor and histamine in response to allergy. The anticoagulant histamine is secreted by basophils. They also contain the vasodilator histamine which dilates blood vessel. Basophils have receptors on their cell surface which bind IgE (immunoglobulin). The receptor bound IgE antibody that confers a selective response of the basophils to pollutants such as pollen proteins or helminth antigens.
5. The increase in basophil count is known as basophilia. It is seen in chronic myeloid leukemia, smallpox and polycythemia.

Agranulocytes: Lymphocytes and Monocytes

Lymphocytes (Fig. 12.5)

1. They are of two types—small and large lymphocytes.
2. The small lymphocyte is 7–10 micron in diameter; having single large oval or round-shaped nucleus which is purple in colour and scanty bluish coloured cytoplasm.
3. The large lymphocytes are 10–14 micron in diameter. The nucleus is mostly indented, and occupies almost entire cell. Nucleus is oval or round-shaped and is purple in colour. The cytoplasm is sky blue and cytoplasm to nucleus ratio is more than small lymphocyte.
4. B lymphocytes produce antibodies. T lymphocytes are differentiated in the thymus and play important role in cell-mediated immunity.
5. The increase in lymphocyte count is known as lymphocytosis. It is seen in tuberculosis, whooping cough, lymphatic leukemia and in physiological conditions in infancy and childhood. The decrease in lymphocyte count is known as lymphopenia: It is seen in acquired immune deficiency syndrome and hypoplasia of the bone marrow which may be secondary to chemotherapy or irradiation.

Monocytes (Fig. 12.5)

1. They are 14–18 micron in diameter.
2. They are the largest in size amongst the WBC.
3. Nucleus is large round, oval or kidney shape and deeply indented, it is pale purplish blue in colour and its location is usually eccentric.
4. The cytoplasm is abundant and greyish blue in colour.
5. The increase in monocytes count is known as monocytosis. It is seen in tuberculosis, brucellosis, malaria and infectious mononucleosis.

Normal Differential Leucocyte Count

1. **Neutrophils:** 50–70%
2. **Lymphocytes:** 20–40%
3. **Eosinophils:** 1–4%
4. **Basophils:** 0–1%
5. **Monocytes:** 2–8%

OSPE SKILLED

Q1. Prepare a blood smear.

I. Preparation of an ideal blood smear

1. Makes a sterile finger prick under aseptic precaution. Discards the first drop of blood and allows the blood to free flow. (Yes/No)
2. Places a drop of blood at one of the end of each of the glass slides. (Yes/No)
3. Places the spreader slide at an angle of 45 degrees just in front of the blood drop. (Yes/No)
4. Moves the spreader slide backwards to touch the blood drop. (Yes/No)
5. The drop spreads out along the line of contact of the spreader slide. (Yes/No)
6. Moves the spreader slide smoothly and evenly in forward direction maintains an angle of 45 degrees. (Yes/No)
7. Let the smear dry in air. (Yes / No)

II. Fixed and stain the blood smear

Fixing and staining of blood smear:

1. Places the slide on the two glass rods which are placed parallel in the arrangement made in the washing sink. (Yes/No)
2. Pours 10–12 drops of Leishman stain on the slide so as to cover it under the stain and waits for two minutes. (Yes/No)
3. Adds adequate amount of buffered water so that it spreads over the smear uniformly. Mixes the water and stain by rocking movement. Ensures that the stain is completely covered with distilled water so as to clear any overstained portion. (Yes/No)
4. Waits for another 10 minutes allowing time for proper staining. (Yes/No)
5. After interval of 10 minutes washes the slide holding it horizontally with tap water. Ensures water does not hit the smear directly, otherwise the stained portion may get removed. (Yes/No)

Focus the peripheral blood smear under microscope and identify a single neutrophil, eosinophil, lymphocyte and monocyte.

Examination of a blood film:

1. Keeps the peripheral blood smear slide under oil immersion objective. (Yes/No)
2. Places a drop of cedarwood oil over the chosen area and move the oil immersion objective into position; making the lower end of the objective touch the drop of oil. (Yes/No)
3. Focuses the cell using fine adjustment knob. (Yes/No)
4. Identifies a single neutrophil, eosinophil, lymphocytes and monocyte (the differential cell count is done by moving the slide along the central and periphery of the smear). (Yes/No)

VIVA VOCE QUESTIONS

Q1. What are the components of Romanowsky stain? What are their prime functions?

Ans. *The components of a Romanowsky stain are*:

1. A basic or cationic dye (methylene blue or azure B), which binds to anionic sites and imparts a blue-grey colour to nucleic acids, nucleoproteins, granules of neutrophils and granules of basophils.

2. An anionic or acidic dye such as eosin Y or eosin B, which binds to cationic sites on proteins and imparts an orange-red colour to eosinophil granules and haemoglobin.

Q2. What are the types of Romanowsky stains?

Ans. *The types of Romanowsky stains are*: May-Grunwald-Giemsa, Giemsa stain, Wright's stain, Leishman's stain and Field's stain (malarial parasites).

Q3. What are the constituents of Leishman stain?

Ans. *The composition of Leishman's stain is*: Powdered Leishman's stain 0.15 gm is dissolve in 100 ml methyl alcohol. Leishman's stain belongs to the Romanowsky group of stains. It is a mixture of methylene blue and eosin in acetone free methyl alcohol.

Q4. What is the function of methylene blue and eosin in Leishman stain?

Ans. Methylene blue which is a basic dye stains cytoplasm, nucleus and granules of basophils. Eosin is an acidic dye which stains the granules of the eosinophils and RBCs.

Q5. What is the function of acetone-free methyl alcohol?

Ans. The acetone-free methyl alcohol is a fixative; it causes precipitation of the proteins, thus aiding the blood smear to get fixed to the slide and is prevented from being washed off.

Q6. Why there should not be any delay in making smear after placing drop of blood on slide?

Ans. There should be no delay in making the smear after placing the drop of blood on the slide because delay will result in non-uniform distribution of the white blood cells, and most of the white cells will be visible at the thin edge of the smear.

Q7. Describe the characteristic features of ideal blood smear.

Ans. *The characteristic features of ideal blood smear are*:

1. An ideal smear covers around ¾th of the entire slide area.
2. Ideal smear is tongue shaped, slightly rounded or curved at the feathery edge tail region. It is thick at head end, and thin at tail end.
3. The upper and lower edges of the smear are clear and visible.
4. The smear is smooth without any streaks or vacuoles.
5. It is single cell layer thick.

Q8. Enlist the precautions for preparation of a blood smear.

Ans. *The precautions for preparation of a blood smear are*:

1. Ensure that the complete drop of blood is uniformly spread along the edge of the spreader slide.
2. The gentle pressure should be constantly maintained when drawing the slide forward.
3. The spreader slide should be moved smoothly and evenly in forward direction maintaining an angle of 45 degrees; to make the ideal blood smear.
4. If spreader slide is moved slowly, the leucocytes will be unevenly distribute.
5. Place the slide on glass rods and let it dry.

Q9. What are the biological causes of poor smear?

Ans. *The biological causes of poor smear are*:

A. Cold agglutinin: In which RBCs clump together. This can be resolved by warming the blood at 37°C for 5 minutes, and then remaking the smear.

B. Lipemia: It produces vacuoles like holes in the smear. This cannot be corrected.

C. Rouleaux: RBCs form stacks resembling coins. This also cannot be corrected.

Q10. Enlist the causes for increased and decreased in neutrophil count.

Ans. The increase in neutrophil is called neutrophilia. It is commonly seen in acute bacterial infections, lung abscesses, burns, acute haemorrhage and in physiological conditions such as after muscular exercise, after meals, pregnancy, lactation, etc. The decrease in neutrophil is called neutropenia. It is seen in typhoid, paratyphoid fever, viral infections, malaria, aplastic anaemia and in bone marrow destruction due to irradiation.

Q11. Enlist the causes for increased and decreased eosinophil count.

Ans. The eosinophil count is increased in allergic condition and they by degrading mediators of inflammation such as bradykinin and histamine limit allergic reactions. The increase in eosinophil count is known as eosinophilia. It occurs in allergic conditions like urticaria and bronchial asthma, parasitic infestations (round worm, dermatitis and tropical eosinophilia). The decrease in eosinophil count is known as eosinopenia. It is seen in Cushing syndrome, ACTH or steroid treatment, acute infections, etc.

Q12. What are the causes of increase basophil count?

Ans. The increase in basophil count is known as basophilia. It is seen in chronic myeloid leukemia, smallpox and polycythemia.

Q13. Enlist the causes lymphocytosis and lymphopenia.

Ans. Lymphocytosis (increase in lymphocytes) is seen in children (normal count 40–60%), lymphocytic leukemia, viral infection and tuberculosis. Lymphopenia (decrease in lymphocytes) is seen in hypoplastic bone marrow and acquired immune deficiency syndrome.

Q14. Enlist the causes of monocytosis and monocytopenia.

Ans. Monocytosis (increase in monocytes) seen in tuberculosis, syphilis and some leukaemias. Monocytopenia (decrease in monocytes) is seen in hypoplastic bone marrow.

Q15. What is the normal percentage range of differential leucocyte in peripheral blood smear?

Ans. The leucocytes are divided into five types on the basis of its morphological appearance under a light microscope and pictorial characteristics when stained: Neutrophils (50–70%), eosinophils (1–4%), basophils (less than 1%), monocytes (2–10%) and lymphocytes (20–40%).

Q16. What are fixed leucocytes?

Ans. Some white blood cells move and migrate into the tissues and permanently occupy stay at that location rather than remaining in the circulation. Examples are Kupffer cells of liver, mast cell in basophils, microglia and dendritic cells in central nervous system, and histiocytes (tissue microphages).

Q17. How do you classify proliferative disorders linked with leucocytes?

Ans. WBC proliferative disorders can be classed as myeloproliferative and lymphoproliferative.

Q18. Enlist the medication causing leucocytosis.

Ans. The medications, causing leucocytosis are corticosteroids, lithium and beta agonists, etc.

Snap box 1

Paul Ehrlich (1854–1915): He was a German Jewish physician and scientist who worked in the field of haematology, immunology, and chemotherapy. He invented the precursor technique to gram staining bacteria, and his methods of staining tissue made it possible to distinguish between different types of blood cells, and this helped to diagnose numerous blood diseases. Ehrlich used both alkaline and acid dyes, and also created new, "neutral" dyes. For the first time this made it possible to differentiate the lymphocytes among the leucocytes (white blood cells). He stained their granulation and could distinguish between non-granular lymphocytes, mononuclear and polynuclear leucocytes, eosinophil granulocytes, and mast cells.

Bibliography

Temkin. The era of *Paul Ehrlich*. Bull NY Acad Med. 1954 Dec; 30(12): 958–67.

OBSERVATIONS: Exercise for Students

Results:

N = _____ %, E = _____ %, B = _____ %, L = _____ %, M = _____ %

Observe the cells and note them successively in the squares below.

N	E	N	N	N					

Fig. 12.6