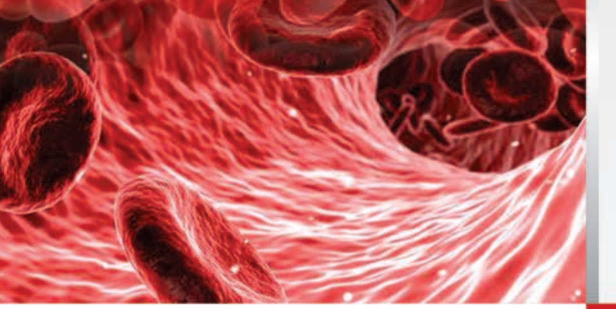


Chapter

1

Blood Collection and Anticoagulants

Blood is the most frequent body fluid used for analytical purpose. Blood is a mesenchymal tissue consisting of a liquid portion called plasma and particulate or formed elements (RBCs, WBCs, platelets) which are suspended in plasma.

Plasma

Blood plasma is a straw-coloured fluid component of blood which normally holds the blood cells in suspension. So, plasma can be called extracellular matrix of blood cells. It makes near about 55% of the total blood value. It is composed mostly of water (up to 95% by volume) and dissolved proteins (6–8%). These proteins are albumin (4.5 g%), globulins (2.5 g%) and fibrinogens (0.3 g%). Apart from proteins, glucose, clotting factors, electrolytes (Na^+, Mg^{2+}, Ca^{2+}, Cl^-, HCO_3^-, etc.). Hormones, carbon dioxide and oxygen are present in the plasma.

Serum

When blood is collected in the test tube or vial without addition of anticoagulants, then blood is clotted. The clot is formed by using blood cells, clotting factor like fibrinogen. The fluid is separated from clot. So, this fluid contains proteins and other elements but lacks fibrinogen (used in the formation of clot).

So, serum is plasma minus fibrinogen.

BLOOD COLLECTION AND PROCESSING

Three general procedures for obtaining blood are (Fig. 1.1A and B):

1. Venipuncture for venous blood
2. Arterial puncture for arterial blood
3. Skin puncture for capillary (peripheral) blood

Venous blood is preferred for most haematological examinations.

Venipuncture or Venous Puncture

Venous blood is best withdrawn from an antecubital vein by means of a dry glass syringe or disposable plastic syringe. The steps are:

i. Position the patient properly, depending on whether the patient is sitting or prone (ambulatory or non-ambulatory). This is to make sure for easy access to the antecubital fossa.
ii. The patient is asked to make a fist, so that veins become more prominent and more palpable.
iii. Select a suitable vein for venipuncture. For veins of the antecubital fossa, the median cubital and cephalic veins may be used alternatively. In case, the patient has an intravenous line, draw venous blood from other arm.
iv. Cleanse the venipuncture site with 70% alcohol (isopropanol) or 1% iodine-

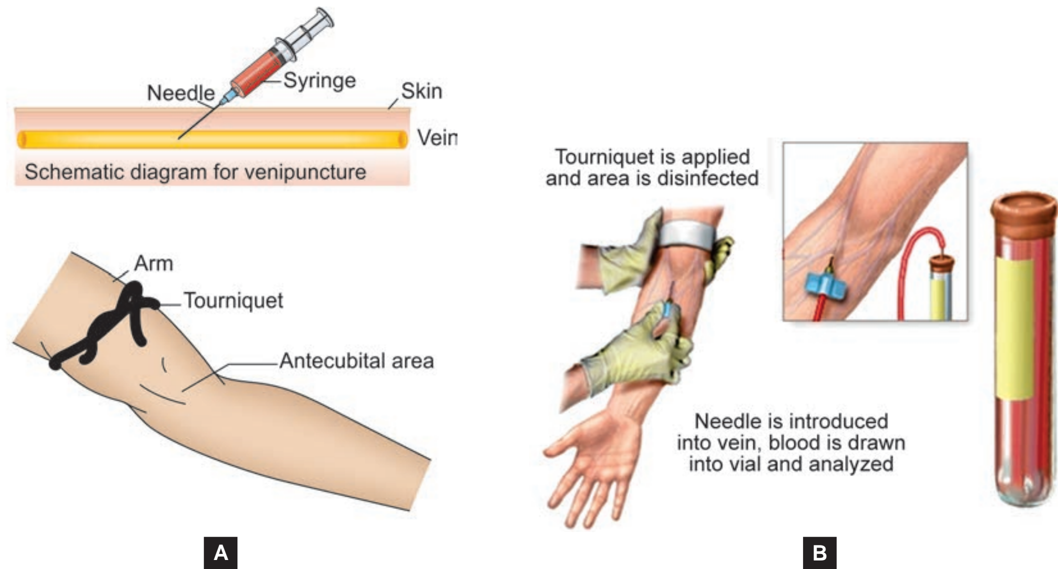


Fig. 1.1A and B: Blood collection procedure

saturated swab stick. Allow the area to dry.

v. Apply a tourniquet few inches above the puncture site. But remember, do not keep the tourniquet for more than one minute.

vi. Hold the vein firmly, both above and below the puncture site. For this, use either thumb and middle finger or thumb and index finger.

vii. Perform the venipuncture. Enter the syringe needle (19 or 20 gauge) at approximately 15° angle to the arm. If using the evacuated system or vacuotainer as soon as the needle is inserted in the vein, ease the collection tube forward in the holder as far as possible, firmly securing the needle holder in place.

viii. Release the tourniquet when blood begins to flow.

ix. After blood collection, place a clean cotton ball or gauze lightly over the site. Withdraw the needle, then apply pressure to site.

x. Now apply an adhesive bandage strip over the cotton ball or gauze to stop bleeding or formation of haematoma.

Note

i. The needles should not be too fine or too long, those of 19 or 20 SWG British standard, American standard 19 SWG = 18 (1.016 mm), 20 SWG = 19 (0.914 mm) are suitable.

ii. If the veins are very small then 23 SWG (= 22 or 0.610) to be used to collect at least 2 ml venous blood.

iii. If veins are selected from dorsum of the hand, it tends to bleed easily. So, care must be taken.

iv. Beware of haemolysis of blood during collection. It can be avoided or minimized by using clean apparatus, withdrawing the blood slowly, not using too fine needle, delivering the blood slowly into the receiver and avoiding frothing during the withdrawal of the blood and subsequent mixing with the anticoagulant.

Arterial Puncture

Arterial blood is used rarely. It is used to measure oxygen and carbon dioxide tension, as well as pH (arterial blood gases or ABGs). These blood gas measurements are critical in the assessment of oxygenation problems encountered in patients with pneumonia, pneumonitis and pulmonary embolism. Also critically ill cardiovascular patients and

patients who are undergoing cardiac or pulmonary surgery are monitored for hypoxaemia.

Arterial puncture is technically more difficult than venous puncture. Increased pressure of the flowing blood, makes it more difficult to stop bleeding with the undesired development of a haematoma. The arteries selected for arterial punctures are radial, brachial and femoral arteries in order of choice. Unsuitable sites are oedematous, irritated, near a wound, or in an area of an arteriovenous (AV) shunt or fistula. Although venous blood yields adequate pH values if properly collected but venous blood yields incorrect values for arterial oxygen saturation and alveolar pCO_2.

Skin Puncture (Capillary Blood)

Skin puncture is the method of choice in paediatric patients especially infants. The large amount of blood collected from repeated venipuncture may cause anaemia (iatrogenic), especially in premature infants. Skin puncture is also preferred in geriatric patients because of thinness of skin and loss of skin elasticity which cause venipuncture difficult.

In the neonates and infants, the heel is often used for skin puncture. A deep heel prick is made at the distal edge of the calcaneal protuberance following 5–10 minutes prior exposure to prewarmed water. In the older paediatric population, and in geriatric patients, earlobes or fingers are preferred.

This capillary blood collected by skin puncture is good for making blood smear or for a single routine haematological test. A blood smear prepared from capillary blood without anticoagulant gives better information about blood cell morphology and differential count. But the total count is not very accurate. This is because of dilution by the tissue fluid and sometimes also due to lack of free flow of capillary blood. But if free flow capillary blood is received, then it is as satisfactory as venous blood.

Capillary blood obtained from heel puncture is not good for pCO_2 and pO_2 determination in the first day of life, probably owing to vasoconstriction and poor perfusion of the extremities. In infants with respiratory distress syndrome, heel blood deviates significantly from arterial blood in all parameters except standard bicarbonate and base excess.

The discrepancies between peripheral (capillary) and venous blood are more pronounced if earlobe than finger is chosen for skin puncture. However, if the ear is rubbed well with a piece of cotton or lint until ear is warm and pink, then a good spontaneous flow of blood can be obtained using sterile lancets as prickers. In this case, RBC count, leucocyte count and haemoglobin content almost close to venous blood.

Heel Puncture Method (Fig. 1.2)

A deep puncture in the heel is made after heel is really warm (5–10 minutes prior

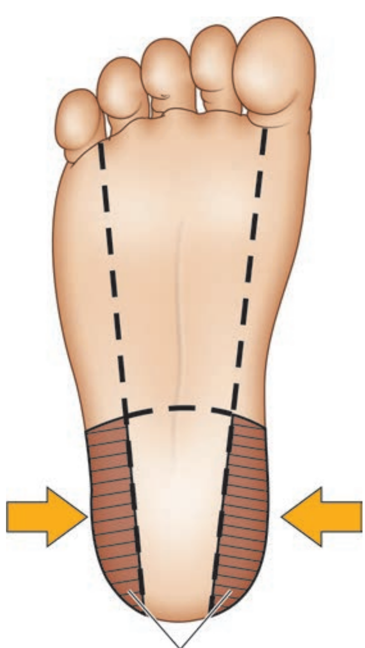


Fig. 1.2: Heel puncture method

exposure to prewarmed water) by using a steel lancet. Ideal sites are the medial or lateral parts of the plantar surface of the heel. Remember, the central plantar area and posterior curvature are not chosen in infants because of risk of injury to the underlying tarsal bones.

Earlobe Puncture Method

Rub the ear until it becomes warm and pink. Then with a sterile lancet prick the earlobe (as it has no bone or cartilage) to a depth of 2–3 mm by a single stab. Wipe and discard first few drops of blood. Collect the blood sample when it flows spontaneously (usually in about 30 seconds). Always use different lancet for different patients.

Finger Prick (Stick) Procedure (Fig. 1.3)

The best locations for finger sticks are the 3rd (middle) and 4th (ring) fingers of the non-dominated hand. Do not select tip of the finger or the centre of the finger. The second (index) finger tends to have thicker and calloused skin, so not preferred. The fifth finger (little finger) tends to have less soft tissue overlying the bone.

1. After selection of site of the finger, put on gloves and cleanse the puncture site with 70% alcohol (isopropanol).
2. Massage the finger toward the selected site prior to puncture.
3. Then with a sterile safety lancet make a skin puncture just off the finger pad.
4. Wipe away the first drop of blood which contains excess tissue fluid/plasma. Take subsequent blood drop into collection tube/device by gentle pressure on the finger; or put the blood drop onto a glass slide.
5. Cap, rotate and invert the collection device/tube.
6. Label it.

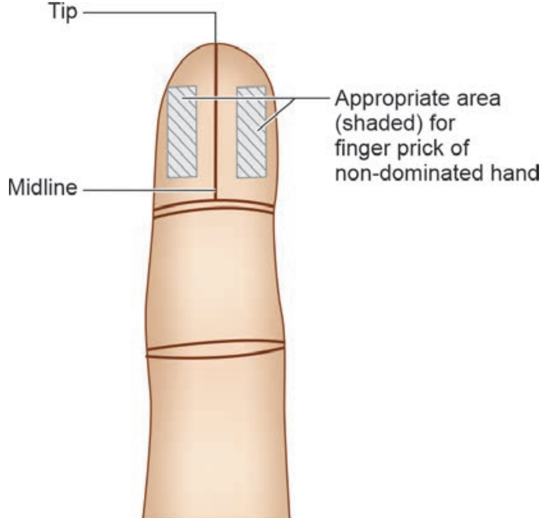


Fig. 1.3: Finger prick (stick) procedure

Differences between Venous and Peripheral (Capillary) Blood

- The platelet count is usually higher in venous than in peripheral blood (average 9% higher, but may go up to 32%). This is probably due to adhesion of platelets to the skin puncture site resulting in lower platelet count in peripheral blood.
- Venous blood and peripheral blood are not same, even though capillary blood is free-flowing which comes from arterioles after skin puncture.
- The RBC count, haemoglobin content and packed cell volume (PCV) are slightly higher in peripheral blood than venous blood. The total leucocyte count (TLC) and neutrophil counts are also higher about 8%, and monocyte count by 12% in peripheral blood. In children, it may be up to 100% higher, both for heel and earlobe punctured capillary blood.
- But the monocytes and neutrophils tend to accumulate in the earlobe if the blood is not free-flowing.

ANTICOAGULANTS

The anticoagulants prevent blood from clotting and as a result, plasma is formed (Table 1.1). Most anticoagulants bind with calcium ions (Ca^{2+}) and remove it by calcium chelating. Calcium is a factor in the coagulation cascade. As the calcium is chelated it cannot work. So, clot is not formed. Heparin, on the other hand, directly interferes in the coagulation process by destroying thrombin as well as thromboplastin.

Platelets + clotting factors + calcium → thrombin which converts fibrinogen → fibrin clot.

1. EDTA (Ethylenediaminetetra-acetic Acid)

It is also called sequestrene and probably the best anticoagulant for routine haematological investigations.

Mechanism of action: It is a powerful calcium chelating or binding agent and acts by binding the calcium in blood. So, active

Table 1.1: Some blood anticoagulants and their use

Name of anticoagulants (requirement per ml of blood)	Mechanism of action	Diagnostic use
1. No anticoagulant for serum in plain vial/tube	No anticoagulant; blood is clotted	Serum: Liver function test (protein, bilirubin, SGOT/AST, SGPT/ALT, alkaline phosphatase γGT, etc.), lipid profile (chole-(sterol, triglyceride, HDL, LDL, VLDL), urea, creatinine, etc.
2. EDTA (1–1.5 mg/ml)	Binds Ca^{2+} and chelates it	Haemoglobin estimation, PCV, TLC, DLC, platelet count, parasite detection (microfilaria, malaria)
3. Trisodium citrate (3.2% aqueous solution; blood to anticoagulant ratio of 9:1 or 4:1 for coagulation studies and ESR respectively)	Binds Ca^{2+} and precipitates it as double salt (calcium sodium salt)	Coagulation studies, prothrombin time, ESR
4. Heparin (0.1–0.2 mg/ml)	Inhibits thrombin in the presence of antithrombin III. Also, it inhibits thromboplastin formation	• Osmotic fragility test • Plasma iron estimation • Demonstration of LE cells • Lymphocyte culture for karyotyping/genetic studies • Lymphoma/leukaemia panel for flow cytometry/ immunophenotyping
5. Sodium or potassium oxalate (2 mg/ml)	Binds Ca^{2+} and chelates it	Blood urea and creatinine
6. Double oxalate (ammonium and potassium oxalate, 2 mg/ml)	Binds Ca^{2+} and chelates it	Hb, TLC, PCV, specific gravity
7. Sodium fluoride (6 mg powder/ml of blood)	• Blocks RBC enzymes for glycolytic inhibition of glucose • Also, chelates calcium	Blood glucose (sugar) estimation

calcium ions are not available for coagulation process.

Concentration of EDTA: A concentration of 1.2 mg of anhydrous salt per ml of blood is required. For dipotassium salt, a concentration of 1.5 ± 0.25 mg/ml of blood is recommended as per International Council for Standardization in Haematology (ICSH). The dipotassium salt is very soluble and is preferred over disodium salt which is less soluble.

Advantages of EDTA

i. Very good anticoagulant for routine haematological investigations. EDTA has the advantage over oxalate anticoagulant because it prevents clumping of platelets *in vitro*. So, platelet count can also be performed on venous blood.

ii. The dilithium salt of EDTA has the advantage that same blood sample can be used for chemical investigations apart from haematological investigations. But dilithium salt is less soluble compared to dipotassium salt and is less preferred.

Disadvantages of EDTA: Excess of EDTA (>2 mg/ml), irrespective of its salts, cause shrinkage and degenerative changes in RBCs and WBCs. Also, excess salt causes significant decrease in PCV and increase in MCHC (mean corpuscular haemoglobin concentration). Excess EDTA causes platelets to swell and then disintegrate, resulting in spuriously high platelet count as the swollen platelet fragments are large enough to be counted as normal platelets.

2. Trisodium Citrate

A 3.2% aqueous solution of trisodium citrate ($Na_3 C_6H_5O_7, 2H_2O$) is the anticoagulant of choice **for coagulation studies**. It is also most widely used anticoagulant **for ESR** (erythrocyte sedimentation rate) also. But for coagulation studies (prothrombin time) 9:1 blood to anticoagulant is used, whereas in ESR determination 4:1 venous blood to anticoagulant is used.

Mechanism of action: Coagulation is prevented by precipitation of blood calcium in the form of a double salt (calcium sodium salt) which is very weakly dissociated.

3. Heparin

Heparin powder or liquid is used in a concentration of 10–20 IU (0.1–0.2 mg) per ml of blood.

Mechanism of action: It acts by inhibiting thromboplastin formation. Also it has antithrombin activity, i.e. inhibiting the action of thrombin on fibrinogen in the presence of plasma of co-factor antithrombin III.

Uses

i. Osmotic fragility test

ii. Chemical investigation like plasma iron estimation

iii. Demonstration of LE cell in SLE patients

iv. Lymphocyte culture for karyotyping/genetic study

v. Lymphoma/leukaemia panel for flow cytometry or immunophenotyping

vi. Nitro blue tetrazolium (NBT) test to assess phagocytic activity of phagocytes.

Advantages: It is an effective anticoagulant and does not alter the size of RBCs. It minimizes chances of haemolysis. Some consider heparin in the form of lithium salt is the ideal universal anticoagulant for blood.

Disadvantages

i. It is expensive

ii. It is inferior to EDTA as anticoagulant

iii. Heparinized blood should not be used for making blood films as it gives a faint blue colouration to the background when blood smears are stained with Romanowsky stains. Also, it causes leucocytes to clump, so TLC and DLC will be erroneous.

4. Oxalate

Potassium, sodium and ammonium oxalates act as calcium chelating agent like EDTA and trisodium citrate. They interact with blood

calcium and form calcium oxalate after chelation.

i. Potassium and sodium oxalate: Used mainly for chemical analysis. Concentration of anticoagulant 2 mg/ml of blood.

ii. Double oxalate (Wintrobe's mixture): It is a mixture of two types of oxalates (ammonium oxalate and potassium oxalate in a ratio of 3:2). It is used in a concentration of 2 mg/ml of blood. Preparation of double oxalate mixture is as follows:

- Ammonium oxalate: 1.2 g
- Potassium oxalate: 0.8 g
- Distilled water: 100 ml

This solution contains 20 mg of oxalates (both ammonium and potassium oxalate)/ml solution. So, 0.2 ml of this solution containing 4 mg of oxalates is sufficient for 2 ml of blood (up to 5 ml blood) as anticoagulants.

Uses

i. Determination of haematocrit value or PCV.
ii. Determination of haemoglobin and total leucocyte count (TLC)
iii. Determination of specific gravity of whole blood or plasma.
iv. Single powder in the form of sodium or potassium oxalate can be used for blood urea and creatinine estimation.

5. Sodium Fluoride

It is used in the concentration of 30 mg powder/5 ml of blood or 6 mg powder/ml of blood. **Mechanism of action:** (i) It chelates calcium and forms calcium fluoride, (ii) it prevents glycolysis by blocking acid phosphorylase enzymes in RBCs but increases amylase activity.

Uses: It is anticoagulant of choice for blood sugar estimation.

For glucose estimation, fluoride may be added to heparin also.

> **Note**
>
> Fluoride inhibits glycolysis of blood cells (RBCs) which may otherwise destroy glucose at the rate of about 5%/hour.

BLOOD COLLECTION TUBES/VIALS AND COLOUR CODE (Figs 1.4 and 1.5)

1. **Red or gold top clot tube:** Contains no anticoagulant. Blood will clot and serum will be formed.
2. **Purple top tube:** Contains EDTA anticoagulant.
3. **Blue top tube:** Contains 3.2% buffered sodium citrate anticoagulant for coagulation studies.
4. **Black top tube:** Contains 3.2% sodium citrate for ESR only.
5. **Light green top tube:** Contains lithium heparin anticoagulant.
6. **Dark green top tube:** Contains sodium heparin anticoagulant used for amino acid and cytogenetic studies.
7. **Gray top tube:** Contains glycolytic inhibitor or sodium fluoride for glucose estimation.
8. **Yellow tube:** Contains acid citrate dextrose (used in blood banking).

SOURCES OF BLOOD COLLECTION ERROR

1. Wrong patient identification or labelling error.
2. Haemoconcentration: Prolonged tourniquet time (>1 minute) restricts blood flow causing false high results, e.g. cell counts.
3. Haemodilution: If blood is collected from an arm with an IV (intravenous), the blood can be diluted and/or contaminated causing false low cell counts.
4. Haemolysis: Caused by traumatic blood drawing technique, vigorous shaking of the blood tube or forcing the blood

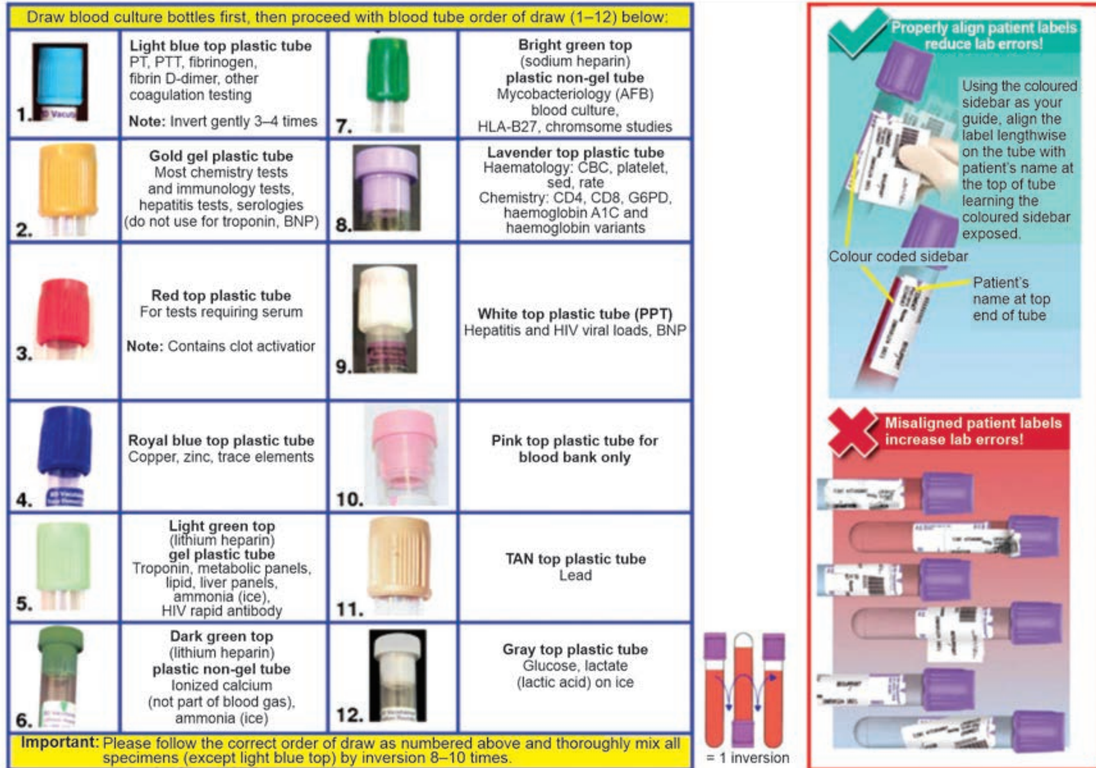


Fig. 1.4: Colour code of top of blood tube/vial and their significance

Fig. 1.5: Different blood collection tubes/vials with colour code

through syringe needle into the tube. Rupture of blood cells causes release of cell constituents like potassium, tissue factors and responsible for low RBC counts.

5. Use of wrong anticoagulant/tube: As for example, heparin causes platelet clumping. Hence, unsuitable for platelet counts.
6. Partially clotted blood draws improper mixing of anticoagulant containing tubes or blood obtained using poor blood drawing technique (e.g. too slow) may clot. Cells are trapped in the fibrin clot causing falsely low cell counts.
7. Insufficient fill: All tubes should have minimum draw amounts to maintain the proper anticoagulant concentration to blood volume. As for example, blue top tubes for coagulation test must be full.
8. Proper instruction for the said test not followed, e.g. certain tests have time limits for testing.

EFFECT OF STORAGE OF BLOOD

- When blood is kept in room temperature (18–25°C) for prolonged time, certain changes take place regardless of the anticoagulant use.

- This is obvious in EDTA blood (tripotassium salt > dipotassium salt).
- RBCs begin to swell; as a result, MCV increases, osmotic fragility and prothrombin time increase slowly and ESR decreases.
- The TLC and platelet count gradually fall. It is best to perform TLC and platelet count within 2 hours.
- The fall in leucocyte count is more if there is excessive amount of EDTA (>4.5 mg/ml).
- Reticulocyte count is unchanged for 24 hours at 4°C but at room temperature, it begins to fall within 6 hours.
- Nucleated RBCs (normoblasts) disappear from stored blood within 1–2 days at room temperature.
- Haemoglobin content is relatively stable for days unless it is infected.

For different investigations, blood is collected in tubes with or without anticoagulants. If there is anticoagulant, after mixing the blood cells of whole blood can be analyzed. Centrifugation of whole blood separates the cells from fluid plasma. In the bottom there will be RBCs. Above, there will be plasma containing fibrinogen. In between these two layers, there will be a **buffy coat** containing WBCs and platelets.

If there is no anticoagulant, fibrinogen will be used up to form fibrin strands which entrap blood cells. Centrifugation of this blood will separate the clot from the fluid serum. Serum lacks fibrinogen.

Serum and plasma can be obtained on standing also and they can be taken out by micropipette without disturbing fibrin clot or blood cells respectively. But, centrifugation will give better quality serum or plasma (Fig. 1.6).

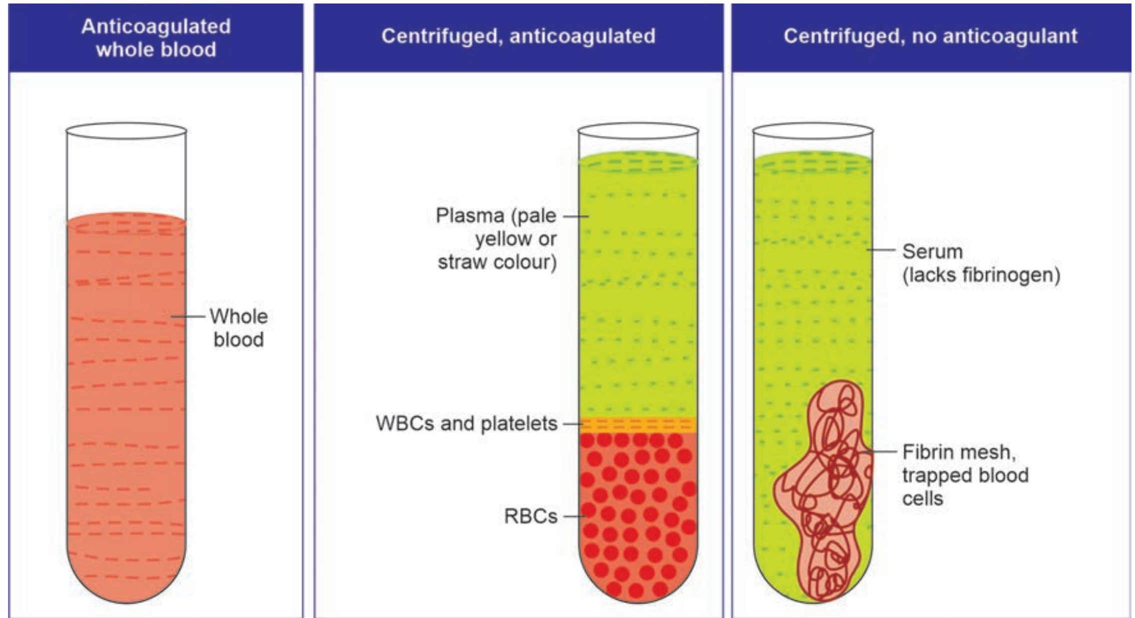


Fig. 1.6: Left tube contains anticoagulated whole blood, middle tube showing plasma and last tube showing serum

Q1. What are differences between serum and plasma?

Ans:

Serum	Plasma
1. Serum is formed as supernatant when blood undergoes clotting	1. Plasma is obtained by centrifugation of anti-coagulant mixed blood. Anticoagulant mixed blood after standing may also give plasma but of poor quality compared to plasma after centrifugation.
2. Serum does not contain fibrinogen, prothrombin and other clotting factors like V, VII, VIII, IX, X, XI and XII which have been used in clotting.	2. It contains all the clotting factors including fibrinogen. But calcium ion is absent.
3. It is used to estimate different biochemical parameters and serum enzymes like uric acid, electrolytes, urea, creatinine, SGOT, SGPT, alkaline phosphatase, etc.	3. It is used mainly for coagulation studies like PT, APTT, TT, etc. Also used for detection of plasma glucose, plasma calcium, plasma ammonia, etc.
4. Serum is clear fluid.	4. Plasma is yellowish or straw coloured.

Q2. What is the ideal gauge needle (bore size) for collection of venous blood?

Ans: If the needle is too large for the vein for which it is intended, it will tear the vein and cause bleeding (haematoma). If the needle, is too small, it will damage the blood cells especially RBCs. So, laboratory tests which require whole blood cells or haemoglobin or plasma will give inaccurate results.

The gauge refers to the inner measurement or opening of the needle. Usually needle gauge of 21G to 23G is preferred for venous blood collection. Small bore needles of 25G or less cannot be recommended and reserved only for problematic venous accesses and newborns. Usually 25G or lesser size may cause haemolysis and inaccurate results of electrolytes especially potassium. Nonetheless, 21G needles are most commonly used for routine tests.

Q3. Why is middle or ring finger preferred for capillary blood collection?

Ans: The best locations for finger sticks are the 3rd (middle) and 4th (ring) fingers of the non-dominated hand. Do not select tip of the finger or the centre of the finger. The second (index) finger tends to have thicker and calloused skin, so not preferred. The fifth finger (little finger) tends to have less soft tissue overlying the bone. Ulnar side of the tip of ring finger is comparatively less innervated. So, needle prick is less painful to the patients.

Q4. Why too much pressure is not given during blood collection from finger prick?

Ans: Needle prick should be deep enough so that free flow blood comes out. Gentle pressure may be applied to start the blood. But too much pressure to finger tip should not be given as tissue fluid will come out which will dilute the blood. So, haematological values will be lowered.

Q5. Why double oxalate is preferred over single oxalate as an anticoagulant?

Ans: Ammonium salt (ammonium oxalate) causes swelling of RBCs while potassium salt (potassium oxalate) causes shrinkage of RBCs. Hence, mixture of these two salts or

double oxalate will cause neither RBC swelling nor RBC shrinkage. Normal shape and size of RBCs are maintained. Potassium oxalate: Ammonium oxalate = 2:3.

But oxalates are not preferred as anticoagulant for Hb/TLC/platelet count as they induce morphologic alterations in WBCs and RBCs. So, smear morphology cannot be studied.

Q6. Why excess EDTA is bad as anticoagulant?

Ans: Excess of EDTA (>2 mg/ml), irrespective of its salts, cause shrinkage and degenerative changes in RBCs and WBCs. Also, excess salts cause significant decrease in PCV and increase in MCHC (mean corpuscular haemoglobin concentration). Excess EDTA causes platelets to swell and then disintegrate, resulting in spuriously high platelet count as the swollen platelet fragments are large enough to be counted as normal platelets.

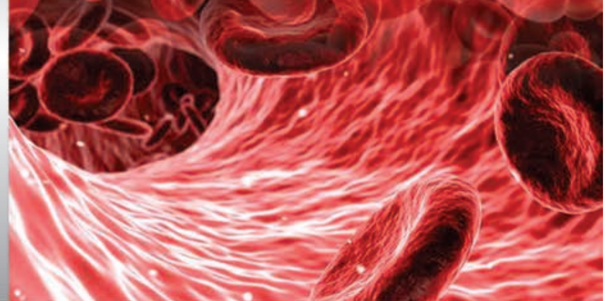
Q7. What is a vacutainer?

Ans: **Vacutainer:** This is a blood collection tube which is sterile glass or plastic tube with a coloured rubber stopper creating a vaccum seal inside of the tube facilitating the drawing of a predetermined volume of blood/liquid. Vacutainer tubes may contain anticoagulant/additives to stabilize and preserve the blood/liquid specimen prior to analytical testing. Tubes containing gel can be easily handled and transported after centrifugation without the blood cells and serum mixing.

Vacutainer tubes were invented by Joseph Kleiner and Becton Dickinson in 1949.

Q8. What are different vacutainers used in haematology?

Anticoagulant/additives	Colour	Blood volume	Uses
1. Plain	Red	6 ml	Most biochemistry including drug levels, serological tests, which uses serum, cross-matching.
2. EDTA-K2	Purple/ Lavender	3 ml	Most haematological tests, HbA1C, molecular genetic tests using blood DNA, blood grouping, crossmatch.
3. Lithium heparin	Green	4 ml	Cytogenetic tests using blood DNA, osmotic fragility test, STAT biochemistry like electrolytes, renal screen, ammonia, etc. After blood is drawn inside by vacuum, it should be inverted gently at least 6 times to prevent clotting.
4. Sodium citrate	Blue	2.7 ml	Coagulation studies. This tube should be inverted at least 3–4 times.
5. Sodium fluoride/ potassium oxalate	Grey	6 ml	Glucose test. It should be inverted gently at least 6 times.
6. SST II, clot activator and serum gel separator, plain	Yellow	5 ml	All tests requiring serum except those few that need red cells as well



Chapter

2

Blood and Bone Marrow Smear Preparation and Staining Methods

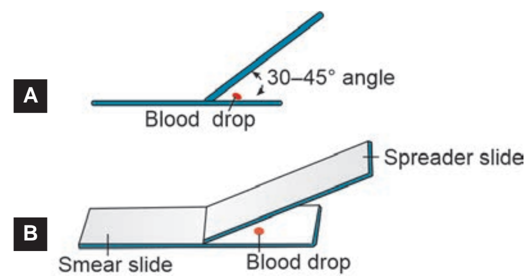
Examination of blood and bone marrow smear/film are important haematologic evaluation and to diagnose a haematologic disease. Blood or bone marrow smears should be prepared immediately as delay can cause spurious results.

Here, three methods of making blood smears/films are described

1. Wedge method
2. Spinner or spin method
3. Cover glass method

WEDGE METHOD

This is perhaps the most common method to prepare a blood film (Fig. 2.1A to D). Place a small drop of blood (2–3 mm in diameter) about 1–2 cm from the end of a clean, dust-free slide which is on flat surface. Then without any delay, a spreader (second slide) is hold between thumb and forefinger of the right hand against the surface of the first slide at an angle of 30–45° and move it back to make contact with the blood drop to spread it. Then push the spreader slide at a moderate speed forward until all the blood has been spreaded over the first slide forming a moderately thin film. Ideally, the spreader slide should be clean, dry and slightly narrower than the first slide so that blood does not cross the edge of the first slide and edges of blood film can be examined under the microscope.



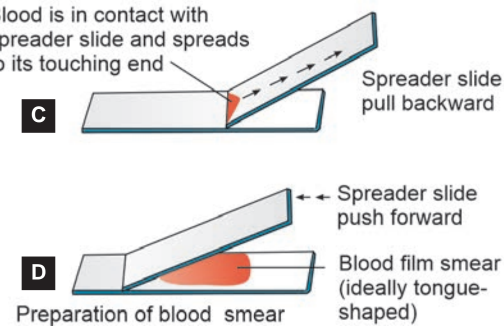


Fig. 2.1A to D: Preparation of blood smears. (A) Small blood drop from 1 to 2 cm from one end of glass slide; (B) Place the spreader slide at an angle of 30° to 45° over the smear slide; (C) Pull back the spreader slide so that it touches the blood drop and spreads throughout the edge of the spreader slide; (D) Push forward to make blood/smear

Fig. 2.2: Ideal thin blood smear

✍ Note

a. The blood drop should be such that it can produce the blood film 3–4 cm in length.
b. The ideal thickness of blood film should be such that there will be some overlap of RBC (red blood cells) throughout much of the blood film's length. But the RBCs are separated at the tail end of the film.
c. The film should not cover the entire surface of glass slide.
d. Ideally, there will be a thick portion and a thin portion in a good film and there will be gradual transition from one to the other (Fig. 2.2).
e. The blood film should have an even, smooth appearance and should be free ridges, waves or holes.
f. The edge of spreader slide must be very smooth. Roughed edges will produce ragged tails containing many leucocytes (WBCs).
g. The thickness of blood film can be adjusted by changing the speed of spreading or by changing the angle of the spreader slide or by using a larger or smaller blood drop (Figs 2.3 and 2.4).
h. At a given angle, increasing the speed of spreader slide will cause increase in the thickness of the film.
i. At a given speed, increasing the angle of spreader slide will also cause increase in the thickness of the film.
j. The faster the blood film is air dried, the better the spreading of the individual cells on glass slide. Slow drying of film (as in humid weather) may cause contraction artifacts of the cells.
k. There may be disproportionate monocytes at the tip of the feather (tail) edge or neutrophils just in from the feather edge and both at the lateral edges of the film.

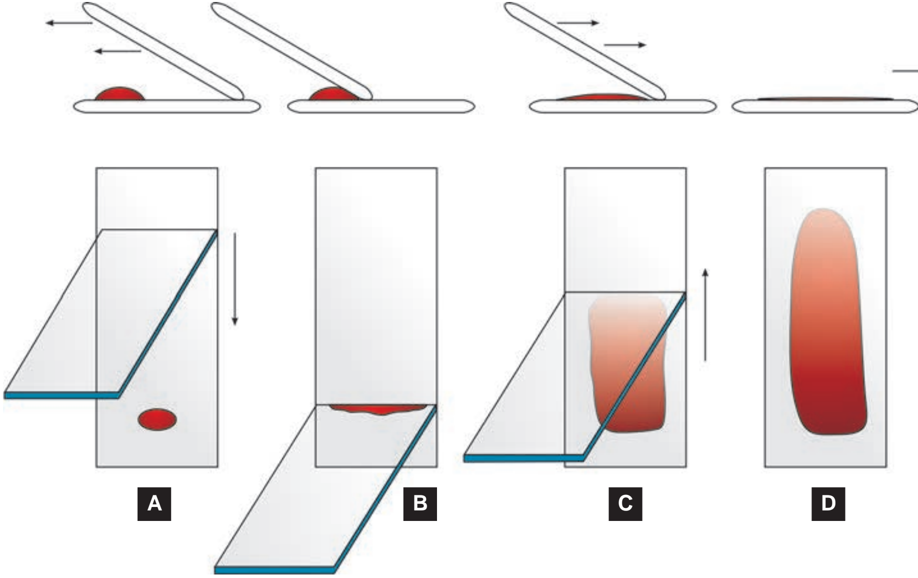
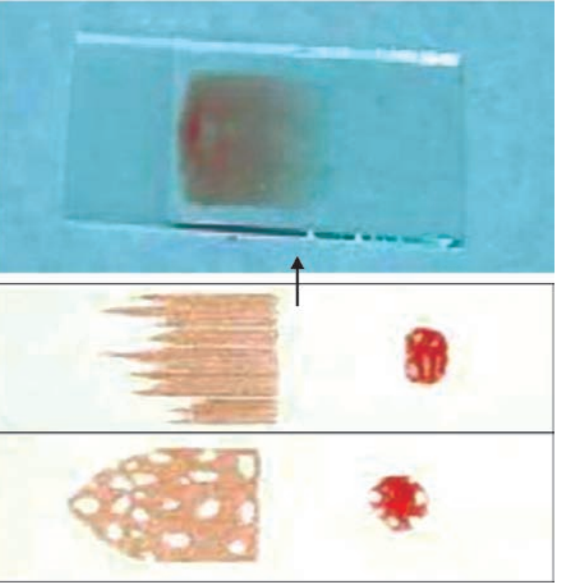


Fig. 2.3A to D: Upper row showing side view of different steps of making a thin blood film. Lower row showing front view of different steps of making a thin blood film

Thin film

- Good preparation—feathered end of the film should be centrally located on the slide with free margins on both sides, when properly prepared, it will be only one cell layer thick at this end.



- Badly prepared smears can cause presence of streaks—as a result of chipped spreader.
- Holes in the film indicate faulty preparation and dirty or greasy slides, respectively.

Fig. 2.4: Good and badly prepared thin blood film

SPINNER OR SPIN METHOD

Spinner or spin method is an automated method in which 1–2 drops of blood drops are placed in the centre of a glass slide. Then it is spun at a high speed in a spherical centrifuge (e.g. cytospin) for a short period. The blood drops are spreaded on the glass slide in a monolayer. With this method leucocytes and platelets are distributed uniformly without any distortion.

✍ Note

1. The RBCs may be distorted.To overcome this problem, mix one volume of 9 g/L NaCl (sodium chloride) to 2 volumes of blood for diluting the blood and then put the diluted blood on the glass slide.
2. White blood cells (WBCs) can be easily examined on any spot in the film made by spinner method.
3. Unlike wedge method, it does not produce disproportionate monocytes and/or neutrophils at the tail or lateral edges.

COVER GLASS METHOD

For this, 22 mm square (number one or one and a half cover glasses) are recommended. Touch a cover glass to the top of a small blood drop without touching the skin. Now place it, blood side down crosswise onto another cover glass, so that the corners appear as an eight point star. The small blood drop will spread out quickly and evenly in a thin layer between the two surfaces. Prepare blood film by pulling the cover glasses quickly and firmly apart on a plane parallel to their surfaces. After that, cover glasses are placed on clean paper (film side up) and are air dried.

Blood film from venous blood may be prepared likewise by placing a blood drop on a coverslip and follow the above mentioned steps.

THICK SMEAR AND ITS PREPARATION

While the blood film mentioned above, is suitable for studies of cellular morphology, sometimes thick smears are prepared to detect microfilariae and malarial parasites (Figs 2.5 to 2.7). Thick smears are very useful

when parasites (malaria and microfilaria) are scanty but identification of the parasites is less than thin films. Mixed infection (both *Plasmodium vivax* and *falciparum*) may also be missed. Thick smear is also useful when there is severe leucopenia. It helps to perform differential count or at least the proportion of polymorphonuclear to mononuclear cells.

Preparation of Thick Film

A drop of blood is placed in the centre of a glass slide and is spreaded out with a corner of another slide to cover an area about four times its original area. The film may be air-dried or dried at 37°C for 30 minutes in an incubator. If the film is satisfactory, then printed matter (small print of newspaper) is just visible.

Alternatively, four small blood drops may be taken in the mid-portion of a glass slide. They are joined together to form a blood film (square shaped) in the mid-portion of the slide.

Sinton proposed to make thin and thick smears onto same glass slide. For this, a large blood drop is taken near one end to make thick smear and one small blood drop is taken in the centre of the slide. Thick smear is prepared from the large blood drop (square shaped) while thin smear is prepared from the small blood drop (tongue shaped) in the same manner as previously described.

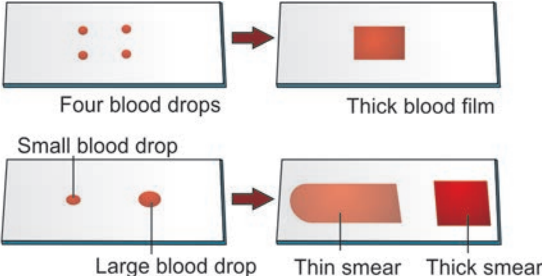


Fig. 2.5: Preparation of thick and thin blood films in the upper panel. Preparation of both thin and thick blood films on the same slide.

Fixation of Blood Films

Blood films need to be fixed before staining to prevent haemolysis when they come in contact with water during water-based (aqueous) stains or water is poured during staining. For this, blood films are coated with acetone-free methyl alcohol for 1–2 minutes. This alcohol (methyl alcohol) denatures the proteins present in the blood and hardens

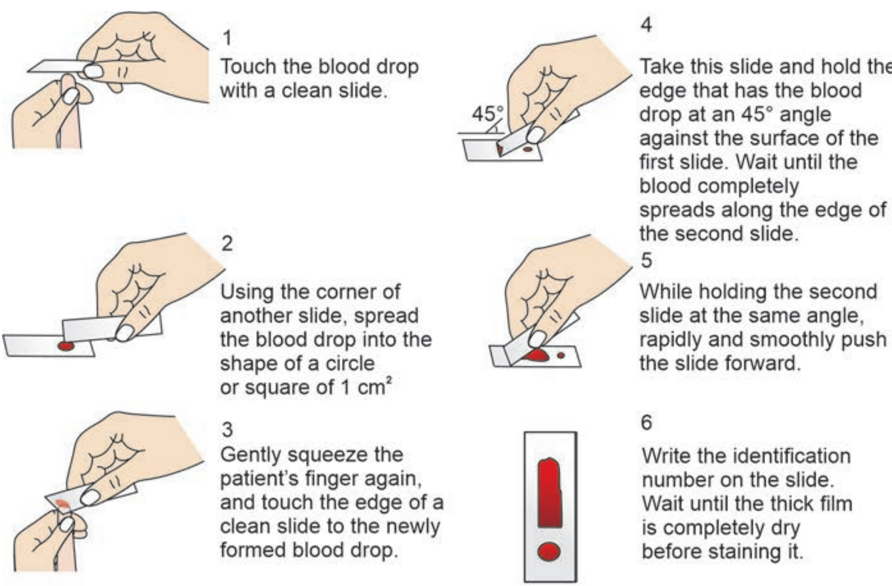


Fig. 2.6: Preparation of a thin and a thick blood film on the same slide

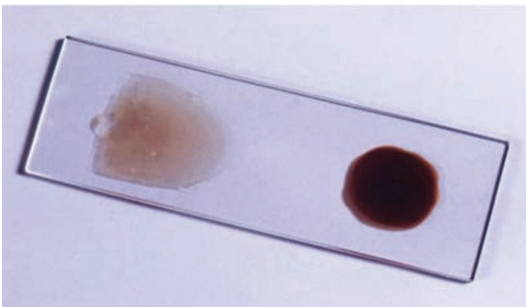


Fig. 2.7: Thin and thick blood films on the same slide. Thin blood film is typically tongue-shaped while thick blood film is circular or square-shaped

the blood cells. As Leishman's stain and Wright's stain contain acetone-free methyl alcohol in the staining solution, the blood films do not require prefixation with alcohol. But Giemsa staining needs prefixation with alcohol as the ready to use staining solution contains only 5% alcohol (suboptimal for fixation).

BONE MARROW ASPIRATE FOR EXAMINATION

1. **Bone marrow films:** Put one drop of aspirate onto slides about 1 cm from one end. Then quickly suck off most of blood present in the aspirate with the help of a fine Pasteur pipette applied to the edge of each drop. Alternatively, keep the glass slides on a slope (to tilt them) for draining away of the blood.

   While the blood is removed, the irregularly shaped marrow fragments adhere to the slide. After that make bone marrow films which will be 3–5 cm in length by using a smooth edged glass spreader of less than 2 cm width. The marrow fragments are dragged behind the spreader and place the marrow cells trailing behind the spreader (trail of cells). The differential count should be made in these cellular trails, starting from the marrow fragment and working back towards the head of the film.

   Fix the bone marrow films and stain them with Romanowsky dyes as for peripheral blood films. But for high quality, a longer fixation time is needed (>20 minutes in methanol).

   Some advocate to add the aspirated marrow material to an anticoagulant like EDTA in a tube and to prepare a marrow films on returning to the laboratory. But there may be a possibility of using excess anticoagulant (only 0.2–0.3 ml of marrow aspirate compared to 2–5 ml of blood). The stained marrow film may show pink-staining amorphous material and some of the erythroblasts and reticulocytes may clump together due to excess anticoagulants.
2. **Bone marrow imprints:** Bone marrow fragments/particles of imprints may also be used for preparation of imprints. One or more visible particles are picked up with a capillary pipette, a toothpick or the broken end of a wooden applicator. The bone marrow particle(s) are transferred immediately to a slide and made to adhere to it by a gentle smearing motion. The slide is air dried rapidly by waving it and then is stained.
3. **Crush preparations:** A small drop aspirate containing a slide near one end. Another slide is placed over the first slide. Slight pressure is given to crush the bone marrow and the slides are separated by pulling them apart in a direction parallel to their surfaces.

   All bone marrow films should be dried quickly by moving them in the air (air dried) or by exposing them to a fan.

   As the bone marrow aspirate is being spreaded, the fat appears as irregular holes and make it sure that the marrow, material not only the blood has been aspirated.

POOR BLOOD SMEARS AND ITS COMMON CAUSES

1. The glass slides should be very clean as dirty slides do not give an even smear.

2. Put an appropriate size of blood drop onto glass slide and make the smear immediately. Delay will cause uneven distribution of WBCs.
3. The spreader slide should be moved steadily and confidently. Jerky movement or loss of contact between spreader slide and smear slide will give poor smears.
4. Angle between the spreader slide and smear slide should be 30° to 45°. Increasing the angle may result in a thick smear, whereas decreasing the result in a thin smear.

FIXING AND STAINING OF BLOOD SMEAR

The smears should be stained immediately after the preparation. Methanol (acetone-free) present in the common Romanowsky stains fix the smear slides in the staining procedure. If staining is delayed then smears must be fixed with methanol for 2–3 minutes. Fixation of smears will prevent distortion of blood cells and smears can be stored for future staining.

In the blood cells, some structural components are acidic while others are basic. Acidic substances stain with basic stain like methylene blue, azure B, etc. and are called basophilic. Examples of basophilic substances are nuclei and nucleic acids. Some basic structures like haemoglobin are stained with acid stains like eosin and are called acidophilic or eosinophilic. Other structures stained by combination of the two are called neutrophilic.

Stains which are composed of both acid and basic dyes are known as "Romanowsky" stains. These stains have the ability to make subtle distinctions during staining of cell and can stain the granules differentially. Neutrophilic granules are weakly stained by azure complexes, whereas eosinophilic granules get stained by acidic component of the dye and basophilic granules which contain acid heparin are stained by basic component of the dye.

The thiazine's basic component consists of methylene blue (tetramethyl thionine) and in varying proportions, its analogues produced by oxidative demethylation: Azure B (trimethyl thionine); azure A (asymmetric dimethylthionine), azure C (monomethyl thionine) and symmetric dimethyl thionine.

As already said most Romanowsky stains are dissolved in methyl alcohol and combine fixation with staining. Various modifications of the original Romanowsky combination of methylene blue (basic stain) and eosin (acid stain) are now used. Usually combination of azure B and eosin Y is used as Romanowsky stain. Common Romanowsky stains are:

1. Leishman's stain
2. Wright's stain
3. Giemsa stain
4. May-Grünwald-Giemsa (MGG) stain
5. Field's stain
6. Jenner's stain
7. MacNeal stain.

Leishman's stain is mostly used in the routine staining of blood film though Wright's stain and Giemsa stain are also very popular (Table 2.1). Giemsa stain is ideal for staining and detecting malarial parasites and other protozoa. Field's stain is used for staining thick film to detect malarial parasites and it offers rapid staining and screening of blood smears. MGG stains are used not only for blood/bone marrow films but also for cytology/FNAC smears.

Leishman's Stain

Reagents

1. Leishman powder (eosin-methylene blue powder): 0.15 g
2. Methyl alcohol (acetone-free): 100 ml

The Leishman powder is placed in a conical flask to which methyl alcohol is added. Then the mixture is warmed to 50°C for 10–15 minutes. It is then filtered. The dye is ripened by keeping the filtrate in sunlight for 3–4 days or in an incubator at 37°C for 7 days.

Method: Dry the film in the air and flood the slide with the stain. After 2 minutes, add double the volume of water and stain the

Table 2.1: Common causes of faulty staining and their corrections

Faulty staining pattern	Causes	Corrections
1. Excessive blue stain	Thick films, prolonged staining time, inadequate washing or too high alkaline pH of stain or diluent.	Staining for less time or using less stain and more diluent. The pH of the buffer should be lowered.
2. Excessive pink stain	Insufficient staining, prolonged washing time, mounting the coverslips before they are dry, too high acidity (very low pH) of the diluents buffer or stain.	Staining time or washing time as advocated pH of buffer and stain should be adjusted.
3. Precipitates on the film	Drying during period of staining, inadequate washing of slide after staining, inadequate filtration of the stain, dust particles on smear or slide and use of unclean slides.	Act as per the cause

film for 7–10 minutes. Then wash the smear in a stream of buffered water until it has acquired a pinkish tinge (up to 2 minutes). After the back of the slide has been wiped clean, set it up right to dry.

Wright's Stain

Reagents

1. Wright's stain powder: 0.2 g
2. Methyl alcohol (acetone-free): 100 ml

The solution is kept at 37°C for a few days before use.

Method: Almost same as in Leishman's staining. When the stain is ripe, a scum of film is formed over the surface of the stain.

Giemsa Stain

Reagents

- Giemsa powder: 0.6 g
- Glycerol: 50 ml
- Acetone-free methyl alcohol: 50 ml

Giemsa powder (0.6 g) is placed in a conical flask. Then 50 ml of glycerol is added. This mixture is warmed at 50°C for 15 minutes with occasional shaking and then 50 ml methanol is added to the mixture. It is now filtered and filtrate is ready for use. But before use the stain should be diluted 1:10 (1 part stain + 9 parts distilled water).

Method: Unlike Leishman or Wright stain, here the blood films should be fixed with methyl alcohol (acetone-free) separately for 3–5 minutes and then dried. Because after 1:10 dilution acetone-free methyl alcohol becomes 5% only (from 50% in original), which is suboptimal for fixation of blood cells. Diluted Giemsa stain (1:10) is poured on the fixed smear and kept for 20–30 minutes. Wash the smear with neutral/ distilled water and dry.

May-Grünwald-Giemsa Stain

Reagents

1. May-Grünwald powder: 0.3 g
2. Acetone-free methyl alcohol: 100 ml

Dissolve the 0.3 g powder (dye) in 100 ml methyl alcohol and warm it at 50°C for 10 minutes. During warming shake it from time to time, filter after 24 hours.

Method: Fixed the smear in methyl alcohol for 3–5 minutes. Then stain the film with diluted (1:10) May-Grünwald stain for 5 minutes. Then stain the film with diluted (1:10) Giemsa stain for 15–20 minutes. Wash with buffered water and dry in the air.

Field's Stain

Reagents

1. **Stain A (polychromed methylene blue)**
   a. Methylene blue: 0.26 g
   b. Azure B (optional): 0.1 g
   c. Disodium hydrogen phosphate: 2.5 g
   d. Potassium dihydrogen phosphate: 1.25 g
   e. Water: 100 ml

Dissolve the phosphates in warm freshly boiled water. Then mix the azure B with phosphate solution and dissolve it well. Lastly the dyes (methylene blue) are added and mix well. Filter it.

2. **Stain B (eosin)**
   a. Eosin Y (yellow eosin, water soluble): 0.26 g
   b. Disodium hydrogen phosphate: 2.5 g
   c. Potassium dihydrogen phosphate: 1.25 g
   d. Water: 100 ml

Dissolve the phosphates in warm freshly boiled water. Then mix the dye (eosin Y) with phosphate solution and dissolve it well, filter it.

Staining Method

1. Fix the film for 10–15 seconds in methanol.
2. Pour off the methanol and put 12 drops of diluted stain B (1:4 dilution in water)
3. Immediately add 12 drops of stain A.
4. Agitate the slides to mix the stains.
5. After 1 minute, rinse the slide in water.
6. Differentiate the slide in phosphate buffer for 5–10 seconds at pH 6.6.
7. Wash the slide in water.
8. Place it on end to drain and then dry.

Note

i. A pH to the alkaline side of neutrality accentuates the azure component of Romanowsky stain at the expenses of the eosin and vice versa.
ii. A pH of 6.8 is usually recommended for general or routine use.
iii. To look, malarial parasites a pH of 7.2 is recommended in order to detect Schuffner's dots of *Plasmodium vivax, ovale* and *malariae*. (Remember Maurer's dots in *P. falciparum* and Ziemann's dots in *P. malariae*).

Chemical Theory of Romanowsky Staining

The mechanism by which certain components or structure of a cell stain with particular dye, depends on complex differences between the different dyes. As for example, azure B in dimer form is bound to anionic molecules, e.g. phosphate groups of DNA, whereas eosin Y is bound as a monomer to cationic sites of proteins.

As early as the dyes are bound to particular structure of the cell, either electron interaction occurs with dye-dye aggregation or the eosin Y molecule is inserted between the azure B molecules and the complex is held together by charge effect.

So, the acidic groupings of the nucleic acids and proteins of the cell nuclei and primitive cytoplasm determine their uptake of the basic dyes (like azure B). On the other hand, the presence of basic groupings on the haemoglobin molecules determines its affinity for acidic dyes and its staining with eosin (acid dye).

Examination of Romanowsky Stained Blood Smear

At first, examine the stained blood smear under low power for screening. Note the background colour and distribution of WBCs. In an ideal stained smear, three zones can be identified visually. The starting area or the "head" of the smear (where blood drop was originally placed), following which is the "body" and the thin end of the smear known as "tail" (Fig. 2.8).

At the tail end, RBCs lie singly and the neutrophils and monocytes predominate. In the body, RBCs overlap each other to a certain extent and lymphocytes predominate. The ideal area is in-between these "body" and "tail" of the smear where blood

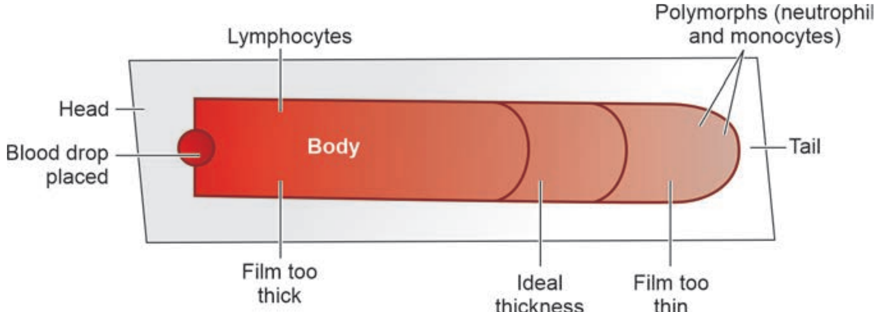


Fig. 2.8: Three zones in stained smears. The head, body and tail

cells are uniformly distributed. Here, the RBCs do not overlap and touch each other slightly.

For differential count of WBCs (Figs 2.9 and 2.10), two methods can be adopted:

1. The original drop of blood spreaded out between spreader and slide (C-C1). The film is made in such a way that representative strips of film like A-A1 and B-B1 are formed from point of application A and B respectively. In order to make an accurate differential count, all leucocytes in one or more strips (like A-A1, B-B1, etc.) should be inspected and classified.

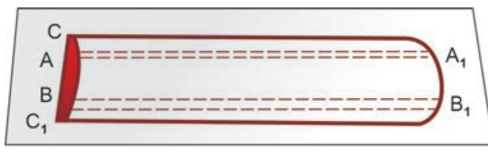


Fig. 2.9: Linear or straight line method of differential count of WBC

2. Choose the ideal thickness of the stained smear. Then inspect and classify all the leucocytes in a serpentine counting pattern (shown diagrammatically) (Fig. 2.10).

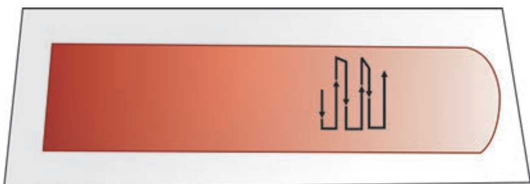


Fig. 2.10: Serpentine counting pattern (area is in between body and tail end of smear)

EXAMINATION OF STAINED BLOOD FILMS

Erythrocytes

The erythrocytes when not crowded together, appear as circular, homogenous discs of nearly uniform size, ranging from 6–8.5 mm in diameter. As for haemoglobinization, normally a small area of central pallor is seen (central 1/3rd) in RBCs.

Colour

1. **Normochromia: Normal RBC** appears pinkish brown due to presence of haemoglobin. Peripheral part looks deep brown while the central part (1/3rd) is pale because of biconcave shape of RBC.
2. **Hypochromia:** When RBC contains less haemoglobin, the central pale area becomes larger and paler. The MCHCs are also decreased.

   Example: **Iron deficiency anaemia.**
3. **Hyperchromia:** The RBCs become thicker and larger and they stain deeply and less central pallor because of increased haemoglobin content (MCH), but the haemoglobin concentration (MCHC) is normal.

   Example: **Megaloblastic anaemia.**
4. **Polychromasia:** Theoretically means many colours but practically RBCs appear bluish grey. This is due to presence of residual RNA in RBC (normally absent in mature RBC). So, young red cell shows polychromasia and larger than mature red cell and may lack central pallor. These

young red cells are called reticulocytes. It is most marked in **haemolysis and blood loss.**

4. **Anisochromia:** It means unequal haemoglobin content due to different populations of RBCs. Hence, different staining patterns of individual RBC. Example: **Iron deficiency anaemia treated with blood transfusion**.

Size

1. Normocytes: **Normal RBC** (6–8 μm in diameter; average 7–7.5 μm)
2. Microcytes: Decrease in size of RBC which may result from fragmentation of normally sized red cells (normocytes) or larger red cells (macrocytes). It occurs in many types of abnormal erythropoiesis, **e.g. iron deficiency anaemia and thalassaemia**.
3. Macrocytes: They are large RBCs having a diameter more than 8 micro mm, a MCV (mean corpuscular volume) more than 95 fl and higher than normal Hb concentration (MCHC). Example: **Megaloblastic anaemia, chronic liver diseases.**
4. Anisocytosis: This is a general term which describes any variation in size of RBC. Example: **Anaemias, thalassaemias**.

Shape

i. **Poikilocytosis:** This is a general term which describes any variation in the shape of RBC.

ii. **Spherocytes:** They are nearly spherical RBC in contrast to normal biconcave disc. Their diameter is smaller than normal and thickness is greater than normal. Tiny bits of membrane (in excess of Hb) are removed from adult RBC resulting the cell with a decreased surface/volume ratio. Example: Hereditary spherocytosis and in some cases of autoimmune haemolytic anaemia.

iii. **Target cells (leptocytes):** These refers to leptocytes (unusually thin red cells), and when stained show a peripheral ring of Hb with a dark, central, Hb containing area. Example: Haemoglobinopathies like thalassaemia, chronic liver disease, following splenectomy, HbC disease.

iv. **Schistocytes (cell fragment)**: It indicates the presence of haemolysis as seen in severe burn, megaloblastic anaemia or in microangiopathic haemolytic anaemia.

v. **Acanthocytes**: These are irregularly spiculated RBCs in which ends of spicules are bulbous and rounded. Examples: Abetalipoproteinaemia, certain liver diseases.

vi. **Burr cells (echinocytes):** These are small cells or cell fragments bearing one or few spines or spicules with regular distribution (unlike acanthocytes where spicules are seen all over RBC surface). Examples: Microangiopathic haemolytic anaemia, severe burns.

Structure or Content

i. **Basophilic stippling (punctate basophilia):** It is characterized by presence within erythrocytes of irregular basophilic granules which vary from fine to coarse. Fine stippling is seen when there is increased red cell production and therefore increased polychromatophilia. Coarse basophilic stippling may be seen in lead poisoning, megaloblastic anaemia or pyrimidine-5-nucleotidase deficiency. This is attributed to an abnormal instability of the RNA in the young red cell.

ii. **Pappenheimer bodies:** These are abnormal granules of iron found inside RBCs and stained by Wright stain and/or Giemsa stain. These bodies are a type of inclusion body formed by phagosomes that have engulfed excessive amounts of iron. They appear as dense, blue-purple granules within RBCs and are usually only one or two, located in the cell periphery. Examples: Sideroblastic anaemia, haemolytic anaemia, and sickle cell disease.

iii. **Cabot rings:** These are ring shaped, figure of eight or loop-shaped structures. These rings are probably microtubules remaining from a mitotic spindle (due to defective erythropoiesis). Examples: Pernicious anaemia, lead poisoning

iv. **Howell-Jolly bodies:** These are smooth, round remnants of nuclear chromatin. Single Howell-Jolly bodies may be seen in haemolytic anaemia, megaloblastic anaemia and after splenectomy. Multiple Howell-Jolly bodies in a single RBC usually indicate megaloblastic anaemia or defective erythropoiesis.

v. **Rouleaux formation:** Rouleaux formation is the alignment of RBCs on one another so that they resemble stack of coins. Examples: Multiple myeloma, other paraproteinaemia (monoclonal gammopathy) and macroglobulinaemia.

White Blood Cells (WBCs)

Differential leucocyte count (DLC) (Fig. 2.11): The DLC is done on the basis of size, cytoplasm with or without granules and type of nucleus of WBCs. The WBC may be divided into:

- **Granulocytes** (WBC with cytoplasmic granules): Neutrophils, eosinophils and basophils.
- **Agranulocytes** (WBC without granules): Lymphocytes and monocytes.

Fig. 2.11: DLC counter (manual) for differential leucocyte count (DLC)

Total leucocyte count (TLC): Normal total leucocyte count in adult person is 4000–11000/mm^3. Leukocytosis refers to TLC more than 11000/mm^3. Leucopenia less than 4000/mm^3.

Neutrophils (Polymorphs) (Fig. 2.12)

This leucocyte averages 12 mm in diameter, they are smaller than monocytes and eosinophils and slightly larger than basophils. **Segmented neutrophil** has at least two of its lobes separated by a filament. **Band neutrophil** has either a U-shaped nucleus of uniform thickness or a strand of nuclear material thicker than a filament connecting the lobes (appearance of "telephonic receiver").

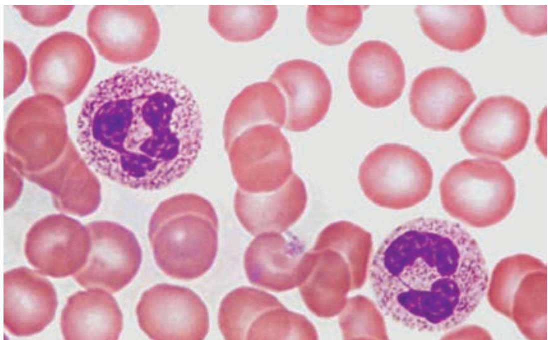


Fig. 2.12: Neutrophils

- The cytoplasm is filled up with tiny granules (0.2–0.3 mm) which stain tan to pink or orange with Romanowsky stains.
- Normal segmented neutrophils: 56% of leucocytes (DLC).
- Normal band neutrophils: 03% of leucocytes (up to 8% may be seen).
- Two lobes neutrophils: 10–30% of neutrophils.
- Three lobes neutrophils: 40–50% of neutrophils.
- Four lobes neutrophils: 10–20% of neutrophils.
- Five lobes neutrophils: ≤5% of neutrophils.
- In women, 2–3% of circulating neutrophils show an appendage at a terminal nuclear segment. This 'drumstick' is connected to the nucleus by a short stalk

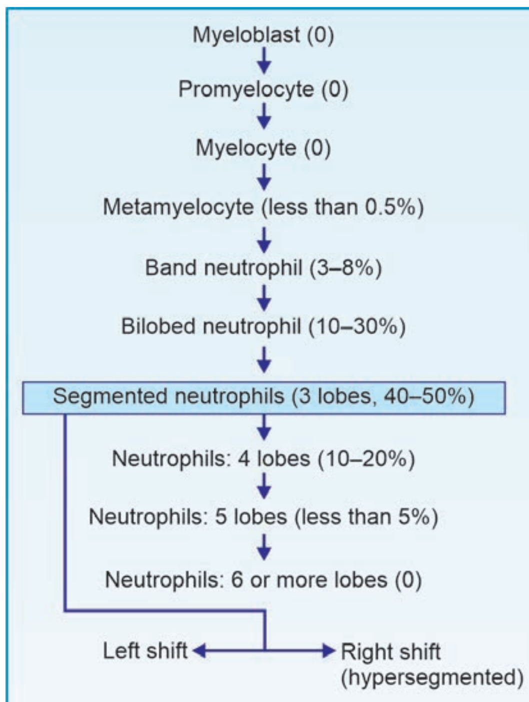
and is about 1.5 mm in diameter. It indicates the inactive X chromosome and corresponds to **Barr body**.

Hypersegmented neutrophils (Fig. 2.13): If the peripheral smear shows ≥5% of neutrophils having 5 lobes or ≥1% of neutrophils having 6 lobes, then the neutrophils are called hypersegmented.

Causes

1. Megaloblastic anaemia
2. Uraemia
3. Hydroxyurea treatment
4. Cytotoxic treatment especially with methotrexate treatment.

Stages of neutrophilic maturation



Segmentation of the nucleus of the neutrophil is a normal separation process. With the three-lobed neutrophil as a marker, shift to the left (less nature) or to the right (hypermature) can be understood. A left shift with band neutrophils, metamyelocytes and occasional myelocytes is common in sepsis and usually neutrophils contain toxic granules in cytoplasm. If myeloblast and promyelocytes are seen in peripheral blood, the causes may be leukaemia or leukoerythroblastic anaemia. Sometimes in pregnancy, a significant number of band forms is seen.

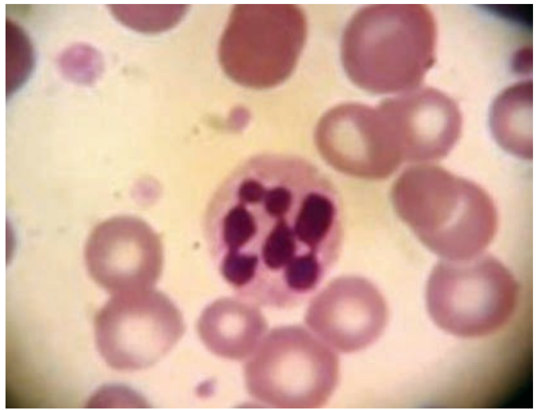


Fig. 2.13: Hypersegmented neutrophils (7 lobes) in megaloblastic anaemia

Arneth count: Neutrophils are divided in five groups according to number of lobe(s), it possesses:

1. Group I : One lobe
2. Group II: Two Lobes
3. Group III: Three lobes
4. Group IV: Four lobes
5. Group V: Five lobes.

One hundred neutrophils are counted in peripheral smear and number of each group of neutrophil is expressed as a percentage.

If there is increase in the group I and II neutrophils (as seen in sepsis) then there is a shift to left. Whereas, if there is more hypersegmented neutrophils (as seen in megaloblastic anaemia), then there is shift to right.

Arneth index: Percentage of neutrophils in groups I, II and ½ of group III is about 60 (normal range 51–65).

Schilling count: In this count, all the granular leucocytes are divided into four groups and the number of each group is expressed as a percentage of the WBCs. The four groups are:

1. Myelocytes
2. Metamyelocytes
3. Band neutrophils
4. Segmented neutrophils

A dividing line is drawn (usually segmented neutrophils' number). A shift to left happens when the number in percent increases, to the left of the dividing line.

Morphologic alterations in neutrophils

Toxic granules: These are dark blue to purple cytoplasmic granules seen in neutrophils (also in metamyelocytes and band forms). Toxic granules are seen in severe bacterial infections and in other causes of inflammation or toxic conditions. These are myeloperoxidase positive and may be numerous or few in number. Toxic granules are azurophil granules that have retained their basophilic staining reaction by lack of maturation or have developed increased basophilia in mature neutrophils. Toxic granules like azurophilic granules seen in neutrophils with prolonged staining time or decreased pH of staining reaction.

Döhle bodies: Döhle inclusion bodies are small, round or oval pale blue-gray structure, usually found at the peripheral cytoplasm of neutrophil. They consist of decomposed ribosomes and endoplasmic reticulum. Originally they were described in scarlet fever, but they are seen in any other infections, in aplastic anaemia, following administration of toxic agents and in burns.

Cytoplasmic vacuoles: It usually indicates severe sepsis, when toxic granules are also present. Cytoplasmic vacuoles will develop as an artifacts with prolonged standing of the blood before smears are made.

May-Hegglin anomaly: Autosomal dominant disease in which pale blue inclusions resembling Döhle bodies are seen. But the inclusions are larger and more prominent than Döhle bodies. Also, they are found in all leucocytes except lymphocytes.

Pelger-Huet anomaly: It is a benign inherited condition in which neutrophil nuclei fail to segment properly. Most of the neutrophils nuclei have two discrete equal-sized lobes connecting by a thin chromatin bridge. The chromatin is coarsely granular and cytoplasmic granular content is normal.

A similar type acquired morphological anomaly, known as pseudo-Pelger cells may be seen in acute myeloid leukaemia (AML). Here, the neutrophils are hypogranular and have irregular nuclear pattern.

Neutrophilia

Definition: When absolute neutrophil count 7500/mm^3 or 72% of DLC.

Relative neutrophilia: It can be divided into primary (clonal) and secondary.

Primary neutrophilia: Myeloproliferative neoplasms (chronic myeloid leukaemia, acute myeloid leukaemia), neutrophilic leukaemia, hereditary neutrophilia.

Secondary neutrophilia: Localized acute infections (pneumonia, tonsillitis, meningitis, acute otitis media), systemic infection (e.g. septicemia), acute rheumatic fever, vasculitis, acute myocardial infarction, burns, leukoerythroblastic reaction.

Leukoerythroblastic reaction: The presence of normoblasts, tear drop cells and immature cells of neutrophilic series (promyelocytes, myelocytes, metamyelocytes, band forms) along with neutrophilia in the blood is known as leukoerythroblastic reaction. It often indicates space-occupying disturbances of the bone marrow such as myelofibrosis with myeloid metaplasia, metastatic carcinoma, leukaemias, multiple myelomas, Gaucher's disease, TB and other granulomatous diseases.

Neutropenia

Definition: When neutrophil count is <43% of leucocytes or an absolute neutrophil and band from <1500/mm^3.