

CHAPTER SIX

HEMOCYTOMETRY

Learning objectives

At the end of this chapter, the student shall be able to:

- Discuss the general principles of manual hemocytometry
- List the materials that are basically required in manual hemocytometry
- Identify the sources of error in manual hemocytometry
- Mention the diluting fluid, dilution factor and areas of counting on the chamber for the RBC, WBC, platelet and eosinophil count
- Perform RBC, WBC, platelet and eosinophil counts
- Discuss the clinical significance and normal values of each of the cell counts.

Introduction

- ✓ Visual counting of blood cells is an acceptable alternative to electronic counting for white cell and platelet counts.
- ✓ It is not recommended for routine red cell counts because the number of cells which can be counted within a reasonable time in the routine laboratory will be too few to ensure a precise result.
- ✓ Yet it is still necessary for the technologist to be able to use this method effectively and to know its limitations.
- ✓ Any cell counting procedure includes three steps: dilution of the blood, sampling the diluted suspension into a measured volume, and counting the cells in that volume.
- ✓ The main principles for such examinations are:
 - Selection of a diluting fluid that not only will dilute the cells to manageable levels but will either identify them in some fashion or destroy contaminant cellular elements.
 - The use of a special glass counting chamber called hemocytometer that will present the cells to the observer in such a way that the number of cells per unit volume of fluid can be counted.

Counting Chambers

- ✓ The hemocytometer is a thick glass slide with inscribed platforms of known area and precisely controlled depth under the coverslip.
- ✓ In the center of the upper surface there are ruled areas separated by moats/channels from the rest of the slide and two raised transverse bars one of which is present on each side of the ruled area.
- ✓ The ruled portion may be in the center of the chamber (single chamber) or there may be an upper and lower ruled portion (double chamber).
- ✓ The double chamber is to be recommended since it enables duplicate counts to be made rapidly. When an optically plane cover glass is rested on the raised bars there is a predetermined gap or chamber formed between its lower surface and the ruled area

(fig. 6.1).

- ✓ This is called the depth of the chamber and it varies with the type of the chamber.
- ✓ The ruled area itself is divided by microscopic lines into a pattern that varies again with the type of the chamber.
- ✓ The counting chamber recommended for cell counts is a metallized surface (Bright-line) double cell Improved Neubauer ruled chamber.
- ✓ Non-metallized hemocytometer are less expensive, but they are not recommended.
- ✓ It is more difficult to count WBCs reliably using this type of chamber because the background rulings and cells are not as easily seen.
- ✓ Not-metallized chambers are also more difficult to fill.
- ✓ Although there are a number of hemocytometer, it is the improved Neubauer counting chamber which is used for most routine cell counts:

I. Ordinary Neubauer counting chamber

- The central platform is set 0.1mm below the level of the two side ones, giving the chamber a depth of 0.1mm.
- The engraving covers an area of 9mm² divided into 9 squares of 1mm² each. The 4 corner squares are divided into 16 squares, each with an area of 1/16 of a mm².
- The central ruled area of 1mm² is divided into 16 large squares by sets of triple lines. These large squares are further subdivided into 16 small squares by single lines. The width of the triple lines dividing the large squares is the same as the width of a small square. Two adjacent sides of the ruled area are bounded by triple lines, the other two by single lines.
- Each side is, therefore, divided into 20 equal divisions (the width of 16 small squares and 4 sets of triple lines).
- Each small square is, therefore, 1/20 of 1mm squared that is 1/400 of 1mm².

II. The Improved Neubauer Counting Chamber

- The depth between the lower surface of the cover glass which is on the raised bars and the ruled area is 0.1mm.
- Each ruled area is a square of 9mm divided into nine large squares each of 1mm side. The central square of these nine is divided by engraved lines into 400 tiny squares of arranged in 25 groups of 16 by triple boundary lines.
- Each large square is 1mm², each of the 25 medium squares is of 0.04mm² area and each of the
- 400 tiny squares has an area of 0.0025mm².

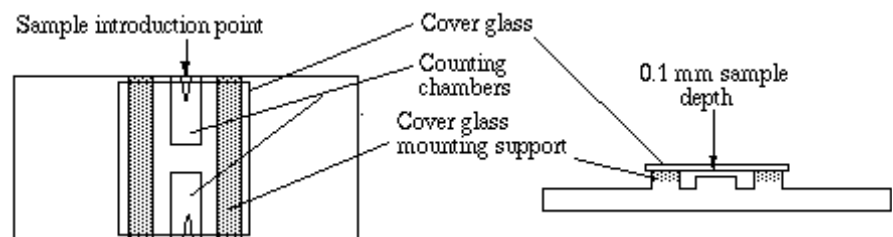
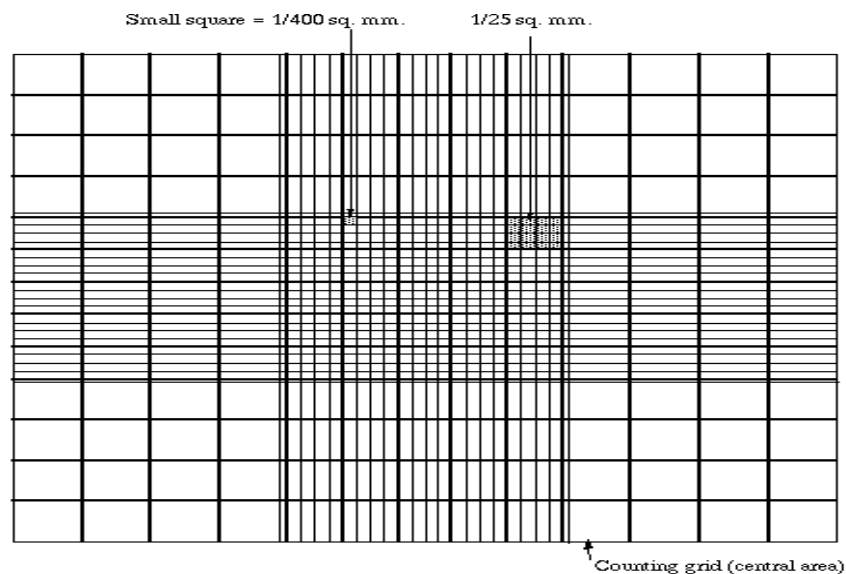


Fig. 6.1a Improved Neubauer ruled counting chamber.
Neubauer counting chamber

Fig. 6.1b: View of the improved

III. Fuchs-Rosenthal counting chamber

- This chamber was originally designed for counting cells in cerebrospinal fluid, but as such a relatively large area
- is covered, it is preferred by some workers for counting leucocytes.
- The depth is 0.2mm and the ruled area consists of 16mm squares divided by triple lines.

- These squares are subdivided to form 16 smaller squares, each with an area of $1/16$ of 1mm^2 (figure 6.2). Another type of Fuchs-Rosenthal chamber is now available, which has the same depth as the one described, but is ruled over 9mm^2 only.

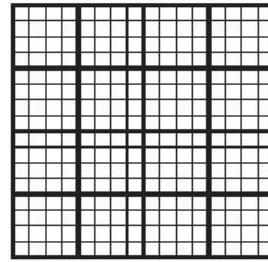


Fig. 6.2: Fuchs-Rosenthal counting chamber

IV. Burkter ruled counting chamber

Like the Neubauer counting chamber, this has a ruled area of 9mm^2 and a depth of 0.1mm . To count white cells using Burkter Chamber, the four large corner squares are used (4mm^2) and the same calculation as describe for the Improved Neubauer ruled chamber is used.

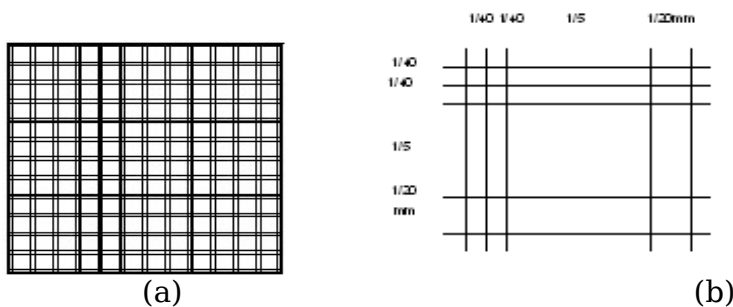


Fig. 6.3 (a) Ruled area of the Burkter counting chamber; (b) enlarged view showing actual measurements.

Dilution of the Sample

Dilution of sample is accomplished by using either a thomma pipette or the tube dilution method. With tubes larger volumes of blood and diluting fluid are used and the greater will be the accuracy as compared with the smaller volumes used in the thomma pipette techniques. Thomma pipettes are small calibrated diluting pipettes designed for either white cell or red cell count.

Counting and Calculation

The diluted cells are introduced into the counting chamber and allowed to settle. They are then counted in the designated area (s). Cells lying on or touching the upper or left boundary lines are included in the count while those on the lower and right boundary lines are disregarded.

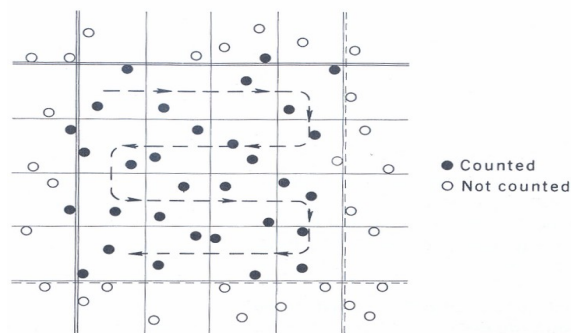


Fig 6.4 Examples of white blood cells counted in a representative area.

Calculation

$$\text{No. of cells/mm}^3 = \frac{N \times \text{DF}}{A \times d}; \quad \text{No. of cells/l} = \frac{N \times \text{DF} \times 10^6}{A \times d}$$

Where, N = no. of cells counted in a given area

DF = dilution factor

A = area of counting in mm²

d = depth of the counting chamber

(Volume of chamber = A × d)

6.1 White blood cell count

- A white blood cell count (total leucocyte count - TLC) is used to investigate infections and unexplained fever and
- to monitor treatments which can cause leucopenia. In most situations when a total WBC count is requested it is usual to perform also a differential WBC count. EDTA anticoagulated blood or capillary blood can be used for counting white cells. Heparin or sodium citrate anticoagulated blood must not be used.

Principle

- ✓ Whole blood is diluted 1 in 20 an acid reagent which hemolyzes the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted.
- ✓ White cells are counted microscopically using an Improved Neubauer ruled counting chamber (hemocytometer) and the number of WBCs per liter of blood calculated.
- ✓ When after examining a stained blood film, many nucleated red cells are present (more than 10%), the WBC count should be corrected.

Diluting Fluid

- ✓ Turk's solution - 2% aqueous solution of acetic acid colored pale violet with gentian violet or pale blue with methylene blue.
- ✓ The glacial acetic acid causes erythrocyte lysis while the gentian violet lightly stains the leucocytes permitting easier enumeration.

Test method

Thomson White Cell Pipette

- ✓ The long stem is divided into 10 equal parts with "0.5" and "1" engraved on it.
- ✓ On the short limb just above the bulb, the mark "11" is engraved.
- ✓ When blood is drawn up to the 0.5 mark and diluent to the 11 mark, the sample of blood (now in the bulb) is diluted 1:20. Once the pipette accurately filled to the mark, the rubber suction (or mouth piece) is carefully removed, with the pipette held horizontally and only one finger sealing the tip.
- ✓ Both ends of the pipette may then be sealed with special small rubber sealing caps or with the middle finger on the tip and the thumb on the other end.
- ✓ The pipette is shaken mechanically or manually for 2 minutes.
- ✓ A bead contained in the bulb of the pipette aids in the mixing.
- ✓ If shaking is done manually, the shaking motions should be varied and alternated.
- ✓ The cover glass is placed on the chamber and a slight pressure applied to the ends of the cover glass until a "rain bow" or Newton's diffraction rings are revealed on either side.
- ✓ Once the diluted blood in the pipette has been thoroughly mixed, a few drops are expelled to discard the cell-free diluting fluid in the long stem of the pipette.
- ✓ With the index finger forming a controlled seal over the end of the pipette, which is held at an angle of 45°, the tip of the pipette is brought up to the edge of the cover glass and by gentle release of index finger pressure, fluid is allowed to run out slowly until the counting platform is covered.
- ✓ The fluid is drawn into the chamber by capillary attraction.
- ✓ Care must be taken not to overfill the chamber which will result in overflow into the channels.
- ✓ If blood is diluted with the tube technique (in which 20µl of blood is taken with a sahli pipette and mixed with 0.38ml of diluting fluid in a small tube).
- ✓ Charging is accomplished by using disposable capillary tubes or long stem Pasteur pipettes.

- ✓ The chamber is placed in position on the microscope stage and is allowed to stand for 2 or 3 minutes so that the cells will settle.
- ✓ All apparatus should be cleaned thoroughly after each use. Pipettes (thomma and sahli) should be washed well with a sequence of water and acetone (filled with each fluid three or four times) and air drawn after the acetone until the inside of the pipette is thoroughly dry.
- ✓ Pipettes should be periodically cleaned with potassium dichromate cleaning solution or hydrogen peroxide. Hemocytometers should be washed in distilled water immediately after use and dried with gauze or tissue paper.
- ✓ They should be stored in such a way as to avoid breakage and scratching of the counting surface.

Performance of the Count

The counting chamber is surveyed with the low power objective to ascertain whether the cells are evenly distributed. Then the number of cells in four large squares is counted.

Calculation

If N is the number of leucocytes in four large squares, then the number of cells per mm³ is given by:

$$\text{No. of leucocytes/mm}^3 = N \times \text{DF Vol.}$$

Where

N is the number of leucocytes in an area of 4mm²

DF is the dilution factor equal to 20

Vol. is the total volume on which the count is done and is given by the total area of count multiplied by the depth of the chamber (0.1mm for the improved Neubauer counting chamber. Substituting these values in the above formula:

$$\text{No. of leucocytes/mm}^3 = N \times 50, N \geq 100^*$$

* 100 cells is a reasonable and practical figure for visual counts. When the leucocyte count is low (below $4.0 \times 10^3/\text{mm}^3$), it is advisable for greater accuracy to use a 1:10 dilution, i.e., take blood to the "1" mark of the pipette and diluting fluid to the 11 mark.

The corrected leucocyte count

Nucleated red cells will be counted and cannot be distinguished from leucocytes in the total leucocyte count. If their number is high as seen on the stained smear, a correction should be made according to the following formula:

$$\text{Corrected leucocyte count} = \frac{\text{Uncorrected count} \times 100}{\text{No. of NRBC} + 100}$$

Where the **No. of NRBC** is the number of nucleated red cells which are counted during the enumeration of 100 leucocytes in the differential count.

Example

The blood smear shows 25 nucleated red cells per 100 white cells in the differential count. The total leucocyte count is 10,000/mm³. Calculate the true leucocyte count.

Tube method

1. Measure 0.38ml of diluting fluid and dispense into a small container or tube.
2. Add 20μl (0.02ml, 20cmm) of well-mixed EDTA anticoagulated venous blood or free-flowing capillary blood and mix.
3. Assemble the counting chamber.
4. Re-mix the diluted blood sample. Using a capillary, Pasteur pipette, or plastic bulb pipette held at an angle of about 45°C, fill one of the grids of the chamber with the sample, taking care not to overfill the area.
5. Leave the chamber undisturbed for 2 minutes to allow time for the white cells to settle.
6. Count as described in thomma white cell count method * When a count is higher than $50 \times 10^9/\text{l}$, repeat the count using 0.76ml of diluting fluid and 20μl of blood. When a count is lower than $2 \times 10^9/\text{l}$, repeat the count using 0.38ml of diluting fluid and 40μl of blood.

Sources of error in manual WBC counts

- Incorrect measurement of blood due to poor technique or using a wet or chipped pipette.
- When using anticoagulated blood, not mixing the blood sufficiently or not checking the sample for clots.
- Inadequate mixing of blood with diluting fluid.
- Not checking whether the chamber and cover glass are completely clean.
- Not using a hemocytometer cover glass
- Over-filling a counting chamber or counting cells when the sample contains air-bubbles.
- Not allowing sufficient time (2 minutes) for the cells to settle in the chamber.
- Using too intense a light source or not reducing the iris diaphragm sufficiently to give good contrast (poor focusing and difficulty in seeing clearly the cells and ruling are common when using non-metallized hemocytometers).
- Not completing counting of the cells before the sample begins to dry in the chamber.
- Counting too few cells. Precision increases with the number of cells counted.
- Not correcting a count when the sample contains many nucleated RBCs.

Interpretation of WBC count

- ✓ Reference ranges for white cell counts vary with age with higher counts being found in children.
- ✓ There are also gender differences with higher total WBC and neutrophil counts being found in women of child-bearing age and during pregnancy.
- ✓ Counts also vary in different populations with lower total WBC and neutrophil counts being found in Africans and people of African descent.
- ✓ Total leucocyte counts are commonly increased in infections and when considered along with the differential leucocyte count can be indicators as to whether the infecting agent is bacterial or viral.

WBC reference range

- | | |
|----------------------------|---------------------------------|
| • Children at 1 y | 6.0 – 18.0 x 10 ⁹ /l |
| • Children 4-7 y | 5.0 – 15.0 x 10 ⁹ /l |
| • Adults | 4.0 – 10.0 x 10 ⁹ /l |
| • Adults of African origin | 2.6 – 8.3 x 10 ⁹ /l |
| • Pregnant women | Up to 15 x 10 ⁹ /l |

Leucocytosis

The main causes of a raised WBC count are:

- Acute infections e.g. pneumonia, meningitis, abscess, whooping cough, tonsillitis, acute rheumatic fever, septicemia, gonorrhea, cholera, septic abortion. Acute infections in children can cause a sharp rise in WBC count.
- Inflammation and tissue necrosis e.g. burns, gangrene, fractures and trauma, arthritis, tumors, acute myocardial infarction.
- Metabolic disorders e.g. eclampsia, uremia, diabetic coma and acidosis.
- Poisoning e.g. chemicals, drugs, snake venoms
- Acute hemorrhage
- Leukemias and myeloproliferative disorders
- Stress, menstruation, strenuous exercise.

Leucopenia

The main causes of a reduced WBC count are:

- Viral, bacterial, parasitic infections e.g. HIV/AIDS, viral hepatitis, measles, rubella, influenza, rickettsial infections, overwhelming bacterial infections such as miliary tuberculosis, relapsing fever, typhoid, paratyphoid, brucellosis, parasitic infections including leishmaniasis and malaria.
- Drugs e.g., chloramphenicol, phenylbutazone,
- Ionizing radiation
- Production failure as in aplastic anemia, megaloblastic anemia

- Anaphylactic shock

6.2. Red Cell Count

- ✓ Although red cell counts are of diagnostic value in only a minority of patients suffering from blood diseases, the advent of electronic cell counters has enormously increased the practicability of such counts.
- ✓ Their value, too, has been increased now that they can be done with a degree of accuracy and reproducibility comparable to that for hemoglobin estimation.
- ✓ Although clearly an obsolete method (because the combined error of dilution and enumeration is high), visual counting will still have to be undertaken for some years to come in the smaller laboratories.

Principle

- ✓ A sample of blood is diluted with a diluent that maintains (preserves) the disc-like shape of the red cells and prevents agglutination and the cells are counted in a Neubauer or Burkner counting chamber.

Diluting Fluid

- 1% formal citrate

Dilution

Thoma Red Cell Pipette

- ✓ Take a well mixed blood or blood from a freely flowing capillary puncture to the "0.5" mark of the pipette and diluent to the "101" mark. Blood will be diluted 1:200.

Tube Dilution

- ✓ Take 20µl blood with sahli pipette and mix it with 4ml diluent in a small tube to give a final dilution of 1:201

Counting and Calculation

After the suspension is charged into the chamber and the cells allowed to settle, cells should be counted using the 40× objective and 10× eyepiece in 5 small squares of the central 1mm² area of the improved Neubauer counting chamber

(4 corner and 1 central squares each with an area of 0.04mm²). If the Burkner counting chamber is used, the count is done in 3 (3mm × 0.05mm) area. It is important to count as many cells as possible for the accuracy of the count is increased thereby; 500 cells should be considered as the absolute minimum.

Calculation

- ✓ No. of RBC/mm³ = $N \times 10,000$ for $N \geq 500$ (Improved Neubauer counting chamber).
- ✓ If the number of RBC in the five small squares is less than 500, then the whole 1mm² central area should be counted.
- ✓ No. of RBC = $N \times 4440$ (Burkner counting chamber).

Normal Values

Adults:

- Men: $4.5-6.2 \times 10^6/\text{mm}^3$
- Women: $4.0-5.5 \times 10^6/\text{mm}^3$

Infants and children:

- at birth: $4.0-6.0 \times 10^6/\text{mm}^3$
- first 3 months: $4.0-5.5 \times 10^6/\text{mm}^3$
- 3 months - 3 years: $4.0-5.2 \times 10^6/\text{mm}^3$
- 3 years - 10 years: $4.0-5.0 \times 10^6/\text{mm}^3$

Significance of Results

- Together with the hematocrit and hemoglobin values it can be used to calculate the red cell indices which provide a valuable guide to the classification of anemias and diagnosis of polycythemia.

6.3 Platelet Count

- ✓ A platelet count may be requested to investigate abnormal skin and mucosal bleeding which can occur when the platelet count is very low.

- ✓ Platelet counts are also performed when patients are being treated with cytotoxic drugs or other drugs which may cause thrombocytopenia.
- ✓ Many methods for counting platelets have been described and their number is doubtless due to real difficulties in counting small fragments which can assume various shapes, which agglutinate and break up easily and which are difficult to distinguish from extraneous matter.
- ✓ The introduction of EDTA as a routine anticoagulant with its ability to inhibit platelet aggregation has to some extent resolved the problem of aggregate formation and the use of phase contrast microscope facilitates platelet identification.

I. Method using formal-citrate red cell diluent

- ✓ Diluents should be prepared using thoroughly clean glassware and fresh distilled water.
- ✓ The solution should be filtered before use.

Method

1. Make a 1:100 dilution of a well mixed EDTA anticoagulated blood using a red cell thomma pipette (blood to the "1" mark and diluent to the "101" mark) or by adding 20 μ l of blood to 2ml diluent in a clean glass tube. EDTA venous blood is preferred to capillary blood since some platelets are unavoidably lost from the latter because they adhere to the edges of the wound and this favors falsely low values.
2. Mix for 2 minutes on a mechanical mixer or manually. Then fill a Neubauer counting chamber and allow the platelets to settle for 20 minutes. To prevent drying of the fluid, place the chamber in a petri-dish or plastic container on dampened tissue or blotting paper and cover with a lid.
3. Count the number of platelets which will appear as small refractile bodies in the central 1mm² area with the condenser racked down.

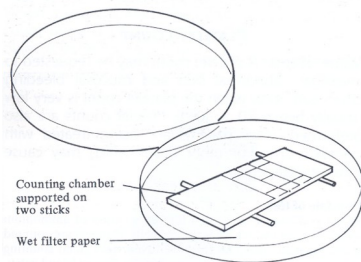


Fig 6.5 Counting chamber in Petri dish to prevent drying of

the preparation

Calculation: No. of platelets/mm³ = $N \times 1000$, $N \geq 100^*$

* The total platelets counted should exceed 100. If the count is less than 100, it is preferable to repeat the count with a lesser dilution of blood.

Disadvantage of the Method

- ✓ Platelets may be obscured by overlying red cells.

II. Method Using Ammonium Oxalate (10g/l; 1%w/v)

- ✓ This diluent causes erythrocyte lysis.
- ✓ Not more than 500ml should be prepared at a time using thoroughly clean glassware and fresh distilled water.
- ✓ The solution should be filtered and kept at 40C. Always refilter the fluid before use.

Method

- ✓ A 1:20 dilution of blood is made using either a WBC thomma pipette or the tube dilution technique.
- ✓ The preparation is mixed, the chamber filled and the cells allowed to settle in a similar fashion as Method 1.
- ✓ The cells are counted in 5 small squares in the central 1mm² of the improved Neubauer counting chamber.

Calculation No. of platelets/mm³ = $N \times 1000$, $N \geq 100$

Disadvantage of the Method

- ✓ Possibility of mistaking red cell debris for platelets

III. Rough estimation of platelet number from astained blood film

- ✓ Normally there are 10-20 platelets per oil immersion field.

Sources of error in counting platelets

Sources of error when counting platelets are similar to those mentioned previously for WBC counts. Special care must be taken when counting platelets:

- To check there are not clots in the blood sample.
- To ensure the blood is well mixed with the diluting fluid.
- Not to mistake debris forms hemolyzed red cells or particles in the diluting fluid for platelets.
- To ensure the platelets are evenly distributed and not in small clumps (if clumps are present, obtain a new blood sample).
- Not to use too intense an illumination.

Interpretation of platelet counts

- In health there are about $150-400 \times 10^9$ platelets/liter of blood. Platelet counts from capillary blood are usually lower than from venous blood and are not as reproducible.
- Platelet counts are lower in Africans.
- The platelet count together with other tests (e.g. bleeding time test, prothrombin time, etc) aids in establishing a diagnosis of coagulation disorders.

Thrombocytosis

Causes of an increase in platelet numbers include:

- Chronic myeloproliferative disease e.g. essential thrombocythemia, polycythemia vera, chronic myeloid leukemia, myelofibrosis.
- Carcinoma (disseminated)
- Chronic inflammatory disease, e.g. tuberculosis
- Hemorrhage
- Sickle cell disease associated with a nonfunctioning spleen or after splenectomy.
- Iron deficiency anemia, associated with active bleeding

Thrombocytopenia

The main causes for a reduction in platelet numbers are:

I. Reduced production of platelets

- Infections, e.g. typhoid and other septicemias
- Deficiency of folate or vitamin B12
- Aplastic anemia
- Drugs (e.g. cytotoxic, quinine, aspirin), chemicals(e.g. benzene), some herbal remedies.
- Leukemias, lymphoma, myeloma, myelofibrosis, carcinoma.
- Hereditary thrombocytopenia

II. Increased destruction or consumption of platelets

- Infections ,e.g. acute malaria, dengue, trypanosomiasis, visceral leishmaniasis
- Disseminated intravascular coagulation (DIC)
- Hypersplenism
- Immune destruction of platelets, e.g. idiopathic thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), other connective tissue disorders, chronic lymphatic leukemia, lymphomas and HIV/AIDS. Also, exposure to rugs, e.g. quinine, mefloquine, penicillin, and some herbal remedies.

6.4 Eosinophil Count

Although total eosinophil count can be roughly calculated from the total and differential leucocyte count, the staining properties of eosinophils make it possible to count them directly and accurately in a counting chamber.

Principle

- ✓ Blood is diluted with a fluid that causes lysis of erythrocytes and stains eosinophils rendering them readily visible.

Diluting Fluid

- ✓ Hinkleman's fluid
- ✓ It has the advantage of keeping well at room temperature and not needing filtering before use.

Method

- ✓ Make dilution of blood using thomma pipette or tube dilution as described for the white cell count.
- ✓ A Fuchs- Rosenthal chamber (with a total area of 16mm² and depth of 0.2mm) is used and counting is carried out as soon as they are settled. Usually 10 minutes in a moist atmosphere petridish will suffice.
- ✓ All the cells in the ruled area are counted (i.e., in 3.2µl volume).

Calculation

If E is the number of eosinophils in 16 large squares (in 3.2µl volume), then the absolute eosinophil count per µl of blood is:

$$\text{Absolute eosinophil count} = \frac{E \times 20}{3.2}; [6.25E]$$

To increase the accuracy at least 100 cells should be counted, i.e., both ruled areas should be counted and if the count is low, the chamber should be cleaned and refilled, average counts per ruled area being used for the calculation.

Reference range

40 - 440 × 10⁶/l

Interpretation of eosinophil counts

Eosinophilia is common in allergic conditions (e.g., asthma) and in parasitic infections.

Review Questions

1. What are the main principles of manual hemocytometry?
2. List the items that are generally required in manual hemocytometry?
3. How do you calculate the number of cells per unit volume of blood after you count the cells in a sample of diluted blood?
4. How do errors in hemocytometry arise? How do you reduce the introduction of such errors in your count?
5. Indicate the diluting fluid, dilution factor, and areas of counting on the chamber for WBC, RBC, platelet and eosinophil count
6. Briefly describe the clinical implications of each of the WBC, RBC, platelet and eosinophil count