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PRACTICAL I

COMPLETE BLOOD COUNT (CBC) OR HEMOGRAM

The Complete Blood Count (CBC) or hemogram is routinely used as an aid in the diagnosis of many diseases. It may be performed manually or by using automated hematology analyzers.

Learning Objectives

In this practical the student will learn to identify the four of the five parts of the complete blood count

1. Total Red Blood Cell (RBC) count
2. Hematocrit determination
3. Hemoglobin determination
4. Differential White Blood Cell count

1. **RED BLOOD CELL COUNT**

The manual count of red cells is made by

(1) diluting a small volume of whole blood 1:200 with a solution which preserves the red corpuscles, and

(2) counting the number of cells in a special counting chamber of known volume.

Obtaining the blood sample

Heparinised venous or capillary blood from a prick of the ball of the finger may be used for this purpose; the latter is more difficult to use as tends to clot quickly.

1. Disinfect the site of the puncture with alcohol or ether which should be allowed to evaporate leaving the skin dry before the puncture is made.
2. With a sterile 'blood lancet' or needle, punctured the finger to a depth of 3 mm
3. The drop of blood should issue freely. **Do not squeeze** the tissues as the relative proportions of plasma and cells will be altered.
4. (Venous blood is obtained by venepuncture using sterile needle and syringe. This technique will be demonstrated to you if venous blood is used for the experiment.)

The accurate counting of erythrocytes (red blood cells, rbc) demands exact and fairly rapid work involving a technique which is acquired by considerable practice. It is necessary that you appreciate the various sources of error in doing an r.b.c count.

The counting chamber:

This is formed by placing a cover slip over the three narrow platforms extending across the middle third of a heavy glass slide (hemocytometer, see fig 1). This cover slip is special with its surfaces ground perfectly plane. It should be clean and grease free. It is applied by first moistening it by breathing over it and then pushing it forwards to the centre of the slide while maintaining steady pressure with the thumbs over its points of contact on the lateral two platforms of the slide as shown in fig 3. When the slide is properly in place rainbow colored rings (Newton's rings) will be seen and the cover slip will not lift off when you fill the chamber with liquid, subsequently. If these rings are not seen to repeat the procedure till a satisfactory application of the cover slip is obtained. During this procedure be very careful not to break the cover slip or slide as they are very expensive. This is particularly liable to happen if you press over the centre platform.

As shown in the diagrams when the cover slip is properly in position there is a space exactly 0.1 mm deep between it and the centre. The platform contains two ruled areas on its surface. The details and dimensions of this ruled area are shown in fig 2 for a type of slide in common use. Examine the ruling by focusing under low power and then high power taking care not to break the cover slip and familiarize yourself with the pattern of ruled lines on your slide. Note in particular the dimensions of the 'large' 'small' and smallest squares as shown in fig 2. Now keep the counting chambers aside carefully till you are supplied with diluted blood.

Procedure for dilution

1. Select a **dry pipette** with the red bead in the bulb.
2. Note the markings on it (fig 6b).
3. Attach the rubber suction tube to the upper end of the pipette as illustrated.
4. Pour diluting fluid into the watch glass ready for use.
5. Disinfect the middle finger and puncture the subject's finger with the sterile needle or lancet.
6. The first drop of blood is usually contaminated with epithelial cells and should be wiped off with dry cotton wool.
7. From the next drop suck the blood up to the 0.5 mark of the pipette holding the pipette more or less horizontal during the process.
8. No drop which has remained in the finger for more than 30 seconds should be used as it may contain particles of fibrin; wipe off such a drop and use the next one. Take care to avoid getting air bubbles into the pipette by attending to the following points:-
9. The finger must be dry before the puncture is made.
10. The drop of blood should be large enough
11. The tip of the pipette should dip well into the blood.
12. When the 0.5 mark is reached block the orifice in the mouth piece with the tongue to prevent the blood running out by accident
13. Quickly wipe the excessive blood adhering to the tip of the pipette and proceed immediately to suck up diluting fluid from the watch glass until the mixture reaches the 101 mark on the pipette. Suction should be gentle and continuous so as to ensure that blood does not leak out into the watch glass when you start to suck the diluting fluid and not to overshoot the 101 mark at the end of the procedure. At no time during this whole procedure should the pipette be taken out of the mouth.
14. As soon as the 101 mark is reached remove the pipette from the solution, hold it horizontal and remove the rubber tubing from the pipette by rolling it off the glass stem with thumb and index finger-care **taking care not to squeeze the rubber tube** during the process as this would displace some of the liquid from the pipette.
15. Close the end of the pipette between the thumb and index finger and mix the contents in the bulb thoroughly by agitation, the red bead in the bulb will facilitate this. Note that the fluid in the stem up to the 1.0 mark is colorless and consists of diluting fluid only. This stem fluid is discharged at the latter stage. The diluting of the blood is then 0.5 in 101-1 or 1:200.
16. Leave the pipette horizontal on the table.

The procedure to be followed with venous blood is similar.
It is an advantage to practice sucking diluting fluid into the pipette and stopping it at a predetermined height before trying to dilute the sample of blood.

Filling the counting chamber:

1. Agitate the contents of the pipette once again to ensure mixing.
2. Hold the pipette vertically closing the upper end with a finger in order to control the flow of liquid out of the pipette.
3. Allow about two drops to flow out and discard them as this contains the diluting fluid in the stem of the pipette.
4. Let ¼ to ½ drop flow out and leave this hanging on the tip by closing firmly the upper end of the pipette with the finger.
5. Now approximate the tip of the pipette to the space between the cover slip and middle platform of the counting chamber which you have already prepared (see fig 4) .The drop runs into the chamber drawn in by capillarity. The drop must not be too large as otherwise the chamber gets overfilled and fluids runs into the gutters surrounding the chamber and this will cause lifting of the cover slip so that the depth of the chambers would not be accurately 0.1mm. If the gutters get filled the counting chamber should be cleaned and the procedure repeated. If your counting chamber is double counting chamber (fig 1) each cell should be filled from its own side and the gutter separating the chamber should be filled. There should be no air bubbles.

Counting procedures:

1. Place the filled counting chamber on the stage of the microscope and allow about 3 min. for the red cells to settle to the bottom. Usually this time can be spent in getting the counting grid under focus.
2. Observe the points illustrated in fig 5 to help you get the ruled lines into focus.
3. You should use the low power first, and then switch over to the high power.
4. Count the cells in 5 small squares, four at the corner and one in the middle (see fig 2b). In counting cells in each 'small' square, one proceeds by counting systematically the cells in the 16 'smallest' squares compromising each 'small' square. When using this chamber prevent counting the same cell twice, It is a useful rule to count only those cells which lie across or touch the top and right hand lines round one of the smallest squares as belonging within that square and not to count the cells which touch the lines at the bottom and left hand edges of the same square. These latter will then automatically be counted in one of the adjacent squares.
5. Counting must be competed rapidly as otherwise the liquid in the chamber starts to evaporate due to the heat of the microscope light.

CALCULATIONS

Let the sum of the corpuscles in 5 small squares be n.

Total number of the smallest squares counted = 16 x 5 = 80.

Each 'smallest' square has a volume: 1/20 x1/20x1/10 =1/4000 c.mm over it.

Hence 80/4000 c.mm of diluted blood contains n corpuscles.

Therefore one c.mm of **diluted blood** contains 4000n/ 80 corpuscles.

Therefore 1 c.mm of undiluted blood contains: 4000n/80 x Dilution factor (1:200)

Which is 4000n/80 x 200 = 10,000 x n corpuscles

Notes

1. The diluting fluid used is usually Haymes fluid 1% NaCI, 2.5% NaSO4 and 25% $HgCI_2$.
2. A diluting fluid that does not have a disadvantage is a solution of 1% (v/v) formalin (40% formaldehyde) in 3 trisodium citrate.

Sources of Error

1. Inaccurate dilution. This is usually due to faulty technique. The error is less with the bulk dilution method described in the notes above.
2. Too slow manipulation. This may allow the blood to coagulate or the filled counting chamber to dry up when counting is being done.
3. Inaccuracy in the depth of the counting chamber due usually to improper application of the cover slip.
4. Uneven distribution of erythrocytes due usually to inadequate raiding with diluting fluid in the pipette.
5. Sampling error.

In this method one is estimating the count in a very large volume by taking a sample which is relatively small and one would not get the same count if one used the count in a different set of 80 square from the 400 available on the counting grid.

This maldistribution has nothing to do with poor mixing but is related to the fact that as each cell enters the chamber it takes a position among 400 squares at -random and as a result there will be a variation in the number of cells which fall into each square. The theoretical mathematical law of the variation is a particular case of the Poisson distribution. According to this law the variation among the different squares in the chamber is given by m +/- s.d. where m is the mean number of cells per unit area and s.d. is the standard deviation of the counts in these areas. The standard deviation for a Poisson distribution is the square root (sqrt) of the mean.

For example, if the mean count in 80 squares is 500, the s.d. of the counts in different sets of 80 squares in the chamber will be sqrt (500) or 22.4. Expressed relatively as a percentage this is 22.4 / 500 x 100 = 4.5%. Between ± 2 S.D are included 95% of repeated counts. This error measured as S.D. = sqrt (m) is the minimal error to which a

single estimate of the count can be considered to be subject even if other sources of error are eliminated.

Berkson, Magath and Hurn have estimated that in that in the hands of an experienced technician the error due to all sources in the region of 16%. If you have time it would be instructive to do repeated counts to see how many times your result fall within +/- 2 s.d. calculated above.

2. THE HAEMATOCRIT_OR PACKED CELL VOLUME

You will use the microhaematocrit method for determining the haematocrit or packed cell volume (PCV).

1. Disinfect and puncture the tip of a finger.
2. Take a heparinised capillary tube and put its end into a drop of blood formed at the tip of the finger soon after puncturing it.
3. The tube may also be filled from the sample of venous blood supplied. The tube will fill more easily if it is held close to the horizontal.
4. The tube should be about 3/4 Full. Tap it gently on one end so that: the column of blood comes clear of either end and is closer to one end than the other.
5. Hold the more empty end of the tube in the flame for a moment so that: the end seals up. Do not hold it in the flame too long as the tube will bend and the blood will boil. The end of the tube will seal and as soon as it does so the blood will move along the tube a little. This is a sign that the tube is sealed. Plastasine can also be used to seal the capillary end.
6. Place the tube in the centrifuge so that the sealed end is towards the outer circumference and record the groove number in your notebook.
7. Run the centrifuge as fast as it will go for 10 minutes.
8. Remove the tube and use the special reader to determine the percentage of packed cells.

ESTIMATION OF HAEMOGLOBIN

One function of the blood is to transport oxygen predominantly bound to the heamoglobin molecule in the RBC. The object of estimating the haemoglobin concentration in the blood is to get a measure of the oxygen carrying capacity of the blood. This can be measured directly but the methods are technically difficult. Haemoglobin, however, is an intense pigment and its concentration can be measured by colorometric methods. The results are usually expressed in terms of grams of Hb per 100ml of blood. Hufner's factor is the volume of oxygen which will combine with 1 gm of Hb.; its value is 1:34.

The haemoglobinometer consists of a color standard and a calibrated tube in which the haemoglobin is converted into acid with hydrochloric acid. The brown color of the acid haematic is then matched against the color standard by dilution with water. The concentration of haemoglobin can then be read off directly.

1. Using a Pasteur pipette, fill the graduated tube up to the 20 mark with 0.1 N HC1. Disinfect your finger, puncture it and suck up blood to the mark.
2. Gently blow the blood into the acid in the tube.
3. Clean the pipette thoroughly by alternately sucking and blowing, with the end held in the blood/acid mixture. This will also mixes the blood and acid.
4. Allow the tube to stand for **exactly five minutes**.
5. Now add water with a dropper until the blood-acid solution matches the standard. Each time you add water, mix carefully with a glass rod, taking care not to loose any of the fluid.
6. Read off the Hb concentration per 100ml of blood and enter the results against your name or group in the results sheet for your class.

From your results calculate the following indices

Mean corpscular volume (M.C.V.) in cubic microns (c.u)

$$= \frac{\text{Volume of packed cells in ml. per 100ml of blood}}{\text{Red cell count in millions per c.mm of blood}}$$

Mean corpuscular haemoglobin content (M.C.M.) In micro micro micrograms (μμg)

$$= \frac{\text{Haemoglobin in g. per 100ml of blood}}{\text{Red count in millions per μ blood}}$$

Mean corpuscular haemoglobin concentration (M.C.H.C.) percentage

$$= \frac{\text{Haemoglobin in g. per 100ml of blood x 100}}{\text{Volume packed cell in ml. per 100 ml of blood}}$$

Things to think about:

1. Relative accuracy of the measurements you have made.
2. Which of the above indices are likely to be most reliable?
3. Learn the normal values for the measurements you have made.

4. DIFFERENTIAL WHITE BLOOD CELL COUNT

(The slides may be pre-prepared from you.)

There are several different types of leucocytes. The estimation of the

relative proportions or percentage of the different types of leucocytes is called differential WBC count.

Preparing a blood film:

1. Disinfect and puncture the finger of the subject with a sterile needle or lancet and touch the drop of blood that issues with two slides near their ends. The slides should, be grease-free.
2. Place the slides on the table with the drops uppermost.
3. Select another slide with a smooth edge to serve as a spreader.
4. Hold the spreader slide against the slide with the drops of blood such that it slopes at about 45 degrees and the drop of blood is on the side of the acute angle formed by the spreader slide.
5. Now draw the spreader backwards till it touches the drop of blood.
6. Allow the blood to flow across between the edge of the spreader and the slide.
7. Then push the spreader along the slide steadily and rapidly without stopping. The blood will then be left behind on the slide as a thin even film. If the drop of blood was the right size the film will end just before the end of the slide in a thin even tail.
8. Immediately pick up the slide by one end and wave it about rapidly till it is dry. This will stop red cells forming rouleaux.

Staining the film:

1. Put the slide across the two bars placed over the sink.
2. Cover the slide with Lieshman's stain, putting the drops in different parts of the film until the whole slide is covered. This plain stain fixes the cells.
3. After about one minute add twice as many drops of distilled water taking care that the liquid does not run off the slide. As the stain contains methyl alcohol the water does not mix easily with the stain.
4. Mix the water and stain by gently blowing on the surface with a Pasteur pipette. Do not blow so hard as to spill the mixture.
5. Wait 5-8 minutes while the mixture of stain and water stains the cells.
6. Flood the stain off the slide with more distilled water and wash the slide with more water.
7. Leave the water on the slide for about 3 minutes till the slide is a pink color and not blue or purple,
8. Tip off the water, dry the 'slide and examine under the low power objective. This will show you a good place to do the differential count. This will be towards the tail of the film as shown in the figure where the cells lie evenly without touching or lying on top of one another.
9. You will probably be able to identify the different kinds of white cells under the high power objective but if this proves to be difficult use the oil immersion objective.

Counting procedure:

Count a total of at least 100 cells and categorize them into the different types of leucocytes. It would be better to count 200 cells but the increase in accuracy achieved by counting more than 200 is usually not worth while. The lymphocytes tend to be distributed in the centre of the smear and the polymorphs in the periphery. It is therefore usual to follow a zig zag path, as shown in the figure, when counting. When attempting to identify a cell direct your attention to the following points: -

a. Relative size of the cell in relation to the red cells seen in the vicinity.
b. Presence or absence of granules in the cytoplasm
c. Relative proportion of cytoplasm to nucleus
d. Shape of the nucleus

The following is a brief summary of the characteristics of the cell type.
Eosinophil: Two to three times the size of an r.b.c.; large corse pink granules filling the cytoplasm. Nucleus usually bilobed (spectacle shaped).

Neutrophil: Very fine purple granules in cytoplsm; multilobed nucleus. Two to three times the size of an r.b.c.

Basophil: Nucleus almost obscured by coarse, unequal blue or purple granules which are not so closely packed together as in the eosinophil. Twice the size of an r.b.c.

Lymphocyte: About the size of a red cell. Consists mainly of nucleus with a thin rim of cytoplasm. Some cells are larger, the large lymphocyte, but the cytoplasm is less than 50% compared to the nucleus. Small and large lymphocytes are counted together.

Monocytes: Two to three times the size of an r.b.c. nucleus, bean shaped , cytoplasm 50% or more of the cell.

Note: Heparin causes clumping of leucocytes. Heparinised blood is therefore unsuitable for leucocyte counts.

Complete Blood Count or Hemogram Laboratory Report

Date of Practical: _____ Group: _____

Student Reg. No.: _____

Group Results	**RBC count /µl**	**WBC differential count %**					**PCV %**	**Hb gm%**
		Eosinophil	Neutrophil	Basophil	Lymphocytes	Monocytes		
1								
2								
3								
4								
5								
6								
7								
8								
Average								
SD								
SEM								
Normal Range								

To be handed in within 7 days after completion of the practical.