Practical Hematology

ISLAMIC UNIVERSITY OF GAZA

HEALTH SCIENCE DEPARTMENT

Medical Laboratory Sciences

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PRACTICAL HEMATOLOGY

LAB NO.	Subject
LAB 1	*Slide preparation and staining, The White Blood Cell Differential , and Platelet estimation.*
LAB2	*Assessing Red Blood Cell Morphology.*
LAB3	*Reticulocyte counts, Alkaline & Acid Hemoglobin Electrophoresis.*
LAB4	*Detection of sickle cell . Quiz 1*
LAB5	*Hemoglobin A2 Determination, Quantification of fetal hemoglobin.*
LAB6	*Hemoglobin F Acid Stain, Screening test for G6PDH Deficiency.*
LAB7	*Quantification of methemoglobin, Blood Sucrose Test. Quiz 2*
LAB8	*Osmotic Fragility test.*
LAB9	*Automated Hematology Cell Counters.*
LAB10	*Normal Cell Maturation, Leukemia Classification.*
LAB11	*Leukemia Classification, Special stains.*

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Content

A Peripheral blood smear preparation and staining

When automated differentials do not meet specified criteria programmed into the automated hematologyinstrument, the technologist/technician must perform amanual differential count from a prepared smear.

There are three types of blood smears:

1. The cover glass smear.
2. the wedge smear
3. thespun smear.

The wedge blood smear will be discussed in this lab. and It is the most common smear preparation in the hematology laboratory and Wright stain, a Romanowsky stain, is the most common dye.

The are two additional types of blood smear used for specific purposes.

1. The *Buffy coat smear* is for use on patient specimens when the patient's white blood cell count is less than 1.0×10^9/L and it is desirable to perform a 100-cell differential. This procedure concentrates the nucleated cells present in the blood.
2. *Thick blood smears* are commonly used when specifically looking for blood parasites such as malaria.

Proper Preparation of a Peripheral Blood Smear

Objective

At the completion of this laboratory, the student will be able to:

1. State the appropriate sample used for preparing a peripheral blood smear.
2. Describe the appearance of a well prepared blood smear.
3. Demonstrate the appropriate technique for preparing a peripheral blood smear.
4. Evaluate prepared blood smears for acceptability in the clinical laboratory.

Principle

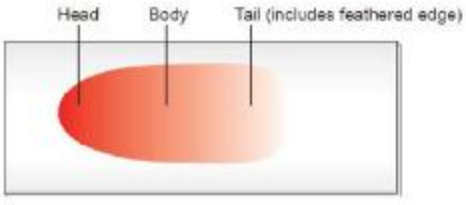
The wedge smear will be discussed in thisprocedure. Smears are prepared by placing a drop ofblood on a clean glass slide and spreading the dropusing another glass slide at an angle. The slide is thenstained and observed microscopically.

Specimen

1. EDTA specimen
2. Smears are made from EDTA
   a. EDTA blood within 2 to 3 hours
   b. Check all Microtainers for clots with applicatorsticks

Requirements for Proper Smear Preparation:

1) Perfectly clean glass slides or coverslips
2) Proper size blood drop
3) Quick, smooth spreading of drop
4) Rapid drying of smear
5) Proper placement of drop
6) Preparation of smear within 3 hours of collection



Procedure:

1. Mix sample well, either by inversion or by mechanical rocker. Remove stopper holding tube away from face. Using two wooden applicator sticks rim the tube and check for fibrin clots.
2. Place a 1 X 3 inch slide on a flat surface, place a 2-3 mm drop of mixed whole blood about 1/4 inch from the right side of frosted area of the slide, utilizing the wooden applicator sticks or filled a capillary tube three-quarter full with anticoagulated specimen .
3. Grasp a second slide (spreader slide) in the right hand between thumb and forefinger.
4. Place the spreader slide onto the lower slide in front of the blood drop, and pull the slide back until it touches the drop.
5. Allow the blood to spread by capillary action almost to the edges of the lower slide.
6. Push the spreader slide forward at approximately a 30-40° angle, using a rapid, even motion. The weight of the spreader slide should be the only weight applied. Do NOT press down. Perform this step quickly. The drop of blood must be spread within seconds or the cell distribution will be uneven. A thin film of blood in the shape of a bullet with a feathered edge will remain on the slide.
7. Label the frosted edge with patient name, ID# and date.
8. Allow the blood film to air-dry completely before staining. (Do not blow to dry. The

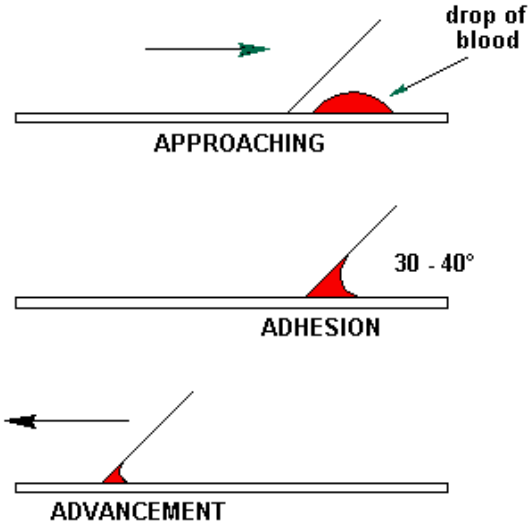


Fig. - How to prepare a blood smear

Procedure Notes

1. Characteristics of a Good Smear
    1. Thick at one end, thinning out to a smooth rounded feather edge.
    2. Should occupy 2/3 of the total slide area.
    3. Should not touch any edge of the slide.
    4. Should be margin free, except for point of application.

    A well made, well distributed peripheral smearwill have a counting area at the thin portion of thewedge smear which is approximately 200 red cells nottouching. A good counting area is an essential ingredientin a peripheral smear for evaluating the numbers ofand types of white cells present and evaluating red celland platelet morphology.

2. As soon as the drop of blood is placed on the glass slide, the smear should be made without delay. Any delay results in an abnormal distribution of the white blood cells, with many of the large white cells accumulating at the thin edge of the smear.Rouleaux of the red blood cells and platelet clumping may also occur.

3. The thickness of the spread when pulling the smear is determined by
    a. Theangle of the spreader slide (the greater the angle, the thicker and shorter the smear).
    b. Size of the blood drop.
    c. Speed of spreading.

   example
   1. If the hematocrit is increased, the angle of the spreader slide should be decreased.
   2. If the hematocrit is decreased, the angle of the spreader slide should be increased.

4. Common causes of a poor blood smear:
    a. Drop of blood too large or too small.
    b. Spreader slide pushed across the slide in a jerky manner.
    c. Failure to keep the entire edge of the spreader slide against the slide while making the smear.
    d. Failure to keep the spreader slide at a 30° angle with the slide.
    e. Failure to push the spreader slide completely across the slide.
    f. Irregular spread with ridges and long tail: Edge of spreader dirty or chipped; dusty slide
    g. Holes in film: Slide contaminated with fat or grease
    h. Cellular degenerative changes: delay in fixing, inadequate fixing time or methanol contaminated with water.

5. Although this is the easiest and most popular methods for producing a blood smear, it does not produce a quality smear. The WBCs are unevenly distributed and RBC distortion is seen at the edges. Smaller WBCs such as lymphocytes tend to reside in the middle of the feathered edge. Large cells such as monocytes, immature cells and abnormal cells can be found in the outer limits of this area. Spun smears produce the most uniform distribution of blood cells.
6. Biologic causes of a poor smear
    a. Cold agglutinin - RBCs will clump together. Warm the blood at 37° C for 5 minutes, and then remake the smear.
    b. Lipemia - holes will appear in the smear. There is nothing you can do to correct this.
    c. Rouleaux - RBC's will form into stacks resembling coins. There is nothing you can do to correct this.

Slide Staining With Romanowsky Stain

Romanowsky staining

Romanowsky stain are routinely used to stain peripheral blood and bone marrow smears. they are considered polychromatic stains in that the dyes present procedure multiple colors when applied on cells and cellular elements.

Principle

The main components of a Romanowsky stain are:

1. A cationic or basic dye (methylene blue or its oxidation products such as azure B*), which binds to anionic sites and gives a blue-grey color to nucleic acids (DNA or RNA), nucleoproteins, granules of basophils and weakly to granules of neutrophils
2. An anionic or acidic dye such as eosin Y or eosin B, which binds to cationic sites on proteins and gives an orange-red color to hemoglobin and eosinophil granules.

*Azure B (trimethylthionin, a product of the oxidation of methylene blue) and eosin Y are the most important components of the stain.

The quantity of dyes used to prepare the stain are controlled in order to yield a neutral compound . When the buffer solution is added to the stain, ionization occurs , during which time staining takes place. The eosin ions are negatively charged and stain the basic components of the cells an orange to pink color. The acid structures of the cells are stained varying shades of blue to purple by the positively charged azure B. neutrophil granules are probably stained by the azure compounds.

The stains include in this category

1. Wright's stain is composed of oxidized methylene blue and eosin azures. It is a simpler method .
2. Giemsa stain is thought to produce more delicate staining characteristics. It combines eosin Y with azure B and methylene blue in methanol with glycerin added as a stabilizer.
3. Leishman's stain is similar to Wright's stain except for the method used to oxidize the methylene blue. It is also a simple method, which is especially suitable when a stained blood film is required urgently or the routine stain is not available (e.g. at night).
4. May-Gruwald is a good method for routine work.
5. Field's stain is a rapid stain used primarily on thin films for malarial parasites.

Fixation is important step, films must be fixed as soon as possible after they have dried to does not allow any further change in the cells and makes them adhere to the Glass slide. It is important to prevent contact with water before fixation is complete. The presence of water during methanol fixation produces refractile body artifacts (water spots) in the erythrocytes.

These water spots persist through staining of the smear and cover items of interest in the smear. Further, they are distracting to the person evaluating the smear. In some cases, the water spots may interfere with diagnosis.
To prevent the alcohol from becoming contaminated by absorbed water, it must be stored in a bottle with a tightly fitting stopper and not left exposed to the atmosphere, especially in humid climates.

The preferred Alcohol is Methyl alcohol (methanol), although ethyl alcohol ("absolute alcohol") can be used as fixative in Fixation.

We use in this Lab *leishmen's stain*, It does not need to step fixation before staining, because the fixative present with the stain, but with the *Wright, Giemsa satin*are needed to it.

LEISHMAN'S STAIN

Preparation of reagents

Staining Solution

Powder stain was weighed (0.2g) and transferred to a mortar. It was ground with about 25 ml of methanol and allowed to settle. The supernatant was transferred through filter paper to a flask. Grinding was repeated with 25ml of alcohol each time until all alcohol was finished and all powder was dissolved .

The flask was placed in a water bath at 50°C for 15 minutes. It was then filtered in a clean brown bottle. Mixture was left for at least 2-3 days to mature. Required amount for daily use was filtered into a smaller dropping bottle every morning.

Buffer pH 6.8

Stock buffer solution -A

1. Anhydrous monobasic potassium phosphate(KH_2PO_4), 0.067 M. 9.1g
2. dissolve and dilute to 1L of distilled water and stored at 40°C.

Stock buffer solution -B

1. Anhydrous Dibasic potassium phosphate(K_2HPO_4), 0.067 M. 9.4g
2. Dissolve and dilute to 1L distilled water and stored at 4°C.

Working buffer solution PH 6.8 was made by mixing 50.8 ml of stock solution A with 49.2 ml of stock solution -B and diluting it up to 2 liters with distilled water.

REAGENTS OFFERED:

1. Leishman's stain – Ready-To-Use
2. Buffer-pH 6.8 phosphate buffer

Staining Procedure

1. Thin smear are air dried.
2. Flood the smear with stain.
3. Stain for 1-5 min. Experience will indicate the optimum time.
4. Add an equal amount of buffer solution and mix the stain by blowing an eddy in the fluid.
5. Leave the mixture on the slide for 10-15 min.
6. Wash off by running water directly to the centre of the slide to prevent a residue of precipitated stain.
7. Stand slide on end, and let dry in air.
8. Staining characteristics of a correctly stained normal film:

- Nuclei Purple
- Cytoplasm
  - Erythrocytes Deep pink
  - Neutrophils Orange-pink
  - Lymphocytes Blue; some small lymphocytes deep blue
  - Monocytes Grey-blue
  - Basophils Blue
- Granules
  - Neutrophils Fine purple
  - Eosinophils Red-orange
  - Basophils Purple-black
  - Monocytes Fine reddish (azurophil)
  - Platelets Purple

Caution !!For using lieshmen's stain

INHALATION

Prolonged or repeated exposure may cause breathing difficulties and irritation.

INGESTION

May cause discomfort if swallowed.

SKIN CONTACT

Prolonged or repeated exposure may cause irritation.

EYE CONTACT

Prolonged contact may cause transient eye irritation.

Discussion

1. The phosphate buffer controls the pH of the stain. If the pH is too acid, those cells or cell parts taking up an acid dye stain will stain pinker and the acid components that stain with the basic dye show very pale staining. If the stain-buffer mixture is too alkaline, the red blood cells will appear grayish-blue and the white cell nuclei will stain very deeply purple. Therefore, to stain all cells and cell parts well, the pH of the phosphate buffer is critical .

Causes & correction
Too Acid Stain:

1) insufficient staining time
2) prolonged buffering or washing
3) old stain

Correction: 1) lengthen staining time
2) check stain and buffer pH
3) shorten buffering or wash time

Too Alkaline Stain:

1) thick blood smear
2) prolonged staining
3) insufficient washing
4) alkaline pH of stain components

Correction: 1) check pH
2) shorten stain time
3) prolong buffering time

2. The staining rack must be exactly level to guard against uneven staining of the smear .
3. Insufficient washing of the smears when removing the stain and buffer mixture may cause stain precipitate on the dried smear .
4. Excessive rinsing of the stained smear will cause the stain to fade .

If it is desirable to restain a slide, the original stain may be removed with methanol. Flood the smear with methanol and rinse with tap water as many times as necessary to remove the stain and then restain the slide according to the previously described procedure. For best results, however, make a new smear.

Performing A Manual differential And assessing RBC Morphology

Introduction

When blood samples are evaluated by the use of automated hematology analyzers, this analysis includes automated differentials. Specific criteria pertaining to normal, abnormal, and critical values have been programmed into the analyzers by the institution, and if the differentials do not meet these criteria, verification is necessary. This is done by performing manual differentials and further evaluating the peripheral smear.

objective

1. To determine the relative number of each type of white cell present in the blood by performing differential cell counts on five relatively normal blood smears and five sets of abnormal blood smears within a ± 15% accuracy of the instructor's values.
2. To determine within one qualitative unit the red cell, white cell, and platelet morphology of each of the above blood smears.
3. To determine within ± 20% accuracy an estimate of the white cell counts and the platelet counts of each of the above blood smears.

Principle

First, a differential white blood cell (WBC) count is performed to determine the relative number of each type of white cell present. Technologists/technicians must recognize and properly record the type(s) of white cell observed. Simultaneously, red cell, white cell, and platelet morphology is noted and recorded. Also, a rough estimate of platelets and WBC counts is made to determine if these numbers generally correlate with the automated hematology analyzer. Technologists/technicians must be proficient at recognizing red and white cell abnormalities, identifying them correctly, and quantifying them.

Reagents and equipment

1. Manual cell counter designed for differential counts
2. Microscope, immersion oil and lens paper

Specimen

Peripheral blood smear made from EDTA-anticoagulated blood. Smears should be made within 1 hour of blood collection from EDTA specimens stored at room temperature to avoid distortion of cell morphology. Unstained smears can be stored for indefinite periods in a dry environment, but stained smears gradually fade unless cover slipped.

Procedure

Observations Under×10

1. Place a well-stained slide on the stage of the microscope, smear side up, and focus using the low-power objective (×10).
2. Find an optimal area for the detailed examination of blood cells in which:
    a. Free of ragged edges and cell clumps.
    b. The RBCs are well separated from each other without touching each other.
    c. Avoid the areas containing large numbers of broken cells or precipitated stain.
3. Check the WBC distribution over the smear.
4. Check that the slide is properly stained.
5. Check for the presence of large platelets, platelet clumps, and fibrin strands.
6. Check for the presence of any large abnormal cells (bone marrow cells).

Observations Under × 40 x-: WBC Estimates

1. Place a drop of immersion oil on the slide and change the objective to ×50 oil. (In cases where no ×50 is available, use the ×40 high dry with no oil.)
2. Choose a portion of the peripheral smear where there is only slight overlapping of the RBCs. Count 10 fields, take the total number of white cells and divide by 10, and refer to Table 1 to determine the WBC estimate.
3. An alternative technique is to do a WBC estimate by taking the average number of white cells and multiplying by 2000.

Table 1 • Estimated WBC Count From Peripheral Smear

WBC/High-Power Field	Estimated WBC Count
2 to 4	4.0 to 7.0 × 10^9/L
4 to 6	7.0 to 10.0 × 10^9/L
6 to 10	10.0 to 13.0 × 10^9/L
10 to 20	13.0 to 18.0 × 10^9/L

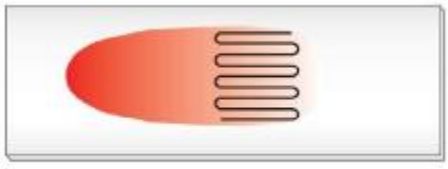
Observations Under × 100: Platelet Estimates

1. Platelet estimates are done under × 100 with the RBCs barely touching, approximately 200 RBCs. This takes place under the × 100 objective (oil). On average there are 8 to 20 platelets per field. See Table 2.
2. Ten fields are counted using the zigzag method. This method of counting is done by going back and forth lengthwise or sidewise (Fig.1).

Platelets per oil immersion field (OIF)

<8 platelets/OIF = decreased
8 to 20 platelets/OIF = adequate
>20 platelets/OIF = increased

Figure1:Zigzag method of performing differential.



3. After the 10 fields are counted, the number of platelets is divided by 10 to get the average. The average number is now multiplied by a factor of 20,000 for wedge preparations. For monolayer preparations, use a factor of 15,000.

Table 2 • Platelet Estimate From Peripheral Smear

Average No. of Platelets per ×100 Field	Platelet Count Estimate
0 to 1	< 20,000
1 to 4	20,000 to 80,000
5 to 8	100,000 to 160,000
10 to 15	200,000 to 300,000
16 to 20	320,000 to 400,000
>21	> 420,000

Example: 120 platelets/10 fields = 12 platelets per field
12 × 20,000 = 240,000 platelets

Manual Differential Counts

1. These counts are done in the same area as WBC and platelet estimates with the red cells barely touching.
2. This takes place under ×100 (oil) using the zigzag method previously described in the platelet estimate (see Fig.1).
3. Count 100 WBCs including all cell lines from immature to mature. Normal values for WBCs can be found in Table 3.

♦ Reporting results
- § Results are expressed as a percentage of the total leukocytes counted.
- § It is also helpful to know the actual number of each white cell type per µL of blood. This is referred to as the absolute count and is calculated as follows:

Absolute number of cells/µl = % of cell type in differential x white cell count

- § Reference values vary depending on age. For this exercise, the following values will be used

Table 3

Cell Type	Birth	1 mo	6 yr	14 yr
Total WBC x 103 /µL	10-26	5-19.5	4.3-13.5	4.5-11.0
Neutrophils %	37-57	25-35	45-55	50-65
Lymphocyte %	25-35	50-65	35-45	30-40
Monocyte %	3-9	2.5-7.5	0-8	0-10
Eosinophil %	1-3	1-4	1-4	0-4
Basophil %	0-1	0-1	0-1	0-1

Observing and Recording Nucleated Red Blood Cells (nRBCs)

1. If nRBCs are observed while performing the differential, they need to be reported. These elements in a peripheral smear are indicative of increased erythropoietic activity and usually a pathologic condition. Additionally, the presence of nRBCs per 100 white cells will falsely elevate the white count and is clinically significant.
2. Correct the WBC count if the nRBC count is greater than 10 nRBCs/100. The following formula is applied for correcting NRBCs:
   WBC × 100/NRBC + 100

Example : If WBC = 5000 and 10 NRBCs have been counted
Then 5,000 × 100/110 = 4545.50
The corrected white count is 4545.50.

Recording RBC Morphology

1. Scan area using ×100 (oil immersion).
2. Observe 10 fields.
3. Red cells are observed for size, color, hemoglobin content or pallor, and shape.
4. Normal morphology
   a. Normocytic: normal cell size and shape
   b. Normochromic: normal hemoglobin content and color
5. Abnormal morphology: Red cell morphology is assessed according to size, shape, hemoglobin content, and the presence or absence of inclusions. See the following sample grading system. Note that red cell morphology must be scanned in a good counting area. Two questions should be asked
   1. Is the morphology seen in every field?
   2. Is the morphology pathologic and not artificially induced?

   Table 4 & 5 represents a system derived to determine a quantitative scale.

Table.4 Qualitative Grading of RBC Morphology

	Grade Degree of Abnormalities
1 to 5 cells/10 fields	Slight
6 to 15 cells/10 fields	Moderate
> 15 cells/10 fields	Marked

Table 5 Grading Inclusions

Rare	0 to 1/hpf
Few	1 to 2/hpf
Mod	2 to 4 /hpf
Many	> 5/hpf

hpf, high-power field.

Discussion

1. A well-made and well-stained smear is essential to the accuracy of the differential count. The knowledge and ability of the cell morphologist is critical to high-quality results.

2. When studying a stained smear, do not .progress too far into the thick area of the slide. The morphologic characteristics of the cells are difficult to distinguish in this area. Conversely, do not use the very thin portion of the smear where the red blood cells appear completely filled with hemoglobin and show no area of central pallor. The cells in this area are generally distorted and do not show a true morphologic picture .
3. Before reporting significant abnormalities such as blasts, malaria or other significant finding on a patient's differential, ask a more experienced tech to review the smear for confirmation. In clinical settings where a pathologist or hematologist is present, the smear is set aside for Pathologist Review.

4. If disrupted cells are present such as smudge cells or basket cells, not them on the report. It may be necessary to make an albumin smear to prevent the disruption of the cells. RBC morphology and WBC morphology must always be performed on the non-albumin smear.

5. When the WBC is very low (below 1,000/µL), it is difficult to find enough WBCs to perform a 100-cell differential. In this situation, a differential is usually performed by counting 50 cells. A notation on the report must be made that only 50 white cells were counted. Multiply each percentage x 2.(Alternatively, a buffy coat smear may be prepared.)

6. When the WBC is very high (>50,000/µL), a 200-cell diff may be performed to increase the accuracy of the diff. The results are then divided by 2 and a note made on the report that 200 white cells were counted.

7. Never hesitate to ask questions concerning morphology or the identification of cells. The differential is one of the most difficult laboratory tests to learn. In fact, learning about cells and their morphology is a process that continues for as long as you perform differentials.

Characteristics of blood cells

- Erythrocyte:

Shape& size: Biconcave disc , size like lymphocyte nucleus.
Nucleus : lost.
Cytoplasm: pinkish hue, small area of central pallor.
Number in man varies between 5 and 5.5 million per cubic mm of blood

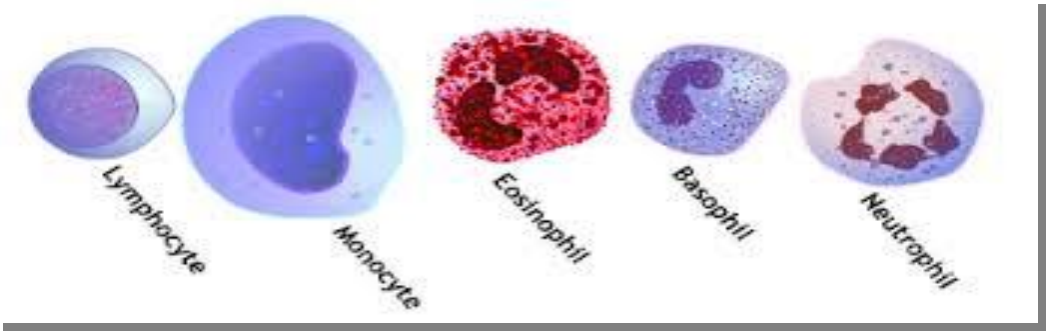
- Platelet (Thrompocytes)

Nucleus: No nucleus.
Cytoplasm: small amount bluish cytoplasm & contains reddish – purple granules.

- White blood cells

White blood cells, or leukocytes, are classified into two main groups; granulocytes and nongranulocytes (also known as agranulocytes).

1. The granulocytes, which include neutrophils, eosinophils, and basophils, have granules in their cell cytoplasm. Neutrophils, eosinophils, and basophils also have a multilobed nucleus. As a result they are also called polymorphonuclear leukocytes or "polys." The nuclei of neutrophils also appear to be segmented, so they may also be called segmented neutrophils or "segs."
2. The nongranulocytes white blood cells, lymphocytes and monocytes, do not have granules and have nonlobular nuclei. They are sometimes referred to as mononuclear leukocytes.



	Segmented Neutrophils	Eosinophil	Basophil
Size	twice size of RBCs.	Like neu. Or slightly larger	slightly largerthan Eos.
Nucleus	2-5 lobes of nucleus (usually 3), purplish-red	Eccentric, usually bilobed, rarely three	Generally unsegmented or clumped bilobed, rarely has 3-4 lobe
Cytoplasm	Light pink	orange-pink	slightly pink to colorless
SPECIFIC granules	• Primary &secondry granule either pink or neutral • fine, numerous,& even distributed	• orange-reddish orange • uniformly round, large, evenly distributed • if poor stained appear crystalloid nature	• violet-blue(or purple-black) • large(obscure the nucleus) ,abundant, varying in size, Coarse and unevenly distributed • vary in number ,shape and color, and less numerous than eosinophil granules • water-soluble and tend to wash out when stained (probably because of improper fixation).

% from the Total WBCs count	40% - 75 %	1 % - 3 %	0.5 % - 1 %
Increased	in inflammation, and they act as the first line of defense against invading pyogenic organisms.	in allergic states and in parasitic infections.	In inflammatory processes , in immediate & delayed hypersensitivity
granules synthesize	nonspecific : lysosomes , acid phosphatase, peroxidase, esterase, lysozyme. Specific: aminopeptidase, collagenase, lactofrrin, lysozyme.	highly metabolic and contain histamine and other substances	heparin and histamine

Lymphocyte

Most lymphocyte are small, there are intermediate sizes and large lymphocytes.
Small lymphocyte are usually round with smooth margins.

Size: approximately the size of RBC
Cytoplasm: thin rim around nucleus, moderate to dark blue
Nucleus:round or oval in shape and may be slightly indented. No nucleoli are visible.

Monocyte

Cytoplasm : Abundant. Blue-gray, outline may be irregular because of the presence of pseudopods. Many fine azurophilic granules, giving a ground glass appearance. Vacuoles may sometimes be present.

Nucleus: round, Kidney shaped, or may show slight lobulation. It may be folded over on top of itself, thus showing brainlike convolutions. No nucleoli are visible.

§ Leukocytosis, a WBC above 10,000, is usually due to an increase in one of the five types of white blood cells and is given the name of the cell that shows the primary increase.

- Neutrophilic leukocytosis = neutrophilia
- Eosinophilic leukocytosis = eosinophilia
- Basophilic leukocytosis = basophilia
- Lymphocytic leukocytosis = lymphocytosis
- Monocytic leukocytosis = monocytosis

1. Neutrophils

- Neutrophils are so named because they are not well stained by either eosin, a red acidic stain, nor by methylene blue, a basic or alkaline stain.

- They are the body's primary defense against bacterial infection. Normally, most of the neutrophils circulating in the bloodstream are in a mature form, with the nucleus of the cell being divided or segmented.

· The nucleus of less mature neutrophils is not segmented, but has a band or rod-like shape. Less mature neutrophils - those that have recently been released from the bone marrow into the bloodstream - are known as "bands" or "stabs".

§ Increased neutrophils count (neutrophilia)

- An increased need for neutrophils, as with an acute bacterial infection, will cause an increase in both the total number of mature neutrophils and the less mature bands or stabs to respond to the infection. The term "shift to the left" is often used when determining if a patient has an inflammatory process such as acute appendicitis or cholecystitis.

- In addition to bacterial infections, neutrophil counts are increased in:

  1. Many inflammatory processes.
  2. During physical stress
  3. With tissue necrosis that might occur after a severe burn
  4. Myocardial infarction.
  5. Granulocytic leukemia.

- Shift to left **ß** Increased bands Means acute infection, usually bacterial. Shift to right **à** Increased hypersegmentedneutrophile.

§ Decreased neutrophil count (neutropenia)

This take place in the following:

- Typhoid fever
- Brucelosis
- Viral diseases, including hepatitis, influenza, rubella, and mumps.
- An great infection can also deplete the bone marrow of neutrophils and produce neutropenia.
- Many drugs used to treat cancer produce bone marrow depression and can significantly lower the neutrophil count.

2. Lymphocytes

Lymphocytes are the primary components of the body's immune system. They are the source of serum immunoglobulins and of cellular immune response. As a result, they play an important role in immunologic reactions.

- All lymphocytes are produced in the bone marrow. The B-cell lymphocyte also matures in the bone marrow and controls the antigen-antibody response that is specific to an offending antigen; the T-cell lymphocyte matures in the thymus gland, the T cells are

the master immune cells of the body, consisting of T-4 helper cells, killer cells, cytotoxic cells, and suppressor T-8 cells.

- In adults, lymphocytes are the second most common WBC type after neutrophils, hence lymphocytosis is usually associated with neutropenia and lymphopenia is associated with neutropeina. In young children under age 8, lymphocytes are more common than neutrophils.

- Lymphocytes increase (lymphocytosis) in:
    - Many viral infections
    - Tuberculosis.
    - Typhoid fever
    - Lymphocytic leukemia.

A decreased lymphocyte (lymphopenia) count of less than 500 places a patient at very high risk of infection, particularly viral infections.

3. Eosinophils

Eosinophils are associated with IgEantigen-antibody reactions.

1. The most common reasons for an increase in the eosinophil count are Allergic reactions such as hay fever, asthma, or drug hypersensitivity.
2. Parasitic infection
3. Eosinophilic leukemia

4. Basophils

- The purpose of basophils is not completely understood.
- Basophils are phagocytes and contain heparin, histamines, and serotonin.
- Tissue basophils are also called" mast cells." Similar to blood basophils, they produce and store heparin, histamine, and serotonin.
- Basophile counts are used to analyze allergic reactions.
- An alteration in bone marrow function such as leukemia or Hodgkin's disease as well as allergic reaction may cause an increase in basophils.

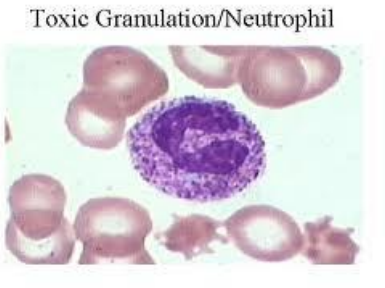
5. Monocytes

- Monocytes are the largest cells in normal blood. They act as phagocytes in some inflammatory diseases and are the body's second line of defense against infection.
- Diseases that cause a monocytosis include:
    - Tuberculosis, Malaria, Brucellosis,Monocytic leukemia,Chronic ulcerative colitis .

Abnormal Changes Of White Blood Cell Morphology

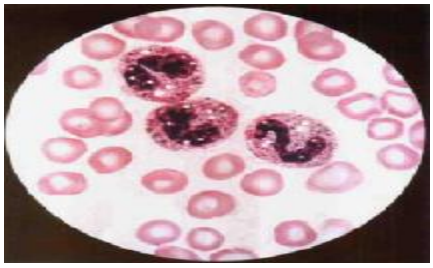
Toxic Granulation

- Morphology:
  Increased granulation. Granulation more basophilic and larger than normal.
- Found in:
  - Severe bacterial infection.
  - Non specific finding - seen in tissue damage of various types.
  - Normal pregnancy.
  - Therapy with cytokines.



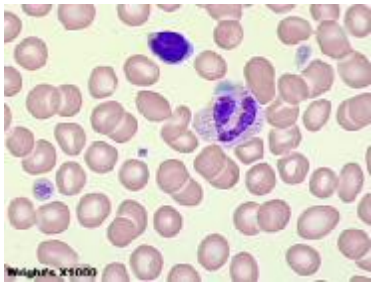
Vaculization

- Morphology:
  Vacuoles in the cytoplasm of granulocytes
- Found in:
  -Infection
  -Toxic effect of ethanol
  -Jordan's anomaly



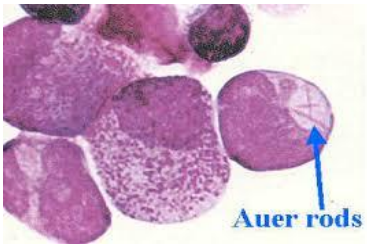
Döhle Bodies

- Morphology:
  Small pale blue cytoplasmic inclusions, often in the periphery of the cell.
- Found in:
  -Infective and inflammatory states
  -Severe burns
  -Tuberculosis
  -Post chemotherapy
  -Pregnancy



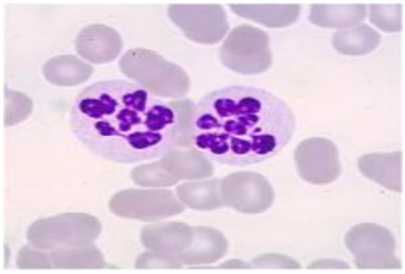
Auer Rods

- Morphology:
  Small azurophil rods in the cytoplasm of myeloblastsand promyelocytes. Sometimes found in mature neutrophils.
- Found in:
  - Acute myeloblastic leukemia
  - Myelodysplastic syndromes



Hypersegmentation or right shift of neutrophil nuclei

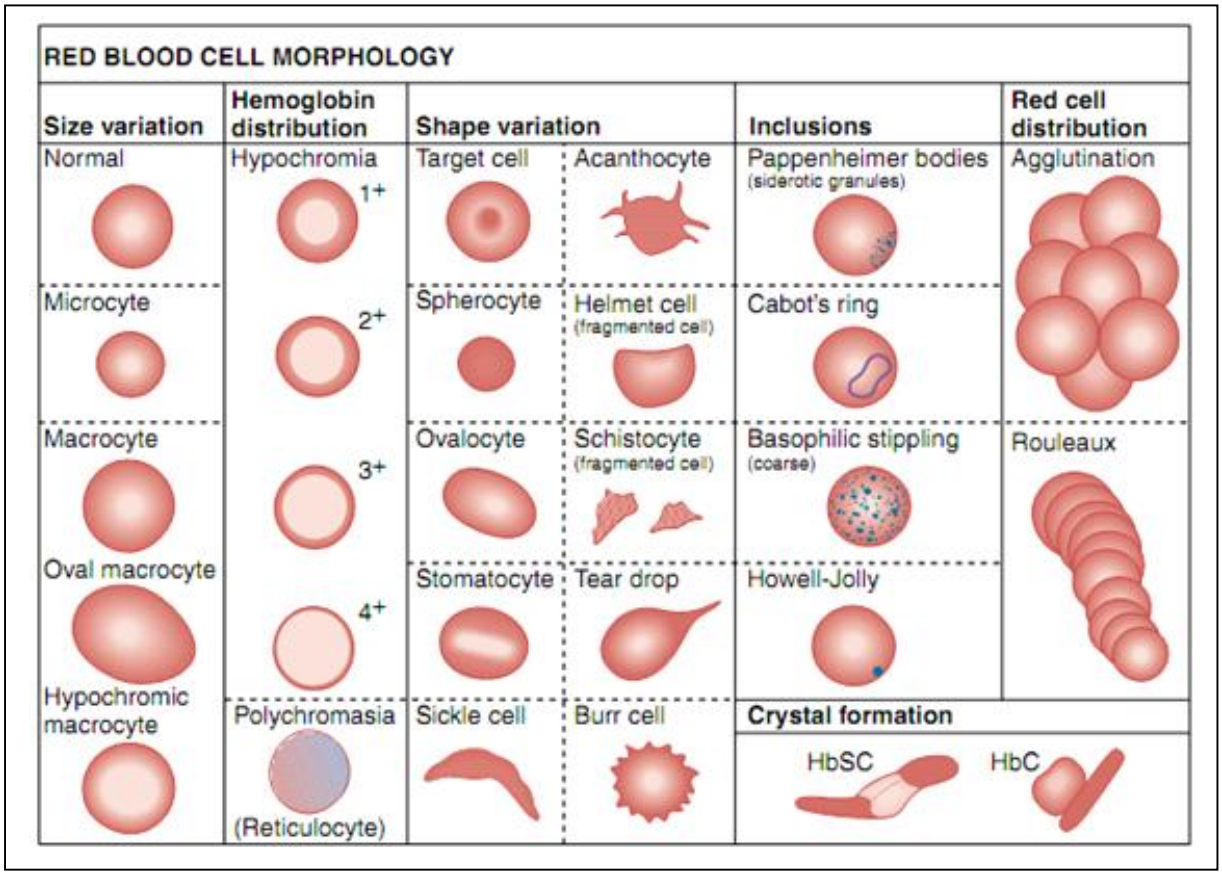
- Morphology:
  Average lobe count increased OR increased % of neutrophils with 5 - 6 lobes OR > 3% neutrophils with 5 lobes or more.
- Found in:
  Megaloblastic anaemia
  Iron deficiency
  Chronic infection
  Liver disease
  Uraemia
  Hereditary



RBCs Abnormal Morphology

Nomenclature of red cell shapes

New Terminology	Old terms, synonyms
Discocyte	Biconcave disc
Echinocyte (I-III)	Burr cell, crenated cell, berry cell
Acanthocyte	Spur cell, acanthoid cell
Stomatocyte	Mouth cell, cup form, mushroom cap, uniconcave disc
Spherocyte	Spherocyte, prelytic sphere, microspherocytes
Schizocyte	Schistocyte, helmet cell, fragmented cell
Elliptocyte&Ovalocyte	
Drepanocyte	Sickle cell
Codocyte	Target cell
Dacryoctye	Teardrop cell, tennis racket cell



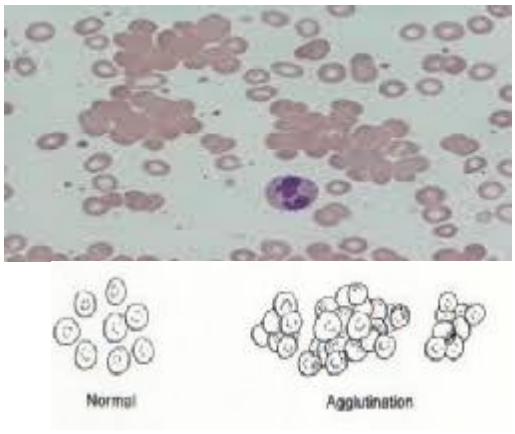
Hypochromia Grading

1+Area of central pallor is one-half of cell diameter
2+Area of pallor is two-thirds of cell diameter
3+Area of pallor is three-quarters
4+Thin rim of hemoglobin

Abnormal erythrocyte morphology is found in pathological states that may be abnormalities in size (anisocytosis), in shape (poikilocytosis), in hemoglobin content or the presence of inclusion bodies in erythrocyte and in Red cell distribution.

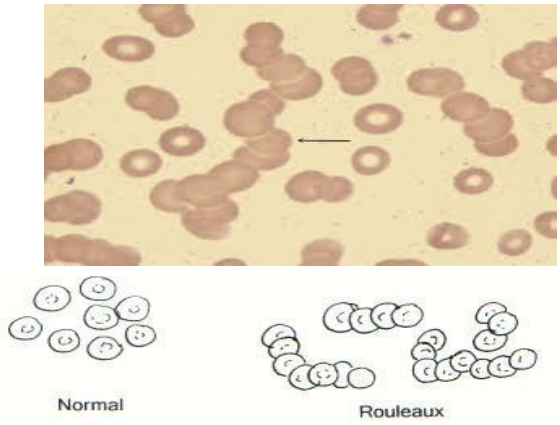
I. Variation in Red cell Distribution

1. Agglutination



Morphology: Irregular clumps of red cells
Found in:
Cold agglutinins
Warm autoimmune hemolysis

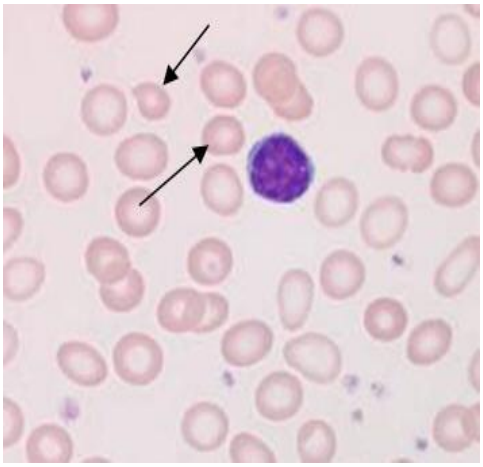
2. Rouleaux



Morphology: Stacks of RBC's resembling a stack of coins.
Found in:
Hyperfibrinogenaemia
Hyperglobulinaemia

II. Variation in erythrocyte size (anisocytosis)

1. Microcytosis

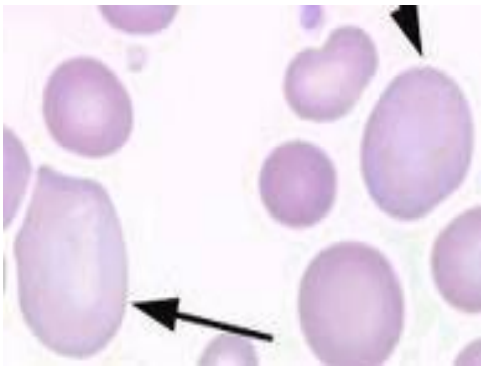


Morphology:
Decrease in the red cell size. Red cells are smaller than ± 7µm in diameter. The nucleus of a small lymphocyte (± 8,µm) is a useful guide to the size of a red

Found in:
Iron deficiency anemia.
Thalassaemia.
Sideroblastic anemia.
Lead poisoning.
Anemia of chronic disease.

2. Macrocytosis

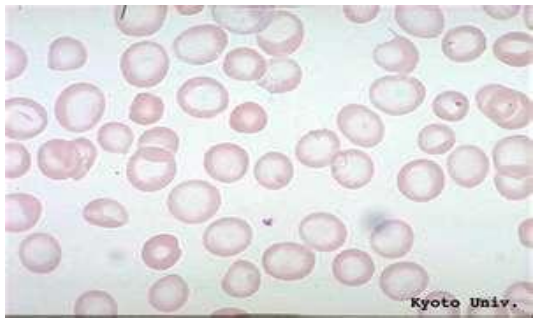
<u>Morphology:</u>
Increase in the size of a red cell. Red cells are larger than 9µm in diameter. May be round or oval in shape, the diagnostic significance being different.
<u>Found in:</u>
Folate and B_{12} deficiencies (oval)
Ethanol (round)
Liver disease (round)
Reticulocytosis (round)



III. Variation in Hemoglobin Content-Color Variation

1. Hypochromasia

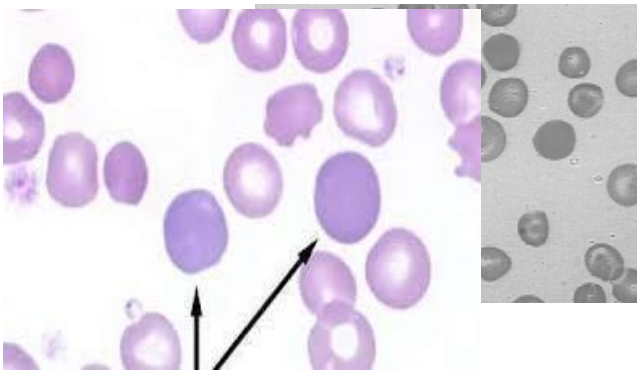
<u>Morphology:</u>
Increase in the red cells' central pallor which occupies more than the normal third of the red cell diameter.
<u>Found in:</u>
Iron deficiency
Thalassaemia
any of the conditions leading to Microcytosis



2. Polychromasia

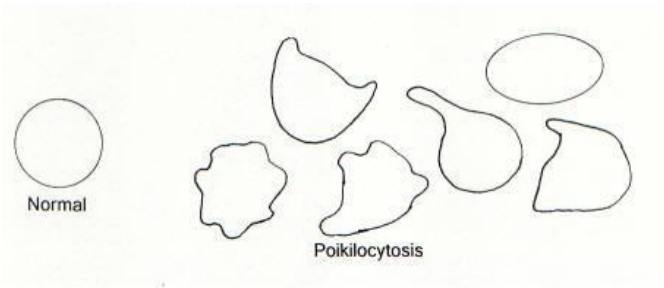
<u>Morphology:</u>
Red cells stain shades of blue-gray as a consequence of uptake of both eosin (by hemoglobin) and basic dyes (by residual ribosomal RNA). Often slightly larger than normal red cells and round in shape - round macrocytosis.

<u>Found in:</u>
Any situation with reticulocytosis - for example bleeding, hemolysis or response to haematinic factor replacement



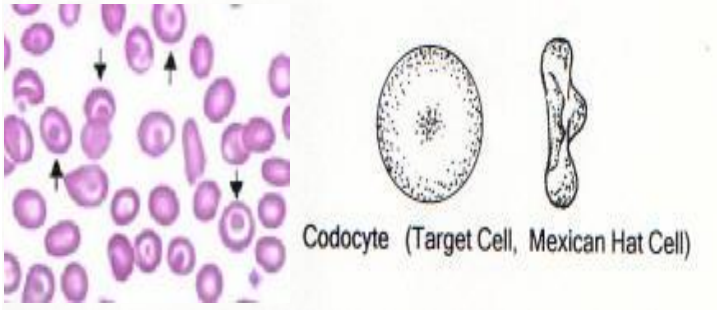
IV. Variation of red cells shape (Poikilocytosis)

RBCs may have different shapes.



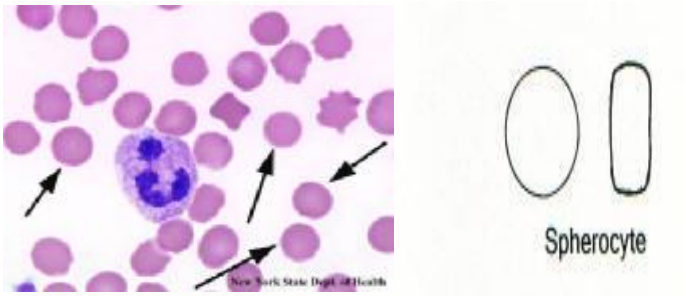
1. Target Cells

<u>Morphology:</u>
Red cells have an area of increased staining which appears in the area of central pallor.
<u>Found in:</u>
Obstructive liver disease
Severe iron deficiency
Thalassaemia
Haemoglobinopathies (S &C)
Post splenectomy



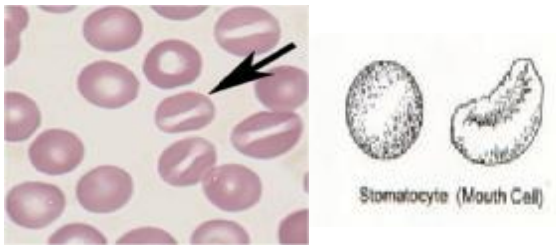
2. Spherocytosis

<u>Morphology:</u>
Red cells are more spherical. Lack the central area of pallor on a stained blood film.
<u>Found in:</u>
Hereditary spherocytosis
Immune haemolytic anemia
Zieve's syndrome
Microangiopathichaemolytic anemia.(MAHA)



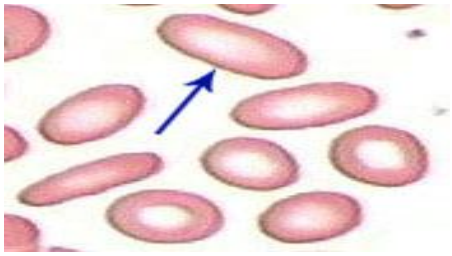
3. Stomatocytosis

<u>Morphology:</u>
Red cells with a central linear slit or stoma. Seen as mouth-shaped form in peripheral smear.
<u>Found in:</u>
Alcohol excess
Alcoholic liver disease
Hereditary stomatocytosis
Hereditary spherocytosis



4. Ovalocytes

Morphology:
oval shape red blood cell
Found in:
Thalassaemia major.
Hereditary ovalocytosis.
Sickle cell anemia.



5. Elliptocytosis

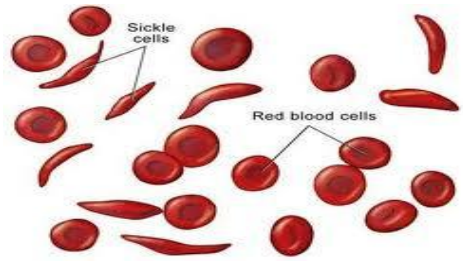
Morphology:
The red cells are oval or elliptical in shape. Long axis is twice the short axis.

Found in:
Hereditary elliptocytosis
Megaloblastic anemia
Iron deficiency
Thalassaemia
Myelofibrosis

6. Sickle Cells

Morphology: Sickle shaped red cells

Found in: Hb-S disease



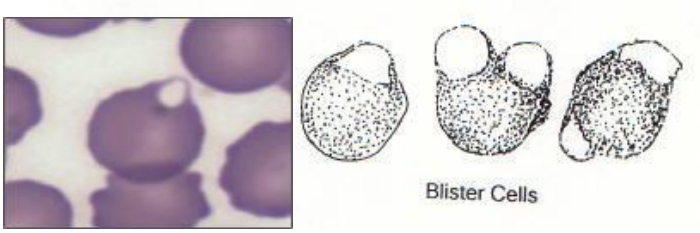
7. Schistocytosis

Morphology: Fragmentation of the red cells.

Found in:
DIC
Micro angiopathichaemolytic anemia
Mechanical haemolytic anemia

a. Blister cell Or prekeratocyte

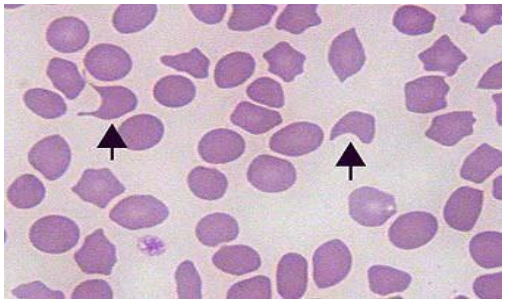
Morphology: Have accentric hallow area. Resemble a women's handbag and may be called *pocket-book* cell.



Found in: Microangiopathic hemolytic anemia

b. Keratocytes : (horn cell)

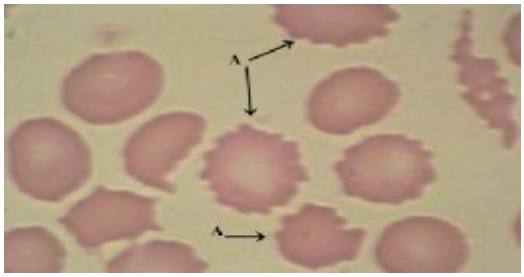
Morphology:
Part of the cell fuses back leaving two or three horn-like projections. The keratocyte is a fragile cell and remains in circulation for only a few hours.



Found in:
Uraemia
Severe burns
EDTA artifact
Liver disease

8. Burr (crenation) cell

Morphology:
Red cell with uniformly spaced, pointed projections on their surface.



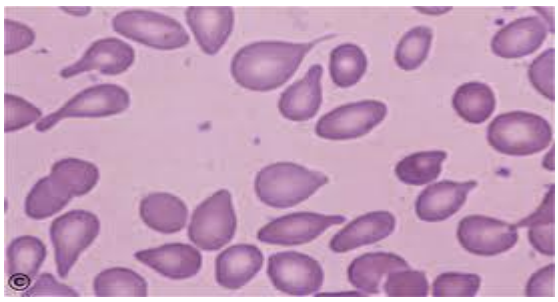
Found in:
hemolytic anemia
Uremia.
Megaloblastic anemia

9. Acanthocytosis

Morphology:
are red blood cells with irregularly spaced projections, these projections very in width but usually contain a rounded end, and lack an area of a central pallor.

Found in:
Liver disease
Post splenectomy
Anorexia nervosa and starvation

10. Teardrop Cells

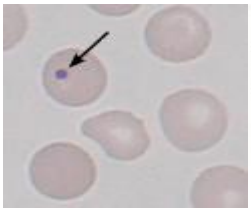


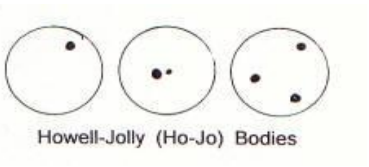
Morphology:
Red cells shaped like a tear drop or pear

Found in:
Bone marrow fibrosis
Megaloblastic anemia
Iron deficiency
Thalassaemia

V. Erythrocyte inclusion bodies

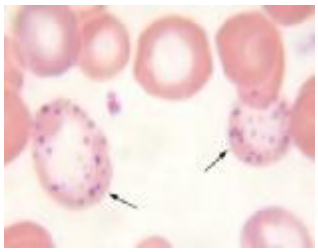
1. Howell-Jolly Bodies

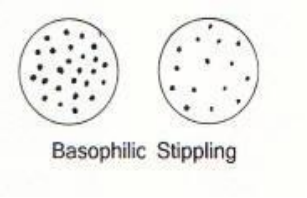




Morphology:
Small round cytoplasmic red cell inclusion with same staining characteristics as nuclei
Found in:
Post splenectomy
Megaloblastic anemia

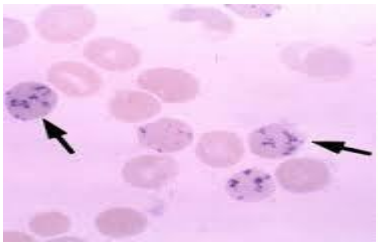
2. Basophilic stippling

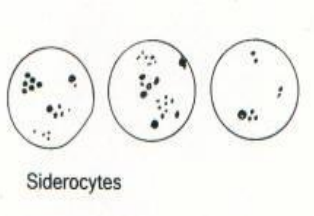




Morphology:
Considerable numbers of small basophilic inclusions in red cells.
Found in:
Thalassaemia
Megaloblastic anemia
Hemolytic anemia
Liver disease
Heavy metal poisoning.

3. Siderotic Granules (Pappenheimer Bodies)

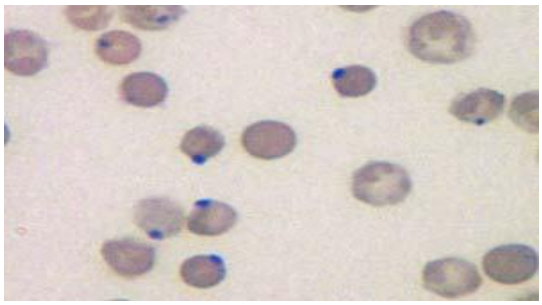




RBCs which contain no hemoglobin iron granules. They appear as dense blue, irregular granules which are unevenly distributed in Wright stained RBCs. Pappenheimer bodies can be increased in hemolytic anemia, infections and post-splenectomy.

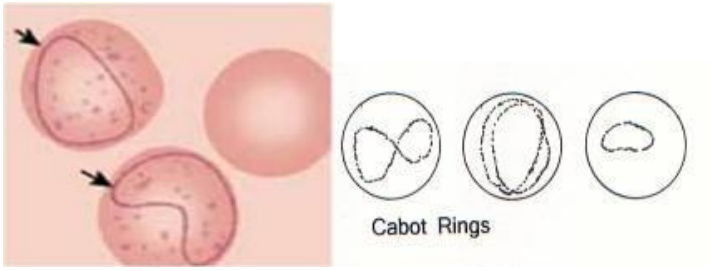
4. Heinz Bodies

Represent denatured hemoglobin (methemoglobin - Fe+++) within a cell. With a supravital stain like crystal violet, Heinz bodies appear as round blue precipitates. Presence of Heinz bodies indicates red cell injury and is usually associated with G6PD-deficiency.



5. Cabot Rings

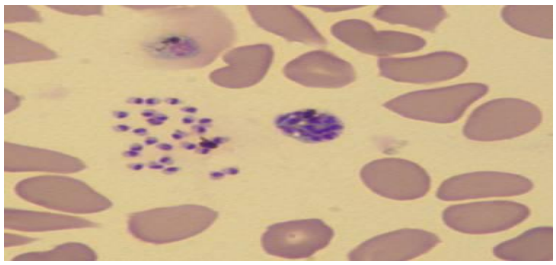
Reddish-blue threadlike rings in RBCs of severe anemia's. These are remnants of the nuclear membrane or remnants of microtubules and appear as a ring or figure 8 pattern. Very rare finding in patients with Megaloblastic anemia, severe anemia's, lead poisoning, and dyserythropoiesis.



6. ProtozoanInclusion

Two organisms are have a tendency to invade the RBCs.

1. All 4 species of the malaria parasite will invade RBCs. We will see the Plasmodium of different species in RBCs.
2. Bebesiamicroti



DETERMINE A QUANTITATIVE SCALE

Table 20.4 Qualitative Grading of RBC Morphology

	Grade Degree of Abnormalities
1 to 5 cells/10 fields	Slight
6 to 15 cells/10 fields	Moderate
>15 cells/10 fields	Marked

GRADING INCLUSIONS

Table 20.6 Grading Inclusions

Rare	0 to 1/hpf
Few	1 to 2/hpf
Mod	2 to 4 /hpf
Many	> 5/hpf

hpf, high-power field.

Reticulocyte Count

Reticulocytes are immature RBCs that contain remnant cytoplasmic ribonucleic acid (RNA) and organelles such as mitochondria and ribosomes. We know that the RBCs have six stages of the development to became mature erythrocyte begins with : Pronormoblast, Basophilic normoblast, ,polychromatophilicnormoblast, orthochromicnormoblast, reticulocyte, and mature red blood cell. The first four stages are normally confined to the bone marrow. The reticulocyte, however, is found in both the bone marrow and peripheral blood. In the bone marrow, it spends approximately 2 to 3 days maturing and is then released into the blood, where it ages for an additional day before becoming a mature red blood cell.

The reticulocyte count is an important diagnostic tool. It is a reflection of the amount of effective red blood cell production taking place in the bone marrow. Since the life span of a red cell is 120 days, ± 20 days, the bone marrow replaces approximately 1 % of the adult red blood cells every day. The normal value for a reticulocyte count is therefore 0.5 to 1.5/100 red blood cells (or, 0.5 to 1.5%), with a range of 25 to 75 X 10^9/L for the absolute count (multiply the red blood cell count by the percentage of reticulocytes).

Decreased reticulocyte count indicates that the bone marrow is not producing a normal number of red blood cells. Low production may be caused by a lack of vitamin B, folic acid, or iron in the diet; or by an illness affecting the bone marrow (for example, cancer). Further tests are needed to diagnose the specific cause. E.g:

1. Aplastic anemia.
2. Exposure to radiation or radiation therapy.
3. Chronic infection.
4. Untreated pernicious anemia, megaloblastic anemia and iron deficiency anemia.
5. Medications such as azathioprine, chloramphenicol, dactinomycin, methotrexate, and other chemotherapy medications.

Increased reticulocyte count when the bone marrow makes more red cells in response to

1. thalassemia, sideroblastic anemia.
2. in acute and chronic blood loss.
3. Hemolytic anemias.
4. Pregnancy.
5. Pernicious Anemia or iron deficiency anemia after treatment.
6. Medications such as levodopa, malarial medications, corticotrophin, and fever-reducing medications.

Counts in newborn may be somewhat higher (2-6%) but return to adult levels in 1-2 weeks.

Reticulocytes are visualized by staining with vital dyes (such as new methylene blue) that precipitate the RNA and organelles, forming a filamentous network of reticulum (Fig. 2). On Wright stain.the reticulocyte appears polychromatophilic or as a macrocytic blue red cell. The reticulocyte is a means of assessing the erythropoietic activity of the bone marrow.

Reagents and Equipment

1. New methylene blue or Brilliant Cresyl Blue (Supravital Stain) solution.
   New methylene blue (CI 52030) 1.0 g
   (certified by the U.S. BiologicalStain Commission)
   Sodium chloride 0.89g
   Distilled water 100mL
   Mix for at least 15 minutes, filter, and store at room temperature. Filter again on the day of use .
2. Glass slides .
3. Microscope.

Specimen

Whole blood (1 mL), using tripotassium EDTA as the anticoagulant. Capillary blood may also be used.

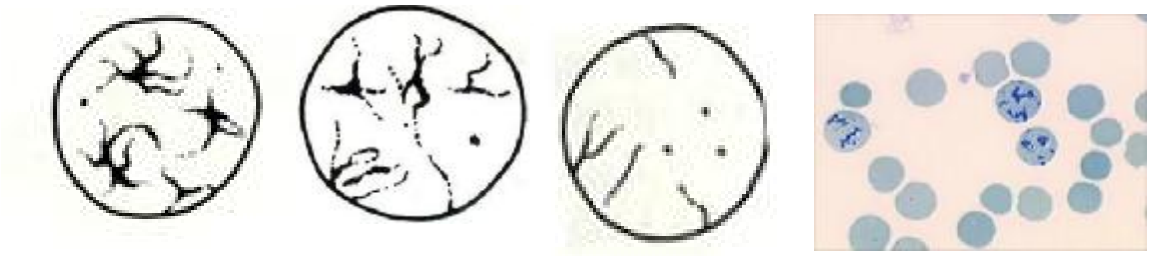
Principle

After the orthochromicnormoblast loses its nucleus, a small amount of RNA remains in the red blood cell, and the cell is known as a reticulocyte. To detect the presence of RNA and organelles, the red blood cells must be stained while they are still living. This process is called supra¬vital staining. Whole blood is incubated with new methylene blue. Smears of this mixture are then prepared and examined. The number of reticulocytes in 1000 red blood cells is determined. This number is divided by 10 to obtain the reticulocyte count in percent.

Procedure:

1. Mix equal amounts of methylene blue and EDTA (two to three drops) on a small test tube. If anemic use a larger proportion of blood; use a smaller proportion of blood if polycythaemic.
2. Mix the tube and allow standing at room temperature or leaving in water bath or incubator at 37^{o}C for 15-20 minutes.This allows the reticulocytes adequate time to take up the stain.
3. Mix blood and stain mixture thoroughly and make two thinwedge or spun smears and allow to air dry.
4. Place the first slide on the microscope stage and, using the low power objective (10X), find an area in the thin portion of the smear in which the red blood cells are evenly distributed and are not touching each other. Carefully change to the "oil immersion objective (100x) and further locate an area in which there are approximately 100 to 200 red blood cells per oil immersion field.

5. As soon as the proper area is selected, the reticulocytes may be counted. The red blood cells will be a light to medium green in color. The RNA present in the reticulocytes stains a deep blue. The reticulum may be abundant or sparse, depending on the cell's stage of development. The youngest reticulocyte shows



6. Average the two results and calculate the reticulocyte count as shown below.

$$\% \text{ Reticulocytes} = \frac{\text{Number of reticulocytes in 1000 RBCs} \times 100}{\text{1000 (RBC's observed)}}$$

EXAMPLE: 25 reticulocytes in 1,000 total RBC's

$$\text{Reticulocyte count} = \frac{25x\ 100}{1000} = 2.5\%$$

Miller Disc Method of Counting

The Miller disc (fig) may be placed in one of the ocular lenses to aid in the counting of the reticulocytes. The disc has two squares; square B is one ninth of square A.

500 RBCs are counted in square B in consecutive fields, while reticulocytes are counted in squares A & B. if a reticulocytes is seen during counting in square B, it is counted both as an RBC and as a reticulocyte. When the counted is finished, the number of reticulocytes in 4500 erythrocytes has theoretically been counted.

Calculate the reticulocyte count:

$$\text{Reticulocyte (\%)} = \frac{\text{total reticulocytes in square A X 100}}{\text{Total RBCs in square B X 9}}$$

Example: 100 reticulocytes were seen in square A and 500 RBCs were counted in square B. ?

$$\text{Reticulocyte (\%)} = \frac{100\ X\ 100}{500\ X\ 9} = 2.2\ \%$$

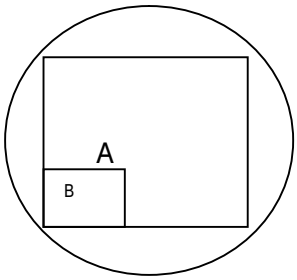


Fig. Miller disk (older model).

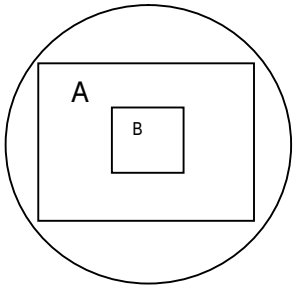


Fig. Miller disk (newer model).

Reporting Results

The reticulocyte percentage may be misleading if one does not consider the degree of anemiaor of intense erythropoietic stimulation. The reticulocyte count may be truly elevated, indicating increased effective erythropoiesis, or it may only appear elevated because the total number of erythrocytes is decreased. To compensate for this the best and simplest method of reporting Retic is the Absolute Retic Count.

Absolute Reticulocyte Count (ARC): is the actual number of reticulocytes in 1L of whole blood. This is calculated by multiplying the retic % by the RBCs count and dividing by 100. Reference values of ARC is 25.0 - 75,0X10^9/L.

$$ARC = \frac{\text{Reticulocyte(\%)X RBCs count } (10^{12}/\text{ L})}{100}$$

For example, a patient's reticulocyte count is 2% and the RBCs count is 2.20X10^{12}/L the normal RBCs count (3.6-5.6) X 10^{12}/L, the ARC would be calculated as follows:

$$ARC = \frac{2 \text{ X } (2.20X1012/L)}{100} = 44.0X10^9/L$$

Corrected Reticulocyte Count

A reticulocyte count should reflect the total production of red blood cells, regardless of the concentration of red cells in the blood (red blood cell count). The reticulocyte count can increase either because more reticulocytes are in the circulation, or because there are fewer mature cells. Therefore, the observed reticulocyte count may be corrected to a normal hematocrit of 45%.

As an example, compare the following two patients. Patient # 1 has a hematocrit of 42% and a reticulocyte count of 1.0%. Patient #2 has a hematocrit of 21 % and a reticulocyte count of 2.0%. Patient #2, theoretically, has 1/2 as many red blood cells as patient # 1 but has the same number of reticulocytes as patient # 1 because the reticulocytes are diluted by only 1/2 the number of red blood cells, as in patient # 1. To compensate for this, a corrected reticulocyte count is calculated based on a normal hematocrit of 45%. The formula for this correction is:

$$\text{Corrected reticulocyte count (\%)} = \frac{\text{Patient'shematocrit}}{45\%} \times \text{Reticulocyte count (\%)}$$

For example, if a patient presenting with a reticulocyte count of 10% with a hematocrit of 22% , the corrected reticulocyte would be:

$$\text{Corrected reticulocyte count} = \frac{10\% \times 22\%}{45\%} = 4.9\%$$

In addition to correcting a reticulocyte count for an abnormally low hematocrit, consideration should also be given to the presence of marrow reticulocytes present in the peripheral blood. In this circumstance, the reticulocyte production index is calculated.

As previously stated, the reticulocytes spend approximately two to three days in the bone marrow before being released into the blood where they spend 1 day maturing in the peripheral circulation. Under some circumstances the marrow reticulocytes are released directly into the blood prior to maturation in the bone marrow. This is detected by nucleated red blood cells and/or polychromatophilicmacrocytes(shift cells) present in the circulating blood. To correct for the increased time spent in maturation in the peripheral blood, the reticulocyte production index is calculated by dividing the corrected reticulocyte count by the number of days the reticulocyte most probably takes to mature in the blood (Tabel 1).

Table 1 Maturation Time of reticulocytes

Maturation Time (Days)	Hematocrit (%)
1	45
1.5	35
2	25
3	15

$$\text{Retic Production Index (RPI)} = \frac{\text{Corrected retic count (\%)}}{\text{\# Days (Maturation time)}}$$

For example, a patient with a reticulocyte count of 12% and a hematocrit of 25% would yield an RPI of :

$$\text{RPI} = \frac{7\% \times (25\%/45\%)}{2} = 3.3$$

Although there is some difference of opinion, correction of the reticulocyte count for shift clearly improves diagnostic results. For the shift correction to be valid there must be a normal relationship between degree of anemia and the increased erythropoietin concentration which produces shift. This is validated by examination of the smear. This index, when compared to the expected marrow response in the anemic subject indicates the state of erythropoiesis. An index equal to or grater than 3 is considered to represent an adequate bone marrow response. An index of less than 2 is inadequate.

Normal results

Reticulocytes Count	
Newborn :	2.5 - 6.0%
Adult:	0.5 - 2.0%
Absolute reticulocyte count:	25 - 75 × 109/L
RPI	3 or greater

Discussion

1. When using EDTA as the anticoagulant, the blood may be stored for 24 hours prior to staining while still obtaining acceptable results. It is thought, however, that the reticulocyte count may tend to drop after 6 to 8 hours after obtaining the specimen .

2. Brilliant cresyl blue also stains reticulocytes but shows too much inconsistency in staining for routine use. Pure azure B, however, may be used in place of new

methylene blue with good results (using the same stain concentration and procedure as described above).

3. The blood-to-stain ratio does not have to be exactly equal. For best results, a larger proportion of blood should be added to the stain when the patient's hematocrit is low. Add a smaller amount of blood to the stain when the patient has an unusually high hematocrit .
4. The time allowed for staining of the reticulocyte is not critical. It should not, however, be less than 10 minutes .
5. The presence of a high blood sugar (glucose) or the use of heparin as the anticoagulant may cause the reticulocytes to show pale staining .
6. It is advisable not to counter stain the reticulocyte smears with Wright stain because any precipitated stain may cause confusion in the identification of reticulocytes
7. It is extremely important that the blood and stain be mixed well prior to making smears. The reticulocytes have a lower specific gravity than mature red blood cells and, therefore settle on top of the red blood cells in the mixture .
8. Careful focusing of the microscope is essential. Platelet granules and leukocyte granules will stain with the dye and these may be easily mistaken for reticulocytes.

9. If the procedure is followed carefully, the distribution of the reticulocytes on the films will be good, and the allowable difference between the number of reticulocytes per 500 RBC's is 5 reticulocytes.

10. Howell-Jolly bodies, Heinz bodies, and iron particles, if present will also take up the stain.

.

11. An automated procedure for counting reticulocytes using flow cytometry with fluorescent dyes . This method is more rapid, precise, and accurate than the manual procedure described here.

Sickle Cell

Overview

Sickle cell anemia is an inherited disorder that leads to the production of an abnormal hemoglobin variant, hemoglobin S (HbS or HgbS). In the red blood cell (RBC), this variant can form polymers in low oxygen conditions, changing the shape of the RBC from a round disc to a characteristic crescent (sickle) shape. This altered shape limits the RBC's ability to flow smoothly throughout the body, limits the hemoglobin's ability to transport oxygen, and decreases the RBCs lifespan from 120 days to about 10-20 days. The affected person can become anemic because the body cannot produce RBCs as fast as they are destroyed. Also, sickled blood cells can become trapped in blood vessels reducing or blocking blood flow. This can damage organs, muscles, and bones and may lead to life-threatening conditions.

Hemoglobin S production arises from an altered (mutated) "S" gene. Hemoglobin S differs from normal adult hemoglobin (hemoglobin A) only by a single amino acid substitution (a valine replacing a glutamine in the 6th position of the beta chain of globing). A person with one altered S gene will have sickle cell trait. In those who have sickle cell trait, 20% to 40% of the hemoglobin is HbS The person does not generally have any symptoms or health problems but can pass the gene on to his children.

When a person has two copies of the S gene (homozygous SS), he has sickle cell anemia. In sickle cell disease, as much as 80% to 100% of the hemoglobin may be HbS. Those individuals who carry both abnormal genes have sickle cell disease. In this condition the person may not experience any symptoms under 'normal' conditions, but may experience episodes called 'sickling crises' brought on by, for example, infection or dehydration. During such episodes symptoms can include joint pain, abdominal pain, fever and seizures. In the long-term, sufferers may experience haemolyticanaemia (breakdown of red blood cells), growth impairment, jaundice and increased risk of serious infections.

Hydroxyurea as a treatment

- HbF, also called fetal hemoglobin, is the form of hemoglobin present in the fetus and small infants.
- Most HbF disappears early in childhood, although some HbF may persist. Fetal hemoglobin is able to block the sickling action of red blood cells.
- Because of this, infants with sickle cell disease do not develop symptoms of the illness until. HbF levels have dropped.
- Adults who have sickle cell disease but still retain high levels of hemoglobin F generally have mild disease.

– Hydroxyurea (Droxia) is a drug that reduces the severity of sickle cell disease by stimulating production of HbF. It is currently the only drug in general use to prevent acute sickle cell crises

Sickle cell test:

A sickle cell test is a blood test done to screen for sickle cell trait or sickle cell disease. Sickle cell disease is an inherited blood disease that causes red blood cells to be deformed (sickle-shaped).

If the screening test is negative, it means that the gene for sickle cell trait is not present. If the screening test is positive, then further haemoglobin testing must be performed to confirm whether one mutated gene or both are present. In unaffected individuals HbS is not present.

General Principle

– We will make the conditions at which oxygen tension decline to induced the sickling process of Hbs in RBCs.

A saline citrate with paraffin oil

Principle:

– a saline citrate suspension of blood is allowed to stand in a test tube under a layer of paraffin oil until sickling takes place.'
– In employing any of the common diagnostic tests for sickling it is desirable to obtain blood which has a low. fraction of oxyhemoglobin.

Sodium Metabisulfite Method

Principle

– When a drop of blood is sealed between a cover slip and a slide, the decline in oxygen tension due to oxidative processes in the blood cells leads to sickling.

In this method added with blood drop a chemical reducing agents. Such as sodium metabisulfite. This rapidly reduces oxyhemoglobin to reduced hemoglobin, then this will be accelerate sickling

Specimen:
Whole blood using heparin or EDTA as anticoagulant. Capillary blood may also be used.

Reagent and equipment:

1. Sodium Metabisulfite 2% (w/v); prepared by dissolving 0.2 gm sodium metabisulfite in 10 ml DW. Stable for 8 hours at room temperature.
2. Petroleum jelly.
3. Cover glass.
4. Microscope.

Procedure:

1. Place one drop of the blood to be tested in a glass slide.
2. Add 1- 2 drops of sodium metabisulfite to the drop of blood and mix well with an applicator stick.
3. Place a cover glass on top of the sample and press down lightly on it to remove any air bubbles and to form a thin layer of the mixture. Wipe of the excess sample.
4. Carefully rim the cover gloss with the petroleum jelly, completely sealing the mixture under the cover slip.
5. Examine the preparation for the present of sickle cells after one hour using 40 X objective. In some instances, the red blood cells may take on a holly-leaf form. This shape is found in sickle cell trait, and, when present, the test is reported as positive.
6. If there is no sickling present at the end of one hour, allow the preparation to stand at room temperature for 24 hours, and examined at that time.
7. When sickle cells or the holly leaf form of the cells are present the results are reported as positive. Normal looking red cells or slightly crenated red blood cells as reported as negative.

Solubility test

Erythrocytes are lysed by saponin and the released hemoglobin is reduced by sodium hydrosulfite in a concentrated phosphate buffer. Under these conditions, reduced HbS is characterized by its very low solubility and the formation of crystals. The presences of HbS or HbC are indicated by the turbid solutions. The normal HbA under these same conditions results in a clear non-turbid solutions.

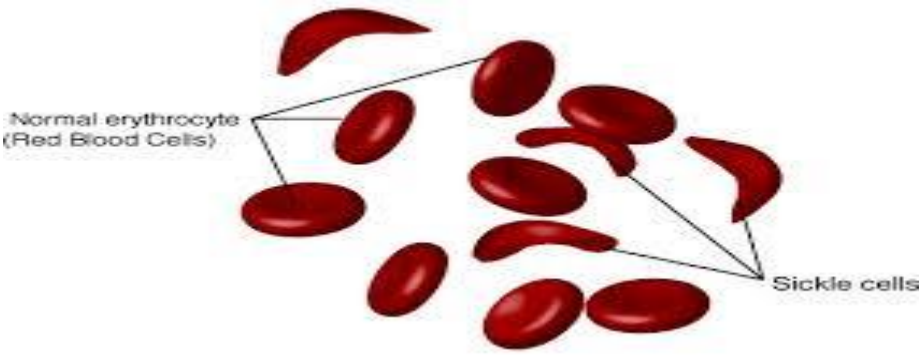
Discussion:

1. The sickle cells or the holly-leaf form of the cell must come to a point or points to be considered positive. Elongated cells with a round end must not be confused with sickle cells.

2. Sickling of the cells is maximum at 37^{o}C and decreased as the temperature lowers.

3. This test should not be performed on infants less than six months old.

4. With this method it is not possible to distinguish sickle cell trait from sickle cell disease. Hence if the test is positive, it is advisable to perform hemoglobin electrophoresis to determine the presence of the trait or the anemia and to positively identified the type of the sickling hemoglobin present.



Gel Electrophoresis

Electrophoresis is a means of separating hemoglobin's. It depends on the migration of the hemoglobin molecules dissolved in a buffer on, or in, a supporting medium when an electric current is passed through them.
Hemoglobin electrophoresis is a test that measures the different types of the oxygen-carrying substance (hemoglobin) in the blood.

Why the test is performed?

Hempoglobin electrophoresis is performed to find out abnormal forms of hemoglobin (hemoglobinopathy).
Many different types of hemoglobin (Hb) exist. The most common ones are HbA, HbA2, HbF, HbS, HbC, Hgb H, and Hgb M. Healthy adults only have significant levels of HbA and HbA2.
Some people may also have small amounts of HbF (which is the main type of hemoglobin in an unborn baby's body). Certain diseases are associated with high HbF levels (when HbF is more than 2% of the total hemoglobin).
HbS is an abnormal form of hemoglobin associated with sickle cell anemia. In people with this condition, the red blood cells have a crescent or sickle shape. These misformed cells then break down, or can block small blood vessels.
HbC is an abnormal form of hemoglobin associated with hemolytic anemia. The symptoms are much milder than they are in sickle cell anemia.
Other, less common, abnormal Hb molecules cause anemias.

Normal Values
In adults, these hemoglobin molecules make up the following percentages of total hemoglobin:

- Hgb A1: 95% to 98%
- Hgb A2: 2% to 3%
- Hgb F: 0.8% to 2%
- Hgb S: 0%
- Hgb C: 0%

In infants and children, these hemoglobin molecules make up the following percentages of total hemoglobin:

- Hgb F (newborn): 50% to 80%
- Hgb F (6 months): 8%
- Hgb F (over 6 months): 1% to 2%

What abnormal results mean
The presence of significant levels of abnormal hemoglobins may indicate:

- Hemoglobin C disease
- Rare hemoglobinopathy
- Sickle cell anemia

Methods of electrophoresis

There are two common methods:

1-Cellulose Acetate At Alkaline pH

Cellulose acetate Hb electrophoresis at alkaline pH is the primary screening procedure used to detect variant (abnormal) Hbs, of which there are several hundred. Hb, is made up of heme and globin, is identified according to the structure of the globin chains. Abnormal globin chains will differ in the number, type, and sequence of amino acids: this gives the Hb its identity. The major portion of normal adult Hb is A. In addition, up to 3.5% Hb A_2 is normally present, along with less than 2% Hb F. The more common mutant Hbs are S, C, E, D, G, and lepore. When an abnormal Hb is detected on cellulose acetate electrophoresis at an alkaline pH (8.2-8.6) further testing is frequently indicated: test for Hb S, quantitation of Hb A_2 and F, and citrate agar gel; acid/alkaline globin chain or neutral pH electrophoresis may also be warranted.

Principle of the test

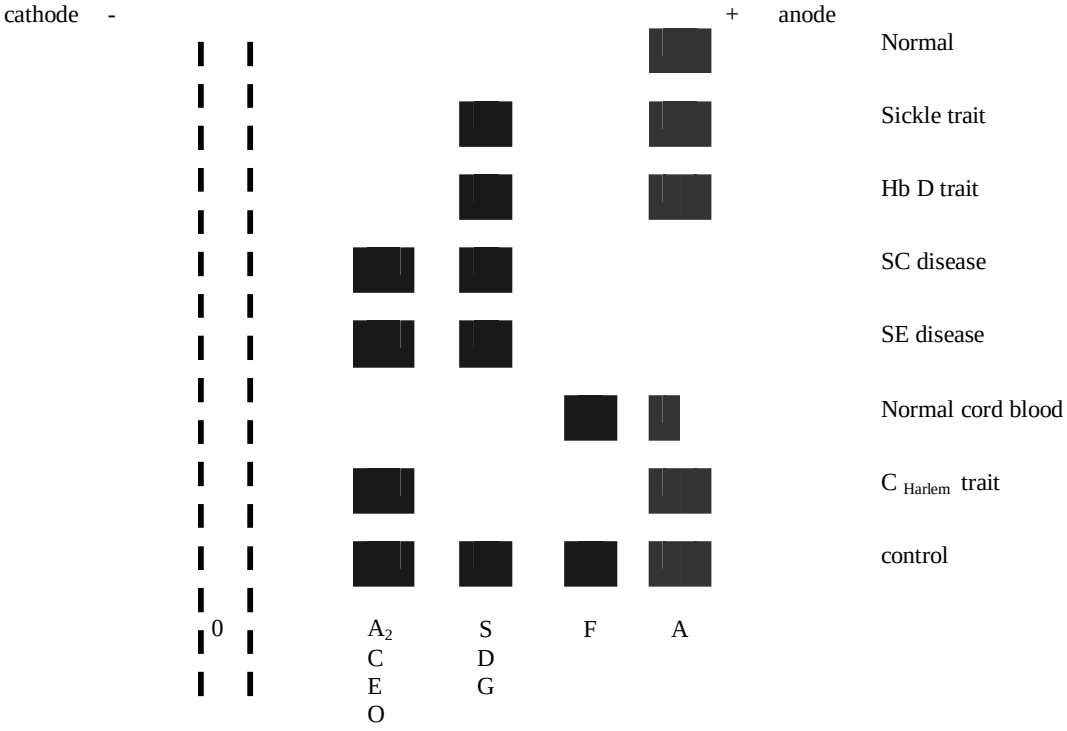
Electrophoresis is the movement charge particles in an electric field. In an alkaline pH (8.2-8.6) Hb is a negatively charged molecule and will migrate toward the anode (+). The various Hbs moves at different rates depending on their net negative charge, which in turn is controlled by the composition (amino acids) of the Hb molecule (globin chain). The red cell hemolysate (red blood cell membranes are destroyed to free the Hb molecules for testing) is placed in a cellulose acetate membrane, which is positioned in an electrophoresis tray with the inoculated hemolysate near the cathode (-).

One end of the cellulose acetate strip is immersed in the buffer (pH 8.2-8.6) on the cathode side and the other end is placed in the buffer on the anode (+) side. An electric current of specific voltage is allowed to run for a timed period. During electrophoresis, the Hb molecules migrate toward the anode because of their negative charge. The difference in the net charge of the Hb molecule determines its mobility and manifests its self by the speed with which it migrates to the positive pole. Example of the fast Hbs are Hb Bart's and the tow fastest variants Hb H and I, while Hb C is the slowest common Hb. The cellulose acetate membrane is then stained in order to color the proteins (Hbs). By noting the distance each Hb has migrated and comparing this distance with the migration distance of known controls, the types of hemoglobins may be identified.

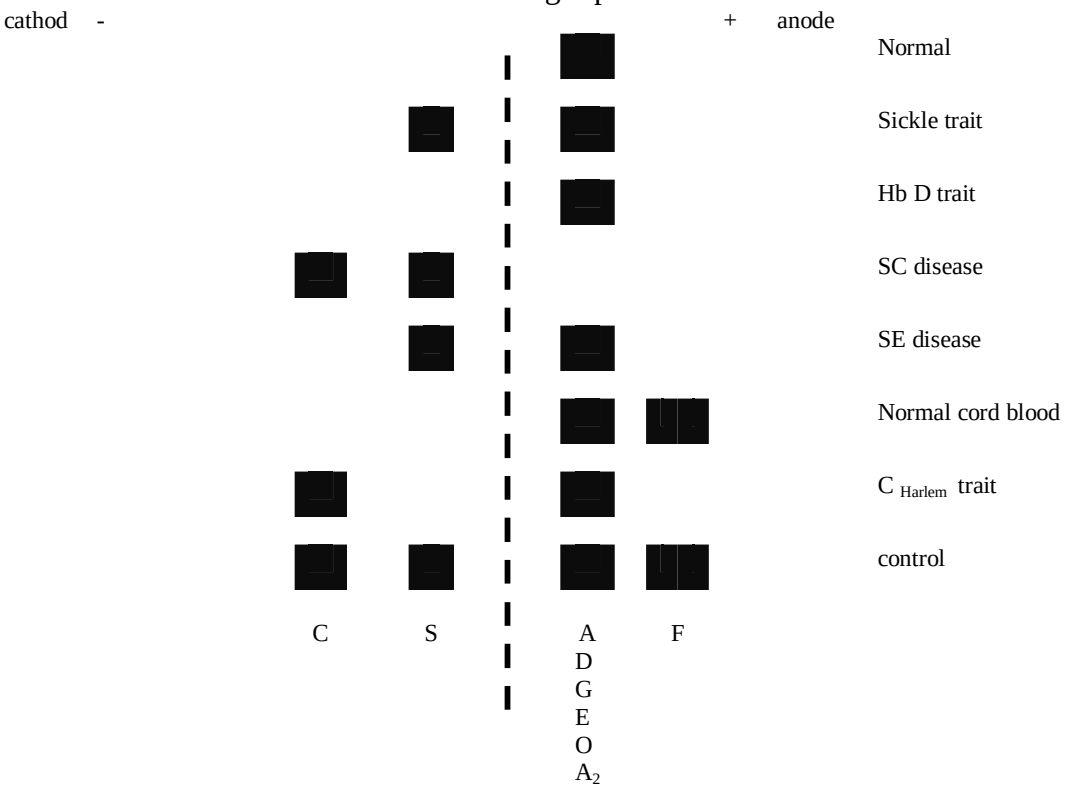
2- Citrate Agar Electrophoresis (acid pH)

Citrate agar separates Hb fractions that migrate together on cellulose acetate agar, all Hb specimens that show an abnormal electrophoretic pattern in alkaline media (cellulose acetate agar) should undergo electrophoresis on an acid citrate agar. Citrate agar electrophoresis is used to confirm variant Hbs and further differentiates Hb S from Hb D and G, and Hb C from Hb E, O_{Arab}, and $C_{Harlem.}$ Theprocedure should not be used as a screening procedure because many abnormal Hbs migrate with Hb A. However, this procedure is the method of choice when examining newborns (cord blood specimens) and infants under 3 months of age for some abnormal Hbs such as S and C because the test is able to detect quantities of Hb not easily seen by other techniques.

Cellulose Acetate Agar pH 8.4



Citrate Agar pH 6.0-6.2



Hemoglobin A_2

Determination of A_2 hemoglobin (Hb A_2) in blood

Introduction

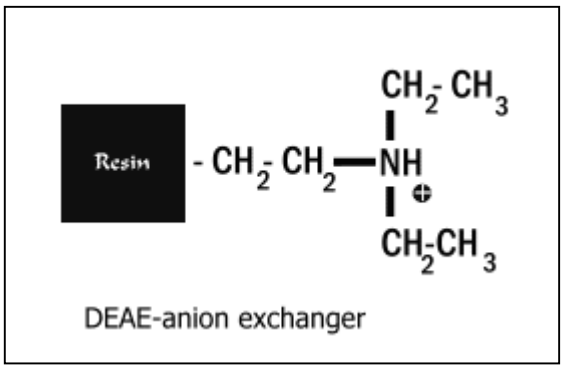
Hemoglobin A_2 is a normal variant of hemoglobin A that consists of two alpha and two delta chains and is found in small quantity in normal human blood.

Normal value of Hb A_2 in the adult is (1.8% – 3.5%). Elevated level (up to 8%) generally indicate β. Thalassemia trait, although some patients with homozygous. Thalassemia may show an increased HbA_2. Decreased level may be found in iron deficiency anemia, Hb H disease, hereditary persistence of Hb F, fibroblastic anemia, and in carriers of α Thalassemia .

The anion exchange micro chromatography procedure outlined below is an accurate and easily performed method for HbA_2 Quantization.

Principle

A hemolysate is prepared from the patients red blood cells. A specific amount of hemolysate is then added to the top of the resin column. The diethyl amino ethyl DEDE resin is a preparation of cellulose attached to positively charged molecules, thus giving the cellulose appositive charge.



DEAE-anion exchanger

When the hemolysate is added to the column, the PH of the buffer present determines the net negative charge of the Hb, which then binds to the positively charged cellulose resin. The Hbare selectively removed from cellulose according to the PH of the developer. In this procedure the HbA_2 (originally bound to the resin) is released from the resin and eluted by the developer as it passes through the column. Most other normal and abnormal HbS remain bound to the resin in the column. The eluted HbA_2 is then measured spectrophotometrically and compared with the amount of total Hb in the specimen to calculate the percent of HbA_2 present.

- © The Kit used in this lab does not need PH developer, because Hb A_2 originally not bounded to the resin. Its free, but using separating Filter for isolation .

Storage and stability

- The reagents are stable up to the expiry date stated on the labels, if stored at 15-25 °C.
- Strong temperature variations may alter resin equilibrium and consequently its functionality; if erroneously stored at 2-8 °C the resin has to remain at room temperature for at least three days before using.
- Tubes containing yellowish-white resin indicate chemical degradation and cannot be used.

Reagent & Equipment

1. Test tubes: 2.5 ml of buffered DAEA resin
2. Lysis reagent : 20 ml of a TritonX100 solution
3. Separating filters
4. HbA_2 DEVELOPER*(not used in this Lab)*

Ingredients: HbA2 Developer contains 0.2 M glycine in deionized water. Potassium cyanide (0.01 %) has been added as a preservative.

5. Automatic pipettes
6. Disposable test tubes
7. Spectrophotometer set at 415 nm

Specimen

Whole blood with Heparin or EDTA.
Hb A_2 is stable 1 week in blood at 4°C or 15 frozen at -20°C.

Procedure

1. Hemolisate preparation

1- Blood 30 µl
2- hemolysis reagent 300 µl, Wait 5 minutes.
3- Good mixing and leaved it for 5 min at R.T.

2. Separation and reading HbA_2

1. Pipet in test tube 1 (resin) Hb $A_2$100µl from hemolisate.
2. Turn upside down test tube 1 till complete resuspension of theresin. Continue to shake gently the test tubes for 5 minutesusing a stirrer or turn upside down al least 6 times at intervals of1 minute.
3. Separate the liquid phase by gently pressing the separating filterin the test tube.
4. Determine at 415 nm the absorbance of the liquid phase of thetest tube (A HbA_2) against a reagent blank made of liquid phasefrom a test tube without hemolisate.

3. Reading of total hemoglobin

1. Pipet in Test tube 2 (empty) Hbtot 20µl from hemolisate and 10 ml from distilled water.
2. read the absorbance of totalhemoglobin (A Hb tot) against a reagent blank made of distilled water.

CALCULATION

$$\% \text{ Hb A2} = \frac{\text{A Hb } A_2}{\text{A Hb tot x 22}} \text{ x } 100$$

Notes:

- This test must not be performed before six months age.
- If the patient heterozygous for β- thalassemia also has iron deficiency, the hemoglobin A_2 may be within the normal range.
- If the patient has received a transfusion recently this test should not be performed.
- Some of the abnormal hemoglobin (Hb S, C, O, E, G, S-G hybrid) are interfere with Hb A_2 in this method. The presence of the abnormal hemoglobin should be confirmed by electrophoretic techniques. Hb F does not interfere with this method.

Hemoglobin F Acid Stain

HEMOGLOBIN F STAIN, ACID ELUTION (KLEIHAUER BETKE TEST)

The acid elution test is employed to assess the distribution of hemoglobin F in the red blood cell:to determine whether hemoglobin F is present in the same amount in all red blood cells, or whether it is present in varying amounts in only some of the red blood cells.This information is useful in helping to diagnose hereditary persistence of fetal hemoglobin and in determining the presence of fetal red cells in the maternal circulation during pregnancy.

Reagents and Equipment

1. Fetal cell fixing solution isEthyl alcohol, 80% (v/v). Stable in the refrigerator for 1 month (or 100 slides), unless the solution becomes cloudy .
2. Fetal cell buffer solutionis Citric acid-phosphate buffer, pH 3.2 to 3.3 .
    a. Dibasic sodium phosphate, 0.2 M
        i. Dibasic sodium phosphate 14.2g (Na_2HPO_4)
        ii. Dilute to 500 mL with distilled water .

© Stable for 6 months when stored in the refrigerator .

    b. Citric acid, 0.1 M
        i. Citric acid ($C_6H_8O_7.H_2O$) 10.5g
        ii. Dilute to 500 mL with distilled water .

© Stable for 6 months when stored in the refrigerator .

Prior to use, prepare the citric acid-phosphate buffer :

    a. Dibasic sodium 13.3mL
        i. phosphate, 0.2 M
    b. Citric acid, 0.1 M 36.7 mL

Check the pH of this mixture on a pH meter. The pH must be within 3.2 and 3.3.

Fetal cell stain:

3. Erythrosin B (eosin B) stain, 0.1% (w/v), aqueous solution. Eosin yellowish, 1.25% (w/v), may be used as an alternative (0.5 g of eosin yellowish in 120 mL of absolute alcohol and 280 mL of distilled water), Add two to three drops of glacialacid .
4. Ehrlich's acid hematoxylin.

Hematoxylin, crystalline	4.0 g
Ethyl alcohol, 80% (v/v)	200 mL
10% aqueous solutionof sodium iodate	8 mL
Distilled water	200 ml

Heat the above solution until it boils, until lukewarm, and add the following :

Glycerine	200
Aluminum sulfate	6.0
Glacial acetic acid	200

Mix, and store at room temper (Mayer's hematoxylin may also be used.)

5. Coplin jars .
6. Waterbath, 37°C.

Specimen

Obtain four blood smears from thefingertip (toe or heel), or make blood smears from venous blood collected in EDTA anticoagulant. Obtain a similar blood specimen for a normal and abnormal control at the same time the patient's blood is collected. For best result blood should be less than 6 hours old, although successful staining has been achieve on specimens refrigerated for up to 2 weeks The smears should be fixed within 2 hours of preparation.

Principle

Blood smears are fixed with ethyl alcohol and then incubated in a citric acid-buffer solution In an acid medium (pH 3.2 to 3.3), hemoglobin F is resistant to elution from the red blood cell, while other types are removed from the red cells. The slides are stained with hematoxylin (stains the white cell nuclei) and erythrosin B (stains the red cells). The smears are then reviewed microscopically to mine the presence of hemoglobin F, and percentage of red blood cells containing fetal hemoglobin may be assessed.

Procedure

1. Prewarm citric acid-phosphate buffer .Place 50 mL of the buffer solution into a coplin jar and cover. Incubate at 37°C for 30 minutes. (Make certain the level of water in the incubator is level with, or above, the level of buffer in the coplin jar.)
2. Preparation of blood smears .
    a. Patient-Make several thin (a monolayer of cells) blood smears .
    b. Normal control-Make two thin blood smears from a normal adult .
    c. Positive control-Mix two drops of cord blood with two drops of normal, ABO compatible, whole blood. Make two thin blood smears. (As an alternative, use cord blood, alone, as the positive control.
3. Allow the blood smears to air-dry for at least 10 minutes .
4. Fix blood smears (patient and controls) in 80% ethyl alcohol for 5 minutes
5. Rinse the smears carefully in distilled water and allow to air-dry .
6. Place the dry smears in the prewarmed citric acid-phosphate buffer solution for 5 minutes. At 1 and 3 minutes (of incubation), carefully lift each slide out of the buffer solution and immediately replace. This action will provide a gentle stirring of the solution .
7. After 5 minutes, remove the slides from the citric acid-phosphate buffer solution and carefully rinse with distilled water. Air-dry .
8. Stain the dry smears in acid hematoxylin for 3 minutes. Rinse with distilled water and remove as much of the water as possible from the smears by gently tapping one end of the slide on an absorbent material .
9. Counterstain the smears with erythrosin B for 4 minutes. Rinse with distilled water, allow to air-dry, and coverslip (if desired).
10. Examine the slides microscopically, (oil immersion objective [1000]), for the presence of hemoglobin F. Red cells containing large amounts of hemoglobin F will stain a deep pink. The intensity of the pink staining is directly proportional to

the concentration of hemoglobin F.Cells containing normal amounts (less than 2%) of hemoglobin F will stain as very pale ghost cells .

11. To determine the percentage of red blood cells containing fetal hemoglobin :
    a. Count the number of red blood cells in three to five microscopic fields and determine the average # of red cells/ field .
    b. Examine 20 to 25 microscopic fields, counting the number of red cells containing hemoglobin F .
    c. Calculate the percentage of RBC containing hemoglobin F as shown below:

$$\text{Number of hgb F RBC/field} = \frac{\text{Number of hgb F RBC counted}}{\text{Number of fields counted}}$$

$$\text{\% RBC with hgb F} = \frac{\text{Number of hgb F RBC/ field}}{\text{Average number of RBC/field}} \times 100$$

Normal value

full-term newborns: Hb F cells are > 90%; normal adults Hb F cells are < 0.01%.

Discussion

1. Reticulocytes may resist elution and would, therefore, give the appearance of cells containing hemoglobin F .
2. The degree of elution of adult hemoglobin may very from patient to patient.
3. In hereditary persistence of fetal hemoglobin, the amount of hemoglobin F in each cell is constant and, therefore, all of the red blood cells are consistently stained. Conversely, in diseases such as sickle cell anemia, thalassemia, acquired aplastic anemia, and several other hemoglobinopathies, the amount of hemoglobin F present in the red blood cells varies. This shows up as an inconsistent staining of the red cells .
4. The pH of the citric acid-phosphate buffer is critical. A pH below 3.1 may cause elution of hemoglobin F from the red cells, while a pH above 3.3 may retard the elution of non-F hemoglobin from the cells .
5. A temperature above 25°C during fixation in the ethyl alcohol will inhibit elution of normal hemoglobin .
6. Ethyl alcohol concentrations above 80% may cause the elution of hemoglobin F, while concentrations below 80% may cause morphologic alterations .

Gluose-6-phosphate dehydrogenase (G-6-PD)

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is the most common human enzyme deficiency in the world; it affects an estimated 400 million people. G6PD deficiency is also known as "favism," since G6PD deficient individuals are also sometimes allergic to fava beans. G6PD deficiency is an allelic abnormality which is inherited in an X-linked recessive fashion.

When someone has G6PD deficiency, complications can arise; hemolytic anemia and prolonged neonatal jaundice are the two major pathologies associated with G6PD deficiency. Both of these conditions are directly related to the inability of specific cell types to regenerate reduced nicotinamide adenine dinucleotide phosphate (NADPH); this reaction is normally catalyzed by the G6PD enzyme.

In G6PD deficient individuals, anemia is usually caused by certain oxidative drugs, infections, or fava beans. When any one of these agents, or their metabolites, enters a G6PD deficient red blood cell, hemoglobin becomes denatured, thus destroying its function as the principal oxygen carrying molecule. In addition to being susceptible to hemolytic anemia, G6PD deficient individuals are also predisposed to prolonged neonatal jaundice. This can be a potentially serious problem as it can cause severe neurological complications and even death.

Principle:

Glucose-6-phosphate dehydrogenase (G6PDH, D-glucose-6-phosphate) catalyzas the first step in the pentose phosphate shunt ,oxidising glucose-6-phosphate (G-6-P)to 6-phosphogluconate(6-PG) and reducing NADP to NADPH, which illustrate by the following equation:

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G-6PDH}} \text{6-PG} + \text{NADPH} + \text{H+}$$

NADP is reduced by G-6-PDH in the presence of G-6-P. The rate of formation of NADPH is directly proportional to the G-6-PDH activity and is measured spectrophotometrically as an increased in absorbance at 340nm. Production of a second molar equivalent of NADPH by erythrocyte 6-phosphogluconate dehydrogenase (6-PGDH) according to the reaction :

$$\text{6-PG} + \text{NADP}^{+} \xrightarrow{\text{6-PGDH}} \text{Ribulose-5- phosphate} + \text{NADPH} + \text{H}^{+} + \text{CO2}$$

Specimen collection and storage

Whole blood collected with EDTA, heparine or acid citrate dextrose .Red cell G-6-PDH is stable in whole blood for one week refrigerated (2-8°c),but is unstable in red cell hemolysates. since activity is reported in term of number of red blood cell or gram hemoglobin, the red cell count or hemoglobin concentration should be determined prior to performing the G6PDH assay.

Procedure

The temperature of the reaction mixture should be maintained at 30°c or some other constant temperature.

1. prepare reaction mixture:
    - Add 0.01ml blood directly to vial containing G-6-PDH assay solution and mix thoroughly to completely suspend erythrocytes, let stand at room temperature (18-25°c) for 5-10min.
    - Add 2.0ml G-6-PDH substrate solution directly to vial and mix gently by inverting several times.
    - Transfer contents of vial to cuvet.
2. Place cuvet in constant temperature cuvet compartment or water bath and incubate for approximately 5min. to attain thermo; equilibrium.
3. Read and record absorbance (A1) of test at 340 nm vs water or potassium dichromate solution. This is initial A .(if using a water bath or incubator ,return cuvet to it)
4. Exactly 5min later, again read and record (A2), this is final A.
5. To determine G-6-PDH activity do the following calculation.

Calculation:

- ΔA per minutes = A2-A1/5
- G-6-PDH activity is expressed as U/1012 erythrocyte (RBC)or as U/g hemoglobin (Hb).
- G-6-PDH (U/1012 RBC) = ΔA per min X 3.01 X 1012 X TCF / 0.01 X 6.22 X (N X 10*6) X 1000

Where:

3.01	= total reaction volume(ml)
1012	= factor for expressing activity in 1012 cells
0.01	= sample volume (ml)
6.22	= millimolar absorptive of NADPH at 340 nm
N X 106	= red cell count (red cells/mm³) determined for each specimen
1000	= conversion of red cell count from mm³ to ml
TCF	=temperature correction factor (1at 30°c)

This equation reduced to: G-6-PDH (U/1012 RBC)= ΔA/min X (48,390/N) X TCF

Where:

N	= red cell count divided by 106
TCF	= temperature correction factor (1at 30°c)

- G-6-PDH(U/gHb) = ΔA per min X 100 X 3.01 / ((0.01 X6.22 X Hb(g/dl)) X TCF
  = ΔA per min X 4839 / Hb (g/dl) X TCF

Where:

100	= factor to convert activity to 100ml
3.01	= total reaction volume (ml)
0.01	= sample volume (ml)
6.22	= mill molar absorptive of NADPH at 340 nm
Hb (g/dl)	= hemoglobin concentration determined for each specimen
TCF	= temperature correction factor (1 at 30°c)

Note: *If anemia and/or leukocytosis is present:* Use buffy coat free blood sample for assay (platelets and WBCs marked activity in this enzyme)

Normal range:

G-6-PDH (U/1012 RBC): (146-376)
G-6-PDH (U/gHb): (4.6-13.5)

Qualitative method in G-6-DP determination:

Principle

Glucose -6-phosphate dehydrogenase,present in the red blood cell hemoysate, act on glucose -6-phosphate and reduces NADP to NADPH which, with the help of PMS, reduces blue colored 2,6Dichlorophenol Indophenol into acolorless form.the rate of decolorization is proportional to the enzynme activity. The reaction can be represented as:

G-6-phosphate +NADP ⟶ 6-phosphogluconic acid +NADPH

NADPH+2,6Dichlorophenol indophenol (DCPIP) ⟶ NADP+ Reduced DCPIP
(Blue color) (colorless)

Rate of declorization is directly proportional to the activity of G-6-PD.

Note:

- Fresh blood sample should be use since refrigeration reduces the enzyme activity.
- Heparine sample should not be use as interfer with enzyme reaction.
- Avoid exposure of substrate vial to the light (it is photosensitive).

Procedure:

Step1: Preparation of red cell hemolysate:

Purified water : 2.5ml
Fresh blood : 0.05ml
Mix well and allow standing for 5min at R.T.

Step2: Assay of the enzyme:

Add 1ml of the hemolysate (step 1) to the vial of solution 1 and mix gently.
Add immediately about 1ml of reagent 3.
Seal the vial with aluminum foil and incubate in water bath at 37°c.

Observe: the time taken for the color change from initial deep blue to reddish purple. Follow up to Amax. Of 6 hours with 30 min intervals.

Results:

Normal : 30-60 min.

G-6-PD deficient (heterozygous males, homozygous female): 140min-24hr
G-6-PD carriers (heterozygous females): 90min-several hours.

QUANTITATION OF METHEMOGLOBIN

methemoglobin (Hi) is a form of hemoglobin In which the ferrous ion(Fe^{2+}) has been oxidized to the ferric state(Fe^{3+}) and is, therefore, incapable of reversibly combining with oxygen (and, therefore, cannot transport the oxygen molecule). Normally, a small amount of methemoglobin is continuously being formed in the red cell, but is, in turn, reduced by the red blood cell enzyme systems: NADH methemoglobinreductase (cytochrome-b5 reductase) (major pathway), NADPH methemoglobinreductase (minor pathway) and to a lesser extent the ascorbic acid and glutathione enzyme systems.

Increased amounts may be found in both hereditary and acquired disorders.

a. The hereditary form of methemoglobinemia is found
    1) in disorders in which the red blood cell reducing systems are abnormal and unable to reduce methemoglobin back to oxyhemoglobin.
    2) in the presence of hemoglobin M, where the structure of the polypeptide chains making up the hemoglobin molecule is abnormal (there is a tendency toward oxidation of hemoglobin, with a decreased ability to reduce it back to oxyhemoglobin).
b. The acquired causes of methemoglobinemia are mainly due to certain drugs and chemicals, such as nitrates, nitrites, quinones, chlorates, sulfonamides and aniline dyes.

Methemoglobin is normally present in the blood in a concentration of 0.03 to 0.13 g/ dL, but a normal range should be determined by each laboratory. Slightly higher levels are present in infants and heavy smokers.

Reagents and Equipment

1. Phosphate buffer, 0.067 M, pH 6.6 .
a. Solution 1 (potassium dihydrogen phosphate, 0.067 M)
    I. Monobasic potassium phosphate(KH_2PO_4) 9.1 g
    II. Distilled water 1L
b. Solution 2 (dibasic sodium hydrogen phosphate, 0.067 M)
    I. Dibasic sodium phosphate (Na_2HPO_4) 5.9g
    II. Distilled water 1 L

For phosphate buffer, 0.067 M, pH 6.6, mix:

    a. Solution 1 63.0 mL
    b. Solution 2 37.0 mL

2. Phosphate buffer, 0.017 M (pH 6.6)
    a. Phosphate buffer (0.067M, pH 6.6) 1 volume
    b. Distilled water 3 volumes
    c. Add approximately 0.2 g of saponin .

3. Sodium cyanide, 10% w/v .
    a. Sodium cyanide 10 g
    b. Distilled water 100 mL
4. Potassium ferricyanide, 20% w/v .
    a. Potassium ferricyanide 20 g
    b. Distilled water 100 mL
5. Acetic acid, 12% v/v .
6. Neutralized sodium cyanide. Prepare this reagent under a fume hood, just prior to use. Place four drops of 10% sodium cyanide in a small (12 x 75 mm) test tube. While carefully shaking the tube, add four drops of 12% acetic acid. Stopper tube as soon as possible. This reagent must be used within 1 hour of preparation .
7. Test tubes, 13 x 125 mm .
8. Pipets, 5 mL, 50 μL, and 20 μL .
9. Spectrophotometer.

Specimen

Fresh anticoagulated whole blood, using EDTA or heparin as the anticoagulant. This test should be perform d within 1 hour of blood collection. However, once the blood has been diluted in the buffer reagent, it may be stored at 2 to 6° C for a maximum of 24 hours.

Principle

Whole blood is diluted with a phosphate buffer solution. Methemoglobin has a maximum absorbance at a wavelength of 630 nm. The diluted specimen is read in a spectrophotometer at 630 nm and the absorbance reading noted (D1). Neutralized sodium cyanide is added to the mixture, converting the methemoglobin to cyanmethemoglobin, and read on the spectrophotometer (D2). The change in optical density is directly proportional to the amount of methemoglobin present. The methemoglobin in g/dL is then calculated using a factor, previously determined, for the spectrophotometer used.

Procedure

1. Determination of calculation factor, F.Before the methemoglobin results can be calculated, a calibration factor for the spectrophotometer being used must be determined. This is done one time only, or whenever a different spectrophotometer is used for the procedure.
    a. Obtain a whole blood sample and determine the hemoglobin concentration in g/dL .
    b. Place 5.0 mL of 0.017 M phosphate buffer into each of two test tubes. Add 50 μL of the whole blood specimen to one tube and mix. The second tube is to be used as the blank.
    c. Add 20 μL of freshly prepared 20% potassium ferricyanide to both tubes. Mix and allow to stand for 2 minutes.

d. Read on the spectrophotometer at 630 nm, using the blank to set the optical density at 0, and record the absorbance (D_x).

e. Add 20μLof neutralized sodium cyanide to both tubes. Mix and allow to standfor 2 minutes. Read on the spectrophotometer as above and record the optical density (D_y).

f.
$$F = \frac{\text{Hgb (g/dL)}}{D_x - D_y}$$

2. Procedure for testing patient specimen .
   a. Label and place 5 mL of 0.0 17M phosphate buffer into one tube for each specimen and control to be tested and one additional tube to serve as a blank .
   b. Add 50 μL of whole blood to the appropriately labeled tube and mix well. Allow to sit at room temperature for 5 minutes.
   c. Read on the spectrophotometer at a wavelength of 630 nm, using the blank to set the optical density at 0. Record the absorbance of the solution (D_1)
   d. Add 20 μL of neutralized sodium cyanide reagent to each tube including the blank, and mix. Allow to sit for 2 minutes. Read as in step c above record the optical density (D_2).
   e. Calculation of results :

Methemoglobin (g/dL) = ($D_1 - D_2$) x F

Normal value:

Methaemoglobin<2% of total haemoglobin in normal blood, slightly higher in infants, particularly if premature.

Discussion

1. Sulfhemoglobin (SHb) is not measured at any time during the above procedures.
2. The presence of a large amount of SHb will result in an erroneously low measurement of total Hb.

SUCROSE HEMOLYSIS TEST

The sucrose hemolysis test is used as a confirmatory test for paroxysmal nocturnal hemoglobinuria (PNH) when the sugar water test is positive.

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired clonal disorder of haemopoiesis in which the patient's red cells are abnormally sensitive to lysis by normal constituents of plasma.

It is characterized by haemoglobinuria during sleep (nocturnal haemoglobinuria), jaundice, and haemosiderinuria.

PNH is an acquired clonal disorder resulting from a somatic mutation occurring in a haemopoietic stem cell.

The characteristic feature of cells belonging to the PNH clone is that they are deficient in several cell-membrane–bound proteins including red cell:

1. Acetylcholine esterase,
2. Neutrophil alkaline phosphatase,
3. CD55 (decay accelerating factor or DAF),
4. Homologous restriction factor (HRF), and
5. CD59 (membrane inhibitor of reactive lysis or MIRL).

CD55, CD59, and HRF all have roles in the protection of the cell against complement-mediated attack.

CD59 inhibits the formation of the terminal complex of complement, and it has been established that the deficiency of CD59 is largely responsible for the complement sensitivity of PNH red cells

PNH type III red cells have a complete deficiency of CD59, whereas PNH type II red cells have only a partial deficiency, and it is this difference that accounts for their variable sensitivities to complement.

PNH red cells are unusually susceptible to lysis by complement. This can be demonstrated in vitro by a variety of tests e.g:

1. Sugar water test.
2. Sucrose lysis test.
3. The acidified-serum [Ham test].

A characteristic feature of a positive test for PNH is that not all the patient's cells undergo lysis, even if the conditions of the test are made optimal for lysis.

This is because only a proportion of any patient's PNH red cell population is hypersensitive to lysis by complement. This population varies from patient to patient.

There is a direct relationship between the proportion of red cells that can be lysed (in any of the diagnostic tests) and the severity of *in vivo* haemolysis.

Reagents and Equipment

1. Sodium phosphate, 50mmol/L ($NaH_2PO_4 \cdot 2H_2O$) 7.8g
   Dissolve and dilute to 1 liter with distilled water.
2. Sodium phosphate‹50 mmol/L (Na_2HPO_4) 7.1g
   Dissolve and dilute to 1 liter with distilledwater.
3. Sucrose solution (isotonic).
   Sucrose (reagent grade) 92.4 g
   NaH_2PO_4 (50 mmol/L) 91mL
   Na_2HPO_4 (50 mmol/L) 9 mL
   Mix and adjust pH to 6.1, if necessary, using dilute NaOH or HCl. Dilute to 1 liter with distilled water. Reagent is stable at refrigerator temperature for 2 weeks .
4. cyanmethemoglobin reagent.
5. Test tubes.
6. ABO compatible serum (or serum fromtype AB blood) from a normal donor.
7. Specimen must be fresh.
8. Sodium chloride, 0.85% w/v .
9. Pipets,Spectrophotometer. 540 nm.

Specimen

Citrated whole blood: 1 part 0.109 M sodium citrate to 9 parts whole blood. Obtain a blood specimen (preferably the same blood type) for the normal control at the same time the patient's blood is collected.

Principle

Washed red blood cells are incubated in an isotonic sucrose solution containing normal ABO compatible serum. At low ionic concentrations, red blood cells absorb complement components from serum. Because PNH red blood cells are much more sensitive than normal red cells they will hemolyzed under these conditions. The normal red blood cells will not. At the end of the incubation period the mixture is examined for hemolysis.

Procedure

- Prepare washed cell
    1. 1 mL patient blood .
    2. Add normal saline (sodium chloride 0.85%)
    3. Mix , centrifuge at high speed for 5 min. carefully remove supernatant.
    4. Repeat step 2,3.
- Prepare washed cell 50% solution
    1. Add from W.C tube 3 drop of cells
    2. 3 drop of N.S, mix.
- Prepare blood-sucrose tube, Blank1 tube

Reagent	blood-sucrose	Blank1
Sucrose solution.	1.7 ml	1.7 ml
ABO Compatible serum.	0.1ml	0.1ml
The 50% W.C, Mix by inversion	0.2ml	
Incubate at R.T 30 min.		

- Prepare of Total ,Test, Blank tube

Reagent	Total	Blank	Test
Drabkin's	4750 µl	4750 µl	4750 µl
After complete 30 min. incubation, & remix blood-serum tube.	250 µl, mix incubation 10 min.	-----	-----
After complete 30 min. incubation, & remix Blank1 tube.	-----	250 µl, mix incubation 10 min.	-----
After complete 5 min. centrifuge of remaining blood-sucrose tube, from the supernatant.	-----	-----	250 µl, mix incubation 10 min.

10. Transfer above mixtures to a cuvet and read in a spectrophotometer at a wavelength of 540 nm. Setting the blank al 0.0 optical density. Record the O.D. readings for each sample.

11. Calculate the percent hemolysis for each specimen as shown below.

Percent Hemolysis = (O.D. test / O.D. total)* 100

Interpretation of results

1. Hemolysis 5%, or less is considered negative within normal limits.
2. Hemolysis of 6 to 10% is thought to beborderline.
3. Positiveresults will show greater than 10%molysis.

Discussion

1. Increased hemolysis (generally less than 10%) may be found in some patients with leukemia or myelosclerosis, whereas patients with PNH show 10% to 80% hemolysis (will only rarely be as Iowa 5%).
2. Results of the sucrose hemolysis test should correlate with the acid serum test.

OSMOTIC FRAGILITY TEST

Definition

The osmotic fragility test is a measure of the ability of the red cells to take up fluid without lysing. Or it is a test to measures red blood cell (RBC) resistance to hemolysis when exposed to a series of increasingly dilute saline solutions.

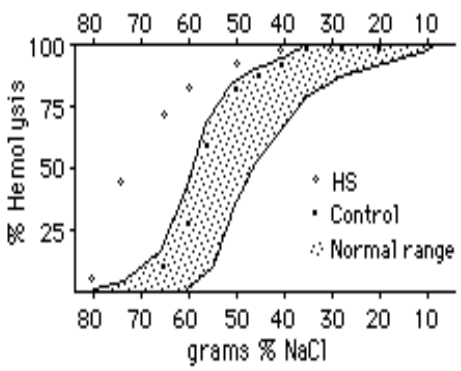
This procedure is employed to help diagnose different types of anemias, in which the physical properties of the red blood cell are altered.

The primary factor affecting the osmotic fragility test is the shape of the red cell, which, in turn, depends on the volume, surface area, and functional state of the red blood cell membrane.

1. Increased surface- to – volume ratios, (it is more resistant to hemolysis and has decreased fragility)
   The larger the amount of red cell membrane (surface area) in relation to the size of the cell, the more fluid the cell is capable of absorbing before rupturing .as
   1. Reticulocytes
   2. iron-deficiency anemia
   3. thalassemia
   4. sickle cell anemia
   5. andoccurs following splenectomy, in liver disease, polycythemia vera, and conditions in which target cells are present.

   The target cell has the largest surface area (amount of membrane) for its size and therefore shows decreased fragility. As the red cell takes in fluid it becomes more round (spherocytic). It therefore follows that the spherocyte has the smallest surface area for its volume, ruptures the most quickly, and has increased fragility.

2. Decreased surface-to – Volume ratios, Increased osmotic fragility (decreased resistance) is found in
   1. hemolytic anemias
   2. hereditary spherocytosis
   3. And wheneverspherocytes are found.
   4. the older red cells are also more fragile.



Reagents and Equipment

1. Buffered sodium chloride stock solution (osmotically equivalent to 10% sodium chloride).
    i. sodium chloride (Dry for 24 hours in a desiccator with calcium chloride prior to weighing out.) 90 g
    ii. Dibasic sodium phosphate (Na_2HPO_4) 13.65 g
    iii. Monobasic sodium phosphate ($NaH_2PO_4 \cdot 2\ H_2O$) 2.43 g
    iv. Dilute to 1 liter with distilled water.

This solution is stable for several months at room temperature if kept well Stoppard.

2. Buffered sodium chloride working solution.
    i. Buffered sodium chloridestock solution 20 mL
    ii. Distilled water 180 mL
3. Distilled water.
4. Erlenmeyer flask (250 mL) and glass beads (3 to 4 mm in diameter), if defibrinatedwhole blood is used .
5. Pipets, 10, 5, and 0.05 mL.
6. Test tubes 13 X 100 mm.
7. Parafilm.
8. Centrifuge.
9. Spectrophotometer.

Specimen

Heparinized venous blood, or, 15 to 20 mL of defibrinated whole blood.The test should be set upwithin 2 hours of collection, or within 6 hours if the blood is refrigerated.

Principle

If red blood cells are placed in an isotonic solution (0.85% sodium chloride), fluid will neither enter nor leave the red blood cell.If red cells are placed in a hypotonic solution (e.g., 0.25% sodium chloride), however.fluid enters the red blood cell until the cell either ruptures or an equilibrium is reached. Aspherocyte, which is almost round, swells up in a hypotonic solution and ruptures much more quickly than a normal red blood cell or more quickly than cells having a large surface area per volume, such as target cells or sickle cells. The fragility of the red blood cell is said to be increased when the rate of hemolysis is increased. When the rate of hemolysis is decreased, the fragility of the red blood cells is considered decreased. In the osmotic fragility test, whole blood is added to varying concentrations of buffered sodium chloride solution and allowed to incubate at room temperature. The amount of hemolysis in each saline concentration is then determined by reading the supernatants on a spectrophotometer.

Procedure

1. Prepare dilutions of buffered sodium chloride and place in the appropriately labeled test tube (Table.).
2. Mix the preceding dilutions well, using Parafilm to cover each test tube whilemixing.
3. Transfer 5 mL of each dilution to a second set of test tubes labeled # 1 through #14. If defibrinated blood is to be used, procedas follows :
    a. place 15 to 20 mL of whole blood into an Erlenmeyer flask containing 15glass beads.
    b. Gently rotate the flask until the hum or noise of the beads on the glass can no longer be heard (about 10 minutes).
4. Add 0.05 mL of the patient's heparinized or defibrinated blood to each of the 14 test tubes. Repeat, adding the normal control blood to the setof 14 control testtubes .
5. Mix each test tube immediately by gentleinversion.
6. Allow the test tubes to stand at roomtemperature for 30 minutes.
7. Remix the test tubes gently and centrifuge at 1200 to 1500 g for 5 minutes.
8. Carefully transfer the supernatants to cuvettes and read on a spectrophotometer at a wavelength of 540 nm. Set the optical density at 0, using the supernatant in test tube #1, which represents the blank, or 0% hemolysis. Test tube #14 represents 100% hemolysis.
9. Calculate the percent hemolysis for each supernatant as follows:

$$\text{Percent of hemolysis} = \frac{\text{O.D. of supernatant}}{\text{O.D. supernatant tube \#14}} \times 100$$

10. The results of the test may then begraphed, with the percent hemolysis plotted on the ordinate (vertical axis) and the sodium chloride concentration on the abscissa (horizontal axis) as shown in Figure (shows normal range).

Test tube	1% buffered NaCl (mL)	D.W. (ml)	Final conc. (%)	% Hemolysis
1	10.0	0.0	1.00	
2	8.5	1.5	0.85	
3	7.5	2.5	0.75	
4	6.5	3.5	0.65	
5	6.0	5.0	0.60	
6	5.5	4.5	0.55	
7	5.0	5.0	0.50	
8	4.5	5.5	0.45	
9	4.0	6.0	0.40	
10	3.5	6.5	0.35	
11	3.0	7.0	0.30	
12	2.0	8.0	0.20	
13	1.0	9.0	0.10	
14	0.0	10.0	0.00	

Normal values

Initial Hemolysis begins 0.45%, complete Hemolysis 0.30 - 0.35%

Discussion

1. This test should be performed immediately because shape change and osmotic conditions change with time.
2. If the patient has a low hemoglobin level, wash the patent with isotonic saline and resuspend with equal volume of RBCs and saline. This will correct for anemia.
3. Completely fill the collection tube and invert it gently several times to mix the sample and anticoagulant thoroughly.
4. Handle the sample gently to prevent accidental hemolysis.
5. Severe anemia or other conditions with fewer RBCs available for testing .
6. The pH of the blood-saline mixture isimportant and should be 7.4.
7. If anticoagulated blood is used for test, use only heparin as the anticoagulant, in order to avoid adding more saltsto the blood such as Oxalate, EDTA, or citrate.
8. Old sample
9. Recent blood transfusion.

Automated Hematology Cell Counters

Current hematology analyzers use a combination of light scatter, electrical impedance, fluorescence, light absorption, and electrical conductivity methods to produce complete red blood cell, platelet, and leukocyte analyses. All the widely used automated instruments analyze cells in flow and are essentially highly specialized flow cytometers.

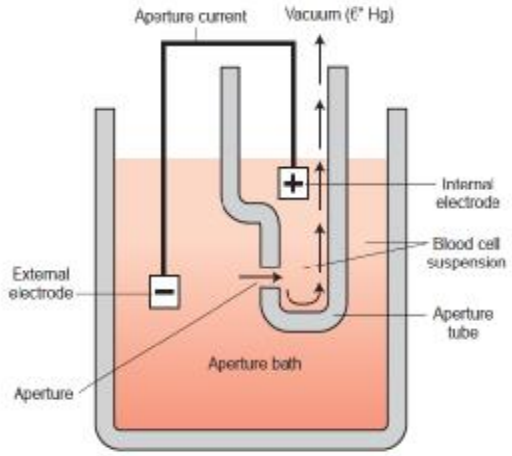
Principles

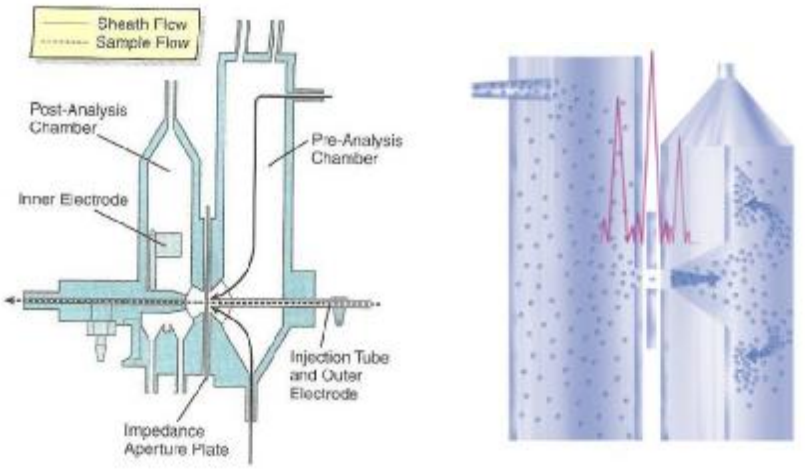
1. The Coulter Principle

Using this technology, cells are sized and counted by detecting and measuring changes in electrical resistance when a particle passes through a small aperture. This is called the electrical impedance principle of counting cells.

A blood sample is diluted in saline, a good conductor of electrical current, and the cells are pulled through an aperture by creating a vacuum. Two electrodes establish an electrical current. The external electrode is located in the blood cell suspension. The second electrode is the internal electrode and is located in the glass hollow tube, which contains the aperture. Low-frequency electrical current is applied to the external electrode and the internal electrode. DC current is applied between the two electrodes. Electrical resistance or impedance occurs as the cells pass through the aperture causing a change in voltage. This change in voltage generates a pulse (Fig.). The number of pulses is proportional to the number of cells counted .The size of the voltage pulse is also directly proportional to the volume or size of the cell.

This was the principal parameter used in earlier analyzers for characterizing all cell types, but it is now used primarily for counting and sizing red blood cells and platelets.





Coulter principle of electric impedance.

Instruments

The newer analyzers include white cell differential counts, relative or percent and absolute number, and reticulocyte analysis. The differential may be a three-part differential that includes granulocytes, lymphocytes, and monocytes or a five-part differential that includes neutrophils, lymphocytes, monocytes, eosinophil's, and basophils. The new generation of analyzers now offers a sixth parameter, which is the enumeration of nucleated RBCs (nRBCs).

Automated full blood counters with a five-part or more differential counting capacity[*]

Instrument and manufacturer	Technology used for differential count
Beckman-Coulter Instrumentation (Coulter STKS, GEN-S, LH 700 series)	*VCS Technology (Volume, Conductivity, and Scatter)* 1. Impedance with low-frequency electromagnetic current 2. Impedance with high-frequency electromagnetic current 3. Laser light scattering
Sysmex Instrumentation (Roche Diagnostics Corporation) (SE series, XE2100)	1. Impedance with low-frequency direct current 2. Impedance with radiofrequency current 3. Hydrodynamic Focusing
Cell Dyne Technology (Abbott Diagnostics Instrumentation) Cell-Dyn 1800	*The Coulter Principle*
Cell-Dyn 3500, 3700	*Multiple-Angle Polarized Scatter Separation (MAPSS)* 1. Four light-scattering parameters: forward light scatter, orthogonal light scatter, narrow-angle light scatter, and

	depolarized orthogonal light scatter 2. Hydrodynamic Focusing 3. Use of Flow Cells
Cell-DynRuby, Sapphire.	1. Multiple-Angle Polarized Scatter Separation (MAPSS) 2. Hydrodynamic Focusing 3. Use of Flow Cells 4. Fluorescence

* In addition to the blood counters listed here, there are an increasing number of instruments on the market that are capable of providing full differential counts using various technologies.

Cell-Dyn 1800 Hematology Analyzer

Cell-Dyn 1800 Hematology Analyzer

The Cell-Dyn 1800 Hematology Analyzer performs a Complete Blood Count (CBC)،Platelet Count, and a Three-Part Differential.

Performance:

Whole blood is aspirated, diluted, and thendivided into two samples. One sample is used to analyze the red blood cells and plateletswhile the second sample is used to analyze the white blood cells and hemoglobin.Electrical impedance is used to count the white blood cells, red blood cells, and plateletsas they pass through an aperture. As each cell is drawn through the aperture, a change inelectrical resistance occurs generating a voltage pulse. The number of pulses during acycle corresponds to the number of cells counted. The amplitude of each pulse is directlyproportional to the cell volume.

- In the RBC chamber, both the RBCs and the platelets are counted and discriminated by electrical impedance Particles between 2 and 20 fL are counted as platelets, and those greater than 36 fL are counted as RBCs.

Lyse reagent is added to the diluted sample and used to count the white blood cells. The lysing reagent also cause WBC's membrane collapse around the nucleus, so the counter actually measuring the nuclear size.Afterthe white blood cells have been counted and sized, the remainder of the lysed dilution istransferred to the Hgb Flow Cell to measure Hemoglobin concentration.

Hemoglobin Measurement

Using cyanide free Hb chemistry methods, rapid RBCs lysis followed by the formation of an imidazole-hemoglobin complex with an absorption peak at 540 nm.

The Cell-Dyn uses electronic sizing to determine a three part automated differential. Thepercentage and absolute counts are determined for lymphocytes, neutrophil, and mid-sizepopulation of monocytes, basophils, eosinophils, blasts, and other immature cells.

Results will be used to monitor patient's cell counts and absolute neutrophil count and todetermine if further chemotherapy should be administered.

Specimen Requirements

1. Whole blood collected in an EDTA tube.
2. Minimum sample volume is 0.5 mL using the Open Sample Mode. The instrument aspirates 30 µL of patient sample.
3. Samples are stable at room temperature for eight hours.

Overview of Analysis Modes

●Whole blood mode

This is the mode of analyzing collected blood sample in the whole blood status. The tube cap is opened and the sample is aspirated through the sample probe one after another.

●Pre-diluted mode

This mode is used in analyzing a minute amount of child's blood, for instance, collected from the earlobe or fingertip. In this mode, blood sample diluted into 1:26 before analysis is used. The sample aspiration procedure is the same as in the whole blood mode.

Note:

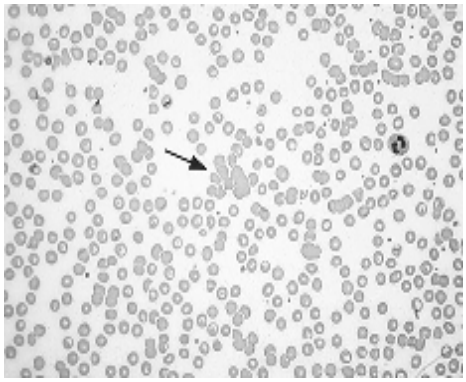
In the pre-diluted mode, particle distribution curve and particle distribution analysis data are not output, and the output is confined to only the CBC 4 parameter (dependent parameter on MCV) but the remainder parameter multiply by dilution factor.

Sources of error

A. In cell count include:

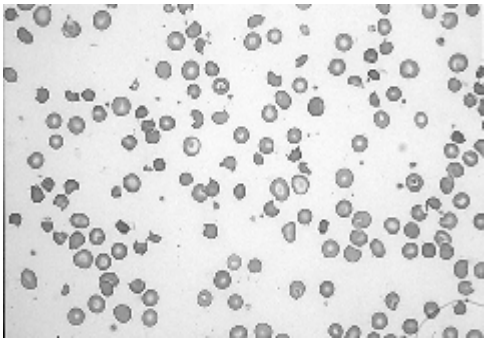
1. Cold agglutinins - low red cell counts and high MCVs can be caused by a increased number of large red cells or red cell agglutinates. If agglutinated red cells are present, the automated hematocrits and MCHCs are also incorrect. Cold agglutinins cause agglutination of the red cells as the blood cools.

   Cold agglutinins can be present in a number of disease states, including infectious mononucleosis and mycoplasma pneumonia infections.

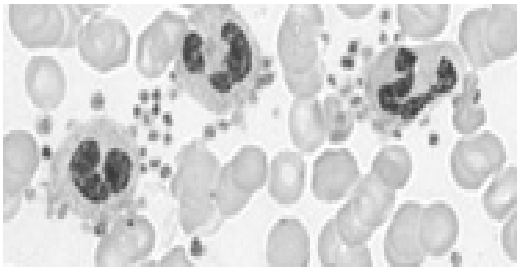


If red cell agglutinates are seen on the peripheral smear, warm the sample in a 37°C heating block and mix and test the sample while it is warm. Strong cold agglutinins may not disperse and need to be redrawn in a pre-warmed tube and kept at body temperature .

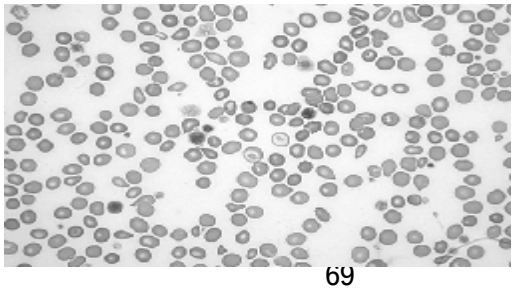
2. Fragmented or very microcytic red cells
   These may cause red cell counts to be decreased and may flag the platelet count as the red cells become closer in size to the platelets and cause an abnormal platelet histogram. The population is visible at the left side of the red cell histogram and the right end of the platelet histogram .



3. Platelet clumps and platelet satellitosis: these cause falsely decreased platelet counts. Platelet clumps can be seen on the right side of the platelet histogram. Decreased platelet counts are confirmed by reviewing the peripheral smear. Always scan the edge of the smear when checking low platelet counts.

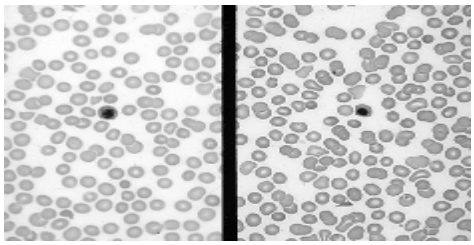


4. Giant platelets: these are platelets that approach or exceed the size of the red cells. They cause the right hand tail of the histogram to remain elevated



and may be seen at the left of the red cell histogram .

5. Nucleated red blood cells: these interfere with the WBC on some instruments by being counted as white cells/lymphocytes.



B. In measuring hemoglobin include

- Anything that will cause turbidity and interfere with a Spectrophotometry method.

Examples are a very high WBC or platelet count, lipemia and hemoglobin's that are resistant to lysis, such as hemoglobin's S and C .

Basic automated hematology analyzers provide an electronic measured

1. red cell count (RBC),
2. white cell count (WBC),
3. platelet count (Plt),
4. mean platelet volume (MPV),
5. hemoglobin concentration (Hb),
6. and the mean red cell volume (MCV).

From these measured quantities, the hematocrit (Hct), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and the red cell distribution width (RDW) are calculated.

RED CELL INDICES

1. Hematocrit calculation

- Hematocrit (Hct) or (PCV) is the volume of the red cells as compared to the volume of the whole blood sample. Hematocrits on the automated systems are calculated .

The volume of each red cell is measured as it is counted and a mean cell volume is derived. The calculations are not precisely the same. But, they can be summarized as mean corpuscular red cell volume (MCV) multiplied by the red cell count (RBC).
Hematocrits are reported in L/L or the traditional .%

Sources of errors in Hct

- Hematocrits calculated by automated instruments depend on correct red cell counts and red cell volumes to arrive at an accurate hematocrit .
- Hence, anything affecting the red cell count or volume measurement will affect the hematocrit .
- This method is not as sensitive to the ratio of blood to EDTA as the centrifuged hematocrit

Correlating Hemoglobin and Hematocrit Values

- The hemoglobin times three roughly equals the hematocrit in most patients.

Example: 14.8 x 3 = 44 (patient's hematocrit result is 45 L/L)

11.0x 3 = 33 (patient's hematocrit result is 32 L/L)

- The exception to this rule is in patients with hypochromic red cells. These patients will have hematocrits that are more than three times the hemoglobin

2. MCV The counter provides us with MCV which is derived from the histogram (sum of pulse height / sum of pulse). Not: 1μL= 10^9fL
3. MCH is Mean Corpuscular Hemoglobin weight in picograms. This is the average weight of the hemoglobin in picograms in a red cell. It is a calculated value.
   Not: 1g = 10^{12}pg, 1L = 10 dL
   MCH =hemoglobin in pg/L / red cell count in pilions/L
4. MCHC is Mean Corpuscular Hemoglobin Content. This indicates the average weight of hemoglobin as compared to the cell size. It is traditionally a calculated value .

MCHC = (Hemoglobin in g/dL / HCT) x 100

5. RDW:The RDW (red cell distribution width) is a measurement of the width of the bases of the RBC histogram the red cell size distribution and is expressed as the coefficient of variation percentage. The RDW is increased in treated iron deficiency, vitamin B12 deficiency, folic acid deficiency, post-transfusion.
6. MPV: The MPV is a measure of the average volume of platelets in a sample and is analogous to the erythrocytic MCV.
7. Pct: (plateletcrit) analogues to HCT for RBCs

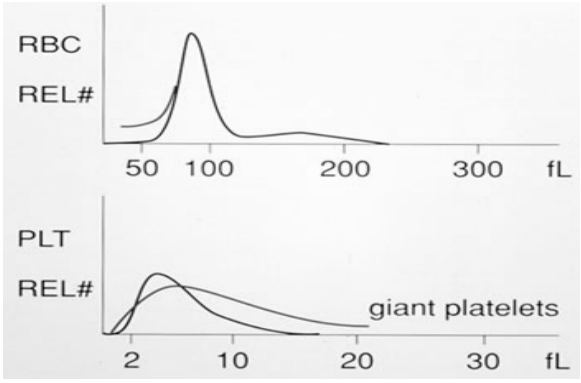
How Data Are Reported

In most automated systems, the complete blood count is numerically reported. The differential is numerically recorded and then graphically displayed.

Red Cells Histogram

- normal red cell histogram displays cells form (36- 360) **fl**

A. (24- 36 **fl**) **fl**ag may be due
   1- RBCs fragments
   2- WBC's fragments
   3- Giant plts
   4- Microcyte



B. Shift to right :
   - Leukemia
   - Macrocytic anemia
   - Megaloblastic anemia

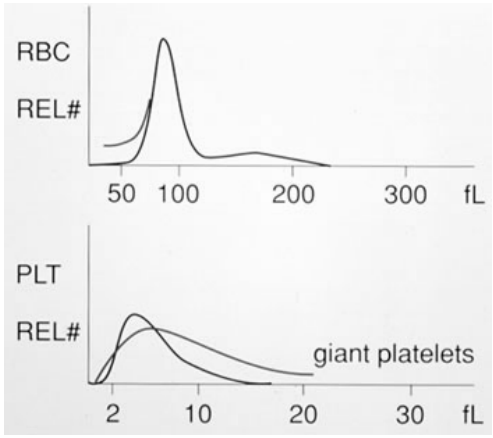
C. Shift to left :
   - Microcytic anemia (IDA)

D. Bimodal
   - Cold agglutinin
   - IDA, Megaloblastic anemia with transfusion.
   -Sideroblastic anemia.

E. Trimodal
   - Anemia with transfusion

Plts histogram

normal platelet histogram displayscells from(2-20 fl).



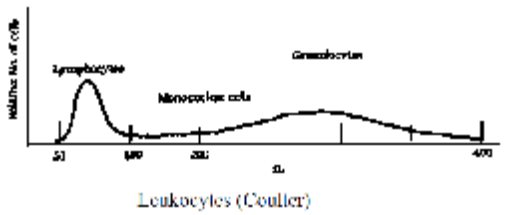
A. (0-2)
   1. Air Babbles
   2. Dust
   3. Electronic and Electricalnoise

A. Over 20 fL
   1. Microcyte
   2. Scishtocyte
   3. WBC's fragments
   4. Giant Plts
   5. Clumped plts

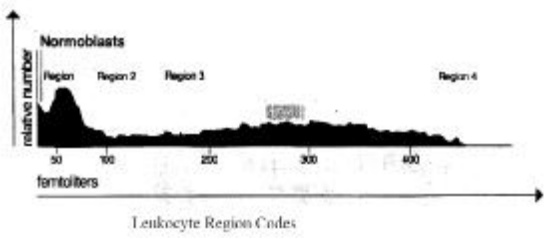
LEUKOCYTE HISTOGRAM ANALYSIS

The histogram is a representation of the sizing of the leukocytes. The differentiation is as follows:

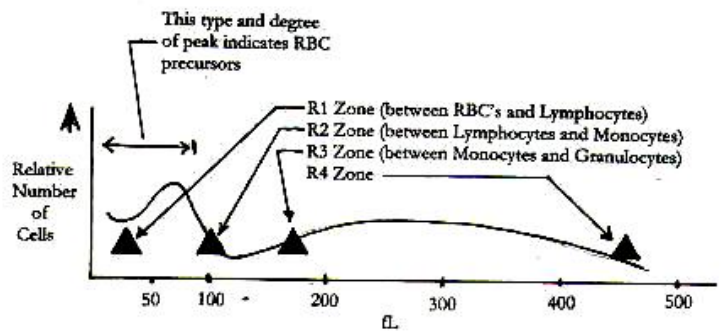
Cell type	Size range	Cells that fall within this size range
Lymphocytes	35-90 fL	lymphs and atypical lymphs
Mononuclear	90-160 fL	Monos, promyelocytes, myelocytes, plasma cells and blasts
Granulocyte	160-450 fL	segs, bands, metas, eos and basos



Leukocytes (Coulter)



Leukocyte Region Codes



The following table lists the region (R) flags andthe abnormalities they may represent:

R Flag	Region	Abnormality
R1	Far left(<35fL)	Erythrocyte precursors (NRBCs) Nonlysed erythrocytes Giant and/or clumped platelets Heinz body Malaria
R2	Between lymphs and monos	Blasts Basophilia Eosinophilia Plasma cells Abnormal/variant lymphs
R3	Between mons and granulocytes	Abnormal cell populations Eosinophilia Immature granulocytes
R4	Far right(>450fL)	Increased absolute granulocytes
RM		Multiple flags

- NORMAL VALUES

REPORTING RESULTS	
Parameter	Normal Range
1. WBC	4.8-10.8 x 10^3/µL
2. RBC	Male 4.7-6.1 x 10^6/µL Female 4.2-5.4 x 10^6/µL
3. Hemoglobin	Male 14-18 g/dl Female 12-16 g/dl
4. Hematocrit	Male 42-52% Female 37-47%
5. MCV	Male 80-94 fl Female 81-99 fl
6. MCH	27-31 pg
7. MCHC	32-36 g/dl or %
8. RDW	11.5-14.5%
9. Platelets	150,000 - 450,000/µL
10. MPV	7.4-10.4 fl

Critical Values

Parameter	Critical Value
WBC (K/mm3)	≤1.0 or ≥30.0
HGB (g/dL)	≤6.5 or ≥19.0
HCT (%)	≤20.0 or ≥60.0
PLT (K/mm3)	≤30.0 or ≥1000

Linearity

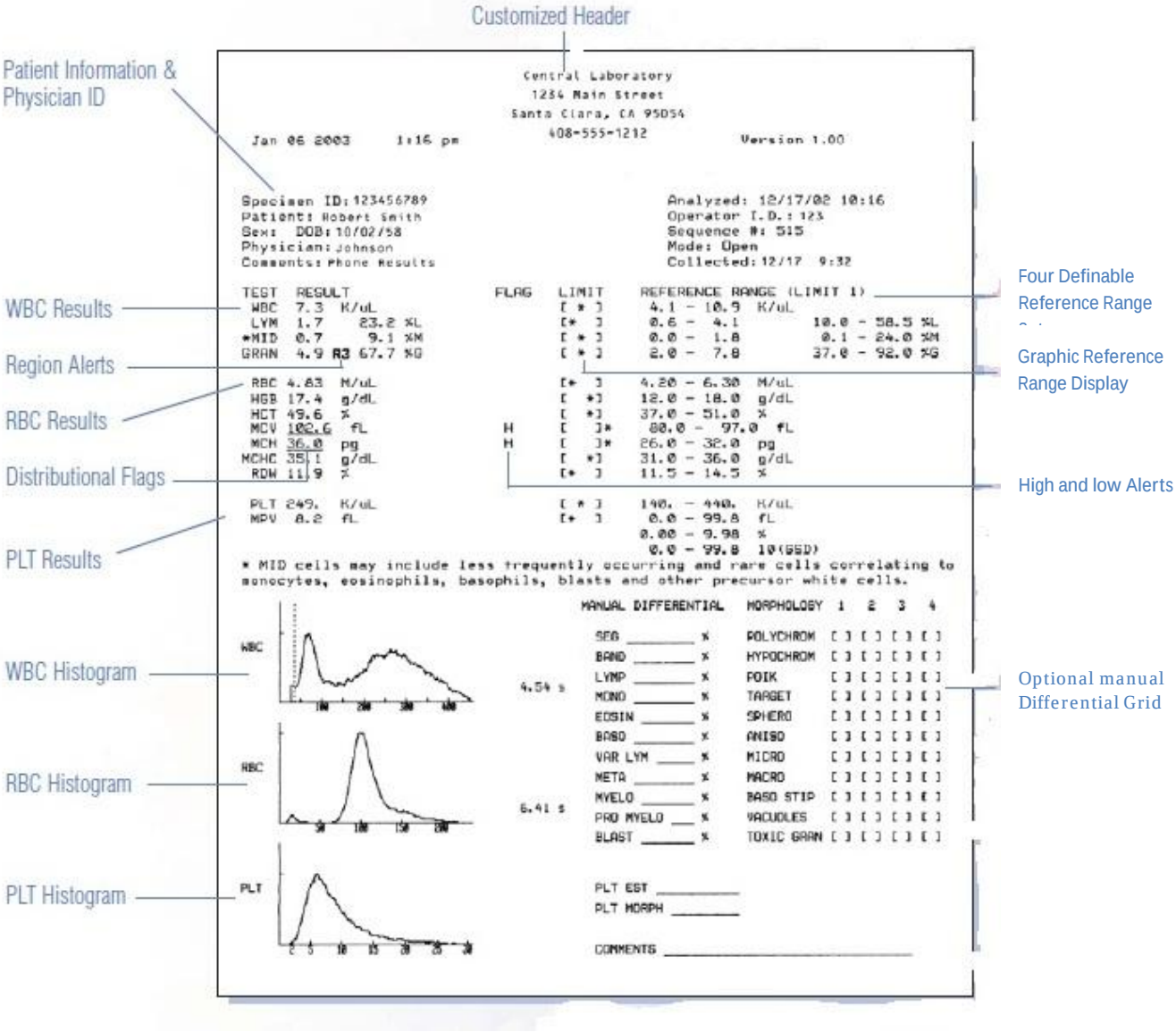
Parameter	Manufacturer's Linear Range
1. WBC (K/µL)	1.0 – 99.9
2. RBC (M/µL)	1.0 – 7.00
3. HGB (g/dL)	2.5 – 24.0
4. MCV (fL)	50 – 200
5. PLT (K/µL)	10 – 999
6. MPV (fL)	5.0 – 20.0

INSTRUMENT CODES

Code	Cause	Action indicated
..... for a single parameter	Incomplete computation	Repeat
..... for all parameters	Partial aspiration	Repeat
____ and no histogram	Total voteout (2 out of 3 measurements do not agree	Repeat
+++++	Result exceeds printable range	Dilute 1:2 and rerun. Continue further dilutions if necessary until the result falls within the linearity range (See "Handling Abnormal Results)
H	Result is higher than the laboratory-set patient high action limit	Review result
L	Result is lower than the laboratory-set patient low action limit	Review result
R next to Plt and MPV result	PDW > 20 or non-positive curve detected, or	Review result
	Plt< 20,000 or	Review result and correlate plt with smear review
	Total voteout of fitted curve, or WBC is overrange.	Review result and repeat
R next to RDW result	Excessive asymmetry in RBC histogram or	Review result
	WBC or MCV overrange	Repeat
R next to MCV; R next to RBC, Hct, MCH, MCHC, RDW, Plt, and MPV	MCV < 50 fL	Repeat
R next to WBC	Check of WBC lower threshold failed	Repeat, review smear

INTERFERENCES THAT MAY CAUSE ERRONEOUS RESULTS	
Parameter	Interfering agent
WBC	1. Unusual RBC abnormalities that resist lysis 2. Nucleated RBCs 3. Fragmented WBCs 4. Unlysed particles greater than 35 fL 5. Very large or aggregated plts 6. Specimens containing fibrin, cell fragments or other debris (esp pediatric/oncology specimens
RBC	1. Very high WBC (greater than 99.9) 2. High concentration of very large platelets 3. Agglutinated RBCs, rouleaux will break up when Istoton is added 4. RBCs smaller than 36 fL 5. Specimens containing fibrin, cell fragments or other debris (esp pediatric/oncology specimens
Hgb	1. Very high WBC count 2. Severe lipemia 3. Heparin 4. Certain unusual RBC abnormalities that resist lysing 5. Anything that increases the turbidity of the sample such as elevated levels of triglycerides 6. High bilirubin
MCV	1. Very high WBC count 2. High concentration of very large platelets 3. Agglutinated RBCs 4. RBC fragments that fall below the 36 fL threshold 5. Rigid RBCs
RDW	1. Very high WBC 2. High concentration of very large or clumped platelets 3. RBCs below the 36 fL threshold 4. Two distinct populations of RBCs 5. RBC agglutinates 6. Rigid RBCs
Plt	1. Very small red cells near the upper threshold 2. Cell fragments 3. Clumped platelets 4. Cellular debris near the lower platelet threshold
MPV	1. Known factors that interfere with the platelet count and shape of the histogram 2. Known effects of EDTA
Hct	Known factors that interfere with the parameters used for computation, RBC and MCV
MCH	Known factors that interfere with the parameters used for computation, Hgb and RBC
MCHC	Known factors that interfere with the parameters used for computation, Hgb, RBC and MCV
Diff parameters	Known factors that affect the WBC count as listed above, high triglycerides that affect lysing

HANDLING ABNORMAL RESULTS	
Plts< 40,000	Check the integrity of the specimen (look for clots, short draw, etc.) Confirm count with smear review for clumps, RBC fragments, giant platelets, very small RBCs
WBC ++++	Dilute 1:2 with Isoton or further until count is within linearity (for final result, multiply diluted result by dilution factor); subtract final WBC from RBC; perform spun hct, calculate MCV from correct RBC &Hct (MCV = Hct/RBC x 10), do not report HGB, MCH, MCHC. Plt counts are not affected by high WBC. Add comment, "Unable to report Hgb, MCH, MCHC due to high WBC."
Plt ++++	Check smear for RBC fragments or microcytes. 1. If present, perform plt estimate. If they do not agree, perform manual plt count. 2. If not present, dilute specimen 1:2 with Isoton or further until count is within linearity, multiply diluted result by dilution factor.
RBC > 7.0	Dilute 1:2 with Isoton or further until count is within linearity, multiply dilution result by dilution factor; perform spun hct, review Hgb, recalculate MCH, MCHC
MCHC > 36.5	Perform manual Hct. 1. If it stays the same, check plasma for lipemia, icteremia or other color interference. If present, perform Isoton replacement. Pour 3 ml blood into 10x75 tube. Mark level of top of blood. Centrifuge at high speed for 10 minutes. Pipette off plasma being careful not to disturb red cells. Replace plasma with Isoton to mark. Mix well and rerun to obtain correct Hgb and MCH, MCHC. RBC should be within ± 0.2 of previous result. Add comment, *"results corrected for lipemia".* 2. If it is significantly higher, check the specimen for cold agglutinin by looking for RBC clumping. If present, warm the specimen at 37C for 5 minutes, mix well and repeat. If results are acceptable, report. If cold agglutinin persists, report the spun hct and mark through the RBC, MCV, MCH, MCHC results. Add comment, *"specimen warmed before running".* 3. If the above conditions are not found, check the smear for spherocytes or lyseresistant red cells. If present, ensure correct instrument operation by running controls and report result.
MCHC < 36.5	Perform spun hct. Verify proper instrument operation by running a previous patient. Decreased MCHC may be caused by swollen hyperglycemic red cells. Perform isoton replacement or correct values using spun hct.
Low plts, "Giant plts" or EDTA clumpers	Confirm with smear review. 1. If clumps are present, check with phlebotomist to see if the phlebotomy was difficult. If not, recollect in blue top tube (Na citrate anticoagulant). If platelet clumps disappear and platelet count is acceptable, multiply plt result by 1.1 to account for the dilution factor. 2. If there are no clumps, but giant platelets are present, perform plt estimate from smear. Perform manual platelet count if smear estimate and instrument count do not agree.



Normal Cell Maturation

Introduction

Red blood cells are the most common type of blood cell and the vertebrate body's principal means of delivering oxygen from the lungs or gills to body tissues via the blood.

- Red blood cells are also known as RBCs, haematids or erythrocytes (from Greek erythros for"red" and kytos for "hollow", with cyte nowadays translated as "cell"). "RBCs" should in factbe referred to as "corpuscles" rather than "cells". Indeed, a 'cell' contains a nuclear element, mature RBC's do not contain a nucleus in mammals.
- Mammalian erythrocytes are biconcave disks: flattened and depressed in the center, with a dumbbell-shaped cross section. This shape (as well as the loss of organelles and nucleus)optimizes the cell for the exchange of oxygen with its surroundings.
- Erythrocytes in mammals are a nucleate when mature; meaning that they lack a cell nucleusand thus have no DNA, also lose their other organelles.
- As a result of the lack of nucleus and organelles, the cells cannot produce new structural or repair proteins orenzymes and their lifespan is limited.
- Erythrocytes consist mainly of hemoglobin, and the color of erythrocytes is due to the heme group of hemoglobin.
- The diameter of a typical human erythrocyte disk is 6–8 µm, much smaller than most otherhuman cells.
- A typical erythrocyte contains about 270 million hemoglobin molecules, with each carrying four heme groups.
- Adult humans have roughly 2–3 × 10^{13} red blood cells at any given time (women have about4 to 5 million erythrocytes per micro liter (cubic millimeter) of blood and men about 5 to 6million; people living at high altitudes with low oxygen tension will have more). Red bloodcells are thus much more common than the other blood particles.

Blood cells go through several stages of development; progression from one stage to the next is not abrupt, so frequently the cell being studied may be between stages (when this occurs the cell is generally given the name of the mature stage). As a cell transforms from the primitive blast stage to the mature form found in the blood, there are changes in the cytoplasm, nucleus, and cell size. Normally, all three of these changes occurs gradually and simultaneously. In some disease states, however, changes will take place at different rates. For example, the cytoplasm may mature more quickly than the nucleus. This occurrence is termed asynchronism.

Cytoplasmic Maturation

The immature cytoplasm usually stain a deep blue color (basophilic) because of the high content of RNA present as the cell matures, there is a gradual loss of cytoplasmic RNA and therefore a lessening of blue color. In some cells (for example, the myelogenouscells), granules appear in the cytoplasm as the cell matures. At first, these granules are few and nonspecific. As the cell matures further, these granules

increase in number and take on specific characteristics and functions. The amount of cytoplasm in relationship to the rest of the cell usually increases as the cell matures.

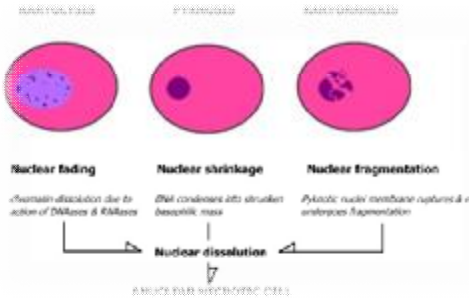
Nuclear Maturation

The nucleus of the immature cell is round oval and is large in proportion to the rest of the cell. As the cell matures the nucleus decreases in relative size and may or may not take on various shapes (depending on the cell type). The nuclear chromatin transforms from a fine, delicate pattern to become more coarse and clumped on the mature form, and the staining properties change from a reddish purple to a bluish purple. Nucleoli present in early stages of cell development usually disappear gradually as the cell ages.

Cell Size

As a cell matures, it usually becomes smaller in size. (for the new student, this change may be difficult to detect. The normal mature red blood cells or small lymphocytes are generally of relatively constant size and may be used as a guide for comparison) the student should know the relative size of each cell type.

<u>Identification of cells</u>

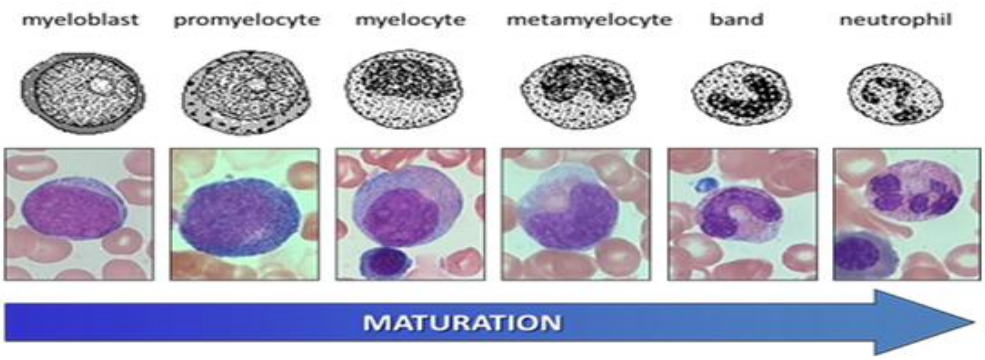


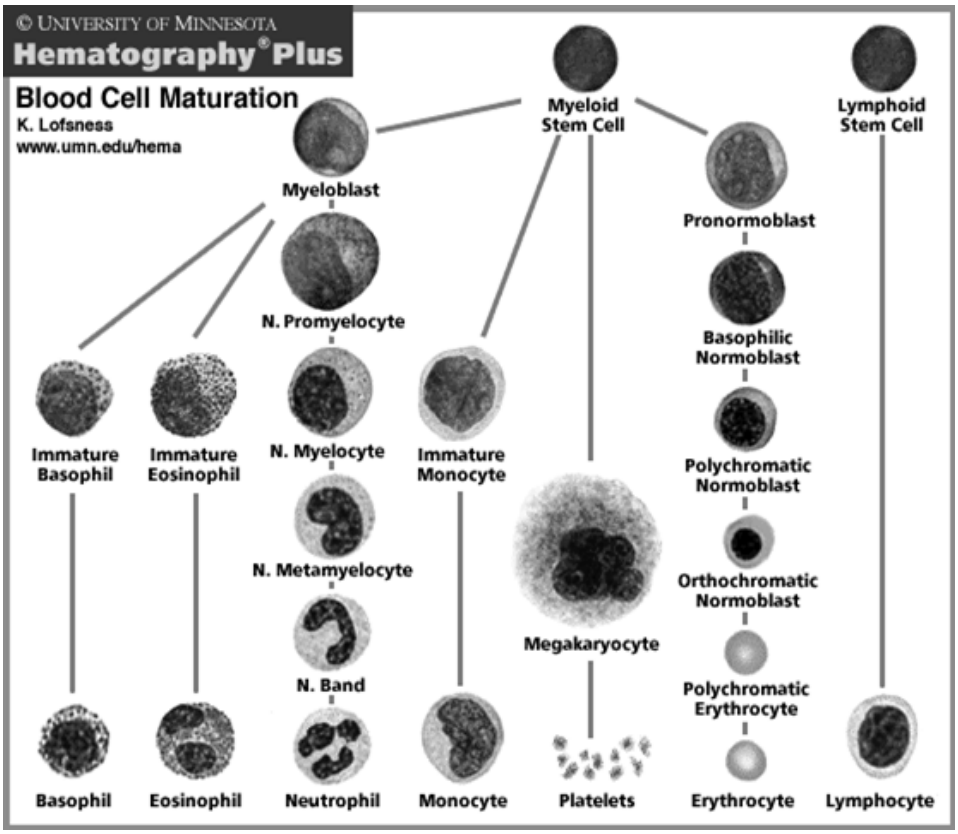
In identifying a cell we should think of the following terms:

1. What is the size of the cell?
   I. Small
   II. Medium
   III. Large

2. What are the characteristics of the cytoplasm?
   I. Granular or nongranular; specific or nonspecific granules
   II. Color (staining properties).
   III. Relative amount.

3. What are the characteristics of the nucleus?
   I. Shape
   II. Relative size. The size of the nucleus compared to the amount of cytoplasm is expressed as the nuclear to cytoplasmic ratio (N/C ratio)
   III. Chromatin pattern: smooth or coarse; Parachromatin visible or not.
   IV. Presence of or absence of nucleoli; number and size.





ERYTHROCYTE MATURATION

The overall trend in RBC maturation is large, pale nucleus to darker, smaller nucleus to loss of nucleus; increase in cytoplasm; gradual decrease in size; cytoplasm from intensely blue (full of RNA) to grayish (mixture of RNA and hemoglobin) to reddish (full of hemoglobin, no RNA). Identify the following cells.

1. Pronormobast (Rubriblast): (14-19 µm): Nucleus is large with fine chromatin and nucleoli; cytoplasm is scant and basophilic.

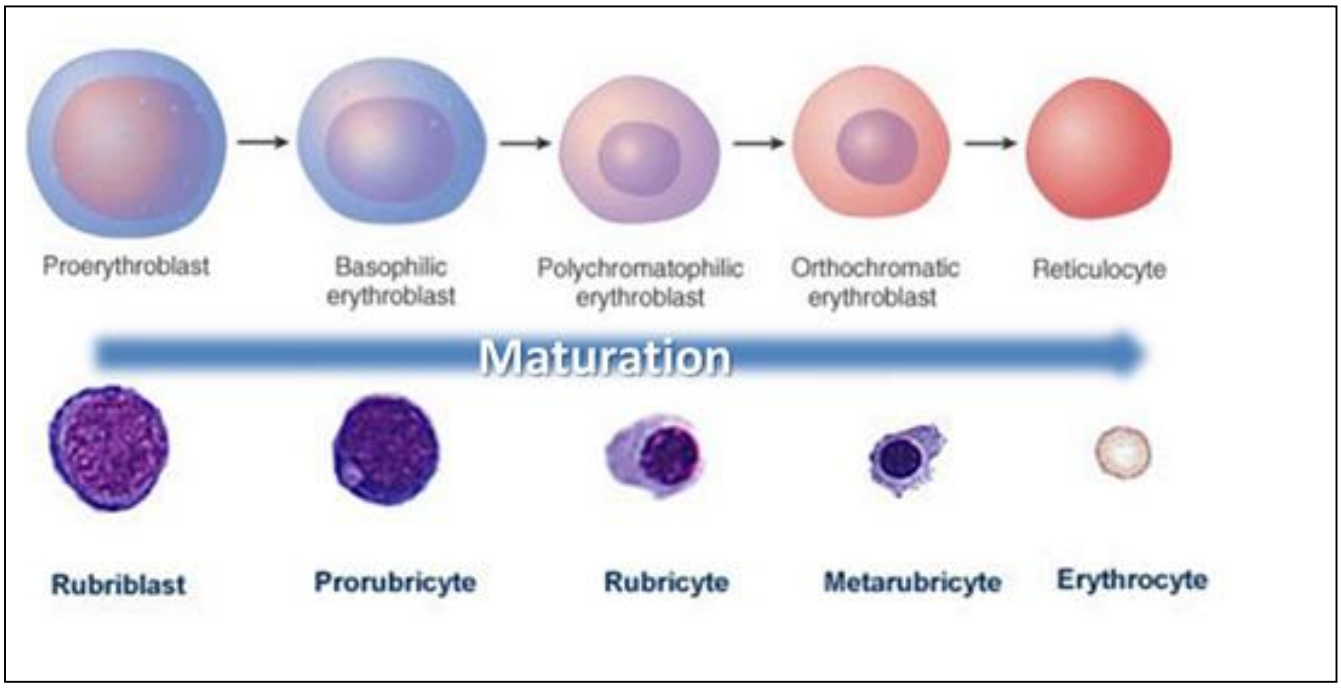
2. Basophilic Normoblast (Prorubriblast):(12-17 µm): Slightly smaller nucleus with slight chromatin condensation; increased cytoplasm and intensely blue (RNA abundance); no granules and no nucleoli present.

3. PolychromatophilicNormoblast (Rubricyte): (12-15 µm): Moderately condensed chromatin; lighter, grayish cytoplasm. The color of the cytoplasm is due to coloring by both acidic and basic components of the stain. Basophilia is from staining of ribosomes and acidophilia from hemoglobin. The nucleus is condensed and intensely basophilic with coarse heterochromatin granules giving a characteristic checkerboard appearance.

4. OrthochromatophilicNormoblast (Metarubricyte): (8-12 µm): Dark, opaque nucleus; gray-red cytoplasm (trace blue). The nucleus has become pyknotic (a homogenous blue-black mass with no structure) and there is abundant acidophilic hemoglobin. In some instances you can detect the nucleus in the process of extrusion.

5. Reticulocyte: (7-10 µm) [Not visible with this preparation. Refer to the laser disc to see an example.]: Nucleus has been extruded; cytoplasm is reddish-pale blue. RNA is still present.

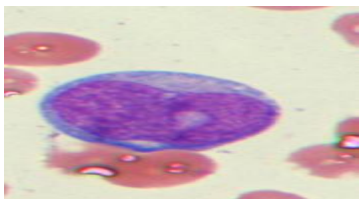
6. Erythrocyte: (7-8 µm): No nucleus; orange-red cytoplasm; RNA is lost.



Leukocyte Maturation

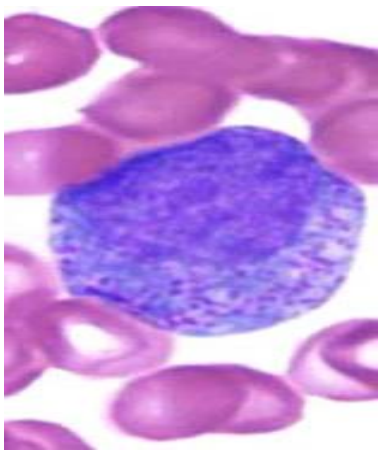
Maturation and Morphology of Immature Granulocytes

Myeloblast:



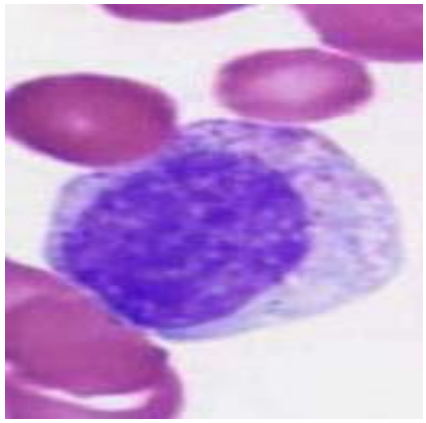
The first and earliest granulocyte, is a large cell (15 µm), high nucleus to cytoplasm (N:C) ratio (5:1), round or oval nucleus with loose light staining euchromatin, 1-2 nucleoli, has minimal light blue cytoplasm, contains no cytoplasmic granules, begins to produce myeloperoxidase granules (MPO), comprises 1% of the nucleated cells in the bone marrow.

Promyelocyte:



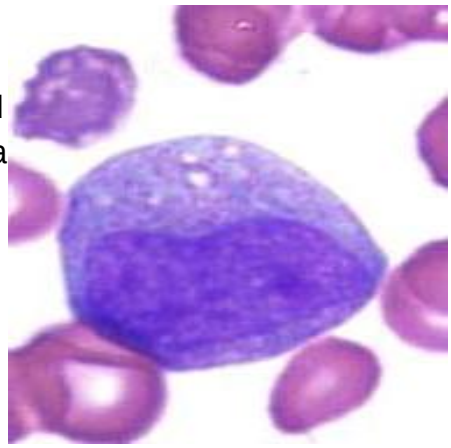
Larger than a myeloblast (20 μm), high N:C ratio (3:1), loose chromatin with nucleoli, dark blue cytoplasm, contains large nonspecific cytoplasmic granules, containing myeloperoxidase (MPO), comprises 3-4% of nucleated bone marrow cells.

Neutrophilic Myelocyte:



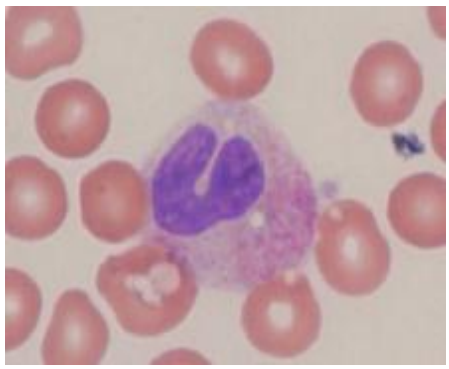
Medium cell size (12 μm), high N:C ratio (3:1), round, oval, or slightly indented nucleus with darker, blue heterochromatin, last stage of cell division, has active RNA, therefore, the cytoplasm is blue, contains MPO and secondary granules containing, leukocyte alkaline phosphatase, comprises 12% of bone marrow nucleated cells.

Neutrophilic Metamyelocyte:



size (11 μm), N:C ratio (2:1), last mononuclear stage, no mitosis, nucleus is kidney or horseshoe shaped, has condensed heterochromatin, has a prominent Golgi apparatus – clear area located at the indentation site of the nucleus, cytoplasm is similar to the mature cell, comprises 18% of bone marrow cells.

Band

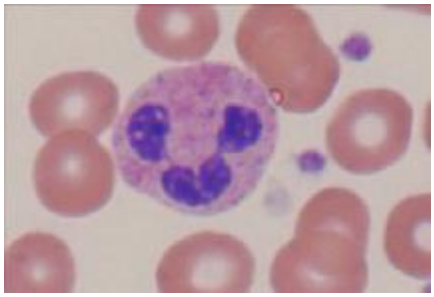


Same size as a mature neutrophil (10-12 μm), N:C ratio has reversed (1:2), nucleus is band- or sausage-shaped without segmentation, cytoplasm is filled with small neutrophilic granules, last immature stage, comprises 11% of bone marrow cells and 0-3% of, peripheral WBCs stored in the bone marrow and released when there is an increased demand for neutrophils.

Note :

Shift to the left is an increase in immature cells indicating increased demand for WBCs in peripheral blood.

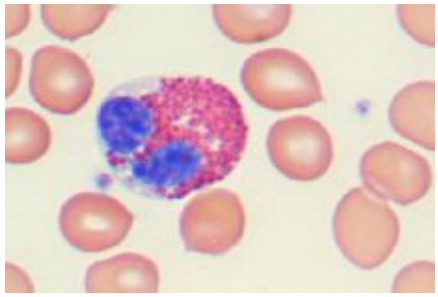
Neutrophil



Also known as segmented neutrophils, segs, polymorphonuclear cells, polys, and PMNs, N:C ratio is 1:3, and the size is 10-12 μm, average nucleus contains 3-5 segments, connected by narrow filaments, hyposegmented is less than 3 segments, and may indicate a shift to the left or an anomaly, hypersegmented is more than 5 segments, and may indicate infection or megaloblastic anemia, cytoplasm contains very small neutral granules, makes up 55-75% of all peripheral WBCs.

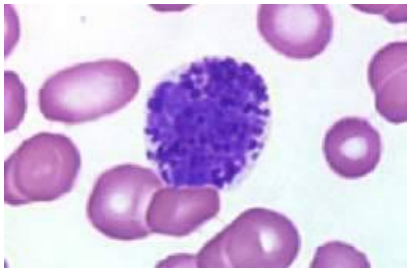
Note : Granules can become larger upon bacterial infection producing toxic granulation, which are numerous, large, basophilic granules.

Eosinophils



Average size is 13 μm, Nucleus is generally bilobed, cytoplasm is bright red or orange which is due tolarge specific, secretory granules containing peroxidase, acid phosphatase, aryl sufatase, beta-glucuronidase, etc. that stain red with the eosin component of Wright's stain, makes up 3% of WBCs in the peripheral blood.

Basophils



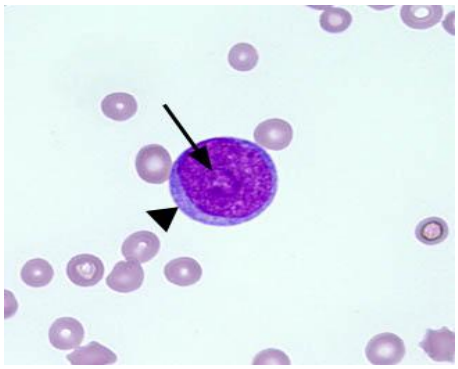
Is the smallest granulocyte at 10 μm, The nucleus is difficult to see due to heavy granulation, cytoplasm contains large specific, secondary granules that contain heparin and histamines, which stain purple with Wright's stain. These granules are water soluble and sometimes appear as holes in the cell if the cells are not fixed well during staining. Makes up to 0.5% of peripheral WBCs.

Note*:*

Tissue mast cells are similar to basophils but are larger and have no developmental relationship with basophils. Mast cells have a mesenchymal (connective tissue) origin and have granules containing serotonin (basophilic granules contain no serotonin).

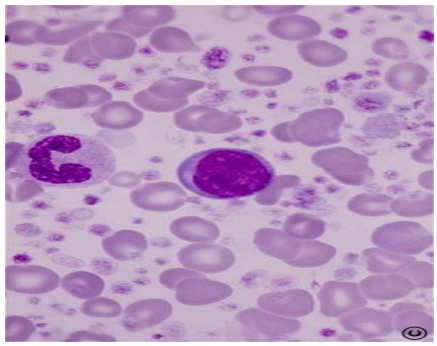
Lymphoid maturation

Lymphoblast



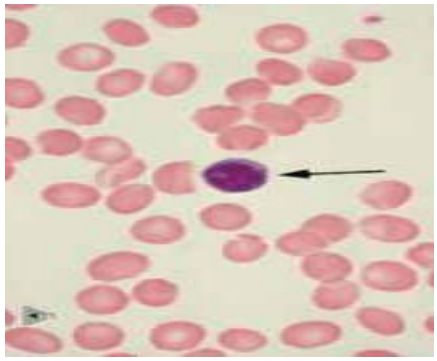
Size : 10 to 18 µm, cytoplasm: no granules present, appears smooth, moderate to dark blue, may stain deep blue at the periphery and a lighter blue near the nucleus, more abundant than in the myeloblast, nucleus: chromatin pattern is somewhat coarse, round or oval in shape, generally contain one to two distinct nucleoli, N/C ratio is 4:1.

Prolymphocyte



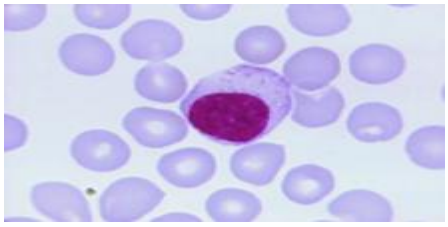
May be the same size as the lymphoblast or smaller, cytoplasm moderate to dark blue, usually nongranular, but may contain occasional azurophilic granules, more abundant than in the lymphoblast, nucleus round, oval, or slightly indented, chromatin pattern is more clumped than in the lymphoblast, may contain one to two nucleoli.

Small Lymphocyte



7 to 18 µm in size, N:C: 4:1, oval nucleus with coarse lumpy chromatin with specific areas of clumping, a compact cell, cytoplasm usually just a thin border with few azurophilic red granules.

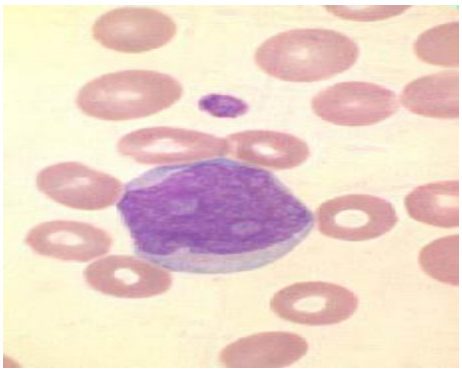
Large Lymphocyte



9 to 12 µm in size, N:C 3:1, looser chromatin pattern, more transparent, larger amount of cytoplasm, lighter in color, Cytoplasm is more abundant with tendency for azurophilic granules.

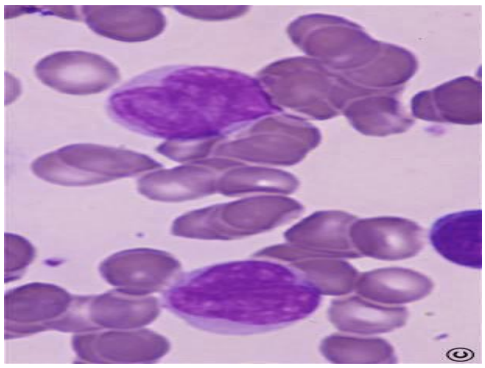
Monopoiesis

Monoblast



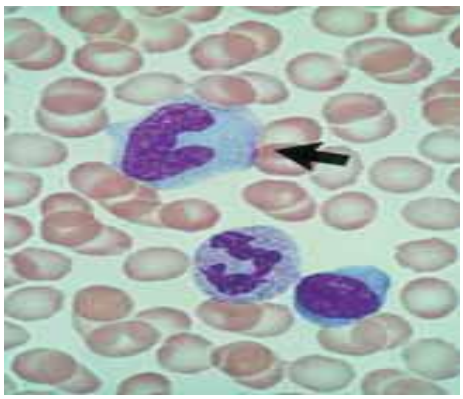
12 to 20 µm in size, moderately basophilic to blue-gray cytoplasm, nongranular, ovoid or round nucleus, light blue-purple in color, fine, lacey chromatin, one to two nucleoli, N/C ratio is 4:1 to 3:1.

Promonocyte (immature monocyte)



14 to 18 µm in size, Blue-gray cytoplasm, may contain fine dustlike azurophilic granules, ground glass appearance, moderate amount, oval nucleus, may have a single fold or fissure, one to five nucleoli, fine chromatin pattern, N/C ratio is 3:1 to 2:1.

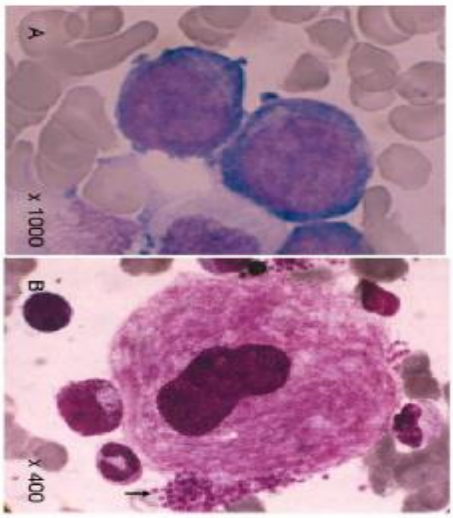
Monocyte



14 to 20 µm in size, abundant blue-gray cytoplasm, outline may be irregular because of the presence of pseudopods. Many fine azurophilic granules, giving a ground glass appearance, vacuoles may some times be present, round kidney shaped nucleus or may show slight lobulation. It may be folded over on top of itself, thus showing brainlike convolution. No nucleoli are visible, chromatin is fine and lacey (not clumpled), arranged in skeinlike strands.

Megakaryocytopoiesis

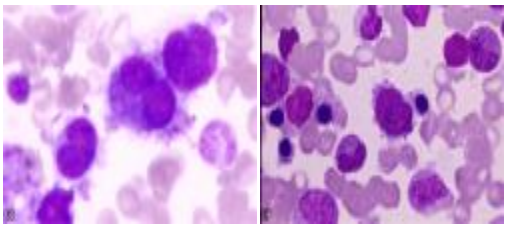
Megakaryoblast (stage 1)



varying shades of blue Cytoplasm, usually darker than the myeloblast, May have small, blunt pseudopods, small to moderate amount, usually a narrow band around the nucleus, as the cell matures, the amount of cytoplasm increases, usually nongranular, Round, oval, or may be kidney shaped nucleus, fine chromatin pattern, multiple nucleoli that generally stain blue, N/C ratio is about 10:1.

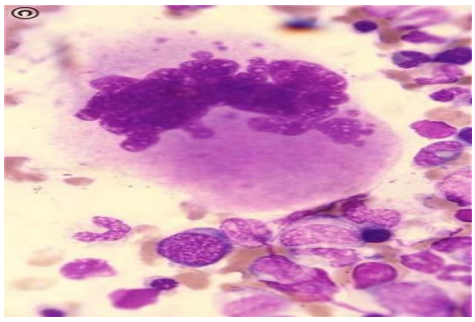
Promegakaryocyte (stage 2)

Cytoplasm more abundant than previous stage, lass basophilic than in the blast. Granules begin to form in the Golgi region, chromatin becomes more coarse, multiple nucleoli are visible, irregular in shape, may even show slight lobulation, N/C ratio is 4:1 to 7:1 depending on the ploidy.



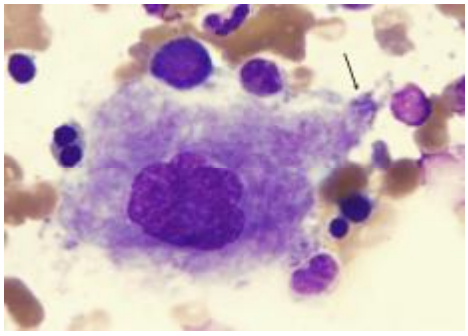
Granular megakaryocyte (stage3)

Abundent cytoplasm, pinkish blue in color, very fine and diffusely granulat, usually has an irregular peripheral border, nucleus small in comparison to cell size, multiple nuclei may be visible or the nucleus may show multilobulation, chromatin is coarser than in the previous stage, no nucleoli are visable, N/C ratio is 2:1 to 1:1.



Mature megakaryocyte (stage IV)

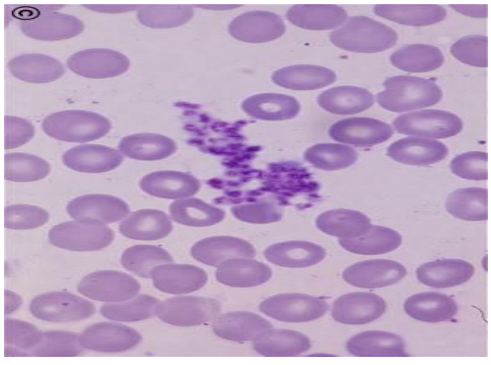
Cytoplasm contain coarse clumps of granules aggregating into little bundles, which bud off from the periphery to become platelets, Nucleus: multiple nuclei are present, or the nucleus is multilobulated. No nucleoli visible, N/C ratio is less than 1:1.



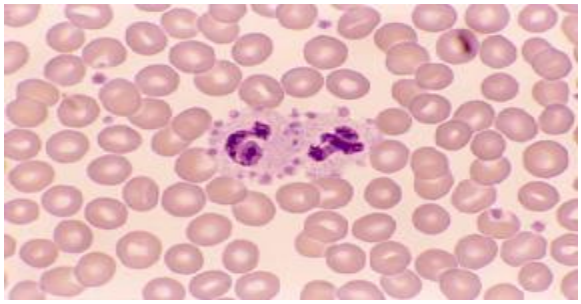
Platelet (thrombocyte)

Size: 1 to 4 µm in diameter, Cytoplasm light blue to purple, Very granular. Consists of two parts:

- The *chromomere*, which is granular and located centrally, and
- The *hyalomere*, which surrounds the chromomere and is nongranular and clear to light blue.



Platelet satellitism:



Platelets adhering to the surface of a neutrophil. In this case, the reported platelet count was only slightly decreased.

Leukemia classification

Introduction

Leukemia is cancer of the blood cells. It starts in the bone marrow, the soft tissue inside most bones. Bone marrow is where blood cells are made.

When you are healthy, your bone marrow makes:

- White blood cells, which help your body fight infection.
- Red blood cells, which carry oxygen to all parts of your body.
- Platelets, which help your blood clot.

When you have leukemia, the bone marrow starts to make a lot of abnormal whiteblood cells, called leukemia cells. They don't do the work of normal white blood cells, they grow faster than normal cells, and they don't stop growing when they should.

Over time, leukemia cells can crowd out the normal blood cells. This can lead to serious problems such as anemia, bleeding, and infections. Leukemia cells can also spread to the lymph nodes or other organs and cause swelling or pain.

There are several different types of leukemia. In general, leukemia is grouped by how fast it gets worse and what kind of white blood cell it affects.

- It may be acute or chronic. Acute leukemia gets worse very fast and may make you feel sick right away. Chronic leukemia gets worse slowly and may not cause symptoms for years.
- It may be lymphocytic or myelogenous. Lymphocytic (or lymphoblastic) leukemia affects white blood cells called lymphocytes. Myelogenous leukemia affects white blood cells called myelocytes.

The four main types of leukemia are:

- Acute lymphoblastic leukemia, or ALL.
- Acute myelogenous leukemia, or AML.
- Chronic lymphocytic leukemia, or CLL.
- Chronic myelogenous leukemia, or CML.

There are less common leukemias, such as hairy cell leukemia. There are also subtypes of leukemia, such as acute promyelocytic leukemia (a subtype of AML).

FAB classification of acute leukemias

Acute lymphocytic leukemia (ALL)

- **L1** Small monotonous lymphocytes
- **L2** Mixed L1- and L3-type lymphocytes
- **L3** Large homogeneous blast cells

Acute myeloid leukemia (AML)

- **M1** : Myeloblasts without maturation
- **M2 :** Myeloblasts with maturation (best AML prognosis)
- **M3** : Hypergranular promyelocytic leukemia (faggot cells)
- **M3V :** Variant, microgranular promyelocytic leukemia
- **M4** : Myelomonocytic leukocytes
- **M5** : Monocytic, subtype

a. Poorly differentiated monocytic leukemia

b. Well differentiated monocytic leukemia

- **M6** : Erythroleukemia/DiGuglielmo syndrome
- **M7** : Megakaryocytic leukemia (Pleomorphic undifferentiated cells with cytoplasmic blebs; myelofibrosis or ↑ BM reticulin; positive for platelet peroxidase antifactor VIII).

Acute lymphocytic leukemia includes :

- L1 – Around 25 to 30% of adult cases and 85% of childhood cases of ALL are of this subtype. In this type small cells are seen with:-
  - regular nuclear shape
  - homogeneous chromatin
  - small or absent nucleolus
  - scanty cytoplasm

- L2 – Around 70% of adult cases and 14% of childhood cases are of this type. The cells are large and or varied shapes with:-
  - irregular nuclear shape
  - heterogeneous chromatin
  - large nucleolus

- L3 – This is a rarer subtype with only 1 to 2% cases. In this type the cells are large and uniform with vacuoles (bubble like features) in the cytoplasm overlying the nucleus.

Acute myelocytic leukemia includes :

Acute myeloblastic leukemia, minimally differentiated (FAB Classification M0)

This AML shows no evidence of myeloid differentiation by morphology

Morphologic and cytochemical features include the following:

- Medium-sized blasts with dispersed nuclear chromatin.
- Agranular cytoplasm.
- Occasionally small blasts that resemble lymphoblasts.
- Cytochemistry negative for myeloperoxidase (MPO), Sudan Black B (SBB), and naphthol ASD chloroacetate esterase (<3% positive blasts).
- Cytochemistry negative for alpha naphthyl acetate and butyrate esterases.
- Markedly hypercellular marrow.

Acute myeloblastic leukemia without maturation (FAB Classification M1)

AML without maturation is characterized by a high percentage of bone marrow blasts with little evidence of maturation to mature neutrophils

Common morphologic and cytochemical features include the following:

- Myeloblasts of 90% or more of the nonerythroid cells in the bone marrow.
- Myeloblasts that may have azurophilic granules and/or Auer rods.
- Myeloblasts that resemble lymphoblasts.
- MPO and SBB positivity in blasts of 3% or more.
- Typically markedly hypercellular marrow.

Acute myeloblastic leukemia with maturation (FAB Classification M2)

AML with maturation is characterized by 20% or more myeloblasts in the blood or bone marrow and 10% or more neutrophils at different stages of maturation. Monocytes constitute less than 20% of bone marrow cells.

Morphologic features include the following:

- Myeloblasts with and without azurophilic granules.
- Auer rods.
- Promyelocytes, myelocytes, and neutrophils 10% or more of the bone marrow cells.
- Abnormal nuclear segmentation in neutrophils.
- Increased eosinophil precursors (frequently).
- Hypercellular marrow (usually).
- Blasts and maturing neutrophils reactive with antibodies to MPO and lysozyme.

Acute promyelocytic leukemia (FAB Classification M3)

APL (Acute Promyelocytic leukemia) AMLis an AML in which promyelocytes predominate. APL exists as two types, hypergranular or typical APL and microgranular (hypogranular) APL

Common morphologic features of typical APL include the following:

- Kidney-shaped or bilobed nuclei.
- Cytoplasm densely packed with large granules (bright pink, red, or purple in Romanowsky stains).
- Bundles of Auer rods within the cytoplasm (faggot cells).
- Larger Auer rods than in other types of AML.
- Strongly positive myeloperoxidase (MPO) reaction in all leukemic promyelocytes.
- Only occasional leukemic promyelocytes in the blood.
- Common morphologic features of microgranular APL include the following:
- Bilobed nuclear shape.
- Apparent scarce or absent granules (submicroscopic azurophilic granules).
- Small number of abnormal promyelocytes with visible granules and/or bundles of Auer rods (faggot cells).
- High leukocyte count in the peripheral blood.
- Strongly positive MPO reaction in all leukemic promyelocytes.

Acute myelomonocytic leukemia (FAB Classification M4)

Acute myelomonocytic leukemia (AMML) is characterized by the proliferation of neutrophil and monocyte precursors

Morphologic and cytochemical features include the following:

- 20% or more blasts in the bone marrow.
- 20% or more neutrophils, monocytes, and their precursors in the bone marrow (to distinguish AMML from AML with or without maturation and to increase monocytes).
- 5 x 10^9/L or more monocytes in the blood.
- Large monoblasts with round nuclei, abundant cytoplasm, and prominent nucleoli.
- MPO positivity in at least 3% of blasts.
- Monoblasts, promonocytes, and monocytes typically nonspecific esterase (NSE)-positive.

Acute monoblastic leukemia and acute monocytic leukemia (FAB classifications M5a and M5b)

Acute monoblastic and acute monocytic leukemia are AMLs in which 80% or more of the leukemic cells are of a monocytic lineage. These cells include monoblasts, promonocytes, and monocytes. These two leukemias are distinguished by the relative proportions of monoblasts and promonocytes. In acute monoblastic leukemia, most monocytic cells are monoblasts (usually ≥80%). In acute monocytic leukemia, most of the monocytic cells are promonocytes

Morphologic and cytochemical features of acute monoblastic leukemia include the following:

- Large basophilic monoblasts with abundant cytoplasm, pseudopod formation, round nuclei, and one or more prominent nucleoli.
- Rare Auer rods.
- Typically intensely NSE-positive and MPO-negative.
- Hypercellular marrow with large numbers of monoblasts.
- Lysozyme positive.
- Morphologic and cytochemical features of acute monocytic leukemia include the following:
- Promonocytes with an irregular nuclear configuration with a moderately basophilic cytoplasm and cytoplasmic azurophilic granules.

- Typically intensely NSE-positive.
- Occasional MPO positivity.
- Lysozyme-positive.
- Hemophagocytosis (erythrophagocytosis).

Acute erythroid leukemias (FAB classifications M6a and M6b)

The two subtypes of the acute erythroid leukemias, erythroleukemia and pure erythroid leukemia, are characterized by a predominant erythroid population and, in the case of erythroleukemia, the presence of a significant myeloid component. Erythroleukemia (erythroid/myeloid; M6a) is predominantly a disease of adults, comprising approximately 5% to 6% of cases of AML. Pure erythroid leukemia (M6b) is rare and occurs in all age groups.

Morphologic and cytochemical features of erythroleukemia include the following:

- 50% or more erythroid precursors in the entire nucleated cell population of the bone marrow.
- 20% or more myeloblasts in the nonerythroid population in the bone marrow.
- Dysplastic erythroid precursors with megaloblastoid nuclei.
- Multinucleated erythroid cells.
- Myeloblasts of medium size, occasionally with Auer rods.
- Ringed sideroblasts.
- Positive PAS stain in the erythroid precursors.
- Hypercellular bone marrow.
- Megakaryocytic dysplasia.
- Morphologic and cytochemical features of pure erythroid leukemia include the following:
- Medium- to large-sized erythroblasts with round nuclei, fine chromatin, one or more nucleoli, deeply basophilic cytoplasm, and occasional coalescent vacuoles.
- Erythroblasts reactive with alpha-naphthyl acetate esterase.
- Acid phosphatase.
- PAS.

Acute megakaryoblastic leukemia (FAB Classification M7)

In acute megakaryoblastic leukemia, 50% or more of blasts are of the megakaryocyte lineage

Morphologic and cytochemical features include the following:

- Medium- to large-sized megakaryoblasts with round or indented nucleus and one or more nucleoli.
- Agranular, basophilic cytoplasm with pseudopod formation.
- Lymphoblast-like morphology (high nuclear-cytoplasmic ratio) in some cases.
- Circulating micromegakaryocytes, megakaryoblastic fragments, dysplastic large platelets, and hypogranular neutrophils.
- Stromal pattern of marrow infiltration mimicking a metastatic tumor in infants.
- Negative stains for SBB and MPO.
- Blasts reactive with PAS, acid phosphatase, and nonspecific esterase.

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a cancer of the lymphocytes, a type of white blood cell involved in the body's immune system.

The leukemia cells found in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. These cells may be found admixed with larger or atypical cells, cleaved cells, or prolymphocytes, which may comprise up to 55% of the blood lymphocytes.

Chronic Myelocytic Leukemia

CML is sometimes called chronic myelogenous leukaemia, chronic granulocytic leukaemia, or chronic myelocytic leukaemia.

CML develops due to a problem with a stem cell in the bone marrow, which becomes abnormal. The abnormal stem cell multiplies and the cells that are made from the abnormal stem cells mature and develop into near-normal white cells - mainly neutrophils, basophils and eosinophils (collectively called granulocytes). Large numbers of these cells are made in the bone marrow and spill into the bloodstream.

Special stains

Cytochemical Stains

Cytochemical stains are very helpful in the diagnosis and classification of acute leukemias (Table1). These stains are usually performed on bone marrow smears but may also be done on peripheral smears.

- The special stains are used to identify enzymes or lipids within the blast population of cells—hence, the reaction in mature cells is not of importance. The positive reactions that occur will be associated with a particular lineage, and with some of the stains (e.g., myeloperoxidase [MPO] and Sudan Black B [SBB]), the fine or coarse staining intensity is an indication of the lineage of blast cells.
- All of the cytochemical stains described below are negative in lymphoid cells (with rare exceptions), so *a positive result with any of these will most often rule out acute lymphoblastic leukem*ia.

Cytochemical Reaction	Cellular Element Stained	Blasts Identified
Myeloperoxidase (MPO)	Neutrophil primary granules	Myeloblasts strong positive; monoblasts faint positive
Sudan Black B (SBB)	Phospholipids	Myeloblasts strong positive; monoblasts faint positive
Specific esterase	Cellular enzyme	Myeloblasts strong positive
Nonspecific esterase (NSE)	Cellular enzyme	Monoblasts strong positive
Periodic acid-Schiff	Glycogen and related substances	Variable, coarse or block-like positivity often seen inlymphoblasts and pronormoblasts, myeloblasts usuallynegative although faint diffuse reaction mayoccasionally be seen

1. Leukocyte Alkaline phosphatase (LAP):

Purpose: Distinguishing the cells of leukemoid reactions with increased activity from these of (CML) with decreased activity.

Principle: Alkaline phosphatase Activity is present in varying degrees in the neutrophil and band form of the granulocytes / sometime in B lymphocytes

* Naphthol As-M or {naphthol AS-BI} $\xrightarrow[\text{Alkaline PH}]{\text{LAP}}$ Hydrolsed Substrate $\xrightarrow[\text{RR dye}]{\text{Fast blue}}$ insoluble precipitate at the site of enzyme activity

Interpretation:

Count 100 neutrophiles and score them (0/+4), then calculate the final score by adding the total scores.

Grading:

*(0) No stain
*(+1) Faint stain
*(+2) Moderate stain
*(+3) Strong stain
*(+4) Strong stain without cytoplasmic background

Normal Range:30-185

LAP elevated in:	LAP decreased in:
1. Leukomoid reaction.	1. CML.
2. Pregnancy	2. Paroxymal Nocturnal Hemoglobinuria.
3. Polycythemia vera.	3. Sickle cell anemia.
4. Aplastic anemia.	4. Hypophosphatasia.
5. Multiuple myeloma	
6. Obstructive juindice.	
7. Hodgkins` disease.	

**The following diseases will not affect LAP result:

1. Untreated hemolytic anemia.
2. Lymphosarcoma.
3. Viral hepatitis.
4. Secodery polycythemia.

2. Peroxidase stain /Myeloperoxidase (MPO):

Purpose: To differentiate a myelogenous or monocytic leukemia from acute lymphocytic leukemia.
Principle: Peroxidase is present in the primary azurophilic granules of neutrophil, eosinophil and monocyte & activity increased with maturation, no activity is found in red cells or lymphocytes.

H_2O_2 + 3-amino-9-ethylecarbazole Or (benzidinedihydrodase) $\xrightarrow{\text{Peroxidase}}$ Insoluble red brown precipitate

Interpretation:

- Red – brown peroxidase found in:
  Neutrophil and eosinophil {promyelocyte – Myelocyte – Metamyelocyte}
- Finely granular staining found in:
  Monocyte
- Negative stain found in:
  Early Myeloblast, lymphoblast, basophiles and plasma cell

Notes:

1. In acute leukemia, infection &myelodysplasia neutrophils show (-ve) stain
2. Increase in CML*
3. Basophile May stain +ve in granulocytic leukemia
4. Peroxidase stain show results similar to those of sudan Black B stain

3. Sudan Black B:

Purpose: To distinguish acute myelogenous and monocytic leukemia from lymphocytic leukemia.
Principle: Sudan black B dye is fat soluble, then it stains fat particles (Steroles, phospholipids and neutral fats) which present in the primary and secondery granules of myelocytic and monocytic cells.

Interpretation:

- Myelogenous cells show coarse staining granules with faint staining pattern for myelobast and increase staining with maturation.
- Auer rods are +vely stained. *
- Monocytic cells show finely scatterd granules.
- -velymphoctic staining except Burkitt`s lymphoma cells, may show +ve staining vacuoles.

4. Acid phosphtase(withtartarate resistance)

Purpose: diagnosis of hairy cell leukemia.

Principle: ACP enzyme present in myelocytic, lymphocytes, monocytic, plasma cell, and platelets in these cells ACP activity will inhibited in the presence of (L-tartarate) and give no color, while hairy cell ACP will not inhibited and give (+ve).

Naphthol AS-BI Phosphoric acid —ACP Acid PH→ naphthol —Parasolinin→ Red precipitate

**ACP isoenzymes → (0, 1, 2, 3a, 3b) inhibited

(L+) → (5) Not inhibited

5. Non Specific Esterase: {with fluoride inhibition}

Purpose: Differentiate myelocytic and monocytic leukemia.

Principle: WBCS contain esterases, a group of lysosomal enzymes

α-naphthyl acetate —Non specific esterase→ naphthyl compound —Parasolinin→ Red ppt.

Interpretation:

- (+ve) brick – red staining which found in: Megakaryocyte and platelets, Histocyte, Macrophage, Monocyte & Lymphoblast of ALL
- (-ve) for granulocytes

**If fluoride added, only monocyte non specific esterase will be inhabited.

6. Periodic Acid – Schiff [PAS] Reaction:

Purpose: -Diagnosis of some acute lymphocytic leukemia
-subtypes of AML
-M_6

Principle: the stain indicates the presence of muccoproteins , glycoproteins and high molecular weight carbohydrates in blood cells.

Glycoprotein —Periodic acid→ Aldehydes —Schiff reagent→ Red ppt

Interpretation:

- Normally all blood cells are (+ve) but Erythroblasts (-ve)
  - Diffused stain pattern (Granulocytes)
  - Granular stain (lymphocytes and monocytes)
  - Plts deeply stained
  - nRBCs (-ve) stain

- In diseases:
  - In CML, lymphosarcoma and Hodgkins` disease (+ve) staining granules will increase.
  - nRBCs in M6, thalassemia and other types of anemia may give [+ve] reaction.

7. Specific esterase or chloroacetate

Principle:

Naphthol (AS-D) $\xrightarrow[\text{/ Esterase}]{\text{Chloroacetate}}$ naphthol $\xrightarrow{\text{Fast blue BB}}$ Blue precipitate

Interetaion:

- Myeloid cells (+ve)
- Monocyte and basophile (–ve) to weak (+ve)
- Other cells {lymph – plasma –megakaryocyte – NRBC } (-ve)
- Auer rods (+ve)

8. Iron stain (Prussian Blue Reaction):

Principle:

Sidrotic granules are found in the cytoplasm of developing cells in [BM] in the form of Ferric [Fe^{+3}].

[Fe^{+3}] + Brussian blue (Perls` reagent) $\longrightarrow$ blue color

Perls' reagent is formed of (Potassium Ferricyanide + HCL)*

* Sidrotic granules are found in nRBCs, some reticulocytes