HEMATOLOGY FOR THE UNDERGRADUATES

By:

Dr. Muhammad Saboor, PhD  
Assistant Professor, Baqai Institute of Hematology  
Director, Baqai Institute of Medical Technology  
Baqai Medical University

and

Dr. Moinuddin, FRCP(C), FRCP (E), PhD (Hons.)  
Professor of Hematology  
Director, Baqai Institute of Hematology  
Baqai Medical University

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Hematology is one of the oldest specialties in conception yet it is the youngest in its inception. Diseases like anemias and leukemias were known to the medical profession in the mid-nineteenth century. The specialty in its simplified version was introduced in the western medicine in the latter half of the 20th century while in Pakistan it was practiced during this period in its more primitive form as a laboratory service only. Teaching the subject in the medical curriculum and its introduction in clinical practice was non-existent. Blood transfusion was given without any concept of its technicalities and adverse effects.

With the surge in technology in the field of hematology and blood transfusion, improvements in the diagnostic and therapeutic services became available only to a small sect of population. Teaching hematology at academic level was still a dream as late as the end of the last century. Technical and professional expertise in this critical but sorely neglected field was a far cry. With the acceptance of hematology as a specialty by the medical profession and the College of Physicians and Surgeons of Pakistan (CPSP) and establishing a society of hematology at the national level provided a strong impetus for this specialty to come to the forefront.

It is however still sadly appreciated that a specialty which alone deals with disorders that collectively afflict half of the mankind is still waiting for its proper acceptance in the academic curriculum at undergraduate level. This is due to lack of appropriate and relevant teaching material for the undergraduates. Minimum contents of the subject in the undergraduate curriculum and insufficient number of health professionals in this field have also contributed to the unsatisfactory state of affairs.

Teaching of academic hematology is assigned only a minimum segment and the practical training at this stage is essentially non-existent. Teaching material is archaic and there is little effort on the part of the teachers and the taught to appreciate the therapeutic dimensions of hematology. There is limited understanding of the basis and pathophysiology of the genesis of laboratory and clinical features of various blood diseases. Rationalization of various therapeutic modalities is also the need of the hour. Reading material that is available to the undergraduates does not address these issues nor does it present the matter in a palatable format.

Purpose of writing this manuscript is to teach hematology to the undergraduates as a continuous spectrum relevant to the stage of curriculum. Every effort has been made to explain the pathophysiological basis of the signs and symptoms, diagnostic avenues and therapeutic modalities for the commonly encountered blood diseases.

In order to achieve these objectives, the manuscript is presented in four parts in conformity with the 3 professional examinations in the MBBS curriculum as listed below:
Part one : Hematophysiology 1st professional curriculum
Part two : Hematopathology 3rd professional curriculum
Part three : Clinical hematology Final professional curriculum
Part four : Laboratory hematology 1st professional curriculum

A useful and informative list of references is provided for acquiring additional knowledge by those who wish to learn the intricacies of various hematological disorders. It is hoped that this book with its novel approach, palatable format, academic information, emphasis on highlighting the basis of the genesis of clinicopathological spectrum of various blood diseases shall be warmly received and acclaimed by the readers.

(Muhammad Saboor & Moinuddin)
ACKNOWLEDGMENTS

Our humble thanks to Almighty Allah for without his kindness and blessings it would not have been possible for us to achieve this goal.

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INTRODUCTION TO BLOOD

Blood may be viewed as a special form of connective tissue. Whereas other tissues are organized as discrete entities with well-defined anatomical localization, blood is in the fluid phase. It fills the vascular compartment and assumes the shape of the blood vessel in which it flows. Blood consists of two components: cells and plasma (fluid part).

Cellular component includes red cells (erythrocytes), various subsets of white cells (leukocytes) and platelets (thrombocytes). Subsets of leukocytes are of two types i.e. granulocytes and agranulocytes. Granulocytes include neutrophils, eosinophils and basophils while lymphocytes and monocytes are agranulocytes.

Plasma is the intercellular fluid which contains dissolved colloids and crystalloids. It is a complex mixture of proteins (7%), electrolytes and other chemical compounds dissolved in water (91%). Colloids are primarily three types of proteins; albumin, globulins and fibrinogen.

Functions of Blood

Since blood is a mixture of cells and plasma, its functions are sums of the functions of its components. Some of the major fractions of blood along with their functions are listed below:

Red cells

- Transport of oxygen
  Oxygen is transported in the blood almost entirely by hemoglobin (a small amount is transported by the plasma in the dissolved state). Association of oxygen with hemoglobin is a reversible phenomenon. Transport of oxygen across the cell membrane is a simple physical process of diffusion whereby gases (O₂ and CO₂) pass from an environment of higher concentration into one of lower concentration.

- Buffer system
  Red cells constitute an important buffer in the body; they play a crucial role in maintaining the internal environment. Buffering capacity of the red cells is almost 70% of that of whole blood.

- Removal of carbon dioxide
  Carbon dioxide is removed from the peripheral tissues by the red cells as carbamino compounds. In this reaction, the hemoglobin combines with carbon dioxide directly and produces carbamino compounds. Compared with the carbonic anhydrase mechanism the amount of CO₂ removed by this mechanism is rather small.

- Maintenance of viscosity of blood
  Viscosity of whole blood differs greatly from that of plasma. Viscosity of plasma is due to plasma proteins. Whereas plasma is 1.8 times more viscous than water (viscosity of water is
taken as 1) the viscosity of whole blood is 4.7. This marked increase in the viscosity of whole blood with respect to plasma is due to the red cells. White cells and platelets play no role in enhancing the viscosity of whole blood. Viscosity of blood is an important factor in maintaining normal peripheral resistance and ensuring normal blood pressure.

**White blood cells**

As mentioned above, white blood cells are further categorized into five subclasses. Each subclass performs its own specific functions as given below:

- Neutrophils: are instrumental in combating bacterial, fungal and other infections. They also play an important role in innate immune response.
- Lymphocytes: play a major role in viral illnesses and synthesis of immunoglobulins.
- Eosinophils: provide defense against helminths and other parasites.
- Basophils: cause allergic reactions by releasing histamine. They also contain heparin which is an anticoagulant and serotonin that mediates vasoactive response.
- Monocytes: are the key cells in phagocytosis; they also play a major role in acquired immune response.

**Platelets**

Platelets form primary hemostatic plug and stabilize the secondary hemostatic plug. In addition they participate in many other biological functions.

**Plasma; components and their functions**

- **Water:** is the major component of blood. It acts as solvent and a vehicle for the transport of blood cells and other components. It also performs some other important and vital functions, as listed below:
  - Regulation of fluid balance
  - Elimination of waste products
  - Regulation of body temperature
  - Maintenance of electrolyte balance

- **Electrolytes:** These include sodium, potassium, calcium, bicarbonate and chloride. Electrolytes maintain the acid base balance of the body. Metabolic process not only produce heat but also a large amount of acids. These acids must be neutralized and eliminated from the body. Electrolytes and plasma proteins are pivotal in buffering and transportation of these toxic substances to the sites of excretion.

- **Proteins** constitute a large amount of dissolved substances in the plasma. Following are some of the major plasma proteins along with their functions:
  - Albumin: It maintains osmotic pressure. It is also a major carrier of drugs, trace elements, hormones and certain other substances e.g. unconjugated bilirubin.
  - Coagulation factors: Plasma contains coagulation factors that play a central role in the clotting of blood.
  - Globulins: They also contribute towards maintaining viscosity, peripheral resistance and hence blood pressure. They also act as a buffer. By combining with CO₂ they produce carbamino compounds which help in the transport of CO₂. They also act as general protein reserves that can be drawn upon as and when the need arises during starvation or extra protein requirements. Certain sub fractions of globulins perform
specific functions i.e. gammaglobulins: antibody fraction, β-globulin: carrier proteins i.e. transcobalamin, ceruloplasmin and haptoglobins.

Following are some of the additional functions of blood:

- Transport of waste products: Blood carries waste products in the body to the kidneys for excretion. Most important of these is urea which is produced from the breakdown of proteins.
- Transport of nutrients: Blood transports nutrients from the gastrointestinal tract to various tissues of the body. These nutrients include glucose, lipids, amino acids etc.
- Transport of hormones and other substances: Blood helps in the transport of hormones, iron, vitamins etc.
- Maintenance of blood pressure: This is maintained through regulation of heart rate and peripheral resistance via centers in the CNS. When blood pressure drops, heart rate increases and peripheral resistance also increases to maintain the blood pressure. The reverse occurs when blood pressure increases. This helps to maintain tissue perfusion.
- Maintenance of fluid balance: Plasma volume is maintained by osmotically active solutes such as sodium and chloride and oncotic pressure provided by plasma proteins especially albumin. Rise in plasma osmolarity stimulates the production of anti-diuretic hormone which causes retention of water.
BONE MARROW

Bone marrow is the soft pulpy tissue present in the bone. It is one of the largest organs of the body. Under physiological conditions it is the principal site of hematopoiesis after birth. Marrow cavity in the newborn is cellular both in the axial as well as in the peripheral skeleton for blood cells production. With the passage of time, the hematopoietic marrow recedes centripetally and at puberty it is mostly confined to the axial skeleton and in the proximal ends of femur and humerus. This is because in absolute terms the volume of marrow cavity increases from 1.4% of body weight at birth to 4.8% in the adult.

Structure of Bone Marrow

Bone marrow exists in the body in two forms; red marrow and yellow marrow.

Red marrow

Red marrow is composed of blood vessels, some fat cells (lipocytes) and large number of mature, immature and primitive hematopoietic cells. It is found mainly in flat bones i.e. hip bone, sternum, skull, ribs, vertebrae, scapula and in the cancellous material at the epiphyseal ends of the long bones (femur and humerus). It is hematopoietically active and is engaged in producing cellular components of blood i.e. red cells, white cells and platelets. Its red color is due to the presence of large number of blood vessels and red cells. Variable numbers of fat cells are also intimately mixed with hematopoietic cells. Their number varies with the age of the individual and the site of marrow sample. Fat cells are scanty in the red marrow of children. Their number gradually increases and at puberty the cell to fat ratio is 1:1. With the advancing age the lipocytes become more numerous and in the elderly, the cell to fat ratio of 3:7 is frequently observed and is considered normal.

Apart from the cellular components red marrow also contains stromal framework of reticulin fibers, nerve fibers and blood vessels etc.

Yellow or fatty marrow

Yellow marrow is the name given to that type of bone marrow which is not actively engaged in the formation of blood. As red cell production becomes restricted to the axial skeleton, marrow in the peripheral parts of the skeleton takes up yellow color. This color is due to the following factors;

- Most of the capillaries in the yellow marrow are closed and only a minimum number of these are patent to supply oxygen to the fatty marrow which has very low metabolic rate.
- Hematopoietic units of the bone marrow progressively become inactive because their function is not required by the body under physiological conditions. These cells do not disappear, they simply ‘hibernate’ and assume a different morphological appearance; they become lipocytes or fat cells.

Functions of Bone Marrow

Primary function of the bone marrow is to produce red cells, white cells and platelets. This procedure can be divided into three phases:

- Blood cells formation
- Cellular release
- Bone marrow reserves

Blood cells formation

All forms of blood cells are derived from a single hematopoietic stem cell called pluripotent hematopoietic stem cell. Each cell has to pass through various stages of division and
maturation in order to become a mature and functional blood cells i.e. stem cell, progenitor, precursor and mature cell.

Cellular release

In the bone marrow, cells are produced in the extravascular compartment. When mature, they enter the sinusoids through a "migration pore" in the endothelial cells of the sinusoid. Release of cells from the bone marrow is controlled by cellular maturity, cellular demand and bone marrow architecture.

Bone marrow reserves

The term bone marrow reserve stands for the ability and capacity of the bone marrow to meet the hematopoietic requirements of the body in times of stress. Normally, the daily loss of red cells, granulocytes and platelets from the circulation is balanced by their production in the bone marrow. Whenever there is an increased demand for any of these cells, marrow responds appropriately by delivering more of those cells in the peripheral blood. In the case of granulocytes, this is achieved by releasing the white cells already present in the marrow without necessarily increasing their rate of production. This is because marrow has a pool of granulocytes which can meet the increased requirements of the body for a certain period. In the case of platelets, there is no significant preformed pool, hence an increased demand is met by their increased production. In acute blood loss or after an acute intravascular hemolysis, marrow shifts its pool of reticulocytes into the peripheral blood to compensate for red cell loss. This response however is neither as marked not as efficient as with granulocytes.
HEMATOPOIESIS

Hematopoiesis describes the process of development of blood cells. It is derived from a Greek word i.e. haima (blood) and poiesis (formation). Hematopoiesis comprises of cellular differentiation, proliferation and maturation in the hematopoietic tissues of the bone marrow under the influence of growth factors and hormones.

In the earliest few weeks of intrauterine life, the blood cells are produced in the yolk sac where it starts by the 19th day (extra embryonically) to a maximum age of 11 weeks. The second organ to play a role in the hematopoiesis is liver where hematopoiesis begins in the 6th week and gradually increases. By the 11th week, liver is the major site of hematopoiesis. It plays this role until the 24th week of gestation. Hematopoiesis in liver then gradually falls off as the marrow takes over the role of blood forming organ. At birth the hepatic phase of blood cell production is nearly completely finished. Other sites of hematopoiesis of minor importance are spleen, thymus, lymph nodes, kidneys and the lymphoid and connective tissues of these minor sites.

All blood cells are derived from a common ancestral stem cell known as pluripotent hematopoietic stem cell (PHSC). PHSC is unique in nature having the capability of self-renewal which is the process of cell division into two daughter cells, one of which is committed to differentiate while the other remains in the stem cell pool. PHSC gives rise to multipotent stem cells which then differentiate into committed stem cells. These committed stem cells differentiate into myeloid stem cells (colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM)) and lymphoid stem cells (CFU-L).

Under the control of specific growth factors, colony forming unit becomes committed to one cell line and is termed as lineage specific committed progenitor cell. Each committed cell is named after the cell line to which it is committed i.e. CFU-E for colony forming unit of erythrocyte, CFU-G for granulocytic cell line, CFU-M for monocytic cell line and CFU-Meg for megakaryocytic cell line; which is the parent cell of platelets. Each cell produces morphologically recognizable maturing cells of their respective lineage. As these cells mature after a series of morphological and functional changes, their proliferative potential decreases or disappears. After maturation the cells are released in the blood stream to perform their specific functions. Diagrammatic representation of hematopoiesis is shown in figure 1.1.

Regulation of hematopoiesis

Hematopoiesis is regulated by certain growth factors and cytokines/ interleukins. Combination of cytokines stimulates the proliferation and/or differentiation of various hematopoietic cells. Bone marrow resident cells are the major source of hematopoietic cytokines. Table 1.1 summarizes various hematopoietic growth factors and cytokines, their source and their target cells.
Figure 1.1: Schematic representation of hematopoiesis

<table>
<thead>
<tr>
<th>Growth factors/Interleukins</th>
<th>Source</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythropoietin</td>
<td>Kidney, liver</td>
<td>Erythroid progenitors</td>
</tr>
<tr>
<td>STF</td>
<td>Fibroblasts</td>
<td>Stem cells</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>T cells, macrophages, endothelial cells, fibroblasts</td>
<td>Progenitors of neutrophils</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Macrophages, endothelial cells, fibroblasts</td>
<td>Stem cells, neutrophil progenitors</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophages, endothelial cells, fibroblasts, B cells, stromal cells</td>
<td>Progenitors of phagocytes</td>
</tr>
<tr>
<td>IL-1</td>
<td>Macrophages, endothelial cells, fibroblasts</td>
<td>Progenitors of phagocytes</td>
</tr>
<tr>
<td>IL-2</td>
<td>T cells, macrophages</td>
<td>T cells, B cells</td>
</tr>
<tr>
<td>IL-3</td>
<td>T cells</td>
<td>Precursors of granulocytes, monocytes and platelets</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>IL-4</td>
<td>T cells</td>
<td>B cells, T cells, mast cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>T cells</td>
<td>B cells, eosinophils</td>
</tr>
<tr>
<td>IL-6</td>
<td>T cells, macrophages</td>
<td>Stem cells, B cells</td>
</tr>
<tr>
<td>IL-7</td>
<td>Stromal cells</td>
<td>Pre B cells, T cells, early granulocytes</td>
</tr>
<tr>
<td>IL-8</td>
<td>Monocytes, T cells, fibroblasts</td>
<td>Neutrophils, basophils, T cells</td>
</tr>
<tr>
<td>IL-9</td>
<td>T cells</td>
<td>BFU-E, T cells, mast cells</td>
</tr>
<tr>
<td>IL-10</td>
<td>T cells, macrophages, B cells</td>
<td>B cells, macrophages, T cells, mast cells</td>
</tr>
<tr>
<td>IL-11</td>
<td>Stromal cells, fibroblasts</td>
<td>Megakaryocytes, B cells, mast cells</td>
</tr>
<tr>
<td>IL-12</td>
<td>B cells, macrophages</td>
<td>T cells, NK cells</td>
</tr>
</tbody>
</table>

**Table 1.1: Growth factors, their sources and target cells**

**ERYTHROPOIESIS**

The term erythropoiesis implies the production of red cells. This is a process of proliferation, differentiation and maturation of red cells under the action of erythropoietin and other growth factors. Normally the process of proliferation and maturation is completed within 3-5 days through 4 successive divisions known as maturational divisions. With each division, there is decrease in the cell size, condensation of the nuclear chromatin, loss of nucleoli, gradual decrease in the basophilia and progressive increase in pink color of the cell due to hemoglobin synthesis. Mature red cell has no nucleus, ribonucleic acid and mitochondria. Morphologically 6 stages of red cell development can be recognized as described below;

**Proerythroblast/Pronormoblast**

This is the earliest recognizable precursor cell of the erythroid series in the bone marrow (see figure 1.2). It is the largest cell of erythroid series with a diameter of 14 - 20 μm. It is usually round in shape. Chromatin pattern of the nucleus is open, finely stippled with one or more prominent nucleoli. A small, pale area known as perinuclear halo may be seen in the cytoplasm; this represents Golgi apparatus. A thin rim of deep blue cytoplasm contains ribosomes, mitochondria, centrioles and rough endoplasmic reticulum (RER). Ear like projections of the cytoplasm may be seen on Romanowsky's stained bone marrow smears.
Basophilic Erythroblast/Early Erythroblast

This is the second member of the erythrocyte generation and develops as a result of mitosis of the proerythroblast (see figure 1.3). The cell size is (12-17 μm) slightly smaller than the proerythroblast. Cell margins are smooth and regular while the cell shape is nearly round or slightly oval. Cell cytoplasm is intensely blue as reflected in its name “basophilic”. Cytoplasm is loaded with polyribosomes, rough endoplasmic reticulum (RER), mitochondria and has a prominent Golgi apparatus.

Nucleus is still quite large and nearly round and it occupies almost 80% of the cell. Its chromatin is still open indicating its activity in synthesizing m-RNA. The nucleoli are also prominent. Nucleus, though still quite open, starts to show a few focal areas of chromatin condensation (heterochromatin). These cells normally constitute about 2% of the total narrow cell population.

Polychromatophilic Erythroblast/Intermediate Erythroblast

This cell arises when basophilic erythroblasts (or early erythroblasts) undergo one mitotic division. Again the cell size becomes smaller and it measures 12-15 μm in diameter. The cell outlines are smooth and regular while the cell shape is nearly round. The nuclear size also becomes smaller and more of the open chromatin starts to change into heterochromatin (dense) indicating that the nuclear activity has also started to decline as the production of m-RNA decreases. Many coarse clumps of chromatin appear in the nucleus which now occupies 50% of the cytoplasm. The obvious change at this stage is the appearance of polychromatophilia in the
cytoplasm with corresponding reduction in the cytoplasmic basophilia as shown in figure 1.4. This is due to the appearance of hemoglobin in the cytoplasm. Polychromatophilic erythroblast (intermediate erythroblast) also undergoes one mitotic division and this is the last cell in the red cell series which is mitotically active.

Orthochromatic Erythroblast/Late or Pyknotic Erythroblast
This is the last stage in the development of the red cells which contains the nucleus. Cell size is much smaller as a result of mitotic division and sharing of the cytoplasm by the daughter cells. Cell boundaries are smooth and the cells are nearly round in shape. Nucleus is markedly pyknotic due to the condensation of the nuclear chromatin into heterochromatin reflecting the inactivity of the nucleus. Nucleoli have now disappeared. Cell cytoplasm is distinctly reddish in color because of hemoglobin which has affinity for eosin dye in Leishman’s stain. Cytoplasmic basophilia is diluted not only because of the presence of large amount of hemoglobin but also because of an actual reduction in the cytoplasmic organelles (RER, polyribosomes and mitochondria). Cell size is about 10 μm in diameter. This cell ultimately extrudes its nucleus and change into a reticulocyte. At this stage of development almost 80% of the final hemoglobin contents has been synthesized. Orthochromatic erythroblast is shown in figure 1.5.

Reticulocyte
After the eosinophilic erythroblast (orthochromatic/late erythroblast) loses its nucleus it becomes a reticulocyte. Reticulocyte is 10 μm in diameter and is slightly larger than the mature red cell as shown in figure 1.6. It is polychromatophilic in color due to the presence of eosinophilic staining of the hemoglobin and basophilic staining of some basophilic radicals (RER, polyribosomes etc.). This cell is capable of synthesizing hemoglobin which it does over 48-72 hours period in the marrow and in the spleen.

Cytoplasm of the reticulocytes contains a few iron granules (siderosomes or pappenheimer bodies), remnants of the nucleus (Howell-jolly bodies), RER, polyribosomes, mitochondria and fragmented Golgi apparatus. These can be demonstrated as reticulum with reticulocyte stain. Reticulocyte matures into RBC in about 48 hours.
Mature Erythrocyte

A mature red cell is approximately 8 μm in diameter. It is non-nucleated and contains the full complement of hemoglobin. It is incapable of synthesizing hemoglobin either in vivo or in vitro. It does not contain any of the cytoplasmic organelles.

It is in the shape of a pliable biconcave disc, a shape which is ideally suited to squeeze through the microcirculation in the body. Because of its shape and the surface characteristics it is best suited to carry on its function as a vehicle for the uptake, transport and delivery of oxygen. Mature red cells are shown in figure 1.7.
Erythropoiesis is controlled by two types of growth factors;

- Locally produced in bone marrow: IL-1, GM-CSF
  These two growth factors are produced by the endothelial cells, fibroblasts and monocytes in the marrow. These factors result in increased recruitment of stem cells to their respective committed cell lines.

- Produced at distant site: Erythropoietin (EPO)
  EPO, a glycoprotein hormone encoded by a gene on the long arm of chromosome has a molecular of 30400D. It has a plasma half-life of 6-9 hours. Normally 90% of the hormone is produced in the kidneys while 10 % is produced in the liver. In embryonic life it is expressed largely by the liver cells. There are no preformed stores in the kidneys. It is produced by tissue hypoxia, a mechanism that may involve the initial hypoxia mediated activation of heme proteins which sense oxygen need and then trigger the synthesis of EPO which is released into blood stream.

**Physiological Variations in the Red Cell Count**

Diurnal variations are an important cause of changes in the red cell count in normal healthy individuals. Red cell count is at its lowest during sleep (horizontal position when the blood volume is at its maximum). It is highest in the late evening when some fluid has extravasated into the tissues as a result of increased hydrostatic pressure at tissue level in upright position during day time. This diurnal variation is therefore a reflection of the changes in the plasma volume which inversely affect the red cell count. The change usually amounts to less than 5% only and is therefore of no physiological consequence.

Exercise also produces slight erythrocytosis as a result of the movement of fluid out of the vascular space and also due to fluid loss through perspiration. Any physiological condition that causes excessive fluid loss and consequent hemoconcentration can produce mild erythrocytosis.

Altitude has an important bearing on red cell count. Under physiological conditions red cell count at high altitude is higher than at sea level. This is because the atmospheric PO$_2$ progressively decreases as the altitude increases.

Age and sex also influence the red cell count. Prior to menopause the red cell count is usually lower in females than in males. After the menopause this difference though still discernible, is insignificant.

**Biological Advantage of the Biconcave Shape of Red Cell**

Shape of the red cells under physiological conditions is eminently suited to enable them to squeeze through the microcirculation. Angular, spherical or even the oval shape does not confer this advantage on the red cells.

Biconcave shape of the red cells allows considerable alteration in the cell volume without any appreciable change in intra-erythrocytic pressure. This is because the concavity can readily balloon out or move in as the cell volume increases or decreases. Cell volume repeatedly increases and decreases as red cells move from the arterial to the venous side of the circulation. As red cells enter the venous circulation, their volume increases by 7.5%. This is brought about by chloride shift which results in an increase in the intra-erythrocytic osmotic pressure and subsequent movement of water into the red cells. It is for this reason that red cells are slightly larger in the venous blood than in the arterial blood and the venous hematocrit is slightly higher.
than the arterial hematocrit. To determine total body hematocrit, venous hematocrit is therefore multiplied by 0.91 to obtain the true body hematocrit.

Another important advantage of the biconcave shape of the red cells is the distribution of hemoglobin in the red cells as a thin layer which is only a few molecules thick. This facilitates the exchange of gases in the peripheral circulation as well as in the lungs.

Factors that maintain the normal biconcave shape of the red cells are:
- Sub membranous cytoskeleton
- Amount and nature of the membrane lipids
- Plasmatic environment of red cells
- Na–K ATP pump

Activity of sodium–potassium pump is dependent upon the amount of ATP generated in the glycolytic and Embden Myerhoff pathways. Any abnormality in these pathways that alters the supply of ATP can adversely affect the shape of the red cells. Deficiency of the enzymes in the EM pathway (Hexokinase, phospho-fructokinase, pyruvate kinase and triose phosphate isomerase) is known to produce changes in the red cell shape.

Suggested further reading
SYNTHESIS OF HEME AND GLOBIN

Heme is an integral part of hemoglobin molecule. It is synthesized from two basic units called glycine and succinate through various enzymatic and non-enzymatic steps. Heme is essential not only for the synthesis of hemoglobin; it is also an integral part of other heme containing compounds like myoglobin, catalase, cytochrome and microsomal P-450 system.

There are seven well defined steps that are involved in the synthesis of heme. Some of them take place in the mitochondria while others are extra-mitochondrial; i.e. they are completed in the cell sap (cell sap is the fluid part of the cytoplasm without any cytoplasmic organelles).

As mentioned above a large number of enzymes participate in this highly complex process. These enzymes and the steps at which they act are shown in figure 2.1.

Formation of δ-ALA

The two building blocks of heme are glycine and succinate. Glycine is an amino acid which is obtained from body’s amino acid pool. Before glycine can complex with succinate, it must be converted into Schiff’s base which is formed when glycine combines with pyridoxal phosphate. The later compound is produced when a phosphate radical is added to pyridoxine (vitamin B₆) under the influence of an enzyme called pyridoxal phosphokinase.

Succinate is obtained from succinic acid which is produced in Kreb’s cycle. In the first instance, succinate combines with Co-enzyme A and forms succinyl Co-A. This in turn combines with Shiff’s base and produces an intermediate compound called α-amino β-keto adipic acids (AKA). This is quickly converted to δ-ALA through a non-enzymatic reaction. This step takes place in the mitochondria. ALA comes out of the mitochondria and the next 3 steps are completed in the cell sap.
Figure 2.1: Pathways of heme synthesis
**Formation of porphobilinogen**

Two molecules of δ-ALA condense to form a ring structure called porphobilinogen. This is an enzymatic reaction in which an enzyme δ-ALA dehydrase participates. Under the influence of an enzyme porphobilinogen deaminase, 4 molecules of porphobilinogen combine to form a tetrapyrrole ring compound called hydroxymethylbilane.

**Formation of uroporphyrinogen I**

Uroporphyrinogen cosynthase acts of hydroxymethylbilane resulting in the formation of either uroporphyrinogen I or uroporphyrinogen III. Uroporphyrinogen I is useless for the purpose of heme synthesis. Uroporphyrinogen III is required for home synthesis. UPG I is excreted through liver and kidneys.

**Formation of coproporphyrinogen III**

This conversion also takes place in the cell sap; the enzyme involved is UPG decarboxylase.

**Formation of protoporphyrinogen IX**

This is a mitochondrial step. Coproporphyrinogen produced in the cytosol migrates back to the mitochondria where, in the presence of oxygen and under the influence of an enzyme coproporphyrinogen oxidase, protoporphyrinogen IX is produced. This is an important intermediary compound in the biosynthesis of protoporphyrin IX which serves as the substrate in the ultimate step in heme synthesis.

**Formation of protoporphyrin IX**

Protoporphyrinogen oxidase catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX. There are a large number of protoporphyrins which exist in nature. These have been serially numbered using Greek numerals. In Man, it is the protoporphyrin IX which participates in the synthesis of heme.

Formation of protoporphyrin IX sets the stage for the ultimate step in heme synthesis i.e. incorporation of iron in the protoporphyrin IX molecule. This reaction takes place in the cristae of the mitochondria and is catalyzed by the enzyme heme synthetase (also known as ferrochelatase). Only the ferrous form of iron is utilized for the formation of heme molecule.

It is postulated that pyridoxal 5-phosphate also participates as a co-enzymes in the last step as it does in the first step of heme synthesis. Pyridoxin deficiency may thus produce anemia by interfering with heme synthesis at two steps.

Heme, once formed, comes out of the mitochondria into the cell sap where it is attached to the globin chains which are synthesized in the cytoplasm. This completes the synthesis of hemoglobin molecule.

**GLOBIN BIOSYNTHESIS**

Globin fraction of hemoglobin molecule accounts for 97% of the weight of molecule. Under normal circumstances about 8 grams of globin is synthesized in the body per day and its production takes priority over the synthesis of all other proteins in the body. Synthesis of globin chains is carried out separately from heme synthesis; however there is a reciprocal feedback mechanism for the regulation of heme and globin synthesis.

Globin fraction of normal human hemoglobin is composed of large number of amino acids. These are arranged in four polypeptide chains which are grouped in two pairs, which differ from each other in number and sequence of their amino acids.
Each globin chain has two ends; one of them is called the N-terminal end (N stands for nitrogen and signifies the presence of an amino acid at this end). This is the point where globin synthesis begins. After the appropriate numbers of amino acids have been added, the synthesis of globin chain is discontinued. This point is called the C-terminal end (C stands for carbohydrates); at this point carbohydrate is added to the chain and the chain is completed.

Cells, which synthesize globin, have in their cytoplasm certain regulatory factors that control the synthesis of globin chain and are called the initiation factors. Other factors control the growth of the chains and are called the elongation factor while a third factor stops further lengthening of the chain and is called the termination factor.

The ultimate control for globin synthesis (as for other functions and features of life) resides in the double stranded DNA macromolecule which morphologically constitutes a chromosome. Genes for the synthesis of α and α like polypeptide chains reside on chromosome No.16, while those for non-α chains are located on chromosome No.11.

Synthesis of each globin chain is completed in less than 5 minutes. The α and the non-α chains have separate genes and separate m-RNA and are synthesized separately. Once the globin chain synthesis is completed, it is released in the cytoplasm. These chains become associated with heme and form dimmers of α/β chains. These combine with other dimmers to produce tetramers of α2, β2 and the hemoglobin synthesis is completed.

**STRUCTURE OF GLOBIN CHAINS**

The polypeptide chain in the hemoglobin molecule differs from one another in amino acid sequence. The α chain contain 141 amino acids and the non α chains 146. The δ chain differs from β chain in only 10 of the 146 amino acid residues, whereas the γ chain and β chain differ by only 39 amino acids.

**TYPES OF HEMOGLOBIN**

In the red cells of normal adult individual there are three types of hemoglobin. Whereas them heme component is exactly alike, they differ from each other in the composition (not the number) of globin chains; each type of hemoglobin must and does have its full component of globin chains arranged in two pairs as shown below:

- Hemoglobin A (96%) = α2 β2
- Hemoglobin A2 (2.5%) = α2 δ2
- Hemoglobin F (1.5%) = α2 γ2

**Suggested further reading**

METABOLISM AND FATE OF RED CELL

Metabolism of red cells is limited as they lack mitochondria. Transportation of gases, a major function of red cells, does not require energy. However, red cells viability needs a variety of energy dependent metabolic processes. Energy is required for the following important functions of red cells:

- Running of cationic pump that maintains the osmotic balance of red cells by ensuring high intracellular K⁺, low Na⁺ and a very low intracellular Ca²⁺
- Maintaining high level of reduced glutathione
- Keeping the hemoglobin in reduced form
- Maintenance of membrane integrity and deformability by ensuring proper lipid turnover and phosphorylation of membrane proteins

Metabolic pathways of red cells require glucose which is broken down through several routes to produce ATP, NADH and NADPH. These pathways include;

- Embden-Meyerhof pathway
- Hexose-monophosphate shunt
- Methemoglobin reductase pathway
- Rapoport-Luebering shunt

Embden-Meyerhof Pathway

Red cells obtain energy almost exclusively through the anaerobic breakdown of glucose i.e. glycolysis through Embden-Meyerhoff (EM) pathway. Mature RBCs lack mitochondria for oxidative metabolism. Glycolysis is illustrated in figure 3.1. Before splitting a molecule of glucose, it is first activated by ATP. Breakdown of one glucose molecule via EM pathway provides two molecules of ATP and two molecules of NADH.

Intracellular cations, Na⁺, K⁺, Ca²⁺ and Mg²⁺ are important regulators of red cell shape, flexibility and membrane integrity. Increased intracellular calcium leads to excessive loss of potassium from the cell leading to rigid and shrunken red cell membrane. Red cells require large amount of ATP to maintain normal concentration of these cations. Abnormal permeable membrane or decreased production of ATP results in increased osmotic fragility. Exhaustion of glycolytic pathway leads to imbalance of these cations resulting in excessive red cell destruction.
Figure 3.1: Red cell metabolic pathways
Hexose Monophosphate Shunt (HMP Shunt)

Small amount of glucose (5-10%) is metabolized by HMP shunt or pentose phosphate pathway. This pathway produces NADPH from NADP⁺ as summarized in figure 3.1. Glucose-6-phosphate is converted into pentose ribulose 5-phosphate by glucose-6-phosphate dehydrogenase. NADPH thus produced is of vital importance for the integrity of red cell membrane. NADPH is utilized for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH).

Oxidants are produced in the red cells during various metabolic processes of red cells. Reducing potential of the red cells is required for detoxification of the oxidants, reduction of methemoglobin and inhibition of membrane lipid oxidation. GSH protects red cells from this oxidative injury.

Methemoglobin produces highly reactive oxygen radicals (superoxide and hydroxyl ions), hydrogen peroxide which oxidize hemoglobin’s sulfhydryl (-SH) groups and forms oxidized glutathione (GSSG). This is reduced back to GSH by adequate levels of NADPH. Failure of NADPH production results in the denaturation and precipitation of hemoglobin in the form of Heinz bodies due to oxidized SH groups. Heinz bodies are removed in the spleen by macrophages resulting in the lysis of the red cells.

Methemoglobin reductase pathway

Oxidation of the ferrous iron (Fe⁺⁺) in the heme molecule to ferric form (Fe⁺⁺⁺) results in the formation of methemoglobin. Methemoglobin is incapable of transporting oxygen. Small quantities of methemoglobin (about 2%) are formed constantly within the red cells. Red cells have efficient mechanism i.e. methemoglobin reductase pathway to minimize the accumulation of methemoglobin.

With the help of NADH, methemoglobin reductase pathway protects ferrous iron from oxidation as illustrated in figure 3.1. Excessive formation of methemoglobin adversely affects the oxygen carrying capacity of hemoglobin.

Rapoport-Leubering Pathway

Oxygen affinity of hemoglobin is regulated by Rapoport-Leubering pathway that works in conjunction with EM pathway and provides the necessary amount of 2,3-diphosphoglycerate (2,3-DPG). This pathway bypasses the formation of 3-phosphoglycerate and ATP from 1,3-diphosphoglycerate (1,3-DPG). Instead, 1,3-DPG forms 2,3-DPG; this is catalyzed by an enzyme DPG mutase and DPG synthase. In deoxygenated state, hemoglobin binds 2,3-DPG in a 1:1 ratio leading to the release of oxygen from the hemoglobin.

RED CELL MEMBRANE

Amongst all other cells in the body, red cells are unique as they spend their life span of nearly 120 days in constant motion. Though confined to the vascular terrain, they are repeatedly subjected to such adverse environments as hypertonic renal medulla, hypoxic splenic cords and watchful hepatic sinusoids. Extremes of blood pressure in aorta on the one hand and cutaneous micro vessels on the other, subject them to the most testing internal milieu. ‘Resting’ diameter of normal red cells denies their negotiation through microvasculature unless they are amenable to considerable morphological distortion and deformation. The very high intra-erythrocytic oxygen tension is an additional threat to the oxidation of their most valuable assets. Also the red cells must maintain a critical electrolyte gradient on either side of the red cell membrane. These pre-requisites are eminently met by the peculiar and unique composition of the red cell membrane.
Structure of the Red Cell Membrane

Red cell membrane, unlike all other biological membranes has two laminae; the outer lipid layer and the inner protein skeleton. The lipid layer conforms to the structure of other cell membranes in the body. It consists of phospholipids, cholesterol and proteins arranged in a highly organized and complex fashion. Because of the ever migratory character of the red cells, this membrane is supported on its inner aspect by another lamina called the membrane skeleton. This provides strength, deformability and plasticity to the red cells. These characteristics are essential for the unique ‘life style’ of the red cells. It enables them to undergo shape changes in response to their surroundings and to squeeze through the narrow straits of microcirculation. It also ensures their successful negotiation through the testing and adverse splenic environments.

Biochemical composition of the red cell membrane has been well characterized and the structural organization of its constituents has been delineated. Biochemically red cell membrane is composed of proteins (52%), lipids (40%) and carbohydrates (8%).

Membrane Lipids

Phospholipids and un-esterified cholesterol are in an equimolar quantities. Distribution of the membrane lipids is highly characteristic; 75% of the uncharged, phospholipids i.e. phosphatidyl choline and sphingomyelin are present in the outer lamella while 80% of phosphatidyl ethanolamine and 100% of phosphatidyl serine (which are the charged phospholipids) are present in the inner lamina. Unlike phospholipids, cholesterol freely diffuses through the full thickness of the red cell membrane. It may be viewed as ‘packing material’ for other membrane lipids. Sphingomyelin (which makes the membrane rigid) and lecithin (which makes the membrane pliable) are both present in the outer lamina. This maintains a balance between membrane rigidity and resilience.

Red Cell Membrane Proteins

Large numbers of proteins are present in the red cell membrane. Some of these have been identified and characterized while others still await their precise identification. Red cell membrane proteins are divided into two groups; integral proteins and peripheral proteins.

Integral proteins

These proteins span the full thickness of the lipid bilayer to which they are firmly anchored through lateral hydrophobic bonds. They display distinct structural and functional domains both within their intra-membrane part as well as on either side of the lipid bilayer. Since they open on both sides of the membrane, they are also called channel proteins. They perform the important function of transporting material in and out of the red cells. Examples of integral proteins are band 3 and glycophorins.

Band 3 protein, also known as the anion exchanger, constitutes about 25% of the total membrane proteins with a molecular weight of 96kDa. It contains 911 amino acids and exists as homodimers. There are 100,000 molecules of Band 3 protein in each red cell. Its synthesis is controlled by a gene which resides on chromosome no 17. Band 3 protein performs two well defined and important functions:

- It acts as an anion channel for the red cell membrane.
- It links lipid bilayer to the underlying protein skeleton through its association with ankyrin which is a peripheral membrane protein. It also binds indirectly with other membrane proteins called protein 4.1 and 4.2.

Other integral proteins are a group of four closely related proteins called glycophorins A, B, C and D. They are rich in sialic acid hence the name glycophorins. Though they account for
only 2% of the membrane proteins, their carbohydrate content imparts a strong negative charge to the surface of the red cells. This is functionally important because it;

- prevents inter-erythrocytic adhesion
- keeps red cells away from the vascular endothelium
- keeps red cells at a distance from other cells

**Peripheral proteins**

Also known as the skeletal proteins, they from intricate fibrous meshwork which is ‘plastered’ along the ‘under surface’ of the lipid bilayer. The four major proteins in this group are: spectrin, actin, protein 4.1 and ankyrin (band 2.1).

**Spectrin**

Spectrin is the major component of the peripheral proteins. It is a fibrous protein which constitutes the backbone of the red cell membrane skeleton. Total copies of spectrin molecules per red cell are 200,000. Structurally it consists of two intertwined polypeptide chains called α and β chains. Gene that regulates the synthesis of α-spectrin is located on chromosome No 14 while the one which governs the synthesis of β-spectrin is located on chromosome No 1.

Intertwining of α and β chains produces heterodimers (dimers produced as a result of pairing of two non-identical chains) which polymerize to form tetramers and higher order oligomers. They produce an intricate and continuous meshwork along the cytoplasmic aspect of the lipid bilayer. They are linked 'head to head' with each other to form tetramers which further polymerize to form oligomers. 'Cross-linking' of the spectrin chains is achieved through a protein called actin. Spectrin meshwork is attached near the head or the cephalic end to the overlying phospholipid layer through the intermediation of a membrane protein called ankyrin. The other point of attachment of spectrin to the phospholipid lamina is a connection between spectrin and glycophorin-C through a protein called protein 4.1. This ensures flexibility, deformability and maintenance of the biconcave shape of the red cells.

**Ankyrin (band 2.1)**

This protein binds with the β-chain of spectrin on one hand and with a channel protein called Band 3 on the other. Spectrin cannot be incorporated into cytoskeleton if there is deficiency of ankyrin. This may lead to increased spectrin degradation. Gene which regulates the synthesis of ankyrin is located on chromosome no 8.

**Protein 4.1**

It is attached to phosphatidyl serine which is a membrane lipid. Its major contribution is to strengthen and stabilize the spectrin-actin complexes. Gene for this protein is located on chromosome no 8.

**Actin**

One oligomer (12 monomers) of actin combines with the tails of six spectrin chains. Protein 4.1 stabilizes the spectrin-actin complex on the one hand and binds with glycophorin C on the other.
Figure 3.2: Diagramatic representation of red cell membrane

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DEGRADATION OF HEMOGLOBIN

Under physiological conditions destruction of red cells takes place extravascularly in the reticuloendothelial system (RES). Sometimes, as a result of changes in the environments, there is intravascular destruction of red cells with consequent release of hemoglobin in the circulating stream. Disposal of hemoglobin under these two circumstances is quite different.

Hemoglobin, which is released within the reticuloendothelial cells, is split into its components protoporphyrin, iron, and globin. Protoporphyrin, through various degradation steps is converted into unconjugated bilirubin which is released from the reticuloendothelial cells into the circulation as shown is figure 3.2. There it binds with albumin to be transported to the liver for conjugation within the hepatocytes and ultimate excretion from the body. Liver has an extensive capacity to conjugate bilirubin. Approximately 40% of the unconjugated bilirubin delivered to the liver regurgitates back into the plasma. This does not reflect any inadequacy of the liver in conjugating and handling the workload; it only represents a normal physiological phenomenon. It is for this reason that there is unconjugated hyperbilirubinemia in cases of accelerated extravascular destruction of the red cells.

Most of the iron is delivered to the circulation to be transported to the bone marrow for utilization in the synthesis of hemoglobin. Some iron is retained in the reticuloendothelial cells as hemosiderin and ferritin to become part of the iron storage pool of the body. Globin enters into the body's general metabolic pool of proteins.

In conditions of intravascular hemolysis as exemplified by malfunctioning cardiac prosthetic valves, paroxysmal nocturnal hemoglobinuria and microangiopathic hemolytic anemia, free hemoglobin is released in the circulation. Mechanism responsible for the disposal of this hemoglobin is quite different.

In this instance, free hemoglobin is coupled on a 1 to 1 basis with a hemoglobin binding protein called haptoglobin. This protein is synthesized in the liver and has a high affinity for hemoglobin. As a result of the formation of hemoglobin-haptoglobin complex, the level of free plasma haptoglobin is decreased. On serum protein electrophoresis a new band separating hemoglobin-haptoglobin complex appears. Haptoglobin decreases not only in intravascular hemolysis but also in accelerated extravascular hemolysis as in hereditary spherocytosis and other congenital hemolytic anemias. This is because some of the intracellular hemoglobin under these circumstances, leaks out of the reticuloendothelial cells and gets bound to haptoglobins to lower its level in the plasma. It may be appreciated however that plasma haptoglobin levels are also decreased in certain non-hematological systemic disorders especially in liver disease. On the other hand, there are certain conditions in which plasma level of haptoglobins is raised. In order to interpret the significance of plasma haptoglobin levels in relation to intravascular hemolysis it is therefore necessary to take into consideration those conditions which alter the haptoglobin level independently.
Suggested further reading
IRON, FOLATE AND COBALAMIN METABOLISM

IRON METABOLISM
Iron is an essential constituent of hemoglobin, myoglobin and various enzymes in the body. Enzymes that contain iron include catalases, peroxidases, oxidases and cytochromes.

Sources of Iron
Dietary ingredients that are rich in iron include meat (particularly the red meat), apple, pear, date and spinach. Cow’s milk is a poor source of iron. One liter of milk contains a maximum of 1mg of iron. Cereals also have very low iron content.

Daily Dietary Requirements
Supply of iron to the body is regulated at the level of absorption. Any iron which gets absorbed and enters the body’s metabolic pool of iron cannot leave the body except through the obligatory loss or when external bleeding takes place. Under physiological conditions a maximum of 10 % of the dietary iron is absorbed. This implies that a balanced diet must contain at least 15-20 mg of iron in order to enable the absorption of 1.5-2 mg of iron and ensure its homeostasis.

Iron Absorption
Body iron is regulated at the level of absorption from the gut rather than its excretion. In order to understand the absorption of iron from the gastrointestinal tract, it will be necessary to categorize food iron into two types:

- Meat iron (Heme-iron)
- Non-meat iron (Non-heme iron)

Absorption of meat (heme) iron
Iron in the meat is present mostly in the hemoglobin and myoglobin where it is attached with the heme moiety of the hemoglobin and myoglobin molecules. This iron once released from the protein molecule, is absorbed without any further processing in the mucosa. Factors which influence the absorption of non-heme iron have no significant effect in the absorption of heme-iron. It is for this reason that meat is the best source of iron. Cost however is the limiting factor; to obtain 3mg of iron it will be necessary to consume 3 ounces of meat every day which is not feasible for most families.

Absorption of non-meat (non-heme) iron
Non-heme iron is present in all food articles other than meat. Iron in these substances is in ferric form which is poorly absorbed. In order to facilitate iron absorption it must be reduced to ferrous form. Not only that ferric iron has to be reduced to ferrous form, it must be kept in this state for as long as possible to facilitate its absorption. To achieve this objective, hydrochloric acid in the gastric juice, vitamin C and other reducing substances in the diet play an important role.

Among other factors which determine iron absorption is the composition of the diet. Sulphates, phosphates and organic radicals like phytates impair iron absorption by complexing...
with non-heme iron in the diet. Factors that decrease iron absorption are composition diet, iron stores of body and rate of erythropoiesis.

Besides local factors, there are two systemic factors which regulate the absorption of iron from the gut; these are:

- Iron stores in the body

Iron stores in the body exert a strong influence on iron absorption from the gut, through a feedback mechanism mediated by plasma ferritin which is in equilibrium with the storage ferritin. Increased iron stores accompanied by a high % saturation of transferrin and increased plasma ferritin level have an inhibitory effect on iron absorption. Conversely, reduced or absent iron stores in conjunction with a low % saturation of transferrin and decreased plasma ferritin level facilitate iron absorption. Hence the amount of storage iron and absorption of iron from the gut are inter-dependent. This is exemplified by increased iron absorption in iron deficiency anemia and reduced iron absorption in transfusion siderosis.

- Rate of erythropoiesis

Rate of erythropoiesis exerts a much stronger influence on iron absorption than storage iron. Conditions which are associated with increased iron stores and erythroid hyperplasia in the bone marrow are characterized by increased iron absorption as seen in thalassemia major. Conversely when erythropoiesis is suppressed and the iron stores are also reduced, there is a net reduction in iron absorption. This is seen in anemia of chronic renal failure.

**Transport of Iron in Blood**

Iron throughout its existence in the body has to be complexed with a carrier protein in order to transport it safely and to protect the body from the ill effects of free or elemental iron. Carrier protein in plasma for iron is transferrin. Transferrin is synthesized in the liver and secreted in the blood as well as in the bile. In the blood it transports iron from the gastrointestinal tract to the liver and to other sites, its biggest customer being the bone marrow. Transferrin has two binding sites for two atoms of iron. A molecule of transferrin either carries two atoms and is completely saturated or it does not carry any iron and is completely unsaturated. There are no partially saturated molecules of transferrin.

**Body’s Iron Stores**

Total amount of iron in the body of an adult male is 4g. While in an adult female it is approximately 3-3.5g because of a lower hemoglobin level, lesser amount of storage iron and concomitant iron loss due to their reproductive functions. In term of body weight, it amounts to 50mg/kg body weight in normal adult men while in women it is 35 mg/kg body weight. Iron in the body is compartmentalized in table 4.1.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Grams</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>2.50</td>
<td>65</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>0.15</td>
<td>4</td>
</tr>
<tr>
<td>Storage</td>
<td>1.50</td>
<td>30</td>
</tr>
<tr>
<td>Transport</td>
<td>0.003</td>
<td>0.1</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.008</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Table 4.1: Distribution of iron in the body*
Storage sites of iron are bone marrow, liver, spleen and other parts of the reticuloendothelial system. Of these, the bone marrow and the liver take up lion’s share which is distributed in almost equal amounts between these two organs. Iron is stored in the body in two forms; hemosiderin and ferritin. Hemosiderin usually accounts for 50 % of the storage iron while ferritin accounts for the other half. Transport iron (serum iron), ferritin and hemosiderin are in equilibrium with each other as shown below;

\[
\text{Serum iron} \quad \xrightarrow{\text{Ferritin}} \quad \text{Hemosiderin}
\]

**Daily Excretion of Iron**
Small amount of iron is lost from the body under all conditions and in all individuals. This basal loss is called the obligatory loss. In adults this amounts to 1-2 mg per day, in children it is proportionately less. Obligatory iron loss is the result of desquamation of epithelial cells from the skin, GIT and urogenital tract. Body’s secretions like saliva, intestinal and biliary juices also contain minute amount of iron. Similarly small amount of iron is lost through sweat and urine. In women, during their reproductive period, there is an additional loss of 1mg of iron per day. While lactating, an average mother produces one liter of milk every day; this contains 1mg of iron. Lactation therefore causes an-extra loss of approximately 1mg of iron per day. Pregnancy extracts its own share of iron from the maternal iron stores. During one menstrual cycle, an average of 60 ml of blood is lost. Since 2ml of blood contains 1mg of iron, this amounts to a loss of 30 mg of iron each month; spread over 30 days, it amounts to 1mg of iron loss per day.

**FOLATE (FOLIC ACID)**
Folic acid is a water soluble yellow colored substance. It is the parent compound of a large number of substances which together constitute the folate family.

**Dietary Sources of Folate**
Folate is abundant in the vegetable kingdom. Green leafy vegetables are especially rich in folate as they synthesize it in large amounts. Spinach, lettuce, beans and asparagus are rich in folate. Amongst the fruits bananas, lemons and melons contain large amount of folate.

Animal kingdom does not constitute a significant source of folate supply to Man. Liver, being the storage site and kidneys being the excretory organs for folate are the two exceptions. A large proportion of hepatic and renal folate is however destroyed during cooking. Yeast and mushrooms are also a good source of folate while meat, fish, eggs, milk and poultry provide very little folate.

**Daily Dietary Requirement of Folate**
Daily dietary requirement of folate varies with the age and sex of individual. In an adult male it is 150 µg while in children below the age of 5 years the requirement is 500 µg per day. In pregnancy a minimum daily intake of 500 µg is recommended to meet the maternal as well as fetal demands.

During lactation, the dietary folate requirement is also high though it is not to the level required in pregnancy. It is recommended that daily intake of 300 µg of folic acid be maintained throughout lactation.

**Absorption of Dietary Folate**
Folate is mainly absorbed in the jejunum especially in its proximal part. Folate in the diet, as mentioned below is in the form of pteroyl polyglutamates. These have to be converted to monoglutamates prior to absorption. This is achieved in a step wise fashion by the enzymes deconjugases. Exact location of these enzymes is not clear.
Transport of Folate
Folate is transported from the gut through the portal system to the liver. Most of it is present in the form of methyl-tetrahydrofolic acid (CH$_3$H$_4$F). It is postulated that there is a carrier protein which carries folate to the liver. Folate in the portal vein is present mostly as CH$_3$H$_4$F; a small amount is present as H$_2$F.

Storage Site for Folate
Receptors are present on almost all tissues of the body. Every cell contains significant amount of folylpolyglutamate. In the liver, hepatocytes store most of the folate.

Excretion of Folate
Folate is excreted from the body through the kidneys and gut. Large quantities of folate are excreted via bile into the duodenum. Fortunately most of it is reabsorbed and re-enters portal circulation to be re-incorporated into body's metabolic pool. If it were not for the enterohepatic circulation, loss of folate through the biliary passages would be so substantial as to cause critical reduction in plasma folate.

Cannulation of biliary passages for only 6 hours causes 30% reduction in plasma folate level. Because of the enterohepatic circulation, biliary tree is an unimportant route for the net excretion of folate. Under physiological conditions kidneys are the major route of folate loss from the body.

Role of Intestinal Flora in Folate Homeostasis
Bacterial flora in the small intestine in animals synthesizes folate. This is available to the host in return for their share in the dietary cobalamin of the host.

COBALAMIN (VITAMIN B$_{12}$)
Cobalamin in the diet is derived from the animal sources; meat, fish, poultry, sea food, eggs etc are rich sources of cobalamin. Plant kingdom contributes very little, if at all, towards cobalamin supply in Man.

A balanced diet contains approximately 15 µg (5-30 µg) of cobalamin. Of this, about 20% is absorbed while the rest is excreted in the feces. The absorbed amount is sufficient to maintain normal plasma cobalamin level, to keep cobalamin stores replenished and to ensure cobalamin homeostasis.

Daily Body’s Requirement of Cobalamin
In a healthy young adult, about 3-5 µg of cobalamin is required per day for the daily body functions. Enough is present in an average diet to supply the requisite amount. It may be stressed that the daily dietary requirements and the daily body’s requirements are two distinct and independent parameters and clear distinction must be made between them.

Absorption of Dietary Cobalamin
Dietary cobalamin is complexed with food proteins. The first step is the liberation of cobalamin. This is achieved through the action of hydrochloric acid. In the absence of hydrochloric acid dietary cobalamin cannot be released. Any cobalamin which remains bound to the dietary proteins cannot be absorbed. Lack of hydrochloric acid is a major factor that causes reduced cobalamin absorption.
Once released, dietary cobalamin binds with a carrier protein called intrinsic factor (IF). Association of cobalamin with gastric intrinsic factor is a prerequisite for its transport to the site of absorption. Deficiency of intrinsic factor for any reason (gastric resection, pernicious anemia or a congenital defect) also precludes cobalamin absorption and produces cobalamin deficiency and macrocytic anemia.

Besides intrinsic factor, there are certain other substances in the gastric juice which bind cobalamin. These are called R-binders. In the upper small intestine there are enzymes (proteases) which liberate cobalamin from these binders and make it available for its association with intrinsic factor.

IF-cobalamin complex, as it reaches the terminal one or two feet of ileum, meets the ileal receptors, essential for the absorption of cobalamin, which are actually the receptors for the intrinsic factor. Since cobalamin is tightly bound to the intrinsic factor it also gets attached to the receptors as a ‘joy-rider’. As the receptors (which project from the surface of the cells) are internalized, whole of the IF-cobalamin complex enters into the cells.

Inside the cell, cobalamin molecule is liberated from the complex, moves to the serosal surface of the cell and is handed over to transcobalamin in the blood in the periserosal capillaries. Fate of the intrinsic factor is not known; it is either degraded inside the cell or it is extruded from the cell when the receptors move in the opposite direction and are externalized once again. From the foregoing it may be construed that a large number of conditions may cause impairment of cobalamin absorption.

**Stores of Cobalamin in the Body**

In a healthy adult, cobalamin stores amount to 3-5 mg. Of this 1 mg is in the kidneys. Hence liver and kidneys are not only rich sources of folate, they also furnish substantial amount of cobalamin because they are also the storage sites for cobalamin.

**Daily Loss of Cobalamin**

Daily excretion of cobalamin is determined by the body’s cobalamin stores. Since body's cobalamin stores usually amount to 3-5 mg while daily loss is 3-5 µg; this is easily replenished by the average diet.

**Suggested further reading**

PHYSIOLOGY OF LEUKOCYTES
Leukocyte or white blood cell is a catchall name given to various nucleated cells present in the peripheral blood. Leukocytes are divided into neutrophils, eosinophils, basophils, lymphocytes and monocytes. Each cell has different origin, structure and performs unique function. Leukocytes are the major components of immune system. They protect the body against invasive extrinsic pathogens. They also remove neoplastic and other abnormal cells.

Leukopoiesis is a broad term used for the production of leukocytes. Granulopoiesis or myelopoiesis, lymphopoiesis and monopoiesis collectively constitute leukopoiesis. As mentioned earlier, leukocytes are of various types hence proliferation and development of each cell needs special mention.

MYELOPOIESIS / GRANULOPOIESIS
It is the process of proliferation, differentiation and maturation of granulocytes (neutrophils, eosinophils and basophils) in the bone marrow. Granulocytes arise from pluripotent hematopoietic stem cells through a series of progressively more committed progenitor cells or colony forming units. Granulocyte-monocyte colony forming unit (GM-CFU) give rise to granulocyte colony forming unit (G-CFU), which pass through successive divisions and stages and ultimately form mature granulocytes.

Proliferation and maturation of myeloid series in the bone marrow demonstrates a continuum of development from the blast to the most mature cells. Each cell type follow the same pattern of development i.e. blast, promyelocyte, myelocyte, metamyelocyte, band cell and mature cell (neutrophils, eosinophils and basophils). Each cell type can be distinguished at myelocyte stage due to the appearance of specific granules. During maturation, there is reduction in nucleus volume, condensation of chromatin, change in nuclear shape, appearance and disappearance of primary granules, appearance of secondary granules, color changes and change in size of the cell.

During developmental series granulocytes pass through five compartments. Proliferative pool consists of myeloblast, promyelocyte and myelocyte; these cells undergo mitosis and have the capability of division. Metamyelocytes and band cells are the components of maturation pool. These cells do not divide. Segmented neutrophils form the storage and circulating compartments. These cells are stored in the bone marrow and are released in the circulation. After few hours neutrophils marginate along with the vessel wall and is termed as marginating pool. These cells leave the circulation and enter the tissues passing through the endothelial cells by a process known as diapedesis. In the tissue compartment, cells either move freely or are restricted to the site of infection. For understanding the developmental series, neutrophilic maturation is described below;

Myeloblast
It is the earliest recognizable immature cell of myeloid series. It is round to oval in shape with scanty basophilic agranular cytoplasm (see figure 5.1). Nucleus is round to slightly oval with open chromatin. Chromatin material shows even, diffuse distribution with no aggregation into large masses. It contains one or more nucleoli of uniform size. Nucleus to cytoplasm ratio is 4:1.
Figure 5.1: Myeloblast

https://www.google.com.pk/search?q=myeloblast&biw=1366&bih=667&source=lnms&tbm=isch&sa=X&ved=0CAYQUAUw2AI&crci=8&iem=2&num=10&ei=472KXej1K8u-gr2S6P6wCA&biw=1366&bih=667&source=lnms&tbm=isch&sa=X&ved=0CAYQUAUw2AI&crci=8&iem=2&num=10&ei=472KXej1K8u-gr2S6P6wCA

Promyelocyte

This is the second stage of this series. This cell is larger than myeloblast. Cytoplasm is moderate in amount and contains primary azurophilic granules. Nucleus is round to oval and somewhat similar to myeloblast except the chromatin has become coarser. Nucleoli may be visible but are not often distinct. Promyelocyte is shown in figure 5.2.

Figure 5.2: Promyelocyte

Myelocyte

At this stage, the cell can be identified as belonging to neutrophilic series due to the appearance of specific granules. This cell is smaller than promyelocyte (see figure 5.3). Cytoplasm is abundant and has lost its blue coloration and appears more acidophilic (pink) in color due to specific (secondary) granules. Primary granules are also present but their production is limited. Nucleus is round to oval and eccentric. Chromatin has become coarser and nucleoli are no more visible. Nucleus to cytoplasm ratio is 50:50. This is the last cell of myeloid series having the capability of division.
Metamyelocyte

This stage is characterized by the presence of kidney shaped or horse-shoe shaped nucleus as shown in figure 5.4. Chromatin has become more condensed. Nucleoli are absent. Cytoplasm contains secondary granules in larger amount and primary granules are present but almost hidden. This cell is smaller than myelocyte.

Band cell/ stab cell

This cell is known as juvenile neutrophil. Nucleus shows more condensation. Shape of the nucleus is band or sausage shape (see figure 5.5). This is smaller than metamyelocyte. Normally they are 2-6% in circulation. This cell matures into neutrophil.
Segmented neutrophil

This is the most mature form of myeloid series and is also known as polymorphonuclear neutrophil. It has a characteristic nucleus having 2-5 lobes joined by thin chromatin thread. Nucleus has dense chromatin. Cytoplasm is pale in color and appears light pink when stained with Leishman’s stain and contains granules as shown in figure 5.6. There are two types of granules primary or azurophilic and secondary or specific granules. It may be appreciated that primary granules remain obscure.

Figure 5.6: Neutrophil

Regulation of myelopoiesis/granulopoiesis

Growth factors essential for the proliferation, growth, development and maturation of granulocytes include granulocyte-monocyte colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-6 (IL-6), IL-11, IL-1, IL-3 and IL-5.

FUNCTIONS OF NEUTROPHILS

Neutrophils are instrumental in providing the first line of defense against bacterial infections. Bacteria and site of inflammation in the host send out signals in the form of chemoattractants that stimulate neutrophils. Chemotaxis is the movement of neutrophils the influence of chemoattractants at the site of infection under the influence of chemoattractants. Neutrophils then squeeze between two endothelial cells to reach at the site of inflammation. This process of squeezing is known as diapedesis and migrating to the site of inflammation is known as migration. The process through which neutrophils engulf bacteria is known as phagocytosis. Phagocytosis is discussed under the heading of inflammation. After phagocytosis bacteria are killed and degraded by two mechanisms i.e. oxygen dependent and oxygen independent.

Oxygen Dependent Microbial Killing

Free radicals such as superoxide, suboxide, hydrogen peroxide has microbicidal activity. Super oxide $(O_2^-)$ is spontaneously converted to hydrogen peroxide $(H_2O_2)$ by the enzyme dismutase.

$$2O_2^- + 2H^+ \rightarrow \text{Superoxide dismutase} \rightarrow 2H_2O_2 + O_2$$

Myeloperoxidase (MPO) mediated $O_2$ dependent killing with oxidized halogens. Superoxide and hydrogen peroxide are not potent microbicidies, but rather function as starting material to generate more potent oxidizing radicals e.g. oxidized halogens or oxidizing radicals.

$$Cl^- + H_2O_2 \rightarrow \text{MPO} \rightarrow H_2O_2 + OCl^- (\text{Hypohalites})$$
Bacterial killing by this System includes halogenation of the Bacterial cell wall, oxidation of various bacterial components, the decarboxylation of bacterial wall amino acid or the generation of long chains chloramines that have antimicrobial activity.

\[
\text{Ocl} + \text{RNH}_3 \rightarrow \text{OH}^+ + \text{RNH}_2 \text{Cl}
\]

MPO Independent Bacterial killing includes by;

- \(H_2O_2\)
- Superoxide anion (O2\(^-\))
- Hydroxyl Radial OH
- Singlet \(O_2\) (O2\(^-\))

**Oxygen Independent Microbial Killing of Neutrophils**
- Neutrophils also have antibacterial activity through the following components;
- Acids: pH 3.0-6.5 after particle ingestion
- Lysozyme: digestive action
- Lactoferrin: is an iron binding protein that makes less iron available for microbial growth.
- Defensin microbicidal peptides kill a variety of bacteria fungi or viruses.
- Bacteriocidal permeability increasing protein: antibacterial activity against gram negative bacteria that neutralizes the toxic effects of endotoxins.
- Leukin: are derived from nucleohistone of neutrophils that interferes with microbial replication.
- Secretions

Secretory functions of neutrophils synthesis of transcobalamin, interleukins and secretion of lysozymes

- Transcobalamin (TCI, TCIII) is derived from granulocytes that binds and transport cobalamin
- Stimulated neutrophils also synthesize IL–1 and TNF–α, these interleukins have important role in inflammation
- Lysozymes are degradative enzymes secreted by neutrophils

**EOSINOPHILS**

Eosinophils are the second most common granulocytes in peripheral blood. They are produced through the same developmental stages as neutrophils. Time required for eosinophils to be matured in bone marrow is 3-6 days. After maturation they are stored in bone marrow and released in blood where they stay for 6-10 hours. They migrate from blood to tissues such as bronchial mucosa, skin, gastrointestinal tract and vagina and spend about 12 days at these sites. They are either destroyed in the tissue during their function or as a result of aging. Eosinophils are slightly larger than neutrophils. They have bilobed (two lobed) nucleus (see figure 5.7). They have large bright red-orange granules.
Function of Eosinophils

Role of eosinophils in parasitic infections

Major protective role of eosinophils in host defense is the destruction of parasites. Ability of eosinophils to combat helminthes infection is antibody-dependent i.e. they require the presence of IgG to kill the parasites. Eosinophils possess large number of Fc receptors for IgG that allow eosinophils to bind with parasites and release contents of granules. Degranulation of eosinophils results in the destruction of the parasite’s cell membrane and ultimately death.

Eosinophils in asthma and hypersensitivity reaction

Number of eosinophils greatly increases in asthma suggesting their role in allergic condition i.e. asthma. Eosinophils cause degranulation of mast cells and are responsible for bronchial tissue destruction in asthma. They also take part in hypersensitivity reaction.

BASOPHILS

Basophils are the least common cells among granulocytes. They are small, round to oval cells having large blackish-purple granules which cover the entire nucleus. Nucleus, if visible, seems to be dentate rather than segmented as shown in figure 5.8. Major contents of granules of basophils are histamine and heparin. Histamine is a vasoactive amine that plays an important role in acute inflammation. Basophils secrete proteolytic enzymes (elastase and lysophospholipase) and oxidative enzymes (lactate dehydrogenase, glucose-6- phosphate dehydrogenase).
Functions of Basophils
Basophils degranulate their substances in trauma and infection. Basic proteins of neutrophils (which cause stimulation of basophils to release vasoactive amines), activated plasma kinins, leukotrienes cause activation and degranulation of basophils in inflammation.

Cold temperature, heavy smoke, dust or chemical irritants stimulate the parasympathetic nerve endings present in the airways. These nerve endings release acetylcholine which causes activation and degranulation of basophils. As a result, bronchial tree is inflammed and constricted causing difficulty in breathing.

Exposure to allergens leads to allergic conditions or hypersensitivity reactions. Hypersensitivity is an immunological reaction that leads to an abnormally strong response to an antigen that has been met before. Hypersensitivity will be discussed later in detail.

LYMPHOPOIESIS
Lymphopoiesis is the process of development of lymphocytes. Hematopoietic stem cells give rise to lymphoid stem cells under the action of interleukins 1, 6 and 7. Lymphopoiesis can be described on the basis of morphological features and immunological features. Lymphocytes are of three types; B lymphocytes (B cells), T lymphocytes (T cells) and natural killer (NK) cells. B cells and NK cells are produced in the bone marrow. After maturation, B cells migrate to lymphoid organs (lymph nodes and spleen) where they differentiate into plasma cells and memory B cells upon stimulation by antigens. Plasma cells have a specific structure while memory B cells are similar in structure to B lymphocytes. T progenitor cells, derived from bone marrow, migrate to thymus where their maturation take place and develop into T cells.

Morphologically, lymphopoiesis can be divided into three steps as described below:

Lymphoblast
It is the earliest recognizable cell of lymphoid series. It contains large round, nucleus with fair amount of basophilic cytoplasm (see figure 5.9). Cytoplasm of lymphoblast is devoid of granules. Nuclear chromatin pattern is thin, open and evenly stained. One to several nucleoli may be seen.

Prolymphocyte
Prolymphocyte is smaller than lymphoblast. It also has a large nucleus but can be easily differentiated from lymphoblast due to coarse chromatin material and indistinct nucleoli. Amount of the cytoplasm is small without any granules (figure 5.10).
Lymphocyte

Lymphocytes in blood appear as small cells having scanty agranular cytoplasm with a relatively large nucleus which occupies almost 80-90% of the cell volume (see figure 5.11). It has a dense, coarsely clumped nuclear chromatin and no nucleoli. NK cells also known as large granular lymphocytes (LGLs) are large lymphocytes containing bright reddish-violet (azurophilic) granules.

Immunological maturation of B and T lymphocytes can be demonstrated by the expression of specific markers using monoclonal antibodies. Maturation of B cells occurs through passing precursor B cell, early pre B cell, pre B cell and mature B cell stages. Appearance and disappearance of markers is simplified in table 5.1.

<table>
<thead>
<tr>
<th></th>
<th>HLA-DR</th>
<th>CD19</th>
<th>CD10</th>
<th>Cµ</th>
<th>slg</th>
<th>CD7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor B cell</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Early pre B cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pre B cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mature B cell</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.1: Immunological markers of B cells
T cell development in thymus is divided into three stages, early thymocyte, common thymocyte and mature thymocyte. Expression of markers on developing and mature T cell is shown in table 5.2.

<table>
<thead>
<tr>
<th>Cells</th>
<th>TdT</th>
<th>CD38</th>
<th>CD2</th>
<th>CD5</th>
<th>CD7</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>TCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature thymocyte</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Common thymocyte</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mature thymocyte</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mature HT cell</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mature CT cell</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 5.2: Expression of surface markers on T cells

T cells are further divided into two types i.e. cytotoxic T cells (CT) and helper T cells (HT). It may be appreciated that details of immunological maturation of lymphocytes is beyond the scope of this book.

Functions of Lymphocytes

Lymphocytes are at the center of stage of immune system by participating in innate immunity, humoral immunity and cell mediated immunity. They also play important role in the regulation of immune system and hypersensitivity reaction. Following is a brief description of the role lymphocytes in immunity.

Innate Immunity

Natural killer cells (NK cells) are instrumental against viral infected cells and neoplastic cells. Viral-infected cells possess receptors for IFN-γ and IL-2. IFN-γ attracts and stimulates NK cells to destroy viral infected cells. Neoplastic cells release growth factors similar to IL-2, which results in the stimulation of NK cells and destruction of these neoplastic cells.

Humoral Immunity

Upon stimulation, naive B cells develop into plasma cells. Plasma cells produce antibodies against specific antigen. Functions of antibodies include opsonization, agglutination, precipitation, neutralization and complement activation. Opsonization is the process of attachment of antibodies with the receptors on the target cells making them (pathogen) susceptible for phagocytosis. Fab portion of the antibody attaches itself to the antigenic determinant present on the surface of pathogen. Fc portion of the antibody binds with Fc receptors found on phagocytic cells resulting in the phagocytosis of the pathogen by the phagocytes.

Various pathogenic microorganisms release toxic substances (usually enzyme in nature) which damage host tissues. Antibodies produced against these pathogens efficiently detoxify toxins by the process of neutralization. Antibodies easily bind these toxins rendering them unable to react with the host tissues. Neutralization also helps in the removal of viruses. It may be noted that viruses can only replicate inside the host cells. Antibodies produced against a specific virus react with antigenic determinants of viral particles. This process makes viral particles unable to attach themselves to the host cells. Hence they cannot enter the host cells. Opsonized virus particles also become an easy target of phagocytes and are eliminated by phagocytosis.
Cell Mediated Immunity

Cytotoxic T cells are involved in acquired immune response. Cytotoxic T cells produce substances known as lymphokines lethal for the viruses. Cytotoxic T cells contain perforins or cytolysins and serine esterases. Perforins produce pores in the target cells resulting in the lysis of the target cells.

Cell mediated immunity also plays an important role in delayed type hypersensitivity. Antigen presenting cells (APC) present the antigen to helper T cells (CD4+) via class II MHC molecules. Association of APC activates HT cells resulting in the release of interleukin-1. IL-1 proliferates and expands delayed type of hypersensitive T cell (DHT) clone. Whenever DHT cells come in contact again with the same antigen, these cells produce various cytokines. These cytokines recruit and activate macrophages, neutrophils, eosinophils and basophils. These cells cause severe inflammation and excessive phagocytosis. If macrophages remain unable to destroy pathogens, then delayed hypersensitivity reaction is produced resulting in the production of multinucleated giant macrophages. These giant macrophages release large amount of lysosomal enzymes destructive for the surrounding host tissues.

Regulation of Immune Response

Lymphocytes also regulate the acquired immune response. Binding of APCs with HT cells releases IL-1 and IL-2. IL-1 and IL-2 proliferates lymphoid stem cells. Suppressor T (ST) cells, another subset of T cells, suppress the activity of HT cells, CT cells and APC by down regulating these cells.

Hypersensitivity Reactions

Lymphocytes are implicated in hypersensitivity reactions. Hypersensitivity reactions are discussed in detail later in this chapter.

MONOPOIESIS

Like other leukocytes, monocytes also have their precursors in the bone marrow. Monopoiesis is the development of monocytes. They share a common stem cell with neutrophils i.e. granulo-monocyte colony forming unit (GM-CFU). Driven by an appropriate stimulus, GM-CFU differentiates and matures into monocytes through following stages:

Monoblast

Monoblast is the precursor of monocytic cell line. It is a large cell having deep-blue clear scanty cytoplasm. Nucleus is large and contains nucleoli as shown in figure 5.12.
Promonocyte

It is a large cell having a pale bluish-gray moderate amount of cytoplasm. Nucleus is large, irregular, deeply indented and contains nucleoli (see figure 5.13). Unlike monoblast which is difficult to be distinguished from myeloblast, promonocyte can be easily differentiated from promyelocyte.

Monocyte

Promonocyte develops into monocyte which is released into the peripheral blood. These are the largest leukocytes in the peripheral blood. Their size varies from 12-18 µm. Their cytoplasm stains bluish gray when stained with Romanowsky’s stain. Nucleus occupies about half area of the cell. It is indented, irregular and deeply folded; at times it may assume a cerebriform appearance. It is also not unusual to encounter a kidney shaped nucleus in these cells. Nuclear chromatin is fine and has a transparent lacy appearance. Their stay in the bone marrow is very short and unlike granulocytes, there is no monocyte storage pool in the bone marrow for more than 24 hours. They leave the blood stream and enter various tissues where they either become fixed tissue macrophages or move about as wandering macrophages and perform the surveillance function.

Immature tissue macrophage

Monocyte travels to the tissues and transform into immature tissue macrophages. They are much larger than monocytes. Transformation of monocytes into macrophages involves three aspects; morphological, biochemical and functional.
Morphological transformation from monocytes to macrophages is achieved by further synthesis of the cytoplasm, enlargement of the cell size and increase in the cytoplasmic organelles and lysosomes. Nucleus also enlarges and many a times multiple nuclei appear in the cell. These multinucleated cells are called giant cells. Concomitant with the changes in the morphology of the monocytes, there is also an increase in their lysosomal and mitochondrial enzymes; hence the metabolic activity of macrophages is considerably increased. As monocytes become transformed into macrophages, their functional capabilities become many fold. Some of these are motility, phagocytosis, lysosomal enzyme activity, mitochondrial enzyme activity, cytocidal activity and cellular metabolism.

**Mature tissue macrophages**

These mature macrophages are present in the tissues. Their nuclei become more condensed and nucleoli are not visible. Cytoplasm is gray-blue in color. They exist as resident and wandering macrophages. Resident macrophages are present in the following locations and have special names as listed below:

<table>
<thead>
<tr>
<th>Site</th>
<th>Special name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Histiocytes</td>
</tr>
<tr>
<td>Liver</td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>Spleen</td>
<td>Splenic macrophages</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Reticular cells</td>
</tr>
<tr>
<td>Lungs</td>
<td>Alveolar macrophages</td>
</tr>
<tr>
<td>Brain</td>
<td>Microglial cells</td>
</tr>
<tr>
<td>Bones</td>
<td>Osteoclasts</td>
</tr>
<tr>
<td>Serous cavities</td>
<td>Macrophages, mesothelial cells</td>
</tr>
</tbody>
</table>

**Functions of Monocyte**

Monocytes are involved in a wide range of defensive functions of the body that include phagocytosis, removal of infected and neoplastic cells and cytokines production.

**Phagocytosis**

Monocytes phagocytose pathogens more efficiently than neutrophils. Monocytes possess Fc receptors for antibodies and activated complement proteins. On one hand antibody molecule attaches itself with monocyte and on the other hand with pathogen via Fab fragment. This process of opsonization helps internalization of the opsonized pathogen which is killed in the phagolysosomes of monocytes.

**Removal of Infected and Neoplastic Cells**

Activation of monocytes leads to a series of cellular response i.e. activation of HT cells, CT cells and NK cells. These activated lymphocytes secrete cytokines that play an important role in the further production of immune cells and elimination of the pathogens by cytolsins and cytolyis. Monocytes also work in association with complement protein C3b, which enhances the attachment of monocytes with the cancerous cells. This binding leads to the phagocytosis of the cancerous cells.

**Removal of Senescent and Necrotic Cells**

Macrophages play a key role in the removal of senescent blood cells and damaged cells. Macrophages remove injured and necrotic cells, degenerated neutrophils and dead microorganisms.
Monocytes as Antigen Presenting Cells (APC)

Monocyte degrades pathogenic organisms into fragments and expresses some of these fragments (proteins) on their surface bound with major histocompatibility complex (MHC). Binding of degraded proteins with MHC complex depends on the origin of antigen (pathogen). Exogenous antigens bind with class II MHC molecules and are incorporated in the monocyte cell membrane. This complex interacts with helper T cells (CD4+ T cells). Endogenous antigens i.e. antigens produced inside the infected cell (viral DNA or oncogenes), bind with class I MHC molecules, move to the surface of the cell and react with cytotoxic T cells (CD8 T cells). This process leads to the movement of activated monocytes from infected site to lymphoid tissues for further activation of HT cells and CT cells. Activated monocytes secrete IL-1 that helps further activation of lymphocytes. Activation of lymphocytes leads to their proliferation which specifically destroys these pathogenic particles. Processing of the pathogens makes monocyte as antigen presenting cells.

Cytokines Production

Activated monocytes produce various cytokines known as monokines. GM-CSF, G-CSF and M-CSF are produced by monocytes which play a central role in the proliferation and production of precursor cells of granulocytes and monocytes. IL-1 is produced by activated monocytes that activate B cells, HT cells and NK cells. IL-6 secreted by monocytes differentiates myeloid stem cells and enhances the production of antibodies by activated B cells.

Regulation: Monopoiesis is regulated by IL-3, GM-CSF and M-CSF.

MONOCYTE-MACROPHAGE SYSTEM/ RETICULOENDOTHELIAL SYSTEM

Monocytes, mobile macrophages, fixed tissue macrophages and specialized endothelial cells together present in bone marrow, spleen, lymph nodes and other tissues is known as monocytes-macrophages system or reticuloendothelial system. This system is present in all tissues and particularly in those tissues where large quantities of particles, toxins and other unwanted substances are destroyed. Function of these macrophages is phagocytozing large quantities of bacteria, viruses, cancer cells, necrotic tissues or other foreign particles in the tissue. They also respond to chemoattractants, detach from their sites and react at the site of inflammation. Following is a brief description of resident macrophages:

Histiocytes/Langerhan's cells

These cells are present in the skin and subcutaneous tissues. Skin itself acts as a defensive barrier which protects the body from foreign organisms. If injury occurs and skin is broken foreign particles encroach the skin, which results in inflammation. Macrophages present in skin act against these infectious agents and destroy them.

Kupffer cells

Bacteria may easily invade the body through gastrointestinal tract. Large number of bacteria constantly passes through the gastrointestinal mucosa into the portal blood. Portal blood enters the liver sinusoids where macrophages known as Kupffer cells are present. These macrophages remove bacteria and prevent them from entering the general circulation.

Splenic macrophages and stromal macrophages

Macrophages present in spleen and bone marrow are known as splenic macrophages and stromal macrophages respectively. Macrophages present in spleen and bone marrow not only attack the infectious agents but also perform other functions. In spleen, they clear senescent blood cells and phagocytose apoptotic B cells whereas in bone marrow they interact with erythroid cells and remove erythroid nuclei.
**Alveolar macrophages**

Alveolar macrophages are present in the lungs. Lungs are frequently at risk of invading organisms. Large number of tissue macrophages is present in the alveolar walls. When the organisms and particles get entrapped in the alveoli, the macrophages phagocytose these particles. When the organisms are highly resistant e.g. Mycobacterium tuberculosis, these macrophages modify and form epitheloid cells and multinucleated giant cells. These giant cells surround the microorganism to form granuloma.

**Macrophages of the lymph nodes**

Macrophages present in the lymph nodes are present at two sites i.e. subcapsular sinuses and medullary sinuses. When local tissue macrophages are unable to kill the infectious organisms they enter the lymph flow. Lymph enters the lymph nodes through afferent vessels. Macrophages present in the subcapsular and medullary sinuses ingest the bacteria and prevent the dissemination of infection in the body.

**Macrophages present at other sites**

Macrophages present in the brain are known as microglial cells. They interact with neurons and cerebrospinal fluid and clear them from infectious organisms and debris. Macrophages present in the bone are called osteoclasts and are involved in bone remodeling. Macrophages present in the ovary and testis remove the dying cells. Macrophages present in the endocrine glands e.g. pancreas, thyroid, adrenal etc. function to maintain metabolic balance.

**Suggested further reading**

IMMUNITY

The term immunity means protection against any harm. In medical terminology it means ability to resist all noxious stimuli and protect the host from the harmful effects thereof. It must be emphasized that different types of immunities do not necessarily function independent of each other. It is more commonly a reunified system with considerable collaboration and interaction between various components of immune system. Immune system of the body is divided into two types: innate or natural immune system which is a nonspecific type and acquired or adaptive immune system which is a specific type. The two major subdivisions of immune system are described below:

INNATE IMMUNITY

The term innate means in born. This implies that an individual throughout his life enjoys this immunity which has been conferred upon him as part of the homeostasis. There is no contribution or any effort or input on the part of the individual in generating this type of immunity. This type of immunity is also independent of the functional integrity or even the presence of immune system. It is the first line of defense against the invading organisms as it is already present and ready to mobilize upon infection to react with organism attacking the body. It can act against wide range of microorganisms and therefore is known as the nonspecific type. This kind of system includes anatomical barriers which protect the body against various microorganisms. It also contains certain inflammatory cells which release cytokines and act against these infectious agents. Humoral components in the blood are also a part of innate immunity. A brief description of components of innate system is described below:

Anatomical Barriers

Mechanical components

Skin in normally protected against invasion by numerable bacteria which are present in air or gain access to skin through other means. Even though heavily colonized by bacteria, viruses and fungi, skin normally does not show any evidence of disease caused by these microorganisms. This is achieved by structural integrity of cutaneous epithelium and presence of sebum which contains bactericidal substance and various organic acids.

Entire respiratory tract is lined by cilia which by it’s to and fro movement remove the dust particles and organisms adhering the airway. The mucus lining also helps in protection by trapping the organisms. Gastrointestinal tract also has a strong mucus lining for protection. Constant peristaltic movement of gastrointestinal tract helps the removal of microorganisms from it.

Secretory elements released from mucosal lining provide protection to body. Sweat production helps in defense system. It contains fatty acids that inhibit the growth of organisms. Lysozyme and phospholipase present in tears, saliva and nasal secretion breakdown and destabilize the cell wall of bacteria. Low pH of the sweat and gastric secretion prevents the growth and killing of microorganisms. Defensins are low molecular weight proteins present in the
linings of lungs and gastrointestinal tract has antimicrobial activity. Surfactants production in lungs acts as opsonins and helps in phagocytosis.

Continuous flow of lachrymal fluid (tosis) from lachrymal sac to lachrymal duct helps in neutralization of any dust, debris and microorganisms which enter the conjunctiva. In addition, an enzyme called lysozyme, present in the lachrymal fluid in abundance helps killing of invading microorganisms.

In kidneys, continuous flow of urine washes down any bacteria which might venture up the urinary tract. Streptokinkase present in urine and other substances play an important role in protecting the urinary tract from various infections.

Normal flora
Normal flora of skin and gastrointestinal tract are friendly bacteria which do not harm the body and give benefits. It plays a major role in defense system by releasing toxic substances against pathogenic bacteria and also competing with them for nutrients. Presence of normal flora inhibits the attachment of pathogenic organisms to the cell surfaces thus prevent their colonization.

Cellular and Humoral Components
Anatomical barriers provide effective protection against microorganisms inhibiting their colonization. However when these anatomic barriers are breeched, next component of innate immune system comes into play i.e. the inflammatory process. This process contains humoral components (complement and coagulation proteins) which provides immunity. Complement cascade increases vascular permeability helping the inflammatory cells to reach at infectious site. Components of complement system help in opsonization (C3b, C5b) and also form membrane attack complex (MAC) thus killing the microorganism. Some products of coagulation cascade increases vascular permeability and act as chemotactic agents for inflammatory cells. β- lysin, produced by platelets, has antimicrobial activity against gram positive bacteria. Lactoferrin and transferrin bind iron which is an essential nutrient for various bacteria and hence inhibiting their growth.

Acquired immunity
As the name suggests this type of immunity is not a natural prerogative of an organism. It is acquired through the participation of immune system which must be intact not only anatomically but it must also be functionally competent.

Acquired arm is activated when innate arm first recognizes the organisms. Once antigen presenting cells (macrophages) bind to appropriate helper T cells, they undergo blast transformation. They bind with B cells and cytotoxic T cells depending on the type of infection/antigen. This type of immune response requires some time to react with an invading organism. This type of immunity is further divided into two types; humoral and cell mediated as described below:

Humoral immunity
When helper T cells activate B cells, they transform into plasma cells and produce antibodies (IgG, IgM, IgD, IgE or IgA). Each plasma cell releases specific antibody specific for a certain type of antigen. Protection of body with the help of these antibodies forms the humoral immunity and hence B cells are the major cells of this type of immunity. Antibodies provide protection to the body through various mechanisms. Antibodies bind with antigens (pathogens) and neutralize them preventing the release of deleterious substances from antigens (pathogens).
Cell mediated immunity

Cell mediated immunity acts against viral and tumorous cells. Cells involved in this type of immunity are helper T cells (CD4+ T cells) and cytotoxic T cells (CD8+ T cells). APCs present MHC II molecules to CD4+ T cells while MHC I molecules to CD8+ T cells. They release IL-2 which causes recruitment of inflammatory cells. These cells form conjugate with organism leading to their endocytosis. These cells also release lymphokines i.e. toxic free radicals. Cell mediated immunity also plays a major role in delayed hypersensitivity reaction and transplant rejection.

INFLAMMATION

It is a defensive response of body that eliminate the initial cause of tissue injury as well as necrotic cells and tissues. Inflammation may be acute, chronic or granulomatous.

Acute Inflammation

It is an immediate response of leukocytes characterized by their migration at the site of infection, ingestion and digestion of invading microorganisms and removal of dead tissue. Acute inflammation shows five cardinal features i.e. heat (calor), redness (rubor), swelling (tumor), pain (dolor) and loss of function (function laesa). Causes of acute inflammation include bacterial infections and toxins. It may also occur in cases of infarction (due to tissue ischemia) and any external injury. It is associated with vascular changes and cellular events.

Vascular changes

Immediately after tissue injury, nervous stimulation causes contraction of vessels. Vasoconstriction lasts for few seconds and is followed by vasodilation. Vasodilation increases blood flow and engorgement of small capillaries. Redness and heat produced at the site of infection occur due to vasodilation. Expanded vessels increase intravascular hydrostatic pressure leading to increased vascular permeability and movement of fluid from capillaries into interstitium. This type of fluid is an ultra-filtrate of plasma and is poor in protein content called as transudate. Movement of transudate is followed by movement of protein rich fluid known as exudate. Exudate accumulation reduces the osmotic pressure of vessels and increases the osmotic pressure of the interstitial fluid. Change in osmotic pressure results in outflow of water and ions in extravascular tissues leading to edema. A number of mechanisms are involved to increase the vessel wall permeability.

Cellular events

Cellular events are marked by movement of neutrophils at the site of injury, degradation and killing of infectious agent. Neutrophils pass through a number of steps in order to reach the affected site. Altered vascular permeability and congested microcirculation helps leukocytes to marginate along with the vessel walls. Neutrophils roll on endothelial cells i.e. temporarily sticking to one endothelial cell then moving on and sticking to another. Adhesion of neutrophils and endothelial cells leads to transmigration. After transmigration, neutrophils release collagenase which destroy the basement membrane and help them to enter the extravascular space.

Neutrophils migrate towards the site of infection under the influence of cheomattractants, known as chemotaxis. After reaching the affected site, neutrophils neutralize or degrade the injurious agent through phagocytosis.

Neutrophils recognize microbes through opsonins attached with their surfaces. Neutrophils extend their pseudopods around the microbe covering them to form phagosome. Lysosomes attach to phagosome and form phagolysosome. After killing, microorganisms are degraded by hydrolases in the phagolysosome. Fate of acute inflammation may be complete resolution of injury, removal of dead cells or progression to chronic inflammation.
Chronic Inflammation

Chronic inflammation is characterized by active inflammation, tissue injury and healing occurring simultaneously. It is elicited in viral infections. Lymphocytes and macrophages are essential for the removal of viruses. It also occurs as a result of prolonged exposure to toxic agents (silicon) or as a result of autoimmune disorders (rheumatoid arthritis and multiple sclerosis). They can also be a result of acute inflammation.

Granulomatous Inflammation

It is a special form of delayed type of hypersensitivity occurs when the infectious microorganism is un-degradable e.g. Mycobacterium tuberculosis. CD4+ T cells, after activation, release IFN-γ resulting in the recruitment of macrophages. Macrophages modify themselves into epitheloid cells and giant cells (formed by the fusion of epitheloid cells) under the influence of IFN-γ. These macrophages replace T cells and surround the organism completely. T lymphocytes form a collar around these macrophages. Accumulation of macrophages and lymphocytes form granuloma as seen in tuberculosis and leprosy etc. It is a defensive mechanism that does not allow the disease to spread.

IMMUNOGLOBULINS

Immunoglobulins or antibodies are proteins produced by the plasma cells in response to an antigenic stimulus. They may be present either in body fluids, in tissues or in both. Based upon thermal reactivity, antibodies are classified as warm and cold. Warm antibodies show maximal reaction at body temperature (37°C). They may be reactive at a slightly lower temperature i.e. 32°C. They are non-reactive at room temperature. Cold antibodies react best at 4°C. They are also reactive at room temperature though the strength of their reaction is somewhat weaker. Highest temperature at which an antibody may react with the antigen and still be called cold antibody is 30°C.

Based upon the chemical structure, antibodies are classified into five major groups i.e. IgA, IgG, IgM, IgD and IgE.

All classes of immunoglobulins conform to a uniform structure. A molecule of immunoglobulin consists of four polypeptide chains of which two are identical heavy chains and two identical light chains. There are five different types of heavy chains; each class of immunoglobulin has its own specific heavy chain. Names of various immunoglobulins are derived from the names of their respective heavy chains.

Light chains are only of two types, kappa and lambda. Irrespective of heavy chain present in immunoglobulin molecule, there is either a pair of kappa or a pair of lambda chains. There is no antibody molecule which contain one lambda or a mixture of kappa and lambda chains i.e. one kappa and one lambda chain in one antibody molecule. It may be pointed out here that in any one class of immunoglobulin, there are two types of molecules depending upon the pair of light chains in the molecule. This is represented in table 6.1.

Table 6.1 shows that there is homogeneity of light chains in antibody molecules of all classes. Antibody molecules showing heterogeneity of light chain population in the same molecule (κκ2 λκ or 2 κλ) are never synthesized. All immunoglobulins conform to this basic structure.
Heavy chains consist of two terminals or ends, C terminal and N terminal. The word C stands for carbohydrate while N stands for amino acid terminal. C terminal is the portion which contains carbohydrate. It also has the following properties:

- Complement is attached to the antibody molecule at this end
- Ability of the antibody molecule to cross placental barrier is conferred by the structure of C-terminal
- C terminal is the site of attachment of antibody to mast cells
- Ability of antibody molecule to crystallize also resides at C-terminal

<table>
<thead>
<tr>
<th>Immunoglobulin class</th>
<th>Heavy chains</th>
<th>Light chains</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA; Kappa</td>
<td>2 α</td>
<td>2k</td>
<td>α2k2</td>
</tr>
<tr>
<td>IgA; Lambda</td>
<td>2 α</td>
<td>2λ</td>
<td>α2 λ2</td>
</tr>
<tr>
<td>IgG; Kappa</td>
<td>2γ</td>
<td>2k</td>
<td>γ2k2</td>
</tr>
<tr>
<td>IgG, Lambda</td>
<td>2γ</td>
<td>2λ</td>
<td>γ2 λ2</td>
</tr>
<tr>
<td>IgM, Kappa</td>
<td>2μ</td>
<td>2k</td>
<td>μ2k2</td>
</tr>
<tr>
<td>IgM Lambda</td>
<td>2μ</td>
<td>2k</td>
<td>μ2 λ2</td>
</tr>
<tr>
<td>IgD, Kappa</td>
<td>2δ</td>
<td>2k</td>
<td>δ2k2</td>
</tr>
<tr>
<td>IgD, Lambda</td>
<td>2δ</td>
<td>2γ</td>
<td>δ2γ2</td>
</tr>
<tr>
<td>IgE, Kappa</td>
<td>2ε</td>
<td>2k</td>
<td>ε2k2</td>
</tr>
<tr>
<td>IgE, Lambda</td>
<td>2ε</td>
<td>2λ</td>
<td>ε2λ2</td>
</tr>
</tbody>
</table>

Table 6.1: Immunoglobulins, their heavy and light chains

Each heavy chain consists of two regions, constant and variable. Constant region, as the name suggests, has a fixed chemical composition and does not show any difference amongst various classes of immunoglobulins. Specificity of various heavy chains is determined by specific and characteristic changes in the chemical structure of variable part of heavy chain.

Light chains also have two regions, constant and variable region. Constant region is towards the C terminal while variable region is towards the N terminal. Difference in the epitopes between kappa and lambda chains is due to specific amino acid sequence in the terminal end of these chains. Variable region of light chains in each immunoglobulin class contains a characteristic amino acid sequence which is specific for each class of immunoglobulin. Brief description of each immunoglobulin class is given below;

**IgG**

IgG is a monomeric and major immunoglobulin present in plasma and extravascular spaces. It has four types depending on the number of disulphide bonds and length of the hinge region i.e. IgG1, IgG2, IgG3 and IgG4. It is capable of carrying all functions of immunoglobulins. It is the only immunoglobulin that can cross placenta which is mediated by the presence of receptor for Fc region of IgG on placental wall. It fixes complement except for IgG4 which does not. It also
acts as an opsonin by attaching to antigens via Fab portion and binds to inflammatory cells by Fc region. IgG helps inflammatory cells in phagocytosis.

IgM
IgM is a pentamer. It is the third most common immunoglobulin in plasma. It is extremely efficient in binding and activation of the complement proteins and lysis of the microorganism. It is also a potential agglutinin and binds with cells via Fc receptors. IgM also exists in monomer form; attach to B cells and function as a receptor for antigens on B cells.

IgA
IgA is the second most common immunoglobulin in plasma and is also present in secretions such as tears, saliva, colostrum and mucus providing local immunity to mucosa. IgA can also bind with inflammatory cells and lymphocytes although it does not bind with complement efficiently.

IgD
IgD is monomeric found in very low levels in plasma. It is primarily found on B cells where it functions as receptor for antigens. It does not bind with complement.

IgE
IgE is a monomer present in least amount in plasma. It binds with basophils and mast cells even before interacting with antigens. As a consequence of its binding to basophils cells, it produces allergic reactions. When an allergen binds to IgE, it releases chemical mediators from basophils and mast cells causing allergic reaction. IgE plays a major role in the opsonization of parasites leading to their destruction. Therefore, IgE is a useful indicator for helping in diagnosing parasitic infections as serum IgE levels are increased in these infections. It does not bind with complement.

COMPLEMENT SYSTEM
The term ‘complement’ describes a system of plasma proteins which participate in a number of biological, immunological and inflammatory reactions. Complement and its ‘allied’ proteins circulate in blood as inactive components of an integrated system. They constitute about 15% of the plasma proteins. There are two pathways for the activation of the complement;

- Classical pathway
- Alternate or the properdin pathway

Classical Pathway
Activation of complement takes place in an orderly fashion and is reminiscent of the cascade of activation of the coagulation system. As activation of the complement proceeds, various activated factors and certain by-products are released with specific biological functions.

There are nine plasma proteins in the complement system which are numerically labeled from C₁ to C₉. C₁ is further subdivided into three components C₁q, C₁r and C₁s. Besides these substrate proteins, there are activators and inhibitors of the complement system in order to keep its functions under precise control.

Alternate (Properdin) Pathway
Properdin pathway, like the classical pathway, is also an integrated system of proteins and enzymes for complement activation. It includes factors B, D, H, I, C₃ and Properdin or factor P. Factors B, D, C₃ and properdin cause activation of the complement through alternate pathway. Factors H and I act as the inhibitors of the properdin pathway.
Critical step in the activation of complement through properdin pathway is the generation of C₃b. This is brought about by the interaction of hydrated C₃ and factors B and D which are present in the blood in an inactive form.

Activation of C₃ in this instance is not mediated by antigen-antibody reaction. Since the reaction takes place in the plasma, it is also called 'fluid phase activation' of the complement.

Interaction of classical and alternate pathways of complement activation is schematically shown in figure 6.1.

Figure 6.1: Complement system pathways

Functions of Complement System

Complement system is a highly complex, delicately poised and critically integrated system of proteins and enzymes. It participates in acute inflammatory responses in order to curtail and ultimately destroy the offending organisms or any noxious stimuli. Some of the biological functions of this system are:

- Complement mediated effects:
  - Phagocytosis
  - Opsonization
• Anaphylotoxin mediated effects
  – Smooth muscle contraction
  – Increased histamine release
  – Release of granulocytic lysozymes
• Chemotaxis and granulocytic migration
• Maintenance of internal homeostasis

An intact and functional complement system is essential for normal homeostasis. Not only does it participate in numerous inflammatory and immunological responses, it is also responsible for body’s defense against infections. This is supported by an increased susceptibility to infections in congenital defects of the complement system.

HYPERSENSITIVITY REACTIONS
Acquired immune response that leads to the production of antibodies and T cell activation against infectious agents (bacteria, viruses, parasites and fungi) is generally protective. However, excessive or inappropriate activation of the immune system may lead to tissue destruction which is known as hypersensitivity reactions. It is an undesirable response of normal immune system that may be fatal at times. There are four types of hypersensitivity reactions.

Hypersensitivity Type I
This type of reaction occurs immediately (within minutes) when allergen (specific antigen) interacts with a specific antibody. This type of hypersensitivity reaction is IgE mediated and primary cells involved are basophils and mast cells. Conditions associated with type I hypersensitivity reactions are urticaria/eczema, conjuctivitis, rhinorrhea/rhinitis, asthma and gastroenteritis.

Hypersensitivity Type II
This type of reaction is antibody mediated against target antigens. These may be normal molecules present intra cellularly or present in the extracellular matrix or they may be adsorbed exogenous antigens e.g. drug metabolites. This antibody mediated hypersensitivity reaction can occur in three ways i.e complement dependent, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody causing cellular dysfunction.

Hypersensitivity Type III
This type of hypersensitivity reaction is characterized by the formation of immune (antigen-antibody) complexes followed by inflammatory reaction. Antigens present in these antigen-antibody immune complexes may be bacteria, viruses or endogenous antigens such as DNA. Immune complex mediated injury may be either systemic or localized.

Acute serum sickness is a systemic disease characterized by the formation of immune complexes in the vascular beds. Immune complex deposition can occur in skin (systemic lupus erythematosus, Arthus reaction), kidneys (lupus nephritis), lungs (aspergilosis), blood vessels (polyarteritis) and joints (rheumatoid arthritis) causing specific diseases.

Hypersensitivity Reaction Type IV
Type IV hypersensitivity reaction is cell-mediated type initiated by T cells. There are two types of type IV hypersensitivity reaction; one is initiated by CD4+ T cells and is known as delayed- type hypersensitivity reaction (DTH). The other is mediated by CD8+ T cells and is known as direct cell cytotoxicity (CTL). Granulomatous reaction is a specialized group of type VI
hypersensitivity reaction. Transplant rejection is an important example of type IV hypersensitivity reaction characterized by both DTH and CTL.

Suggested further reading
HEMOSTASIS

HEMOSTASIS; AN OVERVIEW

Hemostasis is derived from Greek word Haima i.e. blood and stasis means halt. Hemostasis is the process that maintains the flowing blood in a fluid state and confined to the circulatory system.

Hemostatic system represents delicate balance between procoagulant and anticoagulant mechanisms in addition to fibrinolysis. Systems that maintain homeostasis can be divided into four major and three minor systems. Major hemostatic systems include vascular system, platelets, coagulation system and fibrinolytic system. Minor systems are Kinin system, serine protease inhibitors and complement system.

BLOOD VESSELS

Intact unharmed blood vessels are necessary for smooth blood circulation. Vascular system ensures normal blood flow. Functions performed by vessels in the prevention of bleeding are vasoconstriction, stimulation of adjacent vessels and diversion of blood flow around damaged vasculature. Damaged vascular endothelium initiates contact activation of platelets with subsequent adhesion, release reaction, aggregation and contact activation of coagulation proteins (both extrinsic and intrinsic) leading to fibrin formation.

Non thrombogenic functions of intact vascular endothelium include repulsion of platelets and coagulation proteins, inhibition of fibrin formation, vasodilation, inhibition and activation of fibrinolytic system.

Vascular endothelium synthesizes factors that perform thrombogenic function. Tissue factor is absent from resting endothelial cells but may be expressed after their activation by endotoxin, IL1 or TNF. Tissue factor activates thrombin rapidly. Tissue factor pathway inhibitor is synthesized and secreted by endothelial cells. It is a natural inhibitor of tissue factor mediated pathway of coagulation. Tissue plasminogen activator (tPA) produced by endothelial cells activates fibrinolytic system. Endothelial cells also synthesize plasminogen activator inhibitor type I that exerts inhibitory effect on fibrinolytic system.

Endothelial cells also possess anticoagulant activity. Endothelial cells membrane binds exogenous heparin as well as endogenous heparin like molecules such as glycose aminoglycans and heparin sulphate all of which can associate with antithrombi III (ATIII) and adjuvant its anticoagulant activity.

Endothelial cells possess surface receptors for a variety of physiological substances such as thrombin, angiotensin II etc and a variety of intracellular adhesion molecules e.g. VCAM I, E selectin and P selectin that modulate leukocytes and platelets adhesion, inflammation, phagocytosis and vascular permeability.
PLATELET’S STRUCTURE AND FUNCTIONS
Platelets are small, discoid shaped, anucleated corpuscles with a diameter of 2-4 µm and mean volume of 7-11 fl. They are the second most numerous corpuscles in the blood between 150–450×10⁹/L. Platelets remain in circulation for 8-12 days. Sites of platelets removal are spleen, liver and bone marrow. Approximately one-third of the total platelet mass is stored in the spleen. Splenic pool consists of the youngest (and the largest) platelets.

Structure of Platelets
Structurally, platelets are divided into three major regions i.e. surface membranous structure, cytoskeleton (sol-gel zone) and granular (organelle) zone.

Platelet membrane
Platelet membrane is a trilaminar structure composed of a bilayer of phospholipids and a large number of glycoproteins. It participates in a variety of events such as permeability, agonist stimulation, adhesion, activation, secretion and aggregation. Platelet membrane glycoproteins e.g. GP Ia, Ic, IIb-IIIa complex and GPIb-IX-V complex are essential for the normal functioning of platelets.

Glycocalyx surrounds the outer surface of platelet membrane and open canalicular system. Glycocalyx interacts with plasma and cellular components of blood and blood vessels. It also provides a transfer point for plasma proteins such as fibrinogen as they are taken up into secretory granules by endocytosis. Sialic acid present on the surface of the platelets produces negative charge.

Cytoskeletal zone
Platelet membrane and open canalicular system of resting platelets are supported by a highly structured cytoskeletal system. This system consists of elongated spectrin strands that are interconnected by actin filaments. Microtubules located under the platelet membrane play an important role in platelet formation from megakaryocyte and maintain the discoid shape of platelets.

Granular zone
In the cytoplasm of platelets there is a wide variety of granules. These have been named α granules, dense granules, lysosomal granules and peroxisomes.

Functions of Platelets
Platelets are involved in various biological processes including hemostasis, thrombosis, clot retraction and vessel wall constriction. They also play an important role in inflammation, promotion of atherosclerosis, host defense, suppression of tumor growth and metastasis and many others. Functions of platelets are described below;

Role of platelets in primary hemostasis
Platelets must be adequate in number and normal in function for the formation of primary hemostatic plug. One of the initial events after vascular injury is the adhesion of platelets to the damaged endothelium.

After adhesion, platelets release reaction occurs. α-granules secrete a large number of proteins including β-thromboglobulin, PDGF, fibrinogen, vWF, fibronectin and plasminogen. These perform major functions in hemostasis, host defense and wound repair. Dense granules secrete ADP, serotonin and calcium. ADP amplifies the aggregation process.
Activation results in morphological changes of platelets. Shape change produces finger-like projections known as filopodia. Actin and myosin fibers give strength to the activated platelets. These events squeeze the granules and organelles into the center of the cell and help in platelet release reaction and aggregation.

Interlinking of the platelets via fibrinogen produces aggregation. ADP, epinephrine, collagen and thrombin promote platelet aggregation.

Platelet aggregates are strengthened by the binding of membrane glycoproteins to each other and also to other receptors on adjacent platelets. Platelets aggregation provides a surface for fibrin threads to be laid on and form a stable clot.

**Maintenance of vascular integrity**
Platelets not only form hemostatic plug at the site of injury, they also repair and nourish the damaged endothelium.

**Platelets pro-coagulant activity**
Vascular injury activates coagulation cascade resulting in the formation of tenase and prothrombinase complexes. These complexes are assembled on the negatively charged surface of activated platelets. Platelet surface also protects activated coagulation factors from inactivation by their natural inhibitors. Platelets release reaction plays an important role in thrombus formation. Platelet factor 4 (PF4) has anti-heparin activity. Fibrinogen released from platelets contributes further in the thrombus formation.

**Pro-inflammatory role of platelets**
Platelets interact with large number of cells including endothelial cells, neutrophils, monocytes, dendritic cells, cytotoxic T lymphocytes, malaria-infected red blood cells and tumor cells. Interaction of platelets with endothelial cells and leukocytes initiates the inflammatory processes in the arterial wall resulting in atherosclerosis.

Cytokines and chemokines are also stored in platelets. Platelet membrane phospholipids generate inflammatory mediators. Platelets, leukocytes and endothelial cell interaction result in eicosanoids metabolism, leading to the synthesis of PAF, leukotrienes and TxA₂. Production of inflammatory mediators is enhanced by the up-regulation of COX₂ expression on monocyte through interactions with P-selectin and IL-1. COX₂ up-regulation enables further synthesis of pro-inflammatory eicosanoids. PAF is a potent platelet agonist, inflammatory mediator and regulator of neutrophil adhesion with activated platelets.

**COAGULATION SYSTEM**
Plasma contains coagulation proteins in soluble form which after activation play a major role in secondary homeostasis. Definitive hemostasis is achieved when fibrin is added to the platelet mass and platelets induced clot retraction. Following vascular injury, tissue factor activates factor VII to start the coagulation process by providing abundant membrane phospholipids. Thrombin generated at the injury site converts soluble plasma fibrinogen into fibrin, potentiates platelet aggregation and also activates factor XI, XIII, cofactor V and VIII. The fibrin component of the hemostatic plug increases as the fused platelets plug is transformed into a solid mass of cross linked fibrin.

Coagulation factors are twelve in number and are designated with Roman numeric’s coagulation factors are listed in table 7.1.
Table 7.1: Coagulation factors and their common names

<table>
<thead>
<tr>
<th>Factor (F)</th>
<th>Common names</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>III</td>
<td>Tissue factor/thromboplastin</td>
</tr>
<tr>
<td>IV</td>
<td>Calcium</td>
</tr>
<tr>
<td>V</td>
<td>Labile factor/proaccelerin</td>
</tr>
<tr>
<td>VII</td>
<td>Stable factor</td>
</tr>
<tr>
<td>VIII</td>
<td>Antihemophilic factor</td>
</tr>
<tr>
<td>IX</td>
<td>Christmas factor</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower factor</td>
</tr>
<tr>
<td>XI</td>
<td>Plasma thromboplastin antecedent</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
</tr>
<tr>
<td>XIII</td>
<td>Fibrin stabilising factor</td>
</tr>
</tbody>
</table>

FVI term is not used anymore as it was found that it is activated form of FV. Other factors of coagulation are Fletcher factor which is also known as prekallikrein and Fitzgerald factor also known as high molecular weight kininogen (HMWK).

All these factors together coagulate blood through a cascade known as classical pathway. It is also known as in vitro pathway or lab diagnostic pathway as this pathway also activates when coming in contact with a glass tube. The classical pathway is further divided into three pathways:

- Intrinsic pathway
- Extrinsic pathway
- Common pathway

**Intrinsic Pathway**

Components of this pathway are present in the bloodstream, hence named “intrinsic pathway” that includes FXII, XI, IX, VIII, HMWK and prekallikrein. The time for activation of this pathway is prolonged than that of extrinsic pathway. For its activation, the blood must have direct contact with a foreign surface, such as a glass tube or a damaged vessel wall. A glass surface will activate FXII, hence it is also known as the glass factor or the contact factor. Activation also occurs when FXII is exposed to collagen beneath the damaged endothelium. FXII, FXI and prekallikrein acts as enzymes, while the HMWK is the cofactor necessary for their activation.
Initial exposure of FXII to a negatively charged surface (glass, Kaolin, bacterial cell walls, celite and ellagic acid) may result in a weak non-proteolytic activation of FXII, i.e. the molecule is not cleaved but partially activated. FXII then reacts weakly with FXI and prekallikrein, which are then converted into activated FXI (FXIa) and kallikrein. The products of this reaction particularly kallikrein acts on F XII (inactivated form) and cause enzymatic cleavage, thus producing activated FXII (FXIIa). This activated FXIIa is more reactive than weakly activated FXII and is better able to convert FXI into FXIa, prekallikrein into kallikrein and plasminogen to plasmin. These three activated enzymes activate even more FXII, thus amplifying the process. Kallikrein also converts kininogen into bradykinin which is an important compound in inflammation. Plasmin is important in fibrinolytic system. Plasmin and kallikrein are also capable of activating complement system.

FXIa converts FIX into FIXa in the presence of calcium (Ca**). Ca** assists in binding FIX to the phospholipid surface of the platelet. FIX can also be activated via the activated components of extrinsic pathway, i.e. the complex of FVIIa with tissue factor and Ca**.

This complex can act directly on FIX and chance the amplification process. The main function of FIXa is to activate FX, which is the first factor activated in the common pathway. The activation of FX is a complex process involving atleast three other factors. As soon as FIX activates, it forms a complex with FVIII (which is not an enzyme but a cofactor) and Ca** on the platelet phospholipid surface to activate FX. Diagrammatic representation of intrinsic and common pathway is shown in figure 7.1.

Figure 7.1: Cascade of intrinsic and common pathway of coagulation
**Extrinsic Pathway**

The name extrinsic is derived from that, the activation of this pathway requires a factor not normally present in the blood. It is present, however, in most cells of the body. This factor is known as tissue factor (tissue thromboplastin, FIII) and usually is released only upon cell injury. When a vessel is injured, FIII is released to the bloodstream where it binds to F VIIa and Ca++. This complex activates FX as summarized in figure 7.2.

**Common Pathway**

The common pathway performs its function in three steps:

- Activation of FX
- Conversion of prothrombin to thrombin
- Polymerization of fibrin

**Activation of FX**

FX is activated by two ways by a complex derived from activation of the extrinsic pathway and by a complex derived from activation of the intrinsic pathway after activation, the activated FX (FXa) converts prothrombin (FII) to thrombin (FIIa).

**Conversion of prothrombin to thrombin**

FXa forms a complex with FV and Ca++ on the platelet phospholipid surface. This complex of FXa, FV, Ca++ and PF3 is called prothrombinase complex because it converts prothrombin (FII) to thrombin (FIIa). The major function of activated thrombin is to cleave fibrinogen to fibrin.

**Polymerization of fibrin**

Fibrinogen molecules are first cleaved to form fibrin. These fibrin monomers polymerize to form fibrin polymers by factor XIII or fibrin stabilizing factor.

![Diagram](image-url)

**Figure 7.2: Cascade of extrinsic pathway of coagulation**
Inhibitors of Coagulation System

Blood coagulation system is multifactorial biological pathway consisting of zymogens and accelerators. Because of the natural amplification of the enzyme product of the coagulation cascade there is always a danger that the process may get out of hand and develop systemic disproportions. Therefore, in order to contain the fibrin clot to the site of vascular damage the hemostatic system provides a variety of inhibitory mechanisms. The main function of this system is to:

- localize plug formation to the site of injury
- stop the activation/switch off
- inactivate the extra activated coagulation proteins

Following are the inhibitors of coagulation:

- Antithrombin III; is synthesized in liver and endothelial cells. It inhibits thrombin, FXII, FXI, FX, FIX plasmin and kallikrein.
- Heparin co-factor II; site of synthesis is not known. Its main function is to inhibit thrombin.
- Tissue factor pathway inhibitor; is synthesized in liver, lungs, bladder, endothelial cells, platelets, after activation by calcium and thrombin. It inhibits FVIIa-tissue factor complex.
- Protein C: is produced by liver. It inhibits FVa and VIIIa.
- Protein S: is synthesized in liver, endothelial cells and megakaryocytes. It binds protein C to endothelial cells and platelet phospholipid membrane.
- α2-macroglobulin: is synthesized in endothelial cells, platelets and fibroblasts. It inhibits thrombin, plasmin and kallikrein.
- α2-antitrypsin: inhibits XIa and plasmin.
- C1-inactivator: inhibits the contact factors of intrinsic pathway i.e. FXIIa, FXIa, kallikrein and plasmin.

FIBRINOLYTIC SYSTEM

Physiological function of fibrinolytic system is to dissolve intravascular deposits of fibrin (thrombi) in the vessels, extravascular fibrin present in hemostatic plugs and in inflammatory exudates. It is a multicomponent system composed of plasminogen and plasmin, plasminogen activators and plasminogen activator inhibitors.

Plasminogen

It is a single chain glycoprotein with a molecular weight of 92kDa. It is a serine protease, synthesized in liver, having a half-life of 50 hours. It has a strong fibrinolytic activity and is a potent activator of plasminogenetics.

Plasminogen Activators

Plasminogen activators (PA) are glycoproteins that can be divided into three categories according to their origin i.e. plasma plasminogen activators, tissue plasminogen activator and Urokinase.

Inhibitors of Plasminogen Activators

Most important inhibitors of plasminogen activators are:
tPA inhibitor (PAI-1), PA inhibitor (PA1-2), α2-antiplasmin and histidine rich glycoproteins.

Functions of Plasmin

Plasmin performs various functions. It degrades fibrin and fibrinogen. It produces fibrinogen degradation products (FDPs) which increases vascular permeability and interferes with
thrombin induced fibrin formation. Degradation of cross linked stabilized fibrin polymer results in the production of D dimers. Plasmin also destroys factors V, VIII, IX, XI. Indirectly, plasmin enhances or amplifies conversion of FXII to XIIa. It also enhances or amplifies conversion of kinin from kininogen. It also cleaves C3 into fragments.

FIBRINOLYSIS

When plasmin acts on fibrinogen) it breaks up a number of arginine-lysine bonds and progressively splits the molecules of fibrinogen and fibrin into a heterogeneous family of small peptides collectively known as fibrin degradation products (FDPs).

Degradation of fibrinogen and un-cross linked fibrin molecules by plasmin is a step-wise process. In the first step plasmin removes C-terminal portion of Aα chain by hydrolyzing Arg-Lys bond. This is rapidly followed by removal of the first 42 amino acids from the N-terminal of Bβ chain. This produces a thrombin-clottable form of fibrinogen called “fragment X” (still containing both pairs of chains) and the remaining part i.e. 1-42 Bβ chain + remains of Aα chain called as “Bβ 1-42 fragment”, [** assaying this fragment gives a sensitive index of fibrinogenolytic activity].

In the second step, degradation both pair of chains (or Fragment X) occurs, releasing a “D-fragment” (compressed of one D-domain) and a residual (containing the E-domain & remaining D-domain). Fragment Y is for their fragment “Fragment Y” attacked by plasmin, cleaving a second “D fragment (i.e. D-domain)” and leaving behind E-domain which is the central area where N-terminals of all six chains are linked through disulphide bonds. These smaller fragments are relatively resistant to further proteolysis by plasmin.

Degradation of cross-linked fibrin polymers is however different. It is relatively slower and the products formed are different because of specific bonding in the cross-linked fibrin polymers. Cross-linking of fibrin strands is mainly effected through amide-linkages between the parallel strands, plasmin is unable to break these linkages hence fragments are released as complexes of two or more than two domains. The most important and unique degradation product is “D-dimer” which consists of two D-domain fragments joined together by amide-linkages. [*Presence of D-dimers helps in differentiating secondary DIC from pathological fibrinolysis as they will only occur when a well cross-linked stable fibrin clot is degraded].

Kinin system

This system is activated by both coagulation and fibrinolytic systems. It initiates inflammation, chemotaxis, increases vascular permeability, contract smooth muscles and releases PGI2.

Protease inhibitors

After the activation of coagulation and fibrinolytic system, extra fibrin is degraded and eliminated along with some coagulation factors. However plasmin and Kallikerin still circulates until eliminated by liver hepatocytes, reticuloendothelial system or serine protease inhibitors. Serine protease inhibitors are anti-thrombin III, α2-antiplasmin, α1-antitrypsin C1-esterase inhibitors, protein C and S.

Complement system

This system comprise of 22 proteins. Both coagulation and fibrinolytic systems are interrelated with complement system. Complement system has been described in the immunity section of this book.
Suggested further reading


CHAPTER - 8

BLOOD TRANSFUSION

HUMAN BLOOD GROUP SYSTEMS AND TRANSFUSION REACTIONS

ABO BLOOD GROUP SYSTEM

ABO blood group system is the most important system. In Man there are 4 major types of ABO blood groups, i.e. A, B, AB & O. Minor antigens of ABH system include: A₂, A₃, Aₓ, Aₘ, B₃, Bₘ and Bₜ. Three allelic genes A,B and H(O) in various combinations of two account for the four recognized ABO blood groups A, B, AB and O.

Chemical structure of ABO antigens

Basic precursor chains (type-2 chain) consist of three sugar terminal epitopes on glycolipids in the red cell membrane (red cell ceramide). Both ABO gene and H gene code for specific glycosyltransferases that add specific sugar to precursor substance.

H gene codes for an enzyme α-2-L-fucosyltransferase which transfers one molecule of L-fucose to the terminal galactose of type-2 chains and converts them to H-substance. In the presence of A or B genes most, but not all, of the H-substance is converted to A or B antigens. O gene is an amorph gene hence it does not code for any glycosyltransferase.

Antibody characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline reactivity</td>
<td>Yes</td>
</tr>
<tr>
<td>Thermal amplitude</td>
<td>4°C, room temperature, 37°C</td>
</tr>
<tr>
<td>Immunoglobulin class</td>
<td>IgM (A&amp;B blood groups), IgG (O blood group)</td>
</tr>
<tr>
<td>Complement binding</td>
<td>Yes</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>Only IgG in group O females</td>
</tr>
<tr>
<td>Clinically significant</td>
<td>Yes</td>
</tr>
<tr>
<td>Transfusions reactions</td>
<td>Yes</td>
</tr>
<tr>
<td>Hemolytic disease of the new born</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Importance in transfusion:

- Immediate hemolytic transfusion reaction is seen if an incompatible whole blood or red cell products are transfused to a patient. Complement mediated intravascular hemolysis also takes place.
- ABO antigens can be detected in a 37 days old fetus though the ABO antigens on the red cells are not fully developed at birth. Exact phenotype is established by the age of 6-18 months.
- ABO antigens are also found on the membranes of platelets, lymphocytes, endothelial cells and epithelial cells.
- Production of ABO antibodies starts at birth but the titer is very low until the child is 3 to 6 months old.
- ABO antibodies have the ability to react at room temperature (20-24°C) or below (4°C). They efficiently activate complement at 37°C. Thus ABO system is unique among other cold blood groups because of its reactivity at both extremes of temperature.
About 80% of individuals secrete A, B and H substances in saliva and other body secretions.

Lack of H-gene results in Bombay phenotype. In these individuals there is no conversion of precursor substance to H-substance. Thus even in the presence of A or B genes, there is no A or B antigens on the surface of the red cells. Serum of these individuals contain anti-A, anti-B and anti-H antibodies.

**Rh BLOOD GROUP SYSTEM**

Rh blood group system has five major antigens; these are D, C, E, c, e. Rh antigens are transmembrane proteins that are an integral part of the red cell membrane. These proteins have 417 amino acids. There are two closely linked genes in the Rh blood group system that encodes a transmembrane protein comprised of over 400 amino-acids. It traverses the red cell membrane 12 times producing six loops that are arbitrarily numbered from 1 to 6 starting from N-terminal. These genes are called RHD and RHCE genes. RHD gene codes for D antigen while RHCE gene carries the epitope for C or c antigen on the second extra-cellular loop; epitope for E or e antigen is located on the fourth extra-cellular loop.

**Antibody Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Saline reactivity</td>
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<td>Thermal amplitude</td>
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<td>Immunoglobulin class</td>
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<td>Complement binding</td>
<td>No</td>
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<tr>
<td>Placental transfer</td>
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<td>Clinically significant</td>
<td>Yes</td>
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<td>Transfusion reactions</td>
<td>Yes</td>
</tr>
<tr>
<td>Hemolytic disease of the newborn</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Importance in transfusion:**

- Rh antigens are highly immunogenic. Rh mediated hemolytic transfusion reactions usually result in extravascular hemolysis of immunoglobulin coated red cells.
- D antigen is the most immunogenic of all Rh antigens.
- Circulating antibodies appear within 120 days of the primary exposure and within 2-7 days after any subsequent exposure.

**BLOOD TRANSFUSION**

In principle, a person should be transfused with blood of his own group. This is called identical blood group transfusion. At times, blood of ABO identical group is not available while the clinical situation does not permit any delay in transfusion. Under these circumstances group compatible blood can be safely transfused. This is called group compatible blood transfusion.

Patients who are group O negative have very little to choose in a life threatening situation because they can only be transfused with group O negative blood. Their only choice is Bombay type Rh negative blood which itself is an extreme rarity (there may be a few thousand Bombay Rh negative individuals across the globe). For these individuals there is no choice but to receive group O Rh positive blood to save their life. All concerned must be fully aware of the risk of Rh sensitization of the recipient and its consequences in later life particularly for young females in their reproductive years.

Another blood group which is only next in importance to ABO blood group in transfusion practice is the Rh blood group. In this system, human race is divided into Rh positive and Rh
negative individuals. Rh positive type is far more common than Rh negative type. Like ABO blood group system every effort should be made to transfuse Rh identical blood.

Whenever there is a choice between Rh negative and Rh positive ABO compatible donors for an Rh positive recipient, it is recommended that Rh positive blood be transfused. This spares the precious units of Rh negative blood which is in short supply. It is permissible to transfuse Rh negative blood to a patient who is Rh positive if Rh positive blood is not available (No harm comes to the recipient except that the valuable Rh negative blood which is already in short supply is unjustifiably used).

Transfusion of Rh positive blood to an Rh negative person is absolutely forbidden under all circumstances. The only exception is a life threatening catastrophic situation.

Even under these circumstances it is important to be certain that there are no preformed anti-D antibodies in the recipient's blood as a result of previous feto-maternal sensitization or transfusion of Rh positive blood in the past either inadvertently or for the management of a life threatening hemorrhage.

Once an Rh negative person receives Rh positive blood, he can NEVER be given Rh positive blood again in life no matter how life threatening the situation may be. This is because Rh antigens especially D antigen are powerful immunogens and produce high titer antibodies when transfused to a person who himself lacks them. For such patients there is no option except to search for Rh negative blood through every possible means. The option to use Rh positive blood for Rh negative individuals is therefore a once-in-a-life time waiver. This option must be exercised very carefully, if at all.

**TRANSFUSION REACTIONS**

The word transfusion reactions, though firmly ingrained in the vocabulary of transfusion medicine, needs reappraisal. It was initially coined to describe the few well known febrile, allergic and hemolytic reactions during or in the immediate post transfusion period. With the advancement in knowledge and recognition of additional adverse effects related to blood transfusion the spectrum of transfusion associated diseases has expanded vastly and has assumed the dimensions of a specialized field in its own right. It is therefore suggested that the term transfusion reactions which have a rather limited connotation be changed to transfusion related adverse effects or hazards of blood transfusion which includes all immediate reactions, delayed manifestations which appear 7-10 days after transfusion and late consequences that develop after a few months to a few years.

It is estimated that nearly 10% of all blood/blood component transfusions are accompanied by one or the other adverse effect. Because of the high incidence of adverse reactions, it is prudent to view all unusual or unexpected manifestations which the patient experiences or the medical staff observes during or after transfusion as transfusion reactions unless proven otherwise.

It is a common belief that most of the serious transfusion reactions take place within the first few hours of the start of the transfusion while late reactions are of lesser clinical importance. This notion has been dispelled since the recognition of post-transfusion purpura and transfusion associated graft versus host disease (GVHD) which is of serious import. Also awareness about transfusion transmitted viral illnesses of extremely serious consequences has radically changed the significance of the temporal association with the gravity of transfusion reactions.
Following are some of the well-recognized and better-documented consequences of blood transfusion. For the sake of clarity and chronology these are grouped into immediate early and late adverse effects:

- **Immediate reactions**
  Adverse effects that become manifest either during or within 1-2 hours after the transfusion is completed are classified as immediate transfusion reactions. Because of the temporal relationship the term ‘reactions’ is eminently suited for these adverse effects. Transfusion reactions that fall in this category are;
  - Acute hemolytic transfusion reactions
  - Non-hemolytic febrile reactions (NHFTR)
  - Acute allergic reactions
  - Acute anaphylactic reactions
  - Circulatory overload
  - Bacterial sepsis
  - Non-cardiogenic pulmonary edema (TRALI)

- **Early sequelae**

  Adverse effects of blood/blood components transfusion that manifest 7-10 days after the completion of blood transfusion are classified as early transfusion reactions. These are listed below:
  - Delayed hemolytic reactions
  - Post-transfusion purpura
  - Transfusion associated graft versus host disease (TA-GVHD)

- **Late sequelae**

  These are the consequence of transfusion that manifest after many weeks to years.
  - Transfusion siderosis
  - Disease transmission: HBs, HCV, HIV I & II, EBV, CMV, HTLV- I & II

It must be pointed out here that detail of each type of transfusion reaction at this stage is beyond the realm of the readers. A brief description of hemolytic transfusion reactions has been discrised in the section of immune hemolytic anemia.

**Suggested further reading**

ANEMIA; AN OVERVIEW

Anemia is one of the commonest ailments that afflict Man. Its importance lies in the morbidity that accompanies it. To counter the physical and economic impact of anemia it is imperative to diagnose it early and to institute appropriate therapy.

Diagnosis of anemia can be established by four parameters i.e. level of hemoglobin, number of red cells, packed red cell volume and oxygen carrying capacity /100 ml of blood. By convention and for reasons of practicality, reproducibility and economy, it is the level of hemoglobin in blood which is used as the yardstick for the diagnosis of anemia. Its level not only establishes the diagnosis of anemia it also indicates the severity of anemia.

Anemia is defined as reduction in the level of hemoglobin in the blood below normal for the age and sex of the individual. In this definition, two riders have been incorporated i.e. the age and the sex. Hemoglobin levels at various ages and for both sexes under physiological conditions are given in table 9.1. Elevation from the sea level has been recently suggested as a third rider; it has so far not met universal application.

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Normal Hb g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>M &amp; F</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>3 Months</td>
<td>M &amp; F</td>
<td>11 ± 1.5</td>
</tr>
<tr>
<td>10-15 yrs</td>
<td>M &amp; F</td>
<td>13 ± 1.5</td>
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<tr>
<td>15-60 yrs</td>
<td>M</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>15-60 yrs</td>
<td>F</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>&gt; 70 yrs</td>
<td>M &amp; F</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>-</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

Table 9.1: Normal hemoglobin level in health

Once the diagnosis of anemia is made, the next step is the classification of anemia; this is important because of its diagnostic and therapeutic implications. Anemias can be classified according to the:

- morphology of red cells in the peripheral blood
- pathophysiological mechanism

MORPHOLOGICAL CLASSIFICATION OF ANEMIA

This is based upon two parameters; i.e. size and color of red cells in the peripheral blood. Normal red cells are approximately 8 µm in diameter. By this parameter, red cells are classified as normocytes, microcytes and macrocytes.

A more objective parameter for assessing red cell size is the mean cell volume (MCV). Normal MCV is 76-96 femtoliter. Red cells with normal MCV are called normocytes. Red cells
with an MCV below the lower limit of normal are called microcytes while those with an MCV greater than 100 fl are classified as macrocytes.

Color of the red cells is primarily due to their hemoglobin content. Laboratory parameters which indicate the amount of hemoglobin in the red cells are MCH and MCHC. Red cells which contain normal amount of hemoglobin are called normochromic (the term chromia is derived from the word chrome which means color). Red cells which contain less than the normal amount of hemoglobin look pale and are called hypochromic. Red cells which contain more than the normal amount of hemoglobin stain bright pink and may be called ‘hyperchromic’. (In modern hematology, the term hyperchromia has become obsolete because truly hyperchromic red cells do not exist).

There is a fortuitous correlation between the size of the red cells and their color. Red cells which are normal in size are commonly of normal color while small red cells (microcytes) are usually hypochromic (spherocytes are an exception). Red cells which are larger than normal (macrocytes) are usually normochromic; (hyperchromia, as pointed out earlier, does not exist). These two parameters can theoretically produce nine different morphological types of anemias but in practice only three types are commonly encountered; these are:

- Normocytic and normochromic
- Microcytic and hypochromic
- Macrocytic and normochromic

**Normocytic and Normochromic Anemia**

This is the commonest type of anemia which is seen in clinical practice and accounts for nearly 65% of all cases of anemia. Red cells in this type of anemia are of normal size and each red cell contains normal amount of hemoglobin. Anemia is due to low red cell count and the severity of anemia is proportionate to the reduction in the number of red cells. Some of the conditions which produce normocytic and normochromic anemia are:

- Chronic degenerative diseases
- Chronic renal failure
- Chronic infections
- Marrow aplasia

**Microcytic and Hypochromic Anemias**

This is the second most common type of anemia in clinical practice and accounts for almost 30% of all cases of anemia. It is characterized by red cells which are smaller in size and contain less than the normal amount of hemoglobin in them. Anemia is not only due to reduced amount of hemoglobin in the individual red cells, it is also aggravated by a variable reduction in the number of red cells. Some of the conditions commonly associated with significant microcytic and hypochromic anemia are:

- Iron deficiency
- Thalassemia syndromes*
- Anemia of chronic disorders
- Sideroblastic anemias
- Chronic lead toxicity

* Red cell count may be increased in β-thalassemia minor.
Almost 80-85% of all microcytic and hypochromic anemias are due to iron deficiency irrespective of the mechanism thereof. Another 10% are a manifestation of thalassemias (this figure may be higher in Greece, Italy and South-East Asia where as many as 20-30% of patients with microcytic and hypochromic anemias may have thalassemia syndromes).

**Macrocytic and Normochromic Anemias**

These are the least common of the three morphological types of anemias and account for only 3-5% of anemias across the globe. It is however important to diagnose them accurately because of the availability of effective therapy in many instances.

Macrocytic anemias are characterized by the presence of larger than the normal red cells. Morphologically macrocytes are of two types:

- Oval macrocytes
- Round macrocytes

It is important to make this distinction because of their distinct pathophysiology, laboratory features, clinical manifestations and therapeutic modalities.

**Oval macrocytes**

Oval macrocytes in the peripheral blood are usually a reflection of vitamin B\textsubscript{12} and / or folic acid deficiency.

**Round macrocytes**

These red cells are larger than their normal counterparts but they retain the normal shape. They are usually seen in alcoholic liver disease, hemolytic anemias, post-splenectomy and hypothyroidism.

**PATHOPHYSIOLOGICAL CLASSIFICATION OF ANEMIA**

This is based on the mechanism responsible for anemia as seen in;

- Bone marrow failure
- Deficiency of hematinsics
- Reduced red cell survival
- Blood loss

Many a times more than one mechanisms are responsible for anemia in a given instance. It is usually possible to single out one mechanism as the major cause of anemia while role of others is only contributory.

**Anemia due to Bone Marrow Failure**

There are at least four clinical syndromes which may arise because of inability of the bone marrow to produce normal number of blood cells. These are aplastic anemia, hypoplastic anemia, myelodysplasia and myelophthsisic anemia.

**Anemias due to the Deficiency of Hematinics**

This is most commonly due to iron, vitamin B\textsubscript{12} and folic acid deficiency. Uncommonly the deficiency of such substances as vitamin C, pyridoxin, zinc and copper may also produce anemia.
**Anemia due to Reduced Red Cell Survival**

This type of anemia is called hemolytic anemia. Hemolytic anemias are characterized by premature and excessive destruction of red cells. Other mechanisms may contribute towards anemia but these are of secondary importance. Red cell survival may be reduced for two reasons i.e. intracorpuscular defects and extracorpuscular defects.

**Blood Loss Anemia**

Blood may be lost from the body in two ways i.e. acute blood loss and chronic blood loss. Some of the conditions in which acute blood loss may occur are road accidents, bleeding from GIT and obstetrical complications.

Loss of large amount of blood from the body over a short period produces hypovolemic shock and a critical reduction in the oxygen carrying capacity of blood. This results in reduced supply of oxygen to the tissues. Most of the clinical features of acute blood loss are secondary to hypovolemic hypoxemia.

Body compensates for this through various mechanisms; some of these are:

- Redistribution of blood to the tissues
- Increased heart rate
- Increased oxygen extraction from the red cells by the tissues
- Fall in body temperature to reduce oxygen requirement by the tissues.
- Increased rate of respiration to expedite oxygenation of reduced hemoglobin in the red cells as they pass through the pulmonary microvasculature.

Anemia in acute blood loss is of sudden onset and is primarily due to reduced number of red cells in the circulation. Severity of anemia is therefore a reflection of the magnitude of blood loss. Red cells which remain in circulation are of normal size and shape and anemia initially is normocytic and normochromic in type. As bone marrow becomes more responsive, it delivers to the blood peripheral large number of somewhat immature red cells; these are the reticulocytes. Since reticulocytes are larger than the normal red cells, presence of large number of reticulocytes leads to macrocytic and normochromic anemia.

Chronic blood loss, as the name suggests, is a slow process in which small amount of blood is lost from the body constantly or repeatedly. Some of the conditions which cause anemia due to chronic blood loss are hookworm infestation, chronic aspirin ingestion, chronic metrorrhagia, ulcerative lesions in the gastro-intestinal tract and chronic hemoglobinuria.

Loss of blood results in loss of iron from the body. It has been calculated that approximately 2 ml of whole blood or 1 ml of packed red cells contain 1mg of iron. Loss of iron from the body may be of such magnitude that the absorption of iron from the gastro intestinal tract fails to keep pace with the iron lost from the body. As a result, body stores of iron progressively decrease and may ultimately disappear. As this condition progresses, the level of hemoglobin falls and anemia of iron deficiency develops.
MICROCYTIC ANEMIAS

IRON DEFICIENCY ANEMIA

Iron deficiency anemia is characterized by reduced hemoglobin, microcytic and hypochromic red cells, decreased serum iron and absent iron stores in the bone marrow. Iron deficiency anemia is the most common hematological disorder that afflicts Man. It occurs at all ages and in both sexes though its incidence varies amongst the two sexes and in different age groups.

Variable amount of storage iron is present in all individuals under physiological conditions. Body’s demands in the face of reduced supply of iron are initially met by these stores. Once the iron stores are depleted but the demand for iron remains high, iron deficiency anemia develops. It continues to worsen as long as the discrepancy between supply and demand for iron persists. Hemoglobin level stabilizes when demand for iron matches the supply of iron.

It is a general belief that dietary iron is required for the formation of hemoglobin. A careful analysis of iron balance reveals that the synthesis of hemoglobin in an adult requires approximately 21 mg of iron per day. This demand is met by the release of iron from the breakdown of red cells which have completed their life span. Total amount of iron thus released every day is 21 mg. This exactly matches the demand for iron to make the requisite number of red cells every day. It should therefore be clear that erythropoiesis under physiological conditions does not depend on daily dietary supply of iron; it is the daily obligatory iron loss which is replenished by the daily dietary intake of iron as shown in figure 10.1.

As long as the obligatory losses are met by the external iron supply, hemoglobin level is maintained whatever be the iron stores. If iron supply is more than the obligatory losses, the surplus is deposited as storage iron. If on the other hand, the daily dietary supply of iron lags behind the daily obligatory loss, iron is drained away from the iron stores and iron deficiency state begins to ensue. This is because obligatory iron loss has to be met on a priority basis even at the expense of body stores. When body’s iron stores are exhausted, iron is drained away from the

![Figure 10.1: Iron cycle in an adult male](image-url)
iron released by the normally on-going breakdown of the red cells. Hence iron which were to be available for new red cell formation progressively decreases and gradual reduction in hemoglobin level continues. This state is perpetuated till iron supply to the body is increased.

Pathophysiology

Causes of IDA can be grouped into three main classes on the basis of their mechanism i.e. increased demand, poor intake and decreased absorption. Causes of IDA are;

- **Increased demand**
  Increased iron requirement may be physiological or pathological. Physiologically increased iron is required during childhood, pregnancy and lactation. Pathological causes of increased iron demand include increased iron loss due to acute or chronic bleeding (menstrual loss in premenopausal women, GIT blood loss from ulcer, colonic or gastric cancer, hookworm infestation, regular blood donation and dialysis).

- **Decreased intake**
  Inadequate iron due to insufficient dietary intake requirement (iron deficient diet, vegetarian diet and poverty).

- **Decreased absorption**
  Decreased iron absorption as a result of gastric disorders e.g. celiac disease, tropical sprue etc.

- **Combination**
  This is frequently encountered when two or more than two causative mechanisms are operative in the same individual. Some of the common combinations are:

  - malabsorption and bleeding hemorrhoids
  - nutritional deficiency and hookworm infestation
  - multiple pregnancies and metromenorrhagia
  - nutritional deficiency and prematurity

Clinical Features

Clinical manifestations of iron deficiency anemia are as under:

Nonspecific clinical features of iron deficiency anemia include weakness, fatigue, tachycardia, tachypnea and hyperdynamic state of circulation. Some of the features that are historically ascribed to iron deficiency are epithelial changes such as koilonychia (spoon shaped nails), posterior esophageal web and craving for such unnatural items as clay, ice, paint and dirt. The triad of iron deficiency anemia, koilonychia and posterior esophageal web is called Patterson-Kelly’s syndrome; this is also known as Plummer-Vinson syndrome. Posterior esophageal web is presumed to be a pre-cancerous condition. Pruritus in the elderly, many a times, responds to the administration of iron; this may be a manifestation of epithelial changes in iron deficiency. Other changes in the skin and its appendages are probably a reflection of tissue hypoxia rather than sideroepina.

Laboratory Findings

Investigations for iron deficiency anemia are simple, economical and result-oriented. There are two inter-related aspects of the diagnostic work up for iron deficiency anemia.

- To establish that iron deficiency is the basis of anemia
- To determine the cause of iron deficiency
  A systemic approach to establish that iron deficiency is the basis of anemia includes:
- Peripheral blood counts and red cell morphology
Peripheral blood

Peripheral blood smear in iron deficiency anemia is highly characteristic. Morphological abnormalities are confined mostly to the red cell series. The striking abnormality is microcytosis and hypochromia (see figure 10.2) which is supported by the red cell indices. Some pencil cells are commonly seen; their number is however not more than 10% of the total red cell count. Mild thrombocytosis is common particularly in those patients who are bleeding from GIT and GUT. Platelets are small (microthrombocytes) without any abnormality of granulation. In uncomplicated cases of iron deficiency anemia there are no numerical or morphological abnormalities in the leukocytes.

**Figure 10.2: Microcytic and hypochromic red cells**

Bone marrow

Examination of Romanowsky’s stained bone marrow smears in iron deficiency anemia is a useful though not an essential adjunct for the diagnosis of iron deficiency anemia. Cellularity of the marrow is normal. Erythropoiesis is not hyperplastic (contrary to the general belief). This is because iron deficiency dampens the erythropoietic response to anemia. Erythroid precursors are smaller than normal with ragged cytoplasmic edges. These morphological appearances though suggestive, but cannot be accepted as diagnostic of iron deficiency anemia. Myeloid series and megakaryocytes are normal in number, maturation and morphology. There is no stainable iron in the bone marrow in these patients. Figure 10.4 shows absent iron stores in a patient with deficiency anemia as compared with normal control (figure 10.3). Presence iron of any stainable iron excludes iron deficiency anemia.

Bone marrow examination for the diagnosis of iron deficiency anemia is not mandatory. It was an important diagnostic tool in the past when other means for the assessment of storage iron were not available. With the advent of serum ferritin as a marker for iron stores, bone marrow examination for the diagnosis of iron deficiency anemia has fallen into disrepute.
Figure 10.3: Normal iron stores (Perl’s stain positive)

Figure 10.4: Absent iron stores (Perl’s stain negative)

Serum iron profile
Table 10.1 highlights the value of serum iron profile in differentiating iron deficiency anemia from other conditions that produce microcytosis and hypochromia.

Determining the Cause of Iron Deficiency Anemia
When the diagnosis of iron deficiency anemia is established, it becomes mandatory to identify its cause. There is a whole battery of investigations that is available to establish the cause of iron deficiency; this includes:

- Ultrasonic examination of the pelvis for gynecological disorders
- Upper and lower GI endoscopy
- Stool examination for occult blood
- X ray barium meal and follow through
- Radio tracer studies to establish GI blood loss

Differential diagnosis
A number of conditions produce microcytosis and hypochromia; some of the more important ones are:

- Iron deficiency
- Thalassemia syndromes
- Anemia of chronic disorders
- Chronic lead toxicity
- Sideroblastic anemias
### Table 10.1: Discriminatory laboratory parameters in conditions that produce microcytosis and hypochromia

<table>
<thead>
<tr>
<th>Features</th>
<th>Iron deficiency</th>
<th>β-thalassemia minor</th>
<th>Anemia of chronic disorders</th>
<th>Chronic lead toxicity</th>
<th>Sideroblastic anemia</th>
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<td>↑</td>
<td>N</td>
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<td>Serum iron</td>
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<td>TIBC</td>
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<td>Transferrin saturation</td>
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<td>Serum ferritin</td>
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<tr>
<td>Ringed sideroblasts</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>Marrow hemosiderin</td>
<td>0</td>
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<tr>
<td>Hb. electrophoresis</td>
<td>↓ A₂</td>
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**Features:**
- Iron deficiency
- β-thalassemia minor
- Anemia of chronic disorders
- Chronic lead toxicity
- Sideroblastic anemia

**Parameters:**
- MCV: Mean Corpuscular Volume
- MCH: Mean Corpuscular Haemoglobin
- MCHC: Mean Corpuscular Haemoglobin Concentration
- RDW: Red Cell Distribution Width
- Serum iron
- TIBC: Total Iron Binding Capacity
- Transferrin saturation
- Serum ferritin
- Ringed sideroblasts
- Marrow hemosiderin
- Hb. electrophoresis: Hb. electrophoresis
SIDEROBLASTIC ANEMIA

Sideroblastic anemias are a group of disorders characterized by the presence of variable number of microcytic and hypochromic red cells in the peripheral blood and ringed sideroblasts in the bone marrow. Numerically they are an uncommon group of anemias; their importance lies in the insight which they provide into the pathophysiology of iron metabolism. They also furnish information about the plethora of the enzymes that participate in heme synthesis.

The term sideroblasts refers to the nucleated red cells which contain iron granules in their cytoplasm. Under physiological conditions iron which is delivered to the proerythroblasts and the basophilic erythroblasts is utilized for the synthesis of hemoglobin; it is not incorporated into any of the cytoplasmic organelles. Iron in the cytosol is present in combination with a protein called apoferritin which is synthesized within the red cells. These iron granules can be stained with a special stain called Prussian blue stain. In a normal marrow approximately 50-70 % of the nucleated red cells contain iron in their cytoplasm. These are called physiological sideroblasts. In iron deficiency state their percentage decreases in parallel with the reduction in the level of hemoglobin. Presence of sideroblasts in the bone marrow is therefore a physiological phenomenon; it is their absence which is pathological.

There is another type of sideroblasts that are called ringed sideroblasts or pathological sideroblasts. In these sideroblasts, iron is present not only in the cytosol in association with apoferritin it is also present in large quantities in the mitochondria. Since mitochondria are arranged like a ring around the nucleus, these iron granules, when stained with Prussian blue stain, appear as blue dots around the nucleus; hence the name ringed sideroblasts.

Ringed sideroblasts are defined as nucleated red cells which, on iron stain, show iron granules around the nucleus encircling 1/3rd to 2/3rd of its periphery. On electron microscopy iron is present between the cristae of the mitochondria.

Pathophysiology

Sideroblastic anemias may be viewed as a consequence of the non-availability of sufficient amount of protoporphyrins to complex with iron. This causes accumulation of iron in the cristae of the mitochondria that is the hallmark for the diagnosis of sideroblastic anemias. There is no abnormality in the iron metabolism nor is there any abnormality in the globin chain synthesis (this may be secondarily affected because of the inter-dependence of the regulatory mechanisms for heme and globin chain synthesis). Common denominator is an impaired synthesis of heme primarily due to disturbances in porphyrin synthesis. It may therefore be said that sideroblastic anemias are a reflection of abnormal porphyrin synthesis.

Formation of ringed sideroblasts

Under physiological conditions, iron is delivered to the early forms of erythroblasts through transferrin receptors on their surface. This is utilized for the synthesis of hemoglobin as follows:

- Globin chains are synthesized by the rough endoplasmic reticulum in the cytoplasm.
- Protoporphyrin IX is synthesized in the mitochondria.
- Iron is transported to the mitochondria where it is incorporated into protoporphyrin IX to produce heme.
- Completed heme molecules are transported back to the cytosol to combine with globin chains and form hemoglobin.
In conditions where protoporphyrin synthesis is impaired, iron which is delivered to the mitochondria is not properly utilized. Instead it is held in the cristae of the mitochondria causing structural and functional damage.

A comprehensive classification of sideroblastic anemias is given in figure 10.5:

![Figure 10.5: Etiological classification of sideroblastic anemias](image)

Some of the clinico-pathological disorders that are associated with sideroblastic anemias are:

- **Drugs**
  - Isoniazid, pyrazinamide, cysloserine
  - Chronic lead toxicity
  - Chloramphenicol
  - Cytotoxic drugs
  - Alcohol

- **Nutritional disorders**
  - Malabsorption
  - Folate deficiency

- **Malignant disorders**
  - Lymphomas
  - Plasma cell dyscrasias
  - Myeloproliferative syndromes

- **Metabolic disorders**
  - Uremia

- **Immunological disorders**
  - Polyarteritis nodosa
  - Rheumatoid arthritis
  - Myxedema
Clinical Features

Clinico-pathological spectrum of sideroblastic anemias is primarily due to ineffective erythropoiesis, intra-medullary hemolysis and consequent increased iron absorption from the GIT. Anemia is usually insidious in onset and is of moderate severity. Because of the chronicity of the process, patients develop appreciable hemodynamic adjustment and tissue oxygenation is usually adequate. In advanced stages and with severe anemia, there may be evidence of cardiovascular decompensation.

In uncomplicated cases, there is no predisposition to infections or hemorrhage. However due to associated folate deficiency, hypersplenism or myelodysplasia, the leukocyte and the platelet counts may fall and predispose to infections and bleeding.

Laboratory Findings

Laboratory features of sideroblastic anemias are primarily due to ineffective erythropoiesis and intramedullary destruction of red cells and their precursors. Laboratory abnormalities are therefore predominantly hematological but some biochemical changes are also seen.

Hematological

There is moderate degree of anemia. A characteristic morphological abnormality in the peripheral smear is the presence of dimorphic red cell population. Most of the red cells are normocytic and normochromic while a small percentage (10-20%) are microcytic and hypochromic. There is mild to moderate anisopoikilocytosis. Red cell indices show borderline microcytosis and hypochromia. Reticulocyte count is variable. Initially there are no changes in the white cells and the platelets; later on there may be thrombocytopenia and leukopenia with megaloblastic changes in the bone marrow. This may be due to folate deficiency caused by increased folate utilization, ineffective erythropoiesis or myelodysplasia.

Bone marrow

Bone marrow aspirate shows numerous hypercellular particles with cellular trails. Erythropoiesis may be normoblastic, macro-normoblastic or rarely megaloblastic. Mitotic activity is increased as a reflection of an associated dyserythropoiesis. Usually there are no abnormalities of leukopoiesis and thrombopoiesis except for those due to folate deficiency, myelodysplasia or secondary leukemia.

Marrow iron stores are increased and hemosiderin is present as medium to coarse clumps. A characteristic feature is the presence of variable number of ringed sideroblasts (see figure 10.6) in the bone marrow demonstrated by Prussian blue stain.

Biochemical

Serum iron is increased, as is the saturation of transferrin while total iron binding capacity remains unchanged. Unconjugated hyperbilirubinemia is common as a result of intramedullary hemolysis. Serum LDH is increased. Serum folate is decreased in almost 80% of the cases.
10.6: Ring sideroblasts stained with Perl's/iron stain

**Differential Diagnosis**

Differential diagnosis centres around those disorders which are characterized by the presence of ringed sideroblasts in the bone marrow. In this context a detailed history and thorough physical examination may provide some insight into the underlying disease.

Since peripheral blood smear in sideroblastic anemias shows a small population of microcytic and hypochromic red cells, iron deficiency and thalassemia syndromes need be considered in the differential diagnosis.

Other disorders that should be differentiated from sideroblastic anemias are megaloblastic anemias, pre-leukemia and myelodysplasias. They all produce morphological changes similar to those seen in advanced sideroblastic anemias. At times this distinction may become difficult because of the marked variations in the morphological expression of sideroblastic marrows.

Diagnosis of sideroblastic anemia is established by documenting the presence of a characteristic clinico-pathological triad, which comprises of:

- Red cell dimorphism in the blood smear
- Ringed sideroblasts in the bone marrow
- Characteristic serum iron profile

**ANEMIA OF CHRONIC DISEASE**

Anemia of chronic disease (ACD) is a hypo proliferative anemia associated with chronic infections, inflammation or neoplastic disorders not due to bleeding, hemolysis or marrow involvement. It is characterized by decreased levels of plasma iron with normal iron stores. ACD is usually mild and non-progressive. Severity of anemia is directly related to the severity of chronic inflammatory or malignant disease.

**Etiology**

Following are some of the most common causes of anemia of chronic disease;

- Chronic inflammatory conditions
  - Tuberculosis, pneumonia, sub-acute bacterial endocarditis, meningitis, osteomyelitis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease etc.
- Malignant diseases
  - Carcinoma, Hodgkin's disease, lymphosarcoma
Pathophysiology

Several mechanisms are involved in the pathogenesis of ACD that include decreased red cells survival, impaired iron metabolism, cytokines induced suppression of erythropoiesis and decreased erythropoietin levels.

Decreased red cell survival

Decreased life span of red cells has been observed in patients with ACD. The exact mechanism is unclear however it may be due to the activated monocytes and macrophages of the reticuloendothelial system. These cells have increased capabilities of phagocytosis that leads to the hemophagocytosis and shortened survival of red cells.

Impaired metabolism of iron

In ACD, iron stores in the reticuloendothelial system are adequate. However, the release of iron from macrophages is inhibited by the cytokines released from activated monocytes and lymphocytes. This leads to hypoferrimia (low serum iron). Less iron is available to erythroid precursors in bone marrow that causes iron deficient erythropoiesis despite sufficient iron stores.

One of the most important iron regulators is hepcidin. In ACD it is increased in response to inflammation. Hepcidin inhibits release of iron from macrophages and iron absorption.

Cytokines induced suppression of erythropoiesis

Cytokines play a pivotal role in the pathogenesis of ACD. IL-1, IL-6, TNF-α, transforming growth factor-β (TGF-β) and IFN-γ are produced in inflammatory conditions. These cytokines interacts with the early erythroid progenitors and reduce their sensitivity to erythropoietin. Reduced sensitivity to erythropoietin leads to the suppression of erythropoiesis.

Decreased erythropoietin levels and decreased red cell production

IL-1, IL-6, tumor necrosis factor (TNF) and TGF-β also inhibits the production of erythropoietin. Diminished erythropoietin level leads to decreased production of red cells.

Clinical Features

Clinical features of ACD depend upon the type of condition. Features of anemia include mild anemia, weakness, headache, pallor, shortness of breath and fatigue.

Laboratory Findings

Hematological

Complete blood counts usually show normocytic and normochromic anemia. The anemia may be hypochromic if the disease progresses. Red cell indices may be normal or reduced. Leukocyte count may be normal or increased secondary to underline condition. Platelet count remains normal.

Bone marrow

Bone marrow usually shows an increased myeloid to erythroid ratio (M:E) due to suppressed erythropoiesis. Erythropoiesis may be normoblastic or microblastic. Myelopoiesis and megakaryopoiesis remain normal. Prussian blue/ Perl’s stain shows increased iron stores. This feature differentiates ACD form iron deficiency anemia. Iron stores are absent in IDA.

Biochemical

Biochemical findings show decreased serum iron, decreased total iron binding capacity, low transferrin saturation and normal or increased ferritin levels. Care must be taken into account
in the interpretation of ferritin. As it is an acute phase protein and may be increased during infection or inflammation. Serum hepcidin levels are increased in ACD.

**Suggested further reading**

CHAPTER -11

MACROCYTIC ANEMIAS

MEGALOBLASTIC ANEMIA
Megaloblastic anemia is characterized by defective nuclear maturation caused by impaired deoxyribonucleic acid (DNA) synthesis, manifested by the presence of megaloblasts in the bone marrow and oval macrocytes in the peripheral blood. Megaloblastic changes are not limited to red cell precursors; granulocytic precursors also are larger than normal, with giant metamyelocytes being a striking feature. Other nucleated actively proliferating cells i.e. skin, intestinal mucosa, vaginal, uterine, cervical and buccal cells also show megaloblastic changes.

Etiology
Major causes of megaloblastic anemia are folate deficiency, vitamin B\textsubscript{12} deficiency or both. Megaloblastic anemia can also be a result of myeloproliferative disorders, acute leukemias, myelodysplastic syndrome and drugs associated.

Causes of Folate Deficiency
- **Inadequate intake**
  Nutritional deficiency of folate may arise not only when total folate content of the diet is low but also when food is cooked for prolonged periods. This is because naturally occurring folates are heat labile and are destroyed during prolonged cooking. Nutritional folate deficiency is also encountered frequently in infants, alcoholics, elderly and disabled persons. It is important to note that isolated folate deficiency is rather uncommon on nutritional basis alone. It is more common to see folate deficiency as part of a spectrum of multiple deficiencies.

- **Malabsorption**
  Conditions associated with folate malabsorption are tropical sprue, non-tropical sprue, resection of the jejunum, leukemic or lymphomatous infiltration of small bowel, progressive systemic sclerosis, Whipple's disease, systemic amyloidosis, diabetes mellitus and drugs.

  Drugs that cause folate deficiency are anticonvulsants (Dilantin, Primidone and Phenobarbitone), mestranol-containing contraceptive pills, antituberculous drugs (INH, Cycloserine) and lutethimide.

  Rarely folate deficiency is seen as isolated conditions in which the body is specifically unable to absorb the ingested folate.

- **Increased body requirement**
  This is commonly seen in chronic hemolytic disorders, neoplastic diseases, pregnancy, lactation, adolescence, febrile and septicemic conditions and chronic exfoliative dermatitis.

- **Impaired utilization**
  Causes of impaired utilization are anti-folate drugs (trimethoprim and methotrexate) and inborn errors of folate metabolism.

- **Increased loss in hemodialysis**
Causes of Vitamin B₁₂ Deficiency

- Dietary deficiency
  It is rather unusual to develop vitamin B₁₂ deficiency on nutritional basis alone. Strict vegetarians who do not take any animal food including eggs, milk and the milk products for a prolonged period, may develop vitamin B₁₂ deficiency.

- Gastric
  Gastric causes of vitamin B₁₂ deficiency include total or subtotal gastrectomy, atrophic gastritis and Pernicious anemia

- Intestinal
  - Fish tape worm infestation: These worms preferentially take up dietary vitamin B₁₂ for their own metabolic needs. This is seen in those areas where eating raw fish is a common practice. The degree of anemia is proportionate to the worm load and its location; the higher the location of the worm in the GI tract the severer is the deficiency of B₁₂.
  - Bacterial proliferation in the gut: This is seen in blind loop syndrome, malabsorption syndrome, intestinal strictures, diverticular disease and after Billroth gastrectomy.
  - Diseases of the ileum: Diseases of ileum associated with deficiency of vitamin B₁₂ are tuberculosis, Crohn's disease, ileal resection and tropical sprue. Congenital deficiency of intrinsic factor receptors on ileal mucosa known as Immerlund's disease is associated with the deficiency of vitamin B₁₂.
  - Utilization defects: This is a group of rare metabolic disorders in which vitamin B₁₂ cannot be utilized properly. Supply and stores of vitamin B₁₂ are normal or even greater than normal, but the body is unable to utilize it and thus it "starves in the midst of plenty". Some of these conditions are enzyme deficiencies, congenital absence of transcobalamin II and abnormal transcobalamin II.

Pathophysiology

Defective nuclear maturation and megaloblastic morphology of cells is caused by decreased thymidine triphosphate (TTP) synthesis from uridine monophosphate (UMP). This deficiency interferes with nuclear maturation, DNA replication and cell multiplication. When TTP is not present in adequate amounts deoxyuridine triphosphate incorporates into the DNA. This mis-incorporation causes delayed maturation of the nuclear chromatin, fragmentation of the nucleus and immature cell destruction.

Primary cause of the lack of thymidine and consequently defective DNA synthesis are folate and vitamin B₁₂ deficiency. These vitamins, in the form of cofactors, play important role in the key reactions involved in the DNA synthesis as shown in figure 11.1.

![Thymidine synthesis pathway from uridine nucleotide](image-url)

Figure 11.1: Thymidine synthesis pathway from uridine nucleotide
UDP = uridine diphosphate, dUDP = deoxyuridine diphosphate, dUTP = deoxyuridine triphosphate, dUMP = deoxyuridine monophosphate, dTMP = deoxythymidine monophosphate, dTDP = deoxythymidine diphosphate, dTTP = deoxythymidine triphosphate, CH$_2$THF = methylene tetrahydrofolate.

Clinical features of folate and vitamin B$_{12}$ deficiency

Folate and vitamin B$_{12}$ are required for the production and maturation of DNA. In folate/vitamin B$_{12}$ deficiency, the nuclear dysmaturation produced adversely affects the functions of many organs. The abnormality however, is marked in those organs where cell proliferation is high and uric acid turnover is rapid as in the GI tract.

Glossitis, dyspepsia, anorexia, abdominal pain, distension and diarrhoea are some of the common complaints in this condition. Diarrhoea produces folate deficiency while folate deficiency may itself make the diarrhoea worse. Many a times folate deficiency is due to malabsorption syndrome. In this situation, the features of deficiency of other types of food may also be present.

Nervous system is characteristically involved in vitamin B$_{12}$ deficiency even though its metabolic activity is quite low. Neurological abnormalities include symmetrical paresthesiae in hands and feet, loss of vibration sense, spastic ataxia, cranial neuropathy, psychiatric disturbances and pseudo-tumor cerebri.

Anemia is variable in its severity and is essentially a reflection of the duration and the severity of folate/vitamin B$_{12}$ deficiency. Development and progression of anemia is slow and body has sufficient time to bring into play various compensatory mechanisms to improve tissue oxygenation. It is therefore not unusual for patients to seek medical advice only when anemia is severe enough to cause cardiac decompensation. Common presenting features due to anemia are weakness, palpitations, light-headedness, and dyspnoea on exertion.

In the bone marrow, there is ineffective erythropoiesis with consequent pancytopenia in the peripheral blood. Thrombocytopenia may cause cutaneous and mucosal bleeding and retinal hemorrhages. Leukopenia predisposes to infections when leukocyte count falls below 1000/µl.

Laboratory Findings

Hematological

Peripheral blood counts show anemia, leukopenia, thrombocytopenia and reticulocytopenia. Megaloblastic anemia is a macrocytic anemia characterized by the presence of oval macrocytes (see figure 11.2). Depending upon the severity of anemia, the MCV may range from 100-160 fl. Other red cells morphological abnormalities include anisocytosis and poikilocytosis, fragmented cells, tear drop cells Howell-Jolly bodies, basophilic stippling and Cabot’s ring. Neutrophils characteristically show hyper segmentation.
Bone marrow

Oval macrocytosis is a morphological manifestation of systemic megaloblastosis. Marrow is an eminently suited organ to reflect this process. Changes in the marrow are quantitative as well as qualitative.

Marrow cellularity in megaloblastic anemias is markedly increased; at times the marrow particles are packed due to erythroid hyperplasia. Red cell precursors are larger than normal and show the characteristic cytonuclear dissociation. Majority of the erythroid precursors are at basophilic and polychromatophilic stages; orthochromic erythroblasts are less prominent as shown in figure 11.3.

Mitoses are increased and some binucleate or even trinucleate erythroblasts may be seen. Evidence of dyserythropoiesis in the form of abnormal nuclear shapes, nuclear fragmentation, delayed maturation and intracytoplasmic inclusions are frequently seen.

Hallmark of megaloblastic erythropoiesis are the polychromatophilic megaloblasts which can be identified unmistakably. Basophilic megaloblasts are also numerous and constitute an important feature of megaloblastic marrows. Diagnosis based on promegaloblasts is less convincing.

Abnormalities in the granulocytic series also constitute an important component of megaloblastic hematopoiesis. Whereas all stages of development of granulocytes are abnormal, it is the giant metamyelocytes, giant bands and hypersegmented neutrophils which are the hallmark of megaloblastosis.

Giant bands as the name suggests, are large in size but the characteristic feature is their large sized nucleus with arms of the U-shaped nucleus being very broad and thick; nuclear chromatin is also quite open. Neutrophils characteristically show hyper segmentation. Megakaryocytes are reduced in number and show polyploidy.

Marrow iron is increased and is present in the form of fine to medium sized granules of hemosiderin. Ringed sideroblasts are not a feature of megaloblastic erythropoiesis.
Biochemical

Those parameters which are decreased in vitamin B₁₂ deficiency are serum vitamin B₁₂, serum alkaline phosphates, serum uric acid, serum cholesterol and serum haptoglobins.

Biochemical parameters increased in vitamin B₁₂ deficiency are serum unconjugated bilirubin, serum potassium, serum iron, % saturation of transferrin, serum LDH, Methyl-malonic acid in the urine, serum β-leucine and serum muramidase.

Biochemical findings in folate deficiency are low serum folate, low red cell folate, increased excretion of Figlu after histidine load, rapid plasma clearance of the intravenously administered folic acid, decreased urinary excretion of orally administered folic acid, increased serum LDH and increased serum iron.

Differential Diagnosis

Once oval macrocytes and hypersegmented neutrophils are found in the peripheral blood along with megaloblastic erythropoiesis in the bone marrow the differential diagnosis then narrows down to conditions which produce folate and vitamin B₁₂ deficiency.

On morphological grounds alone, it is not possible to distinguish between these two conditions with any certainty. Clinical features and family history may suggest the underlying condition. Neurological deficit in the form of peripheral neuropathy, cranial neuropathy, motor disturbances and disturbed cortical functions suggest vitamin B₁₂ deficiency.

Other conditions in the differential diagnosis are those disorders which produce folate deficiency. No major work up is usually required to determine the cause of folate deficiency. A careful history and physical examination and perhaps a small bowel biopsy are sufficient in most of the cases to establish the cause of folic acid deficiency.

Once it is established that the basic defect is vitamin B₁₂ deficiency, the next step is to determine the cause of its deficiency. Conditions which produce vitamin B₁₂ deficiency have already been enumerated.

PERNICIOUS ANEMIA

Pernicious anemia is a genetic disorder characterized by inability to secrete adequate amount of intrinsic factor in the gastric juice for the physiological absorption of vitamin B₁₂. Whether or not this genetic predisposition leads to a clinically manifest disease depends upon many factors. Some of these are severity of the gastric lesion, duration of the gastric lesion, dietary intake of vitamin B₁₂ and body’s requirements of vitamin B₁₂.
Pernicious anemia can be classified into three types:

- Congenital pernicious anemia
- Juvenile pernicious anemia
- Classical or adult onset pernicious anemia

These three apparently separate entities may for the sake of simplicity be considered as a spectrum of the same disease in which extra-genetic factors determine the sub type of pernicious anemia. Some of these extra-genetic factors are; abnormalities of serum immunoglobulins, anti-intrinsic factor antibodies, anti-parietal cell antibodies and associated endocrine abnormalities and proteinuria.

**Clinical Features**

Clinical features of pernicious anemia are primarily due to vitamin B\(_{12}\) deficiency. These have already been described in detail under the heading of megaloblastic anemia. In the adult onset pernicious anemia, features of associated immunological diseases like vitiligo, thyroid disorders, Addison's disease etc. may modify the clinical picture. In the late stages of the disease, development of gastric carcinoma may also alter the clinical manifestations.

**Laboratory Features**

Laboratory abnormalities in pernicious anemia are similar to those which are seen in vitamin B\(_{12}\) deficiency due to other causes. Certain laboratory features however, are seen exclusively in pernicious anemia. These include:

- Achlorhydria
- Antiparietal cell antibodies
- Anti-intrinsic factor antibodies
- Antibodies against other endocrine glands
- Immunoglobulin abnormalities
- Abnormal Schilling's test
- High serum gastrin levels
- Lack of hematological and biochemical response to physiological doses of oral vitamin B\(_{12}\).

Criteria for the diagnosis of pernicious anemia may be divided into:

- **Major criteria**
  - Positive Schilling's test
  - Achlorhydria after maximum histamine stimulation
  - Anti-intrinsic factor antibodies
- **Minor criteria**
  - Appropriate clinical features
  - Pancytopenia
  - Oval macrocytosis and hypersegmented neutrophils in the peripheral blood
  - Low serum vitamin B\(_{12}\) levels
  - Anti-parietal cell antibodies
Minor criteria in any combination alone are only suggestive of this disease. The presence of any one of the major criteria in association with some or all of the minor criteria establishes the diagnosis of pernicious anemia.

Achlorhydria

Achlorhydria is defined as resting gastric juice pH greater than 3.5 and its failure to drop by 1 unit on maximum histamine stimulation. Documentation of achlorhydria is a strong evidence for the diagnosis of pernicious anemia.

Anti-parietal cell antibodies

These are present in the serum and the gastric juice of almost all cases of pernicious anemia. They are however also present in certain other conditions. Some of these conditions are: atrophic gastritis, thyrotoxicosis and other auto-immune disorders. The presence of parietal cell antibodies therefore does not confirm the diagnosis of pernicious anemia; their absence however casts strong doubts on the accuracy of the diagnosis. Anti-intrinsic factor antibodies

These are also present in the serum, saliva and the gastric juice of patients with pernicious anemia. Salivary antibodies are of IgM type while those in the serum belong to IgG group. In the stomach both IgM and IgG antibodies are present. These antibodies are usually present in only 50% of the cases who have pernicious anemia documented by other criteria. Since they are not seen in any other condition, their presence establishes the diagnosis of pernicious anemia. Their absence, however, does not exclude this diagnosis.

Abnormal Schilling's test

This test is designed to separate the gastric causes of vitamin B₁₂ deficiency from the intestinal causes.

Conditions in which the second stage of Schilling's test is positive are:

- Pernicious anemia
- Total or subtotal gastrectomy
- Chronic non-specific atrophic gastritis

Schilling's stage II is negative in:

- Resection of the terminal ileum
- Diseases of the terminal ileum
- Bacterial proliferation in the small bowel (Blind loop syndrome)
- High titre intrinsic factor antibodies in the gastric juice
- Zollinger—Ellison's syndrome
- Congenital absence of intrinsic factor receptors (Immerslund's disease)

Suggested further reading

HEREDITARY HEMOLYTIC ANEMIAS

HEREDITARY SPEHROCYTOSIS

This disease is characterized by excessive and premature destruction of the red cells in the spleen. Red cells are spherocytic or even microspherocytic with somewhat decreased MCV, normal MCH, high MCHC and reduced surface area to volume ratio.

In hereditary spherocytosis it is the structural abnormalities of the membrane which are at the heart of the membrane changes in the red cell shape and functions. Hereditary spherocytosis shows considerable genetic heterogeneity. There are two genetic abnormalities which are inherited as autosomal dominant trait. These are the ankyrin and protein band 3.

Pathophysiology

Hereditary spherocytosis due to ankyrin gene defect

This, in fact, is an outfall of a mutation in the gene which codes for ankyrin. Deficiency of ankyrin impairs the assembly of spectrin meshwork and weakens the vertical interactions between spectrin and the overlying phospholipid lamina. Therefore it leads to combined deficiency of ankyrin and spectrin.

Hereditary spherocytosis due to protein band 3

This is another group of hereditary spherocytosis in which mutation is in the gene which is concerned with the synthesis of protein 3. It accounts for nearly 20% of all cases of hereditary spherocytosis. This variant is also inherited as autosomal dominant trait.

Red cells in hereditary spherocytosis at the time of release from the bone marrow into the circulation are of normal size and shape. Subsequent journey through the spleen and microcirculation causes irreversible damage to the red cell membrane which continuously shreds off causing transformation of the initially normally looking red cells into spherocytes.

To negotiate this journey successfully, deformability and plasticity of red cells is a prerequisite. Red cells in spherocytosis being spherocytic, fail to complete their transit through the spleen within the allocated period. Delayed transit through the hypoxic, acidic and mechanically testing environment in the open circulation of spleen inflicts additional damage to their membrane during each transit. Extreme pliability and deformability are essential prerequisites for the red cells to come out of the Billroth cords unscathed. Spherocytes because of their restricted deformability get trapped in the cords of Billroth. As more and more spherocytes become trapped in the cords, the spleen becomes enlarged and increasingly detrimental to the red cells.

The defect lies in the red cell membrane which is excessively permeable to sodium. As a result of this increased permeability, sodium enters into the red cells much more freely. In order to maintain electric neutrality and intracellular cation balance, sodium is actively pumped out of the
cells by the ATP-ase dependant Na-K pump. This increased work load on the pump is accompanied by an excessive loss of lipids from the cell membrane leading to spherocytes formation. Despite extensive work in this area, no clearly defined membrane defect appears to be a functional abnormality of the membrane cytoskeleton hence the exact cause for this functional modification is not known.

**Clinical Features**

Clinically, the disease manifests the classical triad of anemia, jaundice and splenomegaly. There are however considerable variations from patient to patient depending upon the severity of hemolysis. Pigment stones in the gall bladder, chronic leg ulcers, aplastic crisis, hemolytic crisis and megaloblastosis due to folic acid deficiency are some of the other characteristic of this disease.

**Laboratory Findings**

Laboratory features of hereditary spherocytosis are a reflection of the excessive red cell destruction in the spleen and compensatory erythroid hyperplasia in the bone marrow.

**Hematological**

Peripheral blood smear shows polychromasia and variable number of spherocytes amongst normocytic and normochromic red cells. Spherocytes in this instance are a reflection of the primary disease rather than an indicator of hemolysis. Leukocytes and platelets are normal in number and morphology. There are usually no red cell fragments, intra erythrocytic inclusions are target cells. Nucleated red cells, Howell-Jolly bodies and reticulocytes may appear in large numbers if hemolytic crisis supervenes. Oval macrocytes and hypersegmented neutrophils may appear in the peripheral blood if folate deficiency complicates the clinical picture. In aplastic crisis there is pancytopenia in the peripheral blood.

![Figure 12.1: Spherocytes](image)

**Bone marrow**

Bone marrow shows marked erythroid hyperplasia of the normoblastic or macronormoblastic type. Mitotic index is increased as is the erythroid/myeloid ratio. Marrow morphology may vary considerably according to:

- Severity of the disease
- Folate stores in the body
- Iron stores in the body
- Presence of complicating factors such as infections, aplastic crisis and hemolytic crisis
Biochemical
- Unconjugated hyperbilirubinemia
- Increased urobilirubinemia
- Increased strencobilinogen
- Decreased haptoglobin

Osmotic fragility test
Osmotic fragility test shows increased red cells fragility at low saline concentration. It is of diagnostic importance.

Differential Diagnosis
Hereditary spherocytosis should be differentiated from all those disorders which are characterized by anemia, splenomegaly and spherocytosis. Immunologically mediated hemolytic anemias usually pose the greatest diagnostic problems. Coombs test is always negative in hereditary spherocytosis while in the majority of cases of immune hemolytic anemias, this test is positive. Hematological investigations of the family members may also help in making this distinction.

HEREDITARY ELLIPTOCYTOSIS
This is a family of genetically determined red cell disorders transmitted as an autosomal dominant trait. Red cells, as the name suggests, are characteristically elliptical in shape. The abnormality does not manifest until the reticulocyte stage is reached and is not fully expressed until three months after birth. The basic defect is in the red cell membrane in which spectrin or spectrin-associated proteins are abnormal. This causes membrane permeability defect which leads to increased rate of ATP utilization and red cells thus become more prone to destruction in the spleen.

Hereditary elliptocytosis is a heterogeneous group of disorders characterized by clinical and laboratory findings ranging from an asymptomatic patient on the one hand to severe hemolytic anemia, jaundice, gall stones and splenomegaly on the other. Elliptocytes vary considerably in number; they may range from 25 to 90% of the total red cell population. Approximately 40 to 50% of the red cells in the peripheral blood are elliptical if the anomaly is inherited from one parent. If on the other hand, the disease is acquired from both parents, virtually all red cells assume elliptical shape. Evidence of hemolysis and marrow response is quite variable. It is important to note that the severity of hemolysis does not correlate with the degree of elliptocytosis.

Pathophysiology
Primary defect in HE lies in the structure of the cytoskeleton. The most frequently documented abnormalities are:

- Abnormal $\alpha$ and $\beta$-chains of spectrin
- Abnormal protein 4.1
- Abnormal protein 3

In the cytoskeleton of normal red cells, the spectrin molecules form dimers which polymerize to form tetramers and oligomers. In hereditary elliptocytosis the major cyto-skeletal abnormality is the failure of the spectrin dimers to polymerize further and to produce tetramers and oligomers. As a result of the abnormalities in the cytoskeletal proteins, a highly characteristic architectural defect becomes apparent. Another structural abnormality is the failure of the $\beta$-chain
of spectrin to bind to another cyto-skeleton protein called ankyrin or protein 4.1. Deficiency of protein 4.1 shows geographical restriction to Southern France and Northern Africa.

A recently described and interesting variant of HE which displays a highly restricted prevalence in South East Asia is caused by an abnormality of protein 3. In this variant, there is deletion of nine amino acids at the junction of the cytoplasmic and transmembrane domain of protein 3. This causes rigidity of the red cell membrane; the condition is however completely asymptomatic. An epidemiologically important association of HE due to protein 3 abnormality is the resistance of the red cells to invasion by the malarial parasites. This most likely is the result of loss of receptors for malarial parasites in the deleted segment of band 3 protein. This natural selection protects the Malanesian Aborigines against malaria.

Clinical Features
Hereditary elliptocytosis manifests a wide spectrum of clinical features with practically asymptomatic abnormality on the one hand to a severe hemolytic anemia on the other. Severe hemolytic disease is clinically indistinguishable from hereditary spherocytosis. Anemia, unconjugated hyperbilirubinemia, splenomegaly, leg ulcers and pigment gall stones occur in severe hereditary elliptocytosis. Hemolytic crises can be precipitated by viral illness, pregnancy and stress.

Laboratory Findings
Diagnosis of hereditary elliptocytosis is made by morphologic examination of blood smear in which 25 to 90 % of the red cells are elliptocytes (see figure 12.2). Other conditions in which elliptocytes are present in the peripheral blood invariably contain less than 15% of the elliptocytes. In severe hemolytic variant of HE the peripheral blood smear shows spherocytosis, polychromasia, poikilocytosis and fragmented red cells. Significant spherocytosis in the peripheral blood is associated with increased red cell osmotic fragility.

Figure 12.2: Elliptocytosis

GLUCOSE 6 PHOSPHATE DEHYDROGENASE DEFICIENCY ANEMIA

Glucose 6 phosphate dehydrogenase, popularly known as G6PD, is an intracellular enzyme. Though present in all cells of the body, it is of vital importance to the red cells for two reasons. Firstly it is the key enzyme in the hexose monophosphate (HMP) pathway which generates reduced glutathione in the red cells for the conversion of oxidized hemoglobin
(methemoglobin) back to its reduced and functional state. Secondly, one molecule of G6PO$_4$ which enters HMP pathway produces 48 molecules of energy in the form of ATP as opposed to 6 molecules of ATP generated through Embden Myerhoff pathway. G6PD deficiency is one of the most common genetic enzymopathies of red cells affecting more than 400 million people worldwide.

**Inheritance**

Structural gene for G6PD is located on long arm of X-chromosome (Xq28) hence the defect is transmitted as an X-linked character. It is for this reason that the defect is fully expressed in males while females are usually asymptomatic carriers. Any mutation or deletion in the structural gene is encoded in the mRNA. The abnormal mRNA thus produced is responsible for the synthesis of the abnormal enzyme. The abnormality can be detected by amino acid analysis of the polypeptide chain of the enzyme.

Some of the common patterns of inheritance are shown in the following examples:

- **Marriage between a normal female and an affected male:** Affected father cannot transmit disease to his sons (because of his contribution of the normal Y-chromosome). All his daughters will be carriers (because of his contribution of the abnormal X-chromosome.
- **Marriage between a carrier female and an affected male:** In this marital setup there will be no normal female off-springs. Fifty percent of the male children will be normal while the rest will be affected.
- **Marriage between an affected male and an affected female:** In this family all sons and daughters will be affected and no normal child will be born.
- **Marriage between a normal male and a carrier female:** In this marital set-up half of the daughters will be carriers while the rest will be normal. Half of the male children will be normal while the other half will be affected.

A female can suffer from overt G6PD deficiency if she is born as a result of the marital set-up II and III. In those communities where the incidence of G6PD deficiency is high and consanguinity is common, female patients with overt G6PD deficiency are frequently encountered.

**Pathophysiology**

One of the most important functions of G6PD is that it offers protection to the red cells against oxidative damage by generating and sustaining the reducing potential in the form of reduced glutathione.

The first step in the chain of chemical reactions that culminates in the regeneration of reduced glutathione is the conversion of glucose-6-phosphate to 6-phosphogluconolactone. In this reaction one molecule of hydrogen is removed from glucose6 PO$_4$ and is transferred to NADP which is reduced to NADPH as shown 12.3.

6-phosphogluconolactone, through a series of enzyme mediated reactions, is converted to fructose-6 P0$\_4$ and 3-phosphoglyceraldehyde which rejoin the glycolytic pathway as shown figure 12.3.

NADPH so generated, participates in the reaction in which glutathione is converted to reduced glutathione as shown in figure 12.4.
Conversion of glutathione to reduced glutathione is an essential step in the reduction of methemoglobin. In G6PD deficiency, conversion of glutathione to reduced glutathione is impaired; this adversely affects the ability of the red cells to convert methemoglobin to reduced hemoglobin.

Methemoglobin cannot deliver oxygen because of the oxidation of its ferrous atom. Normally about 1% of intra-erythrocytic hemoglobin exists as methemoglobin. This level is kept...
constant through an adequate supply of NADPH and regeneration of reduced glutathione. In the presence of some variants of G6PD and under the influence of certain precipitating factors which aggravate the oxidant stress, the concentration of methemoglobin progressively increases to the detriment of red cell survival. Inability of G6PD deficient red cells to transport normal amount of oxygen impairs tissue oxygenation. It also leads to accumulation of methemoglobin in the red cells. Precipitation of methemoglobin in the red cells predisposes them to excessive and premature destruction.

While considering the role of G6PD in the red cell metabolism, it is important to appreciate that red cells are non-nucleated; also they do not contain any synthetic machinery in the form of cytoplasmic organelles. They can not replenish and regenerate their enzymes. There is progressive decline in the concentration and functional capabilities of various red cell enzymes. This is considered to be one of the reasons for the finite span of the red cells.

Clinical Features

Overall incidence of G6PD deficiency across the globe is 10% which makes it the single most common enzymopathy in Man. Compared to the prevalence of the ‘deficiency’ the clinical manifestations are infrequent.

Since G6PD is present in every cell of the body, it is reasonable to expect that clinical manifestations due to the deficiency of this enzyme will be widespread and affect multiple systems. In practice the deficiency mostly produces features of hemolytic anemia and that too under special circumstances. The spectrum of clinical disease due to G6PD deficiency is as follows:

- Asymptomatic
- Non-spherocytic congenital hemolytic anemia
- Neonatal unconjugated hyperbilirubinemia
- Episodic hemolysis associated with
  - Drugs
  - Infections
  - Favism

Laboratory Findings

Peripheral blood counts show decreased hemoglobin with reticulocytosis and features of hemolytic process.

Biochemical

Hyperbilirubinemia, increased lactate dehydrogenase (LDH), decreased haptoglobin and hemoglobinuria. Hemolytic episodes are also characterized by the presence of irregularly contracted red cells (eccentrocytes with hemoglobin puddled to one side of the RBC) and “bite” cells. These eccentrocytes are formed due to the separation of hemoglobin form the membrane leaving an unstained non hemoglobin containing cell membrane. Heinz bodies can be demonstrated using brilliant cresyl blue supra vital stain.

Diagnosis of G6PD deficiency rests with the demonstration of decreased enzyme activity in the red cells. This may be achieved by screening tests which are easier, simpler and commercially available. The most reliable tests are the screening procedures which are based on fluorescence development. Ultimate diagnosis however rests with quantitative assay of the enzyme.
PYRUVATE KINASE DEFICIENCY ANEMIA

Pyruvate kinase (PK) deficiency is the commonest of the enzymopathies of the Embden-Meyerhof (glycolytic) pathway and the second most common erythrocyte enzyme deficiency. It is an autosomal recessive disorder. Anemia manifests in homozygous state. Individuals with heterozygous condition have normal red cell metabolism though their red cells may have slightly lower levels of enzyme activity.

**Pathophysiology**

Red cells synthesize ATP through Embden-Meyerhof pathway. Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate with the generation of ATP. PK deficiency results in the decreased capacity to generate ATP. ATP-requiring membrane pumps (ATPase) that maintain the proper electrochemical gradients begin to fail with decreasing concentration of ATP. It results in the loss of potassium from the cells that leads to cell dehydration, cell shrinkage, distortion of cell shape and increased membrane rigidity. These membrane abnormalities result in premature destruction of red cells in the spleen and liver with consequent anemia.

**Clinical Features**

Severity of anemia associated with PK deficiency varies from mild to severe. Homozygous PK deficiency is characterized by anemia, severe jaundice, kernicterus and non spherocytic hemolytic anemia that may require repeated transfusion during life. In moderated PK deficiency hemolysis, is more pronounced during infections, pregnancy and other stresses.

Gall stones are common in PK deficiency and may lead to bouts of choelcystitis and biliary colic. Excess iron accumulation may occasionally develop even in the absence of transfusion or co-inheritance of a haemochromatosis gene.

**Laboratory Findings**

**Hematological**

Peripheral blood in PK deficiency show normochromic normocytic anemia with reticulocytosis and spur cells or acanthocytes. During hemolytic episode, peripheral smear shows polychromasia, poikilocytosis, anisocytosis and nucleated red cells.

**Bone marrow**

Bone marrow examination shows normoblastic erythroid hyperplasia. Myeloid and megakaryocytic series are normal. Perl's stain demonstrates increased iron stores in patients with PK deficiency; a characteristic finding of hemolytic anemia.

**Biochemical**

Serum unconjugated bilirubin levels are usually increased in PK deficiency anemia. Serum haptoglobin levels are decreased or absent. LDH is usually increased.

Screening tests of PK deficiency include Coombs test, autohemolysis and osmotic fragility test. Coombs test in PK deficiency is negative that helps to exclude immune hemolysis. Definite diagnosis is made on the determination of the enzymatic activity. Enzyme activity rate in most patients with PK deficiency is 5-25% of normal.
THALASSEMA

Thalassemia is a hereditary blood disorder characterized by impaired synthesis of the globin part of the hemoglobin molecule due to abnormalities in the globin genes. It is characterized by reduction in the amount of hemoglobin in each red cell.

The word thalassemia is derived from a Greek word ‘thalas’ which means ‘the sea’. The disease was first diagnosed in Greece which is situated along the coast of Mediterranean sea. It is a well-established fact that thalassemia is widely distributed all over the world. It is one of the most common hemoglobinopathy worldwide. Prevalence of β thalassemia is more in the Mediterranean region while α thalassemia is more common in the Far East.

In 1925, two American pediatricians, Thomas Cooley and Lee first described thalassemia as a disease characterized by severe anemia, splenomegaly and bone deformities. Thalassemia syndrome can be classified on the basis of:

Type of globin chain deficiency:

- α thalassemia
- β thalassemia
- δβ thalassemia
- γβ thalassemia
- Miscellaneous
  - HbS thalassemia
  - HbE thalassemia
  - HbD thalassemia

Clinical severity of disease

- β thalassemia
  - β thalassemia minor
  - β thalassemia intermedia
  - β thalassemia major
- α thalassemia
  - Silent α thalassemia
  - α thalassemia trait
  - HbH disease
  - Hydrops fetalis

β THALASSEMA

β thalassemia is an autosomal recessive disorder characterized by either partial or absolute deficiency of β globin chains of the hemoglobin molecule. As discussed earlier, β thalassemia can be classified into the following three types based upon the severity of the disease.

β Thalassemia Major

β thalassemia major is characterized by complete absence of β globin chain production. This result in complete absence of hemoglobin A that manifests after birth when switch over of hemoglobin F by hemoglobin A occurs. It is characterized by severe anemia, failure to thrive, hepatosplenomegaly and bone deformities.
Pathophysiology

In β thalassemia major there is complete absence of β globin chain, however α globin chain production is not affected. As a result, free α globin chains accumulate in the developing red cells to form α globin chain dimers which precipitate out as intracellular inclusions. These inclusions are pitted out by reticuloendothelial system that results in premature hemolysis. Anemia causes increase erythropoietin secretion from kidney that leads to erythroid hyperplasia in the bone marrow. This results in expansion of medullary cavities of bones specially skull bones.

Clinical Features

At birth, patients with β thalassemia major are asymptomatic due to increased concentration of hemoglobin F. It presents within the first year of life when the switch over of hemoglobin F to hemoglobin A fails due to decreased amount of β chains. These patients present with severe hemolytic anemia. Imbalance of globin chains also results in ineffective erythropoiesis. Splenomegaly with distended abdomen is common in these patients that at times it is associated with hepatomegaly. Thalassemic faces i.e. frontal bossing as a result of expansion of cranial bones and overgrowth of zygomatic bones causes prominent cheek bones leading to mongoloid or chipmunk faces. These patients present with failure to thrive, retarded growth and delayed milestones. Increased susceptibility to infections is also a clinical finding in these patients.

Repeated blood transfusions lead to accumulation of iron in various organs. Myocardial hemosiderosis leads to arrhythmias and cardiac failure. Hemosiderosis of liver and spleen leads to abnormal function of liver and spleen. Diabetes mellitus is caused by iron deposition in islets of Langerhans of pancreas. Iron deposition in endocrine organs leads to hypothyroidism, hypoparathyroidism and growth hormone deficiency. Gonadal dysfunction is associated with delayed puberty and infertility. Due to multi transfusions these patients are at risk of transfusion transmissible infections i.e. Human immunodeficiency virus (HIV), Hepatitis C virus (HCV) and Hepatitis B virus (HBV).
Laboratory Findings

Hematological
Complete blood counts show decreased hemoglobin level. Red cell indices show microcytic and hypochromic anemia as MCV, MCH and MCHC remain below normal in un-transfused patients. Red cell distribution width (RDW) is markedly increased.

Red cell morphology shows marked anisocytosis, pokilocytosis, polychromasia and severe hypochromia. Nucleated RBCs are present. Target cells and fragmented red cells are characteristic findings as shown in figure 12.5. Coarse basophilic stippling can be easily observed. Tear drop cells and occasionally elliptocytes may be seen. Howell jolly bodies are visible. Increased reticulocyte count is a consistent feature of β thalassemia major.

White cell count may be normal or increased in number showing toxic changes due to infection. Usually, platelet count remains normal.

Figure 12.5: Preipheral smear with features of hemolytic process

Bone marrow
Bone marrow examination is not indicated in β thalassemia major. However, bone marrow shows erythroid hyperplasia with marked ineffective erythropoiesis. Myeloid and megakaryocytic cell lines show normal maturation. Increased iron stores can be demonstrated by Perl's/Prussian blue or iron stain.

Biochemical
Hemolytic process leads to decreased serum haptoglobin, increased serum LDH, unconjugated hyperbilirubinemia and increased urobilinogen. Iron profile shows markedly increased serum iron, increased serum ferritin and significantly increased total iron binding capacity (TIBC).

Diagnostic tests of β thalassemia major
- Hemoglobin electrophoresis shows marked elevation of hemoglobin F (10-98%), hemoglobin A₂ (≥3.5%) and low hemoglobin A level as shown in figure 12.6.
- Hemoglobin F can also be demonstrated by acid elution test or by alkali denaturation test.
- Heinz bodies can be demonstrated by supra vital stains.
- DNA analysis specifies the genetic mutation which determines the severity of the disease.

**Radiological findings**

X-ray of skull shows *hair on end* appearance. These are the striations at right angles to the inner table of temporal bone. X-rays of metatarsals, metacarpals and phalanges show mosaic pattern due to trabeculations.

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**Figure 12.6: Location of various hemoglobins on cellulose acetate electrophoresis strip**

<table>
<thead>
<tr>
<th>Disease</th>
<th>H</th>
<th>A</th>
<th>F</th>
<th>S</th>
<th>Aβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>01 Normal (or α-thal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>02 Sickle cell trait</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03 Sickle cell disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>04 β-thalassemia trait</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05 Sickle cell / Hβ-thalassemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06 Sickle cell / Hb C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>07 Hemoglobin H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>08 β-thalassemia major</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>09 Hemoglobin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Line of hemolysate application
Carbonic anhydrase
**β Thalassemia Intermedia**

β thalassemia intermedia is an entity of β thalassemia in which the clinical symptoms are intermediate between β thalassemia major and β thalassemia minor. It is characterized by mild to moderate anemia which is occasionally transfusion dependent.

**Pathophysiology**

β thalassemia intermedia is a genetic defect in the production of β globin chains. It is caused by minor and/or silent mutations in the β globin genes. It can be inherited as homozygous or compound heterozygous state. In this condition there is reduction in β globin chains resulting in low level of adult hemoglobin causing varying degree of anemia.

**Clinical Features**

β thalassemia intermedia manifests a wide clinical spectrum. In cases of silent mutations, patients are completely asymptomatic until adulthood with only mild anemia. Children with β thalassemia intermedia usually present between 2-6 years of age. These patients usually present with moderate anemia, occasionally severe anemia may occur that requires transfusion. Splenic and liver enlargement is also a salient feature.

**Laboratory Findings**

**Hematological**

Complete blood counts shows moderate anemia with decreased MCV, MCH and mean MCHC. RDW is either normal or increased depending upon the clinical severity of anemia. Morphology shows microcytic and hypochromic red cells, target cells, fragmented cells and tear drop cells. Mild reticulocytosis < 5% may be observed in these patients. White blood cells and platelets are usually normal.

Hemoglobin electrophoresis shows decreased amount of Hb A, variable amount of Hb A₂ and usually increased level of Hb F.

Molecular genetic testing determines the specific genetic mutation of β globin gene.

**β Thalassemia Minor (Trait)**

It is the heterozygous carrier state of β thalassemia when β thalassemia gene is inherited from only one parent. It is usually asymptomatic as sufficient amount of hemoglobin A is produced which prevents the adverse effects of hemolytic anemia. Still then it has very characteristic red cell morphology.

**Clinical Features**

β thalassemia minor is usually asymptomatic. However hemoglobin level remains slightly reduced than normal. These patients do not require blood transfusion normally.

**Laboratory Findings**

Complete blood counts shows slightly decreased hemoglobin concentration. Red cell count is relatively increased in relation to hemoglobin concentration. Red cell indices shows microcytic hypochromic anemia as MCV and MCH is reduced. MCHC is normal. RDW is normal. Morphology of red cells shows anisocytosis, hypochromia, poikilocytosis and polychromasia. Numerous target cells, few tear drop cells, occasionally elliptocytes and fragmented cells may be seen. Coarse basophilic stippling and occasionally Howell-jolly bodies are visible. White cell and platelet counts are usually normal.
**Bone marrow**
Bone marrow examination is not recommended for the diagnosis. However, if performed, reveals marked erythroid hyperplasia with increased iron stores.

**Biochemical**
Low serum haptoglobin, increased serum LDH, unconjugated hyperbilirubinemia and increase urobilinogen may be observed during hemolytic episode or during stressful conditions. Serum iron may be increased secondary to increased absorption and hemolysis.

**Diagnostic tests**
Hemoglobin electrophoresis is the diagnostic test for β thalassemia minor. This shows marked elevation of HbA₂ (> 3.5%), Hb F (≥ 1%) and low HbA.

**α THALASSEMIA**
α thalassemia is characterized by reduced production of α-globin chains. Like β thalassemia, α thalassemia is also a genetic disorder. In this condition the genes which govern the synthesis of α chains are usually absent (gene deletion) as opposed to β thalassemia in which the corresponding genes are present but they are abnormal and non-functional (gene mutation). Genes for α-globin chain are located on chromosome no 16.

Unlike β chains which are synthesized according to one gene-one chain hypothesis, there are two α-genes for the synthesis of one α globin chain. Clinical features of thalassemia in children are dictated by the type of thalassemia and also by the series of their gene defect. Hence for two α chains in one molecule of hemoglobin there are four alpha genes. Depending upon the number of α genes that are deleted, there are four types of α-thalassemias as listed in table 12.1.

<table>
<thead>
<tr>
<th>Clinical nomenclature</th>
<th>Genetic defect</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>α thalassemia silent carrier</td>
<td>One gene missing</td>
<td>αα/α-</td>
</tr>
<tr>
<td>α thalassemia trait</td>
<td>Two genes missing</td>
<td>α-/αα</td>
</tr>
<tr>
<td>Hb. H disease</td>
<td>Three genes missing</td>
<td>α/-/αα</td>
</tr>
<tr>
<td>Hydrops fetalis</td>
<td>All four genes missing</td>
<td>--/--</td>
</tr>
</tbody>
</table>

**Clinical Features and Laboratory Findings**
Clinical features depend upon the absence of number of α gene. Types of α thalassemia with common clinical features and laboratory findings are as under;

**Silent carrier [-α/αα]**
These individuals have no anemia, splenomegaly or any detectable clinical symptoms. It is characterized by the presence of very small amount of (up to 2%) hemoglobin Bart’s at birth. After disappearance of hemoglobin Bart’s, no recognizable hematologic abnormality is seen except for the borderline low MCV of red cells.
α thalassemia trait [-α/-α, --/αα]

There is mild anemia with slight microcytosis and hypochromia. After disappearance of hemoglobin Bart’s the electrophoretic pattern of hemoglobin appears normal. α thalassemia trait is usually a mild and asymptomatic disease. Patients generally lead a normal life. They also function normally in every respect including reproduction. They may, however remain pale and slightly jaundiced and their spleen may be a little larger than normal. Women with thalassemia minor may become more anemic during pregnancy. These patients are diagnosed either on a routine hematological examination or during family studies because of the birth of a baby with hydrops fetalis.

Hb H disease [α/-–]

Children with hemoglobin H disease present with variable degree of microcytic and hypochromic anemia. Anemic patients may develop the physical and bony characteristics of thalassemia major. Hepatomegaly and splenomegaly are common in these patients. These patients rarely need blood transfusion.

Adults with hemoglobin H disease may have hemoglobin H from 5-40%, with the remainder being mostly hemoglobin A, with small amount of hemoglobin A2 and hemoglobin Bart’s. Infants who later develop hemoglobin H disease usually have 19-27% hemoglobin Bart’s at birth with the remainder composed of hemoglobin F and A.

Hydrops fetalis [-/-–]

In this type of α thalassemia, no α chains are produced. Because of the complete suppression of α globin gene no Hb F or Hb A is produced. At birth, these infants are severely anemic and edematous and present with ascites, marked hepatomegaly and splenomegaly. Death of the baby takes place either in utero due to fetal hypoxia or very soon after birth.

SICKLE CELL ANEMIA

Sickle cell disorder is a member of hemoglobinopathies characterized by the production of abnormal sickle hemoglobin (HbS). Sickle cell anemia is inherited as an autosomal codominant trait which represents the homozygous form. Hemoglobin S is produced when nonpolar valine is substituted for polar glutamic acid at 6th position in the β chain. In homozygous state the individual inherits a double dose of the abnormal gene (ββS) that codes for hemoglobin S. Individuals heterozygous for HbS (ββS/ββA) produce more than 50% of their hemoglobin in the form of HbA. Usually amount of HbS is only 25-35% of the total hemoglobin present.

Pathophysiology

Hemoglobin S is soluble and usually causes no problem when properly oxygenated. When the red cells containing HbS passes through microcirculation of spleen, alteration in the solubility of hemoglobin occurs. HbS tends to polymerize to form tactoids or fluid polymers in reduced O2 tension. This results in sickling of red cells which can be reversed in good O2 tension. Repeated sickling and unsickling of red cells causes membrane damage and ultimately they become permanently sickled. These sickle cells get trapped in the splenic macrophage that results in chronic extravascular hemolysis. As these cells are less deformable in circulation due to mechanical fragility they get destroyed leading to intravascular hemolysis but its percentage is minimal. These sickle cells also adhere to the vascular endothelium that leads to vasoocclusive crisis. This leads to hypoxia, painful crises and infarction of the organs. Sickle cells also aggregate in vessels that result in increased blood viscosity, vascular stasis and ischemia.
Factors that influence the sickling phenomenon are intracellular concentration of HbS, increased MCHC, reduced oxygen tension, acidosis, cold temperature and association with thalassemia and other hemoglobinopathies.

**Clinical Features**

Sickle cell anemia usually presents at about 6 months of age when levels of hemoglobin F falls. At the time of presentation patients with sickle cell anemia presents with moderate to severe hemolytic anemia. Stunted growth, delayed milestones and delayed puberty are the features of sickle cell anemia. Initially there is progressive splenic enlargement but in later life due to multiple infarcts as a result of vasoocclusion, autosplenectomy occurs. Recurrent leg ulcers are common as a result of vascular stasis and are prone to get infected.

Patients with sickle cell anemia also show delayed wound healing. Patients with sickle cell anemia also present hand foot syndrome (dactylitis): painful, swollen as a result of vasoocclusive crisis. Avascular necrosis of femoral and humeral head is common. These patients show increased susceptibility to infections. Chronic anemia may leads to high cardiac output, cardiomegaly and ejection systolic murmer.

Hepatomegaly usually develops which is at times accompanied by hemosiderosis and cirrhosis. Patients may develop pigmented gall stones as a result of hemolysis. Acute abdominal pain is found as a result of vascular occlusion of abdominal viscera. Stroke may results from vaso-occlusion of cerebral vessels leading to aphasia, seizures or hemiplegia. Salmon patches are the intra-retinal hemorrhages occur because of occlusion of retinal vessels.

Renal failure, proteinuria, hematuria and glomerular sclerosis are common manifestations in sickle cell anemia. Acute chest syndrome, pleuritic chest pain, fever and lung infiltrates occur as a result of infarction and infection. In pregnancy, there is increase risk of abortion, intrauterine growth restriction (IUGR), prematurity and still birth.

**Laboratory Findings**

**Hematological**

Peripheral smear of patients with sickle cell anemia shows normocytic and normochromic red cells, sickle cells, numerous target cells, fragmented cells, polychromasia and nucleated red cells (see figure 12.7). Red cell inclusions: Howell-Jolly bodies and basophilic stippling can be demonstrated in case of autsplenectomy in patients with sickle cell anemia. Reticulocyte count is usually raised. Erythrocyte sedimentation rate is low because of the inability of sickle cells to form rouleaux.

Total leukocyte count may be raised as a result of infection. Platelet count is usually raised as a result of decrease splenic trapping. Thrombocytopenia may occur as result of vaso-occlusion.
Biochemical
In hemolytic crises, patients with sickle cell anemia have unconjugated hyperbilirubinemia, decreased serum haptoglobin, increased urobilinogen and increased LDH. Serum iron profile in these patients exhibit increased serum iron, increased serum ferritin and increased total iron binding capacity (TIBC).

Special test used for the diagnosis of sickle cell anemia
Common laboratory tests used for the screening and diagnosis of sickle cell anemia include sickling test, solubility test, hemoglobin electrophoresis and molecular studies.

- Sickling test: A reducing agent i.e. 2% sodium metabisulphite or sodium dithionite is added to blood that induces sickling of cells containing HbS.
- Solubility test: Sickle hemoglobin is insoluble in deoxygenated state.
- Hemoglobin electrophoresis at alkaline pH: In homozygous state, HbS constitutes about 80-90% and HbF 10-30% with minimal or no HbA. HbA2 in these patients may be slightly increased with a mean of 3.4%. In sickle cell trait, HbA constitutes 60%, 40% HbS and usually elevated HbA2 (mean 3.6%).
- Molecular genetics of globin chain: This signifies the specific genetic mutation of globin chain.
- Estimation of HbF by alkali denaturation test: It determines the severity of sickle cell anemia or co-existence of hereditary persistence of fetal hemoglobin.

Suggested further reading
ACQUIRED HEMOLYTIC ANEMIAS

IMMUNE HEMOLYTIC ANEMIAS

Immune hemolytic anemias are a group of disorders that result from the premature destruction of red cells by the inappropriate activation of immune system. Hemolysis is mediated by antigen-antibody reaction or activation of complement system. Severity of anemia depends upon the immunological destruction of the red cells. An individual may be anemic or not, however, laboratory findings provide clues of immune hemolytic anemia. Immune hemolytic anemias can be classified into three broad categories;

- Alloimmune
- Autoimmune
- Drug-induced

ALLOIMMUNE HEMOLYTIC ANEMIA

Hemolytic anemia caused by the immunization of an individual with red cell antigens of other individual is termed as alloimmune hemolytic anemia. Alloimmunization is the process in which the immune system of an individual is stimulated by foreign antigens and produces the corresponding antibodies. Antibodies produced by this immune response are termed alloantibodies. These antibodies coat the foreign red cells introduced into the circulation, resulting in destruction of red cells.

Alloantibodies may be produced by three mechanisms;

- Transfusion of blood
- Pregnancy (hemolytic disease of new born)
- Transplantation

Hemolytic Transfusion Reactions

Hemolytic transfusion reactions occur when mismatch blood is transfused to an individual with preformed antibodies directed against foreign red cell antigens located on the transfused cells. There are two types of transfusion reactions;

- Acute hemolytic transfusion reaction
- Delayed hemolytic transfusion reaction

Acute hemolytic transfusion reactions

This is the type of transfusion reaction occurs as soon as the transfusion starts i.e. only few millilitre of blood is required to initiate this process. In a vast majority of cases this is caused by an immunological reaction between antigens of ABO blood group system and the naturally occurring antibodies in this system. Similar reactions may develop involving antigens and antibodies in other blood group systems (Kell, Kidd, Duffy). Antibodies associated with the ABO blood group are IgM or both IgM and IgG. Antigen-antibody reaction activates complement system. Activated complement system results in formation of membrane attack complex (MAC). MAC formation leads to the intravascular destruction of red cells. C3a and C5a are also released
by the activated complement system in the circulation that release vasoactive amines (serotonin and histamine). These components are the mediators of clinical manifestations of acute hemolytic transfusion reactions.

Clinical Features

Amongst all immunological transfusion reactions, hemolytic transfusion reactions produce the widest spectrum of clinical manifestations. The earliest and the most consistent feature irrespective of the severity or etiology of hemolysis is fever. It is almost invariably accompanied by chills and rigors. It is important to pay due attention to fever appearing early in the transfusion process. Shortness of breath, chest pain, backache, dark colour urine (hemoglobinuria) and jaundice are some of the common clinico pathological manifestations of acute hemolytic transfusion reaction. A more severe burst of hemolysis produces hypotension and shock. Anemia and bleeding tendencies due to DIC commonly develop when intravascular hemolysis is marked.

Laboratory Findings

Hematological

Complete blood count shows normocytic and normochromic anemia with schistocytosis and polychromasia. Leukocyte and platelet count remain normal. Reticulocyte count is increased as a compensatory mechanism by the bone marrow.

Biochemical

Biochemical parameters that are abnormal in acute hemolytic transfusion reaction include;

- Hemoglobinemia
- Depleted haptoglobin levels
- Decreased serum hemopexin
- Elevated serum bilirubin level (primarily indirect)
- Elevated LDH
- Methemalbuminemia

Other laboratory findings

- Positive direct antiglobulin (coombs) test
- Hemoglobinuria
- Hemosiderinuria

Delayed hemolytic transfusion reactions

This type of transfusion reactions is associated with antibodies of blood groups other than the ABO blood group. Antibodies in this type of reaction are usually IgG that results in the sensitization of the red cells. These sensitized red cells are removed by the RES resulting in the extravascular hemolysis. Spleen and liver are the two major organs of RES. In the spleen, macrophages with Fc receptors line the splenic cords. Antibody-coated red cells interact with the Fc receptors, resulting in complete or partial phagocytosis. In the case of partial phagocytosis, a part of the red cell membrane is removed resulting in the formation of spherocytes. Spherocytes are round in shape and lack deformability.

Symptoms of delayed hemolytic transfusion reactions include mild fever, mild jaundice and an unexpected fall in hemoglobin.
Laboratory Findings

Hematological
Peripheral blood shows normochromic and normochromic anemia with marked spherocytosis.

Biochemical
- Elevated serum bilirubin levels (indirect)
- Elevated serum LDH

Other laboratory findings
- Elevated urobilinogen concentration in urine and stool
- Positive direct antiglobulin test as well as the indirect antiglobulin test

Hemolytic Disease of the Newborn

Hemolytic disease of the newborn (HDNB) is a clincopathological entity characterized by hemolysis, jaundice, anemia and hepatosplenomegaly. Whereas any blood group incompatibility can cause HDNB, it is the ABO and the Rh blood groups that have been incriminated in almost all cases of HDNB. Other blood groups which sometimes produce HDNB are Kell, Kidd, Duffy and MNS blood group systems.

Pathophysiology

Traditionally the term HDNB implies an immune hemolysis mediated by transplacental transfer of IgG antibodies formed by the maternal immune system against the antigens on the surface of the fetal red cells which accidentally enter the maternal circulation. In order to appreciate the pathophysiology of HDNB it will be appropriate to briefly review the placental circulation.

In the placenta, maternal and fetal circulations are separated from each other by a semipermeable membrane. Under physiological conditions there is virtually no transplacental transfer of red cells between these two circulations. At the time of delivery when vessels are ruptured, a small amount of fetal blood (usually no more than 0.1 to 0.2 ml) enters the maternal circulation. Similar transfer may take place at the time of abortion, amniocentesis and other trans-abdominal manipulations.

This is of no consequence if there is no feto-maternal incompatibility in any of the group systems between the fetus and the mother. At times when mother is Rh negative and the fetus is Rh positive, transplacental transfer of fetal red cells (Rh positive) to the maternal circulation (Rh negative) can initiate an immunological process which may have deleterious effects on subsequent pregnancies.

In ABO blood group incompatibility when incompatible red cells of the fetus enter the maternal circulation, they are quickly destroyed by the naturally occurring group specific antibodies in the maternal circulation and maternal immunization does not take place. ABO incompatibility between the mother and the fetus is shows in table 13.1.
In Rh, Kell, Kidd and Fy<sup>a</sup> incompatibility, fetal red cells possess an antigen which is inherited from the father while the mother lacks this antigen. As a result of entry of fetal cells into maternal circulation, mother sometime produces antibodies against these foreign antigens. The initial response is the primary response which is delayed, slow and attenuated. It is characterized by the formation of IgM antibodies which, because of their higher molecular weight and lack of receptors on the placenta, are unable to cross the placental barrier.

The first baby invariably escapes ‘un-hurt’ though he has played his role as an inducer of immune response. During the next incompatible pregnancy when fetal cells enter the maternal circulation, an anamanestic or secondary response is initiated with rapid, sustained and exuberant production of IgG type immune antibodies.

Because of the presence of specific receptors on the placental membrane, IgG antibodies enter the fetal circulation and sensitize the fetal red cells which carry the corresponding antigen on their surface. These sensitized red cells are destroyed by the RES of the fetus and a chain of events is initiated.

**Clinical Features**
Clinical spectrum of HDNB includes anemia, hyperbilirubinemia, kernicterus and hepatosplenomegaly.

**Laboratory Findings**
Laboratory abnormalities in HDNB can be listed under two separate headings.

- **Cord blood parameters:**
  - Hemoglobin <16 g/dl
  - High reticulocyte count
  - Baby Rh D positive
  - Direct Coomb’s test positive
  - Indirect Coombs test may also be positive (depending upon the amount of antibody transferred from the mother to the baby).
  - Unconjugated hyperbilirubinemia
  - Normoblastemia
  - Polychromasia
  - Spherocytosis
- **Mother's blood parameters**
  - Rh blood group D negative
  - Circulating anti-D antibodies in the serum
AUTOIMMUNE HEMOLYTIC ANEMIA

Autoimmune hemolytic anemia (AIHA) is a clinical condition associated with an abnormal immune response of an individual against his own red cells. During embryogenesis auto-reactive B and T cells are inactivated. However, sometimes these auto-reactive B and T lymphocytes escape the mechanism for tolerance of self. As a result, patients produce antibodies that bind to their own red cells (autoantibody). Based upon the type of antibody involved in the pathogenesis of hemolytic anemia, AIHA divided into two subtypes;

- Warm type autoimmune hemolytic anemia
- Cold type autoimmune hemolytic anemia (cryopathic hemolytic anemia)

Warm Type Autoimmune Hemolytic Anemia

Warm type AIHA is the most common. Autoantibodies involved in this type have optimal serologic reactivity at 37°C. WAIHA may occur:

- Idiopathic
- Secondary
- Secondary causes of WAIHA include:
  - Autoimmune diseases (SLE, rheumatoid arthritis)
  - Lymphoproliferative disorders (chronic lymphocytic leukemia, Hodgkin’s disease, non-Hodgkin’s lymphoma, thymoma)
  - Infections (EBV)
  - Ovarian cysts
  - Some cancers
  - Drugs

Signs and symptoms usually do not appear until significant anemia has developed. Pallor, weakness, dizziness, dyspnea, jaundice and unexplained fever occasionally are presenting complaints. Hemolysis is usually extravascular and occurs predominantly in the spleen. The degree of hemolysis can be acute (hemoglobin less than 70 g/L) or mild. The onset of WAIHA is usually insidious and may be precipitated by a variety of factors such as infection, trauma, surgery, pregnancy, or psychologic stress.

Laboratory Findings

General laboratory investigations and findings are similar to those as described in the section of hemolytic transfusion reactions. In cases of secondary autoimmune hemolytic anemia, laboratory findings show diversity based upon the primary etiological and clinical condition of the patient.

Figure 13.1: Fragmented red cells
Cold Type (Cryopathic) Autoimmune Hemolytic Anemia

This is a rare auto-immune disorder in which ‘cold’ antibodies directed against red cells appear in the patients’ blood. These antibodies preferentially react at temperatures below the normal body temperature; the condition is therefore, called cryopathic. There are two types of cryopathic hemolytic syndromes:

- Cold agglutinin disease (CAD)
- Paroxysmal cold hemoglobinuria (PCH)

Antibodies appear as a result of disturbances in the immunological homeostasis which may develop either spontaneously or follow a systemic illness. Both cryopathic hemolytic syndromes may be further classified as idiopathic or primary and symptomatic or secondary.

Cold agglutinin disease

This is an uncommon form of intravascular hemolysis due to the presence of cold agglutinins in the blood. These antibodies stick to the surface of the red cells at temperatures much lower than the normal body temperature and cause hemolysis. Once cold agglutinins attach to the red cells, they bind the initial components of the complement to the red cells surface. As these red cells return from the cold environment of the extremities to the inner warmer parts of the body, the antibodies come off the red cells but activation of the complement proceeds further. With the activation and attachment of C56789 to the red cell surface, the stage is set for the destruction of red cells in the circulating stream.

Human red blood cells have, amongst others, a surface antigen system called I-i antigen. In the fetal life, red cells have i-antigen only on their surface. After birth this gradually disappears and is replaced by the I-antigen. In conditions associated with cold agglutinin disease, antibodies with immunological specificity against the “I-i” system appear in the blood. These may sometimes, appear without any demonstrable systemic disorder i.e. primary cold agglutinin disease. More commonly, they are associated with certain lymphoproliferative disorders as listed below:

- Benign disorders
  - Infectious mononucleosis
  - Mycoplasma pneumoniae
  - Childhood exanthemas
- Maligannat disorders
  - Chronic lymphocytic leukemia
  - Waldenström’s macroglobulinemia
  - Lymphocytic lymphoma
  - Burkitt’s lymphoma
  - Histiolytic lymphoma

Clinical Features

Clinical manifestations of cold agglutinin disease are determined by:

- Environmental temperature
- Concentration of the antibody
- Immunological class of the antibody
- Any associated systemic illness
  - The disease may clinically present as:
    - Paroxysms of massive intravascular hemolysis with hemoglobinuria
    - Chronic hemolytic anemia
    - Raynaud’s phenomenon and acrocyanosis
Laboratory Findings

The first person to draw attention to the presence of cold agglutinin disease is usually a laboratory technologist who may find that:

- Blood clots in the syringe (even in the presence of anticoagulant) before any tests can be done.
- Blood smear cannot be made because red cells agglutinate quickly.
- Incompatible crossmatch both at room temperature and 37°C with all donor samples.

Reason for these initial but highly significant technical observations is the presence of cold agglutinins in the blood which cause red cell clumping as soon as the blood cools down in the extremities particularly in cold climates. It may be pointed out here that red cell agglutination is different from gelation due to cryoglobulins.

![Figure 13.2: agglutination of red cells in CAD](image)

Laboratory documentation of cold agglutinin disease includes:

- Demonstration of cold antibody in the patient’s serum
- Determination of the antibody specifically

In infectious mononucleosis, it is usually anti-i while in mycoplasma pneumoniae the antibody is commonly anti-I. In malignant lymphomas, the antibody may be anti-I or anti-i. Rarely these antibodies may not show any specificity.

Paroxysmal Cold Hemoglobinuria

Paroxysmal cold hemoglobinuria (PCH) is a member of cryopathic hemolytic syndromes. This disease, as the name suggests, is characterized by episodic hemolysis and hemoglobinuria which is triggered by exposure to cold.

Compared with cold agglutinin disease this disorder is extremely rare. Previously the disease was associated with primary and tertiary syphilis. With better control of syphilis to the point of extinction, diseases like rubella, mumps, chicken pox and other childhood exanthema have assumed greater importance for their association with PCH.

Immunologically the condition is characterized by the development of complement binding polyclonal IgG antibodies which react best at cold temperature. These antibodies are hemolytic in nature and are known as biphasic antibodies because they must be sequentially exposed to cold and warm temperatures for hemolysis to take place.
**Phase I; the cold phase**

In this phase, which is completed at cold temperature, antibodies become attached to their corresponding antigens on patient’s red cells. Antigens in this instance are P-antigen and the corresponding antibodies are cold reactive anti-P antibodies. As a result of the antigen-antibody reaction, the initial or the non-hemolytic components of the complement become activated and are attached to the antibody coated (sensitized) red cells.

**Phase II; the warm phase**

As the antibodies and complement coated red cells return to the warmer parts of circulation, the hemolytic component of the complement becomes activated and causes hemolysis. This biphasic behavior of the antibody forms the basis of Donath Landsteiner test which is an in-vitro test to confirm the presence of the antibodies.

**Clinical Features**

The disease is characterized by episodes of massive intravascular hemolysis and consequent hemoglobinuria after exposure to cold. This is preceded by body aches, muscular cramps, chills, fever and marked prostration. These features may, at times be accompanied by Raynand’s phenomenon and cold urticaria. Shortly after exposure to cold, the dramatic manifestation of hemoglobinuria occurs in the first post-exposure urine. The episode is self-limiting if the patient is immediately shifted to warmer environment.

**Laboratory findings**

Laboratory features of PCH are summarized below;
- Spherocytosis
- Reticulocytosis
- Neutrophilia
- Hyperbilirubinemia
- Elevated levels of plasma free hemoglobin
- Erythrophagocytosis
- Decreased complement proteins

**Serology**

PCH produces highly characteristic serological abnormalities in the serum. These include;
- Positive direct Coombs test with anti complement antiserum
- Positive Donath-Landsteiner test
- Hypocomplementinemia

**Differential Diagnosis**

PCH is a rare disease and is encountered mostly in children following childhood exanthemata. The characteristic triad of exposure to cold, massive intravascular hemolysis with hemoglobinuria and self-limiting course clearly distinguishes this condition from other diseases characterized by episodic hemoglobinuria.

The following conditions may be considered in the differential diagnosis;
- Paroxysmal nocturnal hemoglobinuria
- Cold agglutinin disease
- March hemoglobinuria
- Warm type AIHA

Paroxysmal nocturnal hemoglobinuria and warm type autoimmune hemolytic anemia resemble PCH because of the passage of dark urine. A careful review of the clinical scenario readily excludes these conditions. Cold agglutinin disease with hemoglobinuria is the real differential diagnosis.
DRUG INDUCED IMMUNE HEMOLYTIC ANEMIAS

These, as the name suggests, are mediated by an immunological mechanism (antibody alone, complement alone or both) in which drugs are the prime movers. On the basis of their pathophysiology, drug induced immune hemolytic anemias are sub-classified into three types:

- Penicillin or hapten type
- Quinidine or neo antigen type
- α-methyl dopa or auto-immune type

The following is a brief account of each of the three types of drug-associated immune hemolytic anemias.

Penicillin type

This is also known as ‘drug absorption type hemolytic anemia’. This is a better terminology as it sheds light on the basic homeostatic aberration in the red cell. In this type of hemolysis the drug becomes attached to the red cell membrane. Antibodies, almost invariably of IgG type are formed which are specifically directed against Penicillin which is attached to the red cell membrane. Red cells being coated with IgG type antibody are removed by the spleen. Rarely hemolysis is complement mediated in which the red cells may be destroyed in the circulating stream as well as in the reticuloendothelial system in other parts of the body.

Neo-antigen (drug adsorption) mechanism

Previously it was known as immune complex or innocent bystander mechanism. In the light of recent and better documented informations, it is now believed that the association of the drug with a specific receptor site on the red cell membrane produces a new antigen or ‘neo-antigen’. This induces antibody production which causes activation of the complement and subsequent red cell lysis. Both Penicillin and neo-antigen (adsorption) mechanisms produce drug induced immune hemolysis. The two mechanisms however show significant differences; some of these are given in table 13.2:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Penicillin type</th>
<th>Neo-antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane binding</td>
<td>Tight</td>
<td>Loose</td>
</tr>
<tr>
<td>Hemolytic dose</td>
<td>Very large</td>
<td>Small</td>
</tr>
<tr>
<td>Site of hemolysis</td>
<td>Spleen</td>
<td>Intravascular</td>
</tr>
<tr>
<td>Antibody</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>Effector of hemolysis</td>
<td>IgG</td>
<td>Complement</td>
</tr>
<tr>
<td>Onset of hemolysis</td>
<td>Slow, sub-acute</td>
<td>Sudden, massive</td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Renal failure</td>
<td>Uncommon</td>
<td>Common</td>
</tr>
<tr>
<td>DCT</td>
<td>+ve with IgG</td>
<td>+ve with C only</td>
</tr>
<tr>
<td>Eluate</td>
<td>Reactive</td>
<td>Non-reactive</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>Uncommon</td>
<td>Common</td>
</tr>
</tbody>
</table>

Table 13.2: Differentiation of penicillin type and neo-antigen type IHA
Methyl Dopa or Autoimmune type

Administration of high doses (more than 2g per day for 6 months or more) of this important anti-hypertensive drug has been associated with AIHA in some patients. Why only a small percentage of patients at risk manifest this immunological aberration is not clear.

Administration of methyl dopa causes antigenic changes in the red cell membrane which harbor Rh antigen. It is for this reason that the antibodies encountered in methyl dopa associated hemolysis possess anti Rh antigen specificity in the Rh blood group system.

Non immunological protein adsorption mechanism

Certain drugs notably the cephalosporins promote the adsorption of a wide variety of proteins on the surface of the red cells. These include fibrinogen, complement compartments, \( \alpha_2 \)-macroglobulins and many others. There is no hemolysis though about 3% of the patients receiving cephalosporin have a DCT positive. There is no need to stop the drug and the treatment may be continued with impunity. It may be recalled that cephalosporin besides producing DCT positivity as a result of promoting non-specific protein adsorption or the red cell membrane, may also induce hemolysis through hapten and neo-antigen mechanisms.

Clinical Features

Like all immune hemolytic anemias, drug-induced hemolytic anemia also produce anemia, reticulocytosis unconjugated hyperbilirubenimea and at times splenomegaly. Factors which determine the severity of clinical manifestations include:

- Rate of hemolysis
- Site of hemolysis
- Mechanism of antibody formation
- Titre of the antibody

For the site clarity, clinical manifestations in all four mechanisms of drug-induced hemolytic anemia are discussed below;

Clinical features of penicillin type immune hemolytic anemia

This type of hemolytic anemia is also known as hapten type hemolytic anemia. It is characterized by slow and insidious onset. Low grade hemolysis takes place mostly in the spleen. There is usually no hemoglobinuria and no suggestion of renal failure. Clinical features secondary to extravascular hemolysis are generally mild. There are no constitutional features like jaundice, anemia or general weakness. The episodes are almost invariably self-limiting.

Clinical features of neo-antigen of Drug adsorption type

In this type of mechanisms the red cells are rapidly destroyed in the peripheral blood with consequent hemoglobinuria and hemoglobinemia. This is accompanied by other biochemical manifestation of intravascular hemolysis including increased serum LDH, decreased levels of serum haptoglobins and hemopexin. Hemolysis may be quick and brisk and is accompanied by hemoglobinuria with consequent renal failure. Coombs test is positive with complement and anti IgM antibodies.

Clinical features of Methyl Dopa or auto-immune type

This is warm type immune hemolytic anemia of IgG type. It has all the feature of an AIHA of warm antibody. Hemolysis in most cases is not obvious, while in others it is very small in magnitude. Hemolysis is self limiting and it cases within a week or two after the scession of the drug.
Laboratory Findings

Like other clinical manifestations, laboratory features are also determined by the same factors as mentioned above. Some of the laboratory features are:

- Anemia
- Reticulocytosis
- Positive direct antiglobulin test
- Elevated LDH
- Unconjugated hyperbilirubinemia
- Hemoglobinemia
- Hemoglobinuria
- Renal failure

Suggested further reading


PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

This is a rare form of hemolytic anemia characterized by three features which are reflected in the title i.e. it is episodic, has an association with night (sleep) and hemoglobinuria. It seems appropriate to clarify at least two fallacies which are embodied in the title and which are the legacies of the earlier medical days of limited insight into the pathophysiology of the disease.

Firstly the condition is not strictly paroxysmal; many cases develop chronic, low grade persistent intravascular hemolysis, hemoglobinuria and hemosiderinuria. Secondly the condition is not necessarily nocturnal; the temporal association of episodic hemolysis is with sleep whether it is at night or during day time.

Paroxysmal nocturnal hemoglobinuria (PNH) is an example of hemolytic anemia due to an acquired membrane defect (this is the only example of acquired intra-corpuscular defect). Red cell membrane in this disease becomes extremely sensitive to complement mediated hemolysis. Activation of the complement is not triggered by antigen-antibody reaction; (there are no red cell antibodies in the serum of these patients); it becomes activated through alternate pathway (Properdin pathway) of complement activation. Pre-requisite to episodic hemolysis in PNH in many cases is its association with sleep. Episodes of hemolysis take place after an individual wakes up from sleep and voids urine; the first urine after awakening from sleep contains hemoglobin. Subsequent urine samples are devoid of hemoglobin. As mentioned earlier, there are no antibodies against any of the red cell antigens; it is therefore a non-immunological type of hemolytic anemia.

Pathophysiology

The disease is neither congenital nor it is hereditary; it is an acquired membrane defect. It is due to the emergence of a clone of hematopoietic stem cells which have increased sensitivity to the hemolytic action of the complement. There may be episodic hemolysis or the patients may manifest chronic, low grade intravascular hemolysis.
The defect lays in the pluripotent hematopoietic stem cells. As a consequence, all three hematopoietic cell lines are affected though the brunt of the disease falls on red cells. Some degree of leukopenia, thrombocytopenia and functional abnormalities of the white cells and platelets are also present. Many a times, PNH presents as pancytopenia due to hypocellular marrow. A small percentage of patients develop aplastic anemia and acute leukemia.

PNH is caused by a mutation in and subsequent failure of expression of a gene called Pig-A or phosphatidyl-inositol glycan A which is located on X-chromosome. It is essential for the synthesis of an enzyme (alpha 1, 6 N-acetyl glucosaminyl transferase) which regulates the synthesis of a protein called glycosyl phosphatidylinositol (GPI). It facilitates the attachment of various proteins to the phospholipids of the cell membrane. It is for this reason that GPI is popularly known as GPI anchor. Failure to synthesize the 'anchor' results in failure of attachment of a large number of proteins to the cell membrane. Some of the notable ones are the decay accelerating factor (DAF) or CD 55, the membrane inhibitor of reactive lysis or CD 59 (MIRL) and C8 binding protein. All these proteins are concerned with the inactivation of complement which may become attached to the surface of the red cells as they circulate in the blood stream. Absence of these proteins facilitates excessive adsorption of C3 of the complement system. CD59 is of special importance in hemolysis in PNH because it inhibits the spontaneous in vivo activation of the complement through alternate pathway by inactivating C3 convertase. A quick glance at the alternate pathway of complement activation highlights the key position of C3 convertase in the complement activation through non-immunological pathway of complement activation.

Clinical Features
The disease has a wide spectrum of clinical manifestations; it ranges from the well-known presentation of the disease i.e. paroxysmal nocturnal hemoglobinuria to such subtle features as headaches and ocular discomfort. These are highlighted in figure 13.3:

![Figure 13.3: Clinical spectrum of PNH](image)

Laboratory Features
As expected, most of the laboratory manifestations are in the hematological parameters and affect the peripheral blood as well as the bone marrow.

Hematological
Anemia, thrombocytopenia and leukopenia of varying degree and in variable combinations are frequently encountered; pancytopenia is not an uncommon presentation. Red cells are frequently microcytic and hypochromic. Reticulocytosis of variable magnitude is not uncommon. Red cell morphology shows polychromasia and round macrocytosis (a reflection of
high reticulocyte count). Red cell fragmentation is unusual as is the abnormality in the maturation sequence of granulocytes.

**Bone marrow**
Bone marrow shows variable cellularity which is determined by the:
- Extent of marrow's response to hemolysis
- Iron status
- Evolution of aplasia
- Transformation into acute leukemia

**Biochemical**
- Serum iron decreased
- Serum ferritin decreased
- Serum LDH increased
- NAP decreased
- RBC acetylcholine esterase decreased
- Serum complement decreased
- Serum haptoglobins decreased
- Plasma hemoglobin increased

**Urine analysis**
- Hemosiderinuria
- Hemoglobinuria
- Albuminuria
- Hyposthenuria

Diagnosis of PNH calls for a high index of suspicion because of the diversity of the clinical manifestations. Number of patients who present with classical episodic hemoglobinuria after awakening is rather small; most cases present with other manifestations. Diagnosis of PNH should be suspected in all cases of:
- Pancytopenic hypoplastic anemia
- Hemolytic anemia with microcytosis and hypochromia (iron deficiency)
- Pancytopenia with reticulocytosis
- Multisystem or unusual thrombotic episodes

Diagnosis of PNH is made in the light of the clinical symptomatology supported by appropriate laboratory data. Whereas hematological parameters are supportive of the diagnosis, the ultimate test is the demonstration of increased sensitivity of red cells to complement lysis. Some of the diagnostic laboratory procedures are:
- Sucrose lysis test
- Ham test
- Hemosiderinuria
- Bone marrow examination
- Flow cytometry
Suggested further reading

APLASTIC ANEMIA

Aplastic anemia is a disease of pluripotential hematopoietic stem cells characterized by pancytopenia in the peripheral blood and fatty replacement of hematopoietic tissues in the bone marrow. An etiological classification of aplastic anemias is given below:

![Classification of aplastic anemia](image)

**Figure 14.1: Classification of aplastic anemia**

**Congenital Aplastic Anemias**

Congenital aplastic anemias are characterized by pancytopenia early in life but not necessarily at birth. They account for almost 20-30% of all cases of aplastic anemia in children. Fanconi type anemia is inherited as autosomal recessive trait; it is commonly associated with skeletal abnormalities, growth retardation, microcephaly and pigmentary dermatoses. This type of anemia frequently displays non-specific chromosomal breaks which are best demonstrated in tissue cultures of peripheral blood lymphocytes.

Clinical manifestations are those of slowly progressive anemia with features of systemic abnormalities in Fanconi subgroup. Congenital anemias, if not treated, follow a progressively downhill course and most patients die within 2 years after the diagnosis is made. Nearly 10% of the patients develop acute non-lymphoid type of leukemia. Treatment with androgens has shown some promise and remission for up to 2 years are not uncommon in Fanconi’s type aplastic anemias. Vascularization and liver function abnormalities are troublesome features and must be closely observed. Bone marrow transplantation is curative.
Acquired Aplastic Anemia

Idiopathic aplastic anemia

Idiopathic aplastic anemia is the commonest type of aplastic anemia. There are two views regarding its pathogenesis. Numerical deficiency of the stem cells; According to this hypothesis, there is complete or nearly complete loss of the colony forming units of granulocytic and monocytic (CFU-GM) cell lines in the body. The burst forming units of the erythroid line (BFU-E) are similarly affected.

The other hypothesis for the development of aplastic anemia is an alteration in the microenvironment of the stem cells in the bone marrow. This hypothesis has not enjoyed any scientific or popular support.

Drug-related aplastic anemia

A large number of drugs are associated with the development of aplastic anemia. The ultimate mechanism by which drugs produce marrow aplasia is not known. Drug associated marrow aplasia is of three types:

- Phenothiazine type; idiosyncratic
  This effect is not dose-related; it may appear with the first dose or develop during a prolonged course of drug therapy. It may sometimes appear after the drug has been discontinued. The basis of this type of myelosuppression is thought to be idiosyncrasy to the drug. Drugs which are associated with this type of aplasia are phenothiazine and thioauracil group of drugs.

- Busulphan type; dose-related, predictable
  Certain drugs exert a direct and predictable marrow suppressive effect; they cause variable degree of marrow hypoplasia or aplasia in all those who receive them in sufficient doses. Examples of this group of drugs are myleran, phenylalanin-mustard, vinblastine and other chemotherapeutic agents.

- Chloramphenicol type; dose-related, unpredictable
  Unlike Busulphan type, cytotoxicity although it has not been conclusively shown that chloramphenicol is the cause of marrow aplasia, its use has nonetheless been associated with it. Of all the drugs that have some temporal association with aplastic anemia, chloramphenicol is found to have the highest incidence of such an association.

Chemicals and toxins

Large number of substance both in the household and in the industry have been incriminated in the development of aplastic anemia. Most of these substances have a benzene ring in their structure.

Radiations

High dose radiations to the bone marrow cause variable degree of marrow aplasia; the damage is proportionate to the dose of radiations. Large doses administered to any individual will completely ablate the bone marrow.

Hepatitis-associated marrow aplasia

Recently an association has been established between marrow aplasia and non-A, non-B type infectious hepatitis. It commonly affects young male; 75 % of the affected patients are under 20 years of age. Marrow hypoplasia mostly appears within 2 months after the onset of jaundice. The course is fulminant and the outcome is usually fatal.
**Pregnancy associated aplastic anemia**

A few cases of aplastic anemia have been reported in pregnancy. Usually these patients recover spontaneously after delivery. Those who develop marrow aplasia during one pregnancy, have a high risk of developing it in the subsequent pregnancies. Therapeutic termination of pregnancy usually leads to marrow recovery.

**Paroxysmal nocturnal hemoglobinuria**

This disease is characterized by complement mediated hemolytic anemia in which red cells undergo excessive lysis in the circulating stream in the presence of complement. A small number of cases develop aplastic anemia either due to exhaustion of the marrow stem cells or due to emergence of an abnormal clone of stem cells, as evidenced by the emergence of chromosomal breaks on cytogenetic studies.

**Pathophysiology**

Basic defect in aplastic anemia is the failure of red cells, white cells and platelets production by the bone marrow. Blood cell production in the marrow depends upon the growth, differentiation and self-renewal of pluripotent hematopoietic stem cells (PHSC). For proliferation and differentiation, PHSC responds to interleukins and other growth factors produced by cells in the bone marrow microenvironment. Bone marrow failure may develop as a consequence of a failure of blood cell formation at the level of PHSC or a disturbance of the bone marrow microenvironment.

**Clinical Features**

Signs and symptoms of aplastic anemia are dictated by the number of red cells, white cells and the platelets in the peripheral blood. Severity and rapidity of the onset of the clinical features and the ultimate prognosis are determined by the cause of marrow aplasia.

Clinical manifestations essentially center around all three hematopoietic cell lines. Degree of involvement is usually not uniform and one or the other cell lines may be reduced more than the other. Erythroid line is most commonly and most severely affected and the presenting symptoms are therefore one of chronic slowly progressive anemia with tissue hypoxia and metabolic retardation.

Pallor, lethargy, progressive dyspnea on exertion, black outs especially in the upright posture and aculeemia tinge the clinical picture. Bleeding manifestations are the next most frequent; skin bleeding, epistaxis, gum bleeding, retinal hemorrhage, hemorrhage in the GIT and in the urine are not too uncommon. Menorrhagia is another feature and it adds to the severity of anemia and causes iron depletion.

**Laboratory Findings**

**Hematological**

The most significant laboratory abnormality is the reduction in all three hematopoietic elements. There are usually no significant morphological changes in the red cells, white cells or the platelets. Anemia is primarily due to reduction in the number of red cells which are characteristically normocytic and normochromic; occasionally there is mild macrocytosis. Marked anisopoikilocytosis and normoblastemia speak against the diagnosis of aplastic anemia. Absolute reticulocyte count is invariably low although in rare cases a relative reticulocytosis may be present. Granulocytes are also decreased with relative increase in the number of lymphocytes. Platelets are somewhat small in size and their count is significantly decreased.
**Bone marrow**

Bone marrow aspiration invariably results in dry or blood tap. The few particles which may be obtained are fatty and almost totally devoid of hematopoietic elements. Most of the cells in these aspirates are lymphocytes, monocytes, reticulum cells, macrophages, plasma cells and a few mast cells.

Definitive diagnosis of aplastic anemia is based on the examination of bone marrow biopsy. Ideally two biopsies from two different sites should be taken. Typically the biopsy shows total replacement of the hematopoietic marrow by lipocytes. Occasionally, small foci are present in which erythropoiesis is active. These foci of erythroid hyperplasia explain the occasional and rather unusual reticulocytosis which is sometimes seen in patients who otherwise fulfill all criteria of marrow aplasia.

![Figure 14.2: Normal bone marrow biopsy](image)

![Figure 14.3: Hypoplastic bone marrow biopsy](image)

Stainable iron in the marrow is normal in patients who have not been transfused. It may sometimes be decreased in patients who have severe thrombocytopenia and who bleed as a result thereof. Once transfusion is initiated or patients are inadvertently given oral or parenteral iron therapy, marrow iron stores become increased. Hemosiderin is not only increased in amount but it also becomes deposited in the form of coarse granules and large amorphous clumps. Marrow sideroblasts are characteristically decreased.
Suggested further reading


NON-MALIGNANT DISORDERS OF LEUKOCYTES

DISORDERS OF THE NEUTROPHILS

Disorders of neutrophils are traditionally described as quantitative and qualitative. The latter group is further split into functional defects and morphological defects as illustrated in figure 15.1.

![Figure 15.1: A schematic approach to the disorders of the neutrophils](image)

QUANTITATIVE DISORDERS OF NEUTROPHILS

NEUTROPHILIA

Increase in the number of circulating neutrophils greater than $7.5 \times 10^9/L$ is termed as neutrophilia. Neutrophilia may be acute or chronic. Acute neutrophilia occurs usually within hours after an appropriate stimulation. Stimuli that results in acute neutrophilia include bacterial products, toxins, leukotrienes and components of activated complement system. Chronic neutrophilia lasts from several weeks to many months. It reflects the increased production of neutrophils by the bone marrow. It is usually associated with persistent infections, administration of certain drugs or malignant conditions. Mechanisms that result in the increased number of neutrophils in the peripheral blood are:

- Increased production
- Increased release of neutrophils from the marrow
- Shifting of marginating pool to circulating pool
- Reduced egress of neutrophils from the blood to tissues
- Combination of these mechanisms

Following are the conditions associated with neutrophilia:

- Bacterial infections
  - Pyogenic bacterial infections
- Inflammation and tissue damage
  - Myositis
  - Vasculitis
  - Cardiac infarct
  - Trauma
- Metabolic disorders
  - Uraemia
  - Eclampsia
  - Acidosis
  - Gout
- Myeloproliferative disease
  - Chronic myeloid leukemia
  - Polycythemia vera
  - Myelofibrosis
  - Essential thrombocythemia
- Neoplasms
  - Carcinoma
  - Lymphoma
  - Melanoma
- Acute hemorrhage or hemolysis
- Drugs and chemicals
  - Corticosteroids
  - Epinephrine
  - Histamine
  - Ethylene
  - Lithium carbonate
- Treatment with myeloid growth factors (G-CSF)

**NEUTROPENIA**
Reduction in the absolute neutrophil count (below 2.5X10^9/L) is termed as neutropenia. Recurrent and persistent bacterial infections are the hallmark of neutropenia. Neutropenia may be mild (1.0-1.5X10^9/L), moderate (0.5-1.0X10^9/L) or severe (below 0.2X10^9/L). Mechanisms that may lead to neutropenia include:

- Decreased production of neutrophils
- Increased destruction, removal or utilization of neutrophils
- Abnormal distribution
- Combination of these mechanisms

**Decreased production of neutrophils**
A large number of conditions may lead to inadequate production of neutrophils. These may be grossly divided into two groups i.e. inherited and acquired.

**Inherited**
- Reticular dysgenesis
- Cyclic neutropenia
- Kostmann syndrome
- Chronic idiopathic neutropenia
Acquired

- Aplastic anemia
- Bone marrow infiltration
  - Leukemia
  - Lymphoma
  - Tumors
  - Tuberculosis
- Infections
  - Bacterial: Typhoid, bacillary dysentery, tuberculosis;
  - Viral: Influenza, measles, dengue, infectious mononucleosis, rubella, infectious hepatitis
  - Protozoal: Malaria, kala-azar etc.
  - Drug induced: Cytotoxic chemotherapy, radiation, chloramphenicol, penicillins, cephalosporins, phenothiazine, phenylbutazone, gold, antithyroid, quinidine, anticonvulsants, alcohol)
- Myelodysplastic syndrome
- Vitamin B₁₂ or folate deficiency

Increased destruction

Neutropenia may also occur when the peripheral depletion rate exceeds the bone marrow production rate for neutrophils. A number of conditions may result in increased destruction of neutrophils as listed below:

- Hypersplenism
- Immune mediated
- Drug induced
- Associated with collagen vascular disease (Felty syndrome, systemic lupus erythematosus)
- Complement mediated (hemodialysis, cardiopulmonary bypass)

Abnormal distribution

Abnormal transfer of neutrophils from circulating pool to marginating pool leads to neutropenia known as shift neutropenia or pseudo-neutropenia. This may be observed in:

- Idiopathic hemolytic anemia
- Cirrhosis of liver
- Macroglobulinemia
- Drugs
- Stress

FUNCTIONAL DEFECTS OF NEUTROPHILS WITHOUT MORPHOLOGICAL ABNORMALITIES

Like all other living cells neutrophils are also beset with certain inherited functional defects. Affected individuals present with recurrent severe infections, delayed wound healing, impaired pus formation and resistance to antibiotics. A comprehensive physical examination and detailed history supplemented with special laboratory procedures are required to diagnose these disorders. Some of the well-known and better characterized disorders of neutrophils functions without morphological abnormalities are listed below:

- Chronic granulomatous disease
- Leukocyte adhesion deficiency
Myeloperoxidase deficiency
Specific granules deficiency (this is associated with morphological abnormalities)
Lazy leukocyte syndrome
Severe congenital neutropenia

**Chronic granulomatous disease (CGD)**

It is an inherited immunodeficiency disorder which results from the absence, low expression or malfunctioning of NADPH oxidase enzyme in the phagocytic cells i.e. neutrophils, monocytes, macrophages and eosinophils. Under physiological conditions this enzyme is responsible for generating microbicidal radicals such as superoxide, hydrogen peroxide, hydroxal radicals and hypochlorous acid etc.

Patients with CGD are prone to develop severe recurrent and life threatening infections with catalase positive organisms such as *Staphylococcus aureus* and various gram negative bacilli as well as certain fungi such as *Aspergillus* and *Candida* species.

**Leukocyte adhesion deficiency (LAD) syndrome**

Neutrophilia, recurrent soft tissue infections, delayed wound healing and impaired pus formation are the hallmark of leukocyte deficiency syndrome. Soft tissue infections and impaired wound healing is a reflection of the inability of the neutrophils to enter the tissues. Peripheral neutrophilia is the result of the inability of the neutrophils to adhere to the vascular endothelium and form the marginating pool. As a result, all neutrophils remain in the circulating pool causing spurious neutrophilia.

There are three types of leukocyte adhesion deficiency syndromes; these include: type I, I and III. Inheritance of type I and II deficiencies is autosomal recessive while that of type III is not established.

**Myeloperoxidase (MPO) deficiency**

It is an autosomal recessive disorder of phagocytic cells characterized by impaired MPO dependent microbial killing. MPO deficient individuals’ cannot produce hypohalite radicals that have efficient microbicidal activity. These individuals are usually asymptomatic and do not require any treatment except for the frequently contracted fungal infections.

Description of other disorders of this group is beyond the scope of this text book.

**MORPHOLOGICAL ABNORMALITIES OF NEUTROPHILS WITH OR WITHOUT FUNCTIONAL DEFECTS**

These disorders are characterized by morphological changes in the neutrophils as seen on light microscopy of Leishman-stained blood smears without any functional abnormalities. These are listed below:

- Pelger Hüet anomaly
- Alder Reilly anomaly
- May Hegglin anomaly
- Chediak Higashi anomaly
- Jordan’s anomaly
- Hereditary giant neutrophils
- Hereditary hypersegmented neutrophils
Pelger Hüet anomaly

It is a benign disorder which is inherited as an autosomal dominant trait and is characterized by reduced number of nuclear segments of the neutrophils and marked condensation of their nuclear chromatin. Neutrophils in homozygous individuals show round or oval nuclei while in the heterozygous state they have bilobed, spectacle shaped (Pince-nez) nuclei as illustrated in figure 15.2.

![Figure 15.2: Pelger Hüet anomaly of the neutrophils; Pince-nez nuclei](image)

Alder Reilly anomaly

It is a rare autosomal recessive disorder characterized by the presence of large purple inclusions in the cytoplasm of the leukocytes. Not only the neutrophils but also the monocytes and the lymphocytes contain these granules in the peripheral blood as well as in the bone marrow. These granules may form clusters throughout the cytoplasm of the leukocytes. These are shown in Figure 15.3.

![Figure 15.3: Alder Reilly bodies (peripheral blood smear)](image)

May Hegglin anomaly

May Hegglin anomaly is a rare autosomal dominant disorder characterized by a highly diagnostic triad of large basophilic inclusions in the granulocytes, hypogranular giant platelets and variable degree of thrombocytopenia. These granulocytic inclusions (see figure15.4) morphologically resemble Döhle bodies but they are larger in size (2-5 µm), perfectly round and are present in large number. It does not produce any symptoms at any age. May Hegglin anomaly is the result of a mutation in MYH 9 gene (myosin heavy chain 9 gene) which is located on the long arm of chromosome 22 at position 22q.
Chediak Higashi anomaly

It is a rare autosomal recessive disorder characterized by oculo cutaneous albinism, giant grayish-blue granules in the cytoplasm of the leukocytes, recurrent and severe bacterial infections, mild bleeding tendencies, peripheral neuropathy and abnormal natural killer cell functions. Chediak Higashi anomaly is caused by a mutation in LYST gene (Lysosomal trafficking regulator gene) which located on the long arm of chromosome 1. Granules of neutrophils may form cluster or they may be dispersed uniformly throughout the cytoplasm of the affected cells as shown in Figure 15.5.

EOSINOPHILIA

Increase in the number of eosinophils above $0.5 \times 10^9/L$ is termed as eosinophilia. Eosinophilia may be classified into conditions associated with non-malignant and malignant disorders. Conditions that may present with eosinophilia are listed below:

- Allergic diseases
  - Bronchial asthma
  - Hay fever
  - Urticaria
- Parasitic infestations
  - Amoebiasis
  - Hookworm
- Ascariasis
- Filariasis
- Schistosomiasis
- Trichinosis

- Skin diseases
  - Psoriasis
  - Pemphigus
  - Dermatitis herpetiformis
  - Atopic dermatitis

- Poly arteritis nodosa

- Serum sickness

- Malignant conditions
  - Hodgkin’s disease
  - Metastatic malignancy with tumor necrosis
  - Hypereosinophilic syndrome
  - Chronic eosinophilic leukemia

![Figure 15.6: Eosinophila](image)

**LYMPHOCYTOSIS**

Clinical condition in which the absolute lymphocyte count exceeds more than $4.0 \times 10^9$/L in adults and $9.0 \times 10^9$/L in children is known as lymphocytosis or lymphoproliferative disorders. It may be benign (reactive) or malignant (non-reactive). Conditions associated with lymphocytosis are listed below:

- Acute infections
  - Infectious mononucleosis
  - Rubella
  - Pertussis
  - Mumps
  - Infectious hepatitis
  - Cytomegalovirus mononucleosis
  - Herpes simplex or zoster

- Chronic infection
  - Tuberculosis
  - Toxoplasmosis
  - Brucellosis
  - Syphilis
- Chronic lymphocytic leukemia
- Hodgkin's disease and non-Hodgkin's lymphoma
- Multiple myeloma
- Waldenstrom's macroglobulinemia
- Heavy chain disease
- Thyrotoxicosis

**MONOCYTOSIS**

An increase in monocyte count above $0.8 \times 10^9/L$ in the peripheral blood is termed as monocytosis. Conditions associated with monocytosis are shown below:

- Chronic bacterial infections
  - Tuberculosis
  - Brucellosis
  - Bacterial endocarditis
  - Typhoid
- Connective tissue diseases
  - Systemic lupus erythematosus
  - Temporal arteritis
  - Rheumatoid arthritis
- Protozoan infections
- Chronic neutropenia
- Hodgkin lymphoma
- Acute myeloid leukemia (M4, M5)
- Chronic myelomonocytic leukemia

**Suggested further reading**

LEUKEMIA; AN OVERVIEW

The word leukemia literally means an increase in the number of white cells in the blood. At the outset, it must be mentioned that there are many conditions which raise the white cell count in the blood; the commonest cause is infection in any part of the body. Leukemia is perhaps the least common amongst the many conditions which raise the white cell count. It is therefore important to emphasize that a high white cell count in the blood report should not bring to mind leukemia as the first possibility.

Sometimes the white cell count in the blood is not increased despite the unquestionable existent leukemia. This situation is rather uncommon. Whether or not the white cell count is increased in leukemia, there is always a tremendous increase in the number of white cells throughout the body. This increase in the white cell count in the body appears spontaneously and increases progressively. Also, the white cells in leukemia are different from normal white cells in many ways. This together with a progressive increase in their count constitutes leukemia.

Classification of Leukemias

Leukemias are classified according to cell type i.e. cell maturity and cell lineage (origin). Cell maturity is used to distinguish acute and chronic forms of leukemia. When the malignant cells are immature (stem cells, blasts or pro forms) the leukemia is classified as acute; when the cells are predominantly mature, it is described as chronic.

According to the cell lineage, leukemias are divided into lymphoid and myeloid. The term myeloid (myelo = marrow, eidos = form) encompasses granulocytic, monocytic, erythrocytic and megakaryocytic leukemias. Lymphoid leukemias constitute a group of neoplasms related to lymphocytic cell line. Based upon cell type and maturity, leukemia is divided into four broad categories: acute lymphoblastic, acute myeloid, chronic lymphocytic and chronic myeloid leukemia.

Of these acute lymphoblastic leukemia is most common in children and adolescents. Acute myeloid leukemia and chronic myeloid leukemia is prevalent amongst young adults while chronic lymphocytic leukemia is frequently encountered in the elderly. This distribution is, however, not absolute and any type of leukemia may be seen at any age.

Acute lymphoblastic leukemia in children especially in girls between the ages of 2 and 8 years carries very good prognosis. This is the type of leukemia in which most of the therapeutic success has been achieved to the point where this type of leukemia is considered to be curable in almost 50% of the cases afflicted with this disease.

In acute myeloid and chronic myeloid leukemia there is no real encouraging news and the outcome is fatal in a few months to a few years; though sometimes patients with these types of leukemias have been known to live for many years. Chronic lymphocytic leukemia is a docile type of leukemia and many patients live a productive and comfortable life for many years with or even without treatment. Outcome in leukemias is not always tragic and there is a ray of hope for many leukemic patients some may even look forward to complete cure while a considerable number of cases can lead a comfortable life during which the disease can be kept under control with or without treatment.
Etiology and Risk Factors

Like most other cancerous conditions, the exact cause or mechanism for the appearance of leukemia is not known. Vast amount of literature is available which deals with various possible causes of leukemia. Amongst these are the genetic predisposition and the environmental factors the following have been studied and discussed most extensively;

Environmental factors

X-Rays and other radioactive substances

Use of X-rays has been increased considerably in the recent years. However because of the refinement of the equipment and improvement in the radiological, techniques there is no risk of leukemia even after repeated chest X-rays and other special procedures such as X-rays of gallbladder intestines and kidneys. This is because the dose of X-rays which is now used for various radiological procedures is infinitesimal. Certain radioactive substances which are used for the treatment of certain uncommon disease like polycythemia and over-activity of thyroid gland (hyperthyroidism) may include leukemia in those who are given these substances.

Chemical agents and drugs

Certain chemical substances which are used for the treatment of various cancers like Hodgkin’s disease, lymphomas and other cancers may be leukemogenic. Amongst these chlorambucil and other drugs related are suspected to cause leukemia. These however are uncommonly used and that be under special circumstances.

Drugs for the control of blood pressure blood sugar and other frequently used drugs have not be incriminated in the causation of leukemia and there is no cause for concern and alarm when these are used as prescribed by the physician.

Leukemogenic viruses

There is growing evidence that some leukemias are associated with viruses. It is however not clear how and why these viruses initiate leukemic proliferation.

Host factors

Associated diseases

A patient is more prone to develop leukemia if he is suffering from certain diseases. These mostly belong to different forms of blood cancers and these are the only conditions which for one reason or the other sometimes evolve into leukemia. Commonly occurring maladies like sore throat pneumonia, headache, diarrhea, dysentery or arthritis do not predispose to leukemia. Leukemia itself is a rare disease and those diseases which are associated with leukemia are equally rare. It is therefore not warranted to worry about the emergence of leukemia whenever a person is suffering with common illness.

Hereditary disorders

Certain hereditary disorders show clear association with emergence of leukemia. It is well known that the identical twin of a leukemic child is at a greater risk to develop leukemia. The maximum risk under these circumstances is about 20%. Other diseases which show a high incidence of leukemia are extremely rare. The most well-known amongst these is mongolism or Down’s syndrome which characterized by mental retardation, physical abnormalities in the form of small head, umbilical hernia and many others. The exact mechanism(s) by which all the above
groups of disorders induce or predispose to leukemia is (are) not fully understood. Most experts believe that it is not one single factor which is at the heart of the process. In most instances it is the interplay of host susceptibility, physical or chemical factors and perhaps a viral invasion of the stem cells which predisposes to the emergence of leukemia in an individual.

Leukemia at the Time of Presentation

By the time the leukemic cells first make their appearance in the blood, it is already 18-24 months since the process of leukemia first started in the bone marrow. All types of leukemias start in the bone marrow where the leukemic cells divide repeatedly and increase in number. With the passage of time the leukemic cells appear in the blood and are carried to all parts of the body by the blood stream. They are present in the liver spleen, lymph glands, heart, lungs, kidneys, brain etc. Over months and years the number of leukemic cells becomes very large and the liver, spleen and lymph glands in the groins become enlarged and painful. Sometimes the tonsils also become large.

Leukemic cells infiltrate the bone marrow and suppress erythropoiesis that leads to anemia. These patients as a result of anemia fall weak, become tired easily and look pale. In addition there is a fall in platelet count. In leukemia when the disease progresses the platelet count usually drops. Therefore bleeding is common in leukemia at some stage of the illness. Bleeding is most common from the gums, nose and skin. Bleeding in the brain is most serious and at times it kills the patients.

The basic problem in leukemia is an increase in the number of white cells. These cells do not functions as their normal counterparts. This predisposes the patients to infections of various types. Therefore these patients frequently present with fever due to infections. Many patients lose significant amount of weight. From the foregoing, it would be clear that leukemia commonly presents as fever, bleeding, weight loss, hepatosplenomegaly and enlarged lymph glands in the neck or axilla and/or groin. It must be pointed out here that whereas these are the common features of leukemia this disease per se is a very cause of various features listed above.

Until today, there is no proof for the contagiousness of leukemia. It is not contracted by using patient’s dishes, clothes, towels or bed. Touching or handling the patient too does not transmit leukemia. The occurrence of more than one case of leukemia in a family does not indicate contagiousness. It may be a reflection of the presence of a yet unknown or unidentified factor in the environment of that particular family.
ACUTE LEUKEMIAS

ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is a malignant lymphoproliferative disorder characterized by uncontrolled and relentless growth of lymphoblasts in the bone marrow. These cells subsequently 'spill into' the peripheral blood consuming tissue infiltration and infiltrative organomegaly particularly of the lymphoid organs (liver, spleen and lymph nodes). Whereas acute lymphoblastic leukemias are generally equated with pediatric age group, no age is immune and cases of ALL in the elderly are encountered albeit uncommonly. Most of the cases are however in the pediatric age group. There is no significant sex bias and the disease is almost equally distributed in the two sexes.

Etiology

Like all other leukemias, there is an autonomous proliferation of the early hematopoietic progenitor cells. It may be noted that the level of malignant transformation is not the pluripotent hematopoietic stem cell (cf CML) but a shade more differentiated blast cells along the lymphoid series. This concept has an important bearing on the philosophy of therapeutic intervention in ALL. Like all other leukemias the etiology of ALL is also unknown. The myriads of theories and hypothesis are still in the realm of speculations and at present the etiology of ALL may be accepted as unknown.

As mentioned above, ALL is a disease of the children; maximum number of cases is seen between the ages of 3 and 7; its incidence clearly declines after the age of 10 years. There is a second peak of ALL in the 5th decade, although numerically the cases are much fewer than in the pediatric age group.

Clinical Features

Clinical manifestations in ALL, like in other leukemias, are determined by a number of pathophysiological mechanisms, some of these include;

- Leukemic myelopathy leading to hematopoietic lymphoproliferation and 'crowding at' of the normal hematopoietic element causing variable but usually severe cytopenias in the peripheral blood
- Infiltrative (and? proliferative) organomegaly, particularly the lymphoid organs, liver, spleen, nodes etc.
- Hyper metabolic state secondary to exuberant proliferation of the leukemic cells not only in the bone marrow but also other body tissues.
- Expansion of the marrow cavities with subsequent periosteal stretching and leukemic infiltration.
- Immunological impairment due to suppression of the normal function of B and T lymphocytes.

Laboratory Findings

Laboratory investigations are aimed at achieving three objectives;
• to establish the diagnosis of acute leukemia
• to subtype acute leukemia
• to ascertain the prognostic indicators

Establishment of the diagnosis

Peripheral blood
The hallmark of the diagnosis of acute leukemia is a careful review of the hemogram and the morphology of the blood smear. Anemia is almost invariant while thrombocytopenia is also very common. Total leukocyte count may be increased, normal or decreased. Peripheral blood morphology besides other non-specific changes shows the characteristic and diagnostic blast cells.

Bone marrow
Bone marrow examination is mandatory not only to establish the diagnosis in patients with equivocal peripheral blood finding, it is also essential for cytological, immunological and cytogenetic evaluation.

Bone marrow, as expected, shows infiltration with blast cells with paucity of normal hematopoietic elements. For the diagnosis of acute leukemia, a minimum of 20% blast cell population is a pre requisite.

Characterization of blast cells
In order to accurately characterize blast cells, a set of laboratory investigations has been devised, that include:

• Morphology
• Cytochemistry
• Immunophenotyping
• Cytogenetic
• Gene analysis

It is worth mentioning that it is not necessary to carry out all these investigations in all patients.

Morphology
Morphology of the blast cells in the bone marrow (as well as in the peripheral blood) forms the basis of a popular classification called FAB classification (F = French, A = American, B = British). It is applicable to B cell ALL and has shown good correlation with survival in ALL. According to this classification, lymphoblasts are divided into three distinct morphological entities (with some overlap at times), each cell type producing its own leukemic proliferation.

FAB classification of ALL
According to this classification lymphoblasts are classified into three types:

L1: Uniform, small blasts with scanty cytoplasm
L2: Heterogeneous large blasts with more prominent nucleoli and more cytoplasm
L3: Heterogeneous, larger cells with prominent nucleoli, abundant deeply basophilic vacuolated cytoplasm
Cytochemistry

Cytochemistry is a readily available, economical and reliable means of differentiating ALL from AML. Reliable results are obtained with minimum of expense and expertise. The battery of cytochemical stains can be enlarged in order to include other leukemias (acute myelomonocytic and T cell ALL) for accurate classification. Table 17.1 differentiates AML from ALL on the basis of cytochemical stains.

<table>
<thead>
<tr>
<th>Cytochemical stain</th>
<th>AML</th>
<th>ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non-specific esterase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Periodic acid Schiff</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 17.1: Differentiation of AML and ALL using cytochemical stains
Figure 17.3: Per iodine acid Schiff positive stain in ALL

Immunophenotyping

For immunological subtyping of ALL, a set of surface markers called cluster of differentiation (CD) markers are used. These have been numerically named and seem to have a good and consistent correlation with the stage of development and maturation of leukemic cells. These CD markers with the types of ALL is given in Table 17.2.

<table>
<thead>
<tr>
<th>ALL</th>
<th>HLA-DR</th>
<th>CD19</th>
<th>CD10</th>
<th>Cµ</th>
<th>slg</th>
<th>Pan T</th>
<th>CD7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor B cell</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Early B cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pre B cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B cell</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cell</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 17.2: Immunological classification of ALL

ACUTE MYELOBLASTIC LEUKEMIA

Acute myeloid leukemia is fortunately the least common amongst the four major types of leukemias. It is also the least amenable to the treatment which is available at present.

Acute myeloid leukemia does not show any geographical predilection. Its prevalence across the globe is 3 per 100,000. It is equally distributed amongst the two sexes. The disease is more common in the 2nd to the 4th decade, but no age is immune and acute leukemia has been reported amongst individuals at the extremes of life.

Etiology

Like other leukemias, there is no known cause of AML and as there are unending speculative etiologies of other malignancies, acute myeloblastic leukemia is no exception. Radiations, chemicals, drugs, viruses, genetic predisposition, chromosomal aberrations and a
whole set of other factors have been incriminated in the genesis of acute myeloid leukemia. Whereas certain associations do exist with some of these factors, it is extremely difficult to incriminate any one of them as the sole causative agent. They may act through altered immune mechanism or they may contribute as co-carcinogens. Some of the major factors so far identified include radiations and chemicals.

Radiations
Radiation exposure has the strongest association with acute myeloid leukemia as evidenced by its increased incidence in:

- radiotherapy department personnel
- patients with ankylosing spondylitis treated with radiotherapy
- patients treated with radiomimetic drugs like chlorambucil and P32
- survivors of atomic explosions in Hiroshima and Nagasaki

Chemicals
Amongst the chemicals, benzene and other petrochemical compounds have long been suspected as the cause of leukaemia. Cytotoxic drugs particularly chlorambucil which is an alkylating agent has a strong association with the emergence of acute leukaemia in patients treated with this drug as exemplified by the treatment of Hodgkin’s disease and chronic lymphocytic leukaemia with chlorambucil containing regimens.

Role of viruses in leukemogenesis in man has not been established and to date there is no data to suggest that AML is either caused or predisposed by any known human or animal viruses.

Chromosomal abnormalities
Chromosomal abnormalities have been described in AML. Some of the most important of these include t(8;21), t(15;17), Inv 16, del 16, t(1;19).

Clinical Features
Patients with acute myeloid leukemia are almost invariably symptomatic; incidental diagnosis of AML is unusual. Clinical manifestations of AML are based on at least five pathophysiological mechanisms; these include:

- Autonomous proliferation of the myeloblasts in the bone marrow causes suppression and “crowding out” of the normal hematopoietic tissue. The resulting pancytopenia causes symptom related to thrombocytopenia, neutropenia, lymphopenia and anemia.
- Organ infiltration; extra medullary proliferation of leukemic cells cause organomegaly particularly hepatosplenomegaly. Lymph node enlargement is less common and is also less marked as compared to ALL. Congestive cardiac failure and fatty infiltration of liver may also contribute towards hepatic enlargement. Because of the relatively small size of the spleen, hypersplenism is not a feature of AML.
- Cellular hyperviscosity with consequent microvascular leukostasis and thrombosis; leukostasis is also an uncommon presenting feature in AML. It is caused by a massive increase in the number of blast cells in the peripheral blood. This causes slugging in the microcirculation and predisposes these patients to microvascular occlusion. This is particularly likely to happen in the cerebral capillaries. Because of the diffuse nature of the cerebral microcirculatory impairment, a few, some or a multitude of functional disturbances may be encountered in these patients. Visual disturbance, monoparesis,
headache and impairment of sensorium may appear late in the course of the disease particularly when the disease becomes resistant to treatment.

- **Hypermetabolism;** acute leukemia is a hypermetabolic state with all features of hypermetabolism faithfully reproduced; this includes weight loss, fever, sweating weakness and anorexia. Fever is partly due to hypermetabolism and partly due to bacterial infections to which these patients are excessively prone. Predisposition to infections in acute leukemia is the result of neutropenia, granulocyte dysfunction, lymphopenia and at times hypogammaglobulinemia.

- **Bleeding manifestations** are a common presentation. These are caused by thrombocytopenia and platelet function defects. Coagulation factor abnormalities are not a feature of acute myeloid leukemia nor are there any documented vascular abnormalities in AML. Bleeding is mostly mucosal although internal bleeding may also occur; intra-cranial haemorrhage may be the terminal event in some cases. It is important to note that platelets in AML are a product of ‘sick’ marrow environments. Not only is the platelet count decreased in AML, platelet function may also be impaired. These patients may therefore be more prone to bleed at a platelet count at which normal individuals are unlikely to bleed.

- Bone pains are a common presenting feature in children. Pain mostly affects the hands, the feet and the legs. It is less common in adults in whom chest pain and sternal tenderness seem to be more common.

### Laboratory Findings

#### Hematological

Hematological abnormalities constitute the backbone of the diagnosis of AML. A systematic analysis of the peripheral blood is the most economical and the quickest way of diagnosing acute leukaemia. Peripheral blood for the sake of simplicity and clarity may be studied under; numerical parameters, red cell indices, leukocyte differential count and morphology.

In the numerical values, anemia is almost invariant; it is not unusual for these patients to present with extremely low levels of hemoglobin. Leukocyte count is commonly increased, sometimes remarkably low. In some patients the count is not increased while in other it is actually decreased. The former condition is called subleukemic leukemia while the latter is called aleukemic leukemia. Red cells in the majority of the cases of AML are normocytic and normochromic. Some patients may have microcytic and hypochromic anaemia because of bleeding caused by thrombocytopenia. Macrocytic anaemia due to conditioned folate deficiency is a more likely abnormality.

Differential leukocyte count shows shift to the left; many blasts and comparatively fewer neutrophils are the hallmark of the differential leukocyte count in this disease. In many cases it is easy to suggest the nature of the blast cells but in a sizeable proportion of cases it is not possible to be certain about the origin of the blast cells. In such cases two investigations i.e. cytochemistry (special stains) and flow cytometry can help establish the ancestry of the blast cells.

#### Bone marrow

Bone marrow examination is mandatory in the diagnostic work up of patients with acute myeloblastic leukemia. This is true even in those cases where the diagnosis of acute leukemia is established beyond doubt on the basis of the peripheral blood counts and the differential leukocyte count. This is in contrast with CML where bone marrow examination for the purpose of diagnosis is only secondary to peripheral blood examination. The role of bone marrow examination in the diagnosis of acute myeloblastic leukemia is manifold; it is performed in order to:
• establish the diagnosis of aleukemic and subleukemic variants of acute leukemia.
• use the bone marrow slides for cytochemical stains especially the immuno peroxidase reaction which is best done on marrow biopsy or clot sections.
• determine the percentage of blast cells in the bone marrow at the time of diagnosis (>20% blasts confirms the diagnosis of AML) and for confirming remission in acute leukemias, that is defined as less than 5% blast cells in the bone marrow. This is because in some cases of acute leukemias, chemotherapy may clear the peripheral blood of all recognisable blast cells but without achieving the same result in the bone marrow. This information is important for further management of acute leukemias including bone marrow transplantation.
• assess erythropoiesis and thrombopoiesis at the time of diagnosis
• carry out cytogenetic studies. Bone marrow cultures are sometimes required to study the cytogenetic markers in large number of cells and in greater detail

Cytochemical stains which help establish the lineage of the blast cells include Per iod acid Schiff stain (PAS), myeloperoxidase (MPO) and Sudan black B. Characteristic cytochemical reactions with these stains are shown in table 17.3.

<table>
<thead>
<tr>
<th>Cytochemical stains</th>
<th>Myeloblasts</th>
<th>Lymphoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per iod acid Schiff</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sudan Black B</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 17.3: Cytochemical stains and their chemical reactions

Classification of Acute Myeloid Leukemia

Two major systems are used to classify AML into subtypes i.e. the French-American British (FAB) classification and World Health Organization (WHO) classification.

The French-American-British (FAB) classification

The French-American-British (FAB) classification divides AML into subtypes from M0 to M7. This is based on the type of cell from which the leukemia developed and the level of maturity of the cells. The FAB classification relied on appearance of leukemic cells under the microscope after Romanowsky staining. According to the FAB classification, the subtypes M0 to M5 associate with the precursors of granulocytes and monocytes. AML M6 originates in the very early forms of red blood cells and AML M7 starts in the megakaryocytic cell line. FAB classification with morphological features is shown in table 17.4.
<table>
<thead>
<tr>
<th>Subtype</th>
<th>Name</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Undifferentiated acute myeloblastic leukemia</td>
<td>Undifferentiated blasts with myeloid immunophenotype</td>
</tr>
<tr>
<td>M1</td>
<td>Acute myeloblastic leukemia with minimal maturation</td>
<td>Marrow cells are primarily myeloblast with no azurophilic granules</td>
</tr>
<tr>
<td>M2</td>
<td>Acute myeloblastic leukemia with maturation</td>
<td>Leukemic cells show prominent maturation beyond myeloblast stage</td>
</tr>
<tr>
<td>M3</td>
<td>Acute promyelocytic leukemia (APL)</td>
<td>M3: Promyelocytic; abnormal, hypergranular promyelocytes predominate. Auer rods are common M3m: Microgranular variant; indistinct granules, nucleus often reniform or bilobed</td>
</tr>
<tr>
<td>M4</td>
<td>Acute myelomonocytic leukemia</td>
<td>M4: Myelomonocytic; Both monocytic (monocytes and promonocytes) and myeloid differentiation (maturation beyond myeloblast stage) M4E: M4 with marrow eosinophilia; similar to M4 with marrow eosinophilia (abnormal and immature)</td>
</tr>
<tr>
<td>M5</td>
<td>Acute monocytic leukemia</td>
<td>M5a: Monocytic poorly differentiated; Monoblast predominate with abundant cytoplasm and single distinct nucleus M5b: Monocytic well differentiated; Predominantly promonocytes in marrow and more pronounced maturation in blood</td>
</tr>
<tr>
<td>M6</td>
<td>Acute erythroid leukemia (erythroleukemia)</td>
<td>Dysplastic erythroblasts with multinucleation, cytoplasmic budding, vacuolation and megaloblastoid changes</td>
</tr>
<tr>
<td>M7</td>
<td>Acute megakaryoblastic leukemia</td>
<td>Megakaryocytes show wide range of morphological features with cytoplasmic projections</td>
</tr>
</tbody>
</table>

Table 17.4: FAB classification of AML
Figure 17.5: AML M2

Figure 17.6: AML M3

Figure 17.7: AML M4
The World Health Organization (WHO) Classification of AML

Modern classification of AML is the World Health Organization (WHO) classification that divides AML into several broad groups. These include:

- AML with genetic abnormalities:
  - AML with a translocation between chromosomes 8 and 21
  - AML with a translocation or inversion in chromosome 16
  - AML with changes in chromosome 11
  - APL (M3), which usually has translocation between chromosomes 15 and 17
- AML with multilineage dysplasia meaning involvement of more than one abnormal myeloid cell type
- AML related to previous chemotherapy or radiation
- Unspecified AML including those that do not fall into one of the above groups, this includes:
  - Undifferentiated AML (M0)
  - AML with minimal maturation (M1)
  - AML with maturation (M2)
  - Acute myelomonocytic leukemia (M4)
  - Acute monocytic leukemia (M5)
  - Acute erythroid leukemia (M6)
  - Acute megakaryoblastic leukemia (M7)
  - Acute basophilic leukemia
  - Acute panmyelosis with fibrosis
  - Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)

Biochemical

Blood chemistry has very little to offer towards the diagnosis of acute leukemias. Routine biochemical investigations i.e. urea, creatinine, electrolytes, liver function tests and serum uric acid are determined as pre-therapy investigations. This establishes a baseline for subsequent follow up of the patients. Three biochemical parameters need special mention:

- Serum uric acid
  Uric acid is the end product of nucleoprotein catabolism. In acute leukemia where cell turn over is high, nucleoprotein catabolism is also increased. This results in a rise in the serum uric acid level which becomes particularly high when chemotherapy is administered and massive tumor lysis takes place.
• Serum and urinary muramidase
Hematopoietic cells of monocytic lineage are rich in an enzyme called muramidase. Being a low molecular weight substance, it is readily excreted in the urine. Conditions which are characterised by proliferation of the monocytic series cause a rise in the serum and urinary muramidase levels. This has come to be recognised as an important criterion in the differential diagnosis of acute leukemias. Serum and urinary muramidase levels are increased in acute monoblastic leukemia, chronic myelomonocytic leukemia, Juvenile CML, listeriosis and reactive monocytosis.

• Hypercalcemia
Hypercalcemia is sometimes encountered in acute myeloblastic leukemia. When present, it must be vigorously treated before the institution of chemotherapy lest hypercalcemia in conjunction with hyperuricemia precipitates renal shut down.

Immunophenotyping
Flow cytometry, used for the immunophenotyping, is the latest introduction to the diagnostic tools which are at present available for characterising blast cells. It is helpful even in those cases where light microscopy and cytochemistry fail to make a clear distinction between myeloblasts and lymphoblasts. Characteristic flow cytometric findings in AML are given in table17.5.

<table>
<thead>
<tr>
<th>AML</th>
<th>HLA-DR</th>
<th>CD34</th>
<th>CD33</th>
<th>CD13</th>
<th>CD11c</th>
<th>CD14</th>
<th>CD41</th>
<th>CD235a</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
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<tr>
<td>M3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>M6</td>
<td>+/-</td>
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<td>+/-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M7</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 17.5: CD markers in AML

Cytogenetic analysis
Acute myeloblastic leukemia, being a malignant disease, does show chromosomal aberrations. They are at times of significance not only in diagnosing and prognosticating the outcome of the disease but also in the monitoring any residual disease after therapy. An interesting observation is that patients with AML who manifest cytogenetic abnormalities enjoy a better prognosis than those with no cytogenetic abnormalities. Four cytogenetic abnormalities are of extreme interest and importance. These are:

• 8:21 translocation; It occurs in M2 subset of AML which is seen primarily in children and accounts for 30% of cases of AML. It is associated with good prognosis.
- t(15:17) translocation; This translocation is uniquely associated with acute promyelocyte (M3) leukemia.
- Inv 16; This abnormality is associated with abnormal eosinophilia (M4E) and favourable prognosis.
- Philadelphia chromosome t(9;22) (q34;q11); In approximately 20% of the cases of AML, Philadelphia chromosome can be demonstrated; it imparts a dismal prognosis in Ph positive AML.

**Radiological**

Radiology has little to offer in the diagnosis / prognosis of acute leukemia. X-ray chest, tomography, ultrasound of abdomen to detect hepatosplenomegaly and to ascertain mesenteric lymphadenopathy may be carried out to complete the diagnostic work up. These investigations however become mandatory if there are symptoms attributable to mediastinal, porta hepatis or para-aortic lymphadenopathy. Mediastinal adenopathy is not a feature of AML but it is sometimes seen in acute leukemia of T-cell type in young patients.

**Suggested further reading**

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder characterized by sustained increase in the number of moderately mature lymphocytes beyond 8,000/µl in the peripheral blood. Peripheral lymphocytosis is accompanied by an accumulation of similar cells in the bone marrow, liver, spleen, lymph nodes and other lymphoid organs.

Based upon the cell of origin and the surface markers, CLL is sub-classified into B cell CLL and T cell CLL. In most cases a single clone of B cells undergoes leukemic change but in 2-4% of the cases the transformation affects T cells.

Pathophysiology

Pathophysiology of CLL has two components i.e. intramedullary lymphocytic proliferation and tissue infiltration.

- Intramedullary lymphocytic proliferation
  Lymphocytic proliferation in the bone marrow causes lymphocytosis and variable degree of cytopenias in the blood.

- Tissue infiltration

  Tissue infiltration also constitutes an important basis for the clinico-pathological features of chronic lymphocytic leukemia. Clinical features that may arise as a result of tissue infiltration include cervical, axillary and inguinal lymphadenopathy. Mediastinal nodes may also increase in size and cause signs and symptoms of compression of airways, esophagus, recurrent laryngeal nerve, venous channels and sympathetic trunk. Hepatomegaly and splenomegaly with features of increased splenic pooling and painful and recurrent splenic infarcts are common.

  Chronic lymphocytic leukemia produces varying combinations of cytopenias in the peripheral blood. This is brought about through four mechanisms that include marrow infiltration, splenic pooling, autoimmunity and pure red cell aplasia.

Clinical Features

Patients with CLL are not infrequently diagnosed on routine examination. These patients are usually elderly and CLL is diagnosed when blood is examined for some completely unrelated purpose like pre-operative evaluation or an insurance examination.

Most patients, however, present with features of slowly progressive anemia such as pallor, weakness, shortness of breath on exertion and blackouts in upright position. These symptoms are caused by tissue hypoxia and cerebral anoxia due to a critical reduction in the oxygen carrying capacity of blood.

Recurrent infections frequently affect upper respiratory tract and skin due to increased susceptibility to bacterial, viral or fungal infections. Perineal area is not an infrequent site of infection and must be carefully examined for pyrexia of unknown origin (PUO) in these patients. Predisposition to develop Herpes zoster is common and may be a pointer towards an underlying
CLL or some other malignant disorder of the lymphorecticular system. Candidiasis and other fungal infections are also frequently encountered.

Bleeding is usually not a presenting feature although it may tinge the clinical picture as the disease progresses and thrombocytopenia becomes pronounced.

**Laboratory Findings**

**Hematological**

Whereas red cells remain unaffected (except when AIHA supervenes) it is the rise in the small to intermediate size lymphocytes which gives away the diagnosis. Morphologically, these lymphocytes are small and have relatively mature, well-differentiated appearance with a hyper condensed almost soccer ball appearing nuclear chromatin material. International Workshop on chronic lymphocytic leukemia recommends a minimum peripheral lymphocytosis of 5,000/µl along with 30% lymphocytes in the bone marrow. A common observation and an almost consistent accompaniment of lymphocytosis are the smudge cells. Though not diagnostic on their own, their presence in conjunction with an increase in the number of small and medium sized lymphocytes has come to constitute a highly characteristic morphological feature of chronic lymphocytic leukemia (see figure 18.1).

Smudge cells are not cells in the true sense of the word. They represent the excessively fragile lymphoid cells that break apart. They are consistently seen in CLL but they have also been observed in infectious mononucleosis, infectious lymphocytosis, viral lymphocytosis and lymphoblastic leukemias.

Anemia, when it occurs, is usually normochromic and normocytic with normal or low reticulocyte count. Reticulocyte count may be increased in patients with CLL with autoimmune hemolytic anemia. Platelet count is usually decreased. Neutropenia is common in patients with CLL.

**Bone marrow**

Marrow infiltration is the hallmark of CLL. One of the criteria for the diagnosis of CLL is marrow lymphocytosis accounting for a minimum of 40% of marrow nucleated cells. There are three patterns of marrow lymphocytosis in CLL; nodular, diffuse and mixed.

In the mixed pattern, nodular lymphocytic aggregates and diffuse infiltrates are present simultaneously. It was believed that nodular aggregates implied a better prognosis than the diffuse infiltrate; prognosis in the mixed pattern lay between these two pure patterns. Recent studies have not substantiated these earlier observations.
Erythropoiesis correlates with the marrow lymphocytosis and may be normal or decreased. In patients of CLL with AIHA, erythropoiesis may be increased. Platelet production is not seriously impaired, especially in the early stages.

**Biochemical**

No significant changes occur in biochemical parameters. However, the most common feature is hypogammaglobulinemia. Patients of CLL with AIHA have hyperbilirubinemia. Coombs test Coombs test is usually positive in 10% of the patients with AIHA.

**Immunophenotyping**

Immunophenotypic marker expression helps in the differentiation of B cell CLL and T cell CLL. Characteristic immunophenotype for B cell CLL is the expression of faint or low density sIg with kappa or lambda light chain, B cell associated antigens (CD19, 20, 79a), CD5, CD23, CD43, and faint CD11c.

**Cytogenetics**

There are no specific or diagnostic chromosomal abnormalities in CLL with respect to the general population. Some of the common chromosomal abnormalities in CLL are:

- **Trisomy 12:** This is the most common numerical abnormality and carries the most ominous prognosis. Trisomy 12 implies that there are three chromatids in chromosome No 12 instead of the usual two chromatids. This calls for an early and aggressive chemotherapy.
- **14 q-:** This is the most common chromosomal abnormality and frequently signals the evolution of pro-lymphocytic leukemia.
- **t(11,14) (q13, q32):** Other chromosomal abnormalities have also been described in CLL but none is seen in more than 15–20% of the cases. Since cytogenetic studies are neither easily available nor they are consistently reproducible, these are not incorporated in the diagnostic work up and in the therapeutic protocols for CLL.

**Suggested further reading**

MYELOPROLIFERATIVE DISORDERS

Myeloproliferative disorders are a group of disorders characterized by an overproduction of one or more than one hematopoietic cell lines which are native to the bone marrow. This conceptual definition includes such diverse conditions as leukemias, myelomas, megaloblastic anemias, hemolytic anemias and proliferation of the hematopoietic stem cell. Conventionally the term myeloproliferative disorders is used in a more restricted sense because all benign disorders and all malignant proliferative disorders affecting one hematopoietic cell line are excluded from this group of hematological diseases. The term myeloproliferative disorders was coined for the first time by William Dameshesk in 1951 as a group of disorders characterized by autonomous and relentless growth which results in clonal expansion of one or more of the subordinates in isolation or in variable combination. The disease process usually causes death of the patient in 5-15 years. Diseases which are included in this group of disorders are:

- Chronic myeloid leukemia
- Polycythemia rubra vera
- Essential thrombocythemia
- Myelofibrosis with myeloid metaplasia

These clinically distinct entities were grouped on the assumption that all these disorders were clonal in origin and that the molecular abnormality was a mutation in the pluripotent hematopoietic stem cell. The four diseases not only manifest a host of common clinical and laboratory features, they also show propensity to interchange.

Over the years not much has changed in our understanding of the molecular biology, clinical behaviour and natural outcome of these disorders except for some fine-tuning of their pathophysiology.

A change for the sake of description (but not for exclusion) is the separation of chronic myeloid leukemia from polycythemia vera, essential thrombocythemia and myelofibrosis with myeloid metaplasia which are grouped under the heading of ‘non-leukemic’ myeloproliferative disorder. This point however needs some clarification and deliberation.

The term ‘non-leukemic’ is used to indicate that these disorders are not leukemic at the outset; they however do transform into acute leukemia in a variable number of cases. The division is therefore not absolute and it should be taken in the perspective of cell morphology at the time of presentation. Features which unify these seemingly diverse disorders are:

- Clonal nature
- Autonomous behaviour
- Panmyelosis
- Hepatosplenomegaly
- Peripheral leukoerythroblastosis
- Cytogenetic abnormalities
Clinical features
Clinical features of myeloproliferative disorders are dictated by a host of pathological aberration. These include:

- Myeloproliferation, relentless autonomous and uncontrolled (unregulated)
- Platelet dysfunction
- Coagulation abnormalities
- Natural progression
- Metabolic derangements
- Biochemical changes
- Hypervolemia
- Hypermetabolism
- Hyper viscosity

It must be stressed at the outset that not all mechanisms contribute equally towards the clinical manifestations in all members of the MPD.

CHRONIC MYELOID LEUKEMIA

The term chronic myeloid leukemia (CML) is used interchangeably with the terms chronic granulocytic and chronic myelocytic leukemia. CML is a member of the myeloproliferative disorders as proposed by William Dameshek. It is a clonal disorder of the hematopoietic tissue caused by a mutation in the pluripotential hematopoietic stem cell. It is characterized by relentless and uncontrolled proliferation of granulocytes and their progenitors in the bone marrow with a variable involvement of the megakaryocytes. Red cells do not participate in this process. This is the first neoplasm in which a highly characteristic and diagnostic abnormality called Philadelphia chromosome t(9:22) (q34: 11) was described.

Pathophysiology
Philadelphia chromosome is an acquired, non-transmissible genetic abnormality which is produced as a result of an unbalanced translocation of genetic material between the long arms of chromosome No 9 and No 22. The term unbalanced implies that the amount of genetic material that separates from the two chromosomes is unequal; more genetic material is removed from chromosome 22 (which already is a short chromosome) than the amount of genetic material from chromosome 9 (which is already a long chromosome). Genetic material that is removed from chromosome 22 becomes attached to chromosome 9 and vice versa. As a result chromosome 9 becomes longer while chromosome 22 which was normally a short chromosome becomes even shorter. This altered and shortened chromosome 22 is the Philadelphia chromosome. This translocation was first described by Hungerford in 1960 in the city of Philadelphia (USA) hence the name Philadelphia chromosome. It forms the basis of classification of CML and its presence is associated with better prognosis than those who are Philadelphia chromosome negative.

A schematic diagram to explain the formation of Philadelphia chromosome is shown in the figure 19.1.
Pathophysiology of myeloid hyperproliferation

Under physiological condition myeloproliferation is controlled and regulated by an enzyme tyrosine kinase which is regulated by a protein of 145KDa. This regulatory protein is the product of ABL oncogene on chromosome No 9. As a result of translocation, a part of ABL oncogene is shifted on to chromosome 22. If this was the only change and there were no associated abnormalities in the biological behaviour of the gene, there would have been no analogical consequences. What actually happens is that as a result of the formation of the fusion gene (BCR-ABL) an abnormal fusion protein of 210 KDa is produced. The tyrosine kinase producing potential of this fusion protein is not only heightened, it unfortunately becomes autonomous (defying all controls and feed-back inhibition). As a result, the proliferation of the myeloid stem cells and their progenitors becomes uncontrolled. The genetic abnormality can be demonstrated in the progenitors of granulocytes, megakaryocytes, red cells as well as T and B lymphoid cells. The stimulus affects the granulocyte precursors to a maximum extent while megakaryocytes and fibroblasts participate to a variable extent. Erythroid precursors usually remain unaffected. Erythroid precursors actually become numerically indistinct (M:E ratio 100:1). This is compounded by red cell pooling in the spleen which causes variable degree of anemia in this disease.

ABL is the oncogene

Figure 19.1: BCR-ABL gene rearrangement
**Clinical Features**

Patients with CML may be asymptomatic to symptomatic depending upon the phase or stage of the disease. CML can be categorized into four phases as described below:

**Latent phase**

It is the period which precedes the clinical diagnosis of CML. During this period which is completely asymptomatic, there is neither any atypical feature in the peripheral blood nor is there any change in the bone marrow to arouse suspicion of CML. This phase is therefore a diagnosis in retrospect. From the kinetic studies of the granulocytes and the 'leukemic mass' at the time of diagnosis it is felt that the latent phase of CML spans over a period of approximately two years.

**Chronic phase**

This is the phase in which most of the patients with CML usually present. Some of the common presenting features include weakness, anorexia, early satiety, weight loss, night sweats and low grade fever. There is moderate to massive hepatosplenomegaly. Lymphadenopathy, bone pains or bone tenderness and bleeding manifestations are not encountered in these patients at the time of presentation. This phase usually last from 2 to 8 years and is most amenable to treatment.

**Accelerated phase**

This is the phase which commonly evolves at the end of the chronic phase. This is characterized by aggravation of the symptoms, infections, bone tenderness, resistance to drugs that were effective in chronic phase and significant numerical and morphological changes in the peripheral blood and bone marrow.

Hallmark of the diagnosis is the emergence of blast cells in the bone marrow as well as in the peripheral blood. A minimum of 20% blast cells in the bone marrow is a pre-requisite for the diagnosis of accelerated phase of CML.

**Blast crisis**

Blast crisis is a common terminal event in the natural history of CML. It is characterized by an exuberant proliferation of blast cells in the bone marrow and their spilling over into the peripheral blood. Blast cells in this phase are usually more than 50% of the TLC.

Anemia is a common accompaniment while platelets though usually decreased, may at times be normal or increased. This phase is most resistant to treatment and carries worst prognosis of all acute leukemias.

**Laboratory Findings**

**Hematological**

The most striking feature in the peripheral blood is marked leukocytosis. White cell count is usually greater than 100 X 10^9/L. A spectrum of myeloid forms from blast to mature neutrophil is seen in the peripheral blood known as complete left shift. However, myelocytes and mature neutrophils are the most numerous forms in the differential leukocyte count. The very high leukocyte count in CML is accompanied by various functional and morphological abnormalities of the granulocytes. Some of the morphological aberrations include pseudo Pelger-Hüet anomaly, hypogranulation, abnormal nuclear segmentation and formation of smudge cells as a result of increased fragility of granulocytes. In addition many leukemic cells display granular chimerism.

Normocytic and normochomic anemia is a common feature. Severity of anemia is proportional to increase in leukocyte count. Nucleated red cells may be present with
polychromasia, basophilic stippling and poikilocytosis. Thrombocytosis is found in 50% of patients with CML. As the disease progresses, platelet count decreases with abnormal function.

**Bone marrow**

Diagnosis of chronic myeloid leukemia is made by examining the blood counts and the peripheral blood smear. Bone marrow aspirates show only myeloid hyperplasia which supports the diagnosis of CML made on examination of the peripheral blood smear. Myeloid: erythroid (M:E) ratio is generally at least 10:1 as compared with normal ratio of 4:1.

Marrow fat spaces in chronic myeloid leukemia are almost non-existent. Those present, show considerable variation in their size and shape. Their relationship with the hematopoietic marrow shows considerable disarray. Bone marrow in CML may appropriately be described as “packed marrow” which implies that fat spaces account for less than 10% of the total marrow space.

![Figure 19.2: Peripheral smear showing complete spectrum of myeloid cells](image)

Like bone marrow aspirate, bone marrow biopsy is also not a prerequisite for the diagnosis of CML. Marrow biopsies however do have their contributions to make. Features like the extent of marrow fibrosis, the clustering and location of the megakaryocytes and marrow cellularity can only be assessed accurately by examining the bone marrow biopsy which also has a role in prognosticating the outcome in CML.

Some cases may show megakaryocytic hyperplasia in the marrow and thrombocytosis in the peripheral blood accompanied by morphological abnormalities involving the size and the shape of the platelets. It is unusual for CML to present with thrombocytopenia no matter how large is the spleen and how extensive is the marrow fibrosis.

Red cells do not participate in myeloproliferation in CML. Erythropoiesis in the bone marrow becomes indistinct because of the exuberant proliferation of the granulocyte precursors. M:E ratio may at times exceed 50:1 as opposed to the normal ratio of 4:1. This is reflected in the peripheral blood as normocytic and normochronic anemia of moderate severity.

Secondary myelofibrosis may accompany CML late in the course of the disease. Sea blue histiocytosis may be seen in the marrow of patients with CML.

**Biochemical**

Neutrophil alkaline phosphatase (NAP) level is markedly reduced in about 90% of cases of CML. Normal neutrophil alkaline phosphatase score is between 35-100; in CML it is often reduced to less than 10. This is one of the diagnostic features of CML. NAP score differentiates
CML from other myeloproliferative disorders and leukemoid reactions where the score is increased.

Gene for neutrophil alkaline phosphatase is located on the long arm of chromosome 21. It was originally thought that in CML there was deletion of a part of the long arm of chromosome 21 with concomitant loss of the gene which codes for this enzyme. In the light of the currently established cytogenetic abnormality in CML (translocation, not deletion) this explanation is not acceptable. It is now believed that 'NAP gene' in its new 'environment' is unable to code for the enzyme with resultant reduction of NAP activity in CML.

Chronic myeloid leukemia is commonly associated with an increased serum level of vitamin $B_{12}$ (cobalamin) and vitamin $B_{12}$ binding protein (Transcobalamin III.) This protein is synthesized by the neutrophils and is secreted in the plasma. As it firmly binds vitamin $B_{12}$ and parts with it only reluctantly, serum level of vitamin $B_{12}$ is concomitantly increased.

Serum and urinary muramidase levels are also increased because this enzyme is produced by the granulomonocytes. High serum and urinary muramidase level is a characteristic feature of juvenile CML which displays a strong monocytic component in the differential leukocyte count. Serum uric acid which is the end product of nucleoprotein catabolism is also increased proportionate to the number of leukocytes.

**Cytogenetics**

The hallmark of CML is the presence of Philadelphia chromosome in the karyogram of these patients. Almost 90-95% of the cases of CML in adults display this cytogenetic abnormality. Philadelphia chromosome is an important diagnostic marker of CML. Previous notion that patients with Philadelphia chromosome positive CML enjoyed better prognosis than those who were Philadelphia chromosome negative has not been substantiated by more extensive experience and data.

**Radiological**

Radiological features are uncommon in chronic myeloid leukemia. Ultrasonography may be used to demonstrate organomegaly which nevertheless is clinically apparent in most cases of CML.

**Morphological Variants of CML**

There are four morphological variants of chronic myeloid leukemia; these are

- Chronic neutrophilic leukemia
- Chronic eosinophilic leukemia
- Chronic basophilic leukemia
- Chronic monocytic leukemia

**Chronic Neutrophilic Leukemia**

This is a rare variant characterized by marked increase in the number of neutrophils without a concomitant increase in the number of immature granulocytes. NAP score is high and Philadelphia chromosome is positive. There is marked granulocytic hyperplasia in the bone marrow without any significant myelofibrosis. Liver and spleen are enlarged with evidence of extramedullary hematopoiesis. Median survival is approximately 2 years.

**Chronic Eosinophilic Leukemia**

In some cases of CML, eosinophilia is a striking feature. There is an increase in the number of mature as well as immature eosinophils along with numerous mature and immature
neutrophils. Generally the eosinophils account for more than 30% of the total leukocyte count. Eosinophilic leukemias may be Philadelphia chromosome +ve or –ve.

Eosinophilic leukemias must be distinguished from hypereosinophilic syndromes where the percentage of mature eosinophils may be as high as 90%; other features of CML are however missing. In addition there is the characteristic pulmonary infiltration and the cardiac involvement in hypereosinophilic syndromes.

Chronic Basophilic Leukemia
Clinical features are similar to those of classical CML except for an elevated count of basophils. In extreme cases, basophils may be the only cells present. The disorder is mostly Ph positive though some Ph negative cases have been reported. This variant responds to the same treatment as for classical CML; its course and prognosis are also similar.

Chronic Monocytic Leukemia
This term is sometimes applied to a condition in which the monocyte count in an otherwise classical case of CML is very high. Clinical course, prognosis and response to treatment are similar to that of typical CML.

POLYCYTHEMIA VERA
Polycythemia vera (primary proliferative polycythemia, polycythemia rubra vera or von-Vaques disease) is a clonal disorder of multipotent hematopoietic stem cells and is a member of the myeloproliferative syndromes. It is characterized by an over production of all three hematopoietic cell lines particularly the red cell line.

Etiology
There is no known etiology nor are there any consistent etiologically established associations. Though a disease of trilineage hyperplasia, its clinico-pathological features are dominated by an overbearing expansion of the red cell mass.

Erythroid hyperplasia in PRV is erythropoietin independent as evidenced by the ability of the neoplastic erythroid progenitors to grow in vitro in the absence of erythropoietin. Level of erythropoietin in the serum is markedly reduced. This is because of the inhibition of erythropoietin production by the high oxygen carrying and delivering capacity of blood. This eliminates the element of hypoxia and inhibits erythropoietin production.

Polycythemia vera manifests a wide variety of symptoms ranging from non-specific headache and general weakness to such devastating features as cerebrovascular and cardiovascular catastrophes. These are summarized in figure 19.3.
Clinical Features

Polycythemia vera produces a plethora of clinico-pathological manifestations. These are a reflection of interplay between a host of mechanisms; some of these are listed in figure 19.4.

![Figure 19.3: Spectrum of clinical manifestations in PRV](image)

Fundamental abnormality that underlies the genesis of these clinical features is the markedly expanded blood volume and total body hematocrit. Overproduction of platelets and white cells plays only a limited role in the clinico-pathological spectrum of PRV.

Relentless over-production of red cells expands the total red cell mass in the body. Additional space has to be provided for the expanded blood volume. Since arterial vessels down to the arterioles are the conducting vessels, they do not accommodate much of the additional red cell mass. Similarly larger venous channels also offer very little additional space to accommodate the increased blood volume. It is the micro-circulation that plays a major role in accommodating the expanded red cell mass. Dilatation of the micro-circulation and opening up of all ‘dormant’ capillaries and venules provide the necessary additional space. This becomes evident in the eyes and over the face where a fine network of small vessels is formed with suffusion of the conjunctiva and cyanosis of the face.

To accommodate the additional red cell mass, spleen also enlarges (the other reason for splenic enlargement is its participation in the myeloproliferative process).
Vastly expanded red cell mass with relatively smaller increase in the plasma volume causes cellular hyperviscosity and increased resistance to blood flow. This imposes a greater burden on the heart to pump larger volumes of 'viscous' blood through the expanded microvasculature. As a result, the blood pressure is raised which induces cardiac hypertrophy and increases myocardial O\textsubscript{2} demand.

All myeloproliferative disorders are characterized by hypermetabolism; polycythemia vera is no exception. Severity of hypermetabolism is however not as marked as in CML and myelofibrosis with myeloid metaplasia. Weakness is the cardinal symptom of hypermetabolism in these patients.

Gouty arthritis is precipitated by hyperuricemia due to increased production of uric acid through the degradation of large amount of nucleic acid released from the increased number of red cells produced in this disease.

**Laboratory Findings**

**Hematological**

Being a hematological malignancy, PRV produces a vast array of morphological and numerical abnormalities in hematological parameters. These dominate the laboratory findings in this disease. Some of these are:

- Increased red cell count
- Microcytosis and hypochromia
- Polymorphonuclear leukocytosis
- Increased basophil count
- Thrombocytosis
- Panmyelosis
- Coagulopathies
- Impaired platelet aggregation
- Coagulation abnormalities are not of much clinical significance in PRV; the following abnormalities are sometimes encountered in this disease:

  - Fibrinogen level is increased
  - Platelet aggregation is impaired with epinephrine, ADP and collagen
  - PT and APTT are prolonged

  The latter is due to a relatively increased amount of sodium citrate added for the smaller volume of plasma which is present in these patients. This, therefore, is a technical aberration of no clinical consequence.

**Bone marrow**

Examination of bone marrow has very little role in the diagnosis of PRV. Bone marrow aspirate shows richly cellular marrow with fat free particles hosting all three cell lines particularly the red cell line. There is no dysmaturation of any of the three hematopoietic lineages. Iron stores are characteristically depleted. Marked hypercellularity and panmyelosis are supportive but not diagnostic of polycythemia vera.

Bone marrow biopsy in the proliferative phase shows markedly hyper cellular marrow, paucity of fat spaces and only a minimal increase in the reticulin fibers.
As the disease evolves into fibrotic and leukemic phase, bone marrow examination becomes increasingly important especially in the phase of myelofibrosis where bone marrow biopsy clinches the diagnosis.

Biochemical
Biochemical abnormalities that are commonly encountered in uncomplicated polycythemia vera are:

- Serum uric acid increased
- Serum vitamin B₁₂ increased
- Serum serum B₁₂ binding capacity increased
- NAP score increased
- Serum LDH increased
- Serum potassium (pseudo-hyperkalemia)
- Serum histamine increased
- Serum cholesterol increased
- Serum erythropoietin decreased

Cytogenetics
Cytogenetics has very little contributions to make either in the diagnosis or in the prognosis of polycythemia vera. Being a clonal disorder of the multipotential hematopoietic cells, there are some cytogenetic abnormalities of non-descript and nonspecific nature that are observed with greater frequency in PRV.

JAK2 mutation is the most consistent chromosomal abnormality in patients with polycythemia vera. In untreated PRV, nearly 30% of the patients show some chromosomal abnormalities; their incidence rises to 50% in patients who receive chemotherapy. Some of the common cytogenetic abnormalities are deletion of long arm of chromosome No 5 (5q), deletion of long arm of chromosome No 20 (20q), trisomy-8 and trisomy-9. Deletion of the long arm of chromosome 20 (20q) is seen in 30% of cases. None of these karyotype abnormalities have any bearing on the clinical severity or the outcome of the disease.

PVSG Criteria for the Diagnosis of Polycythemia Vera
Whereas it is easy to diagnose polycythemia (by determining peripheral blood hematocrit), the criteria to establish the diagnosis of PRV are more elaborate and stringent. Polycythemia vera study group (PVSG) in 1985 proposed a set of clinico-pathological criteria for the diagnosis of PRV. These criteria have undergone certain modifications in the light of the current advancements in hematology. The following are the original PVSG criteria for the diagnosis of PRV:

Category A:
1: Total body hematocrit
   > 36 ml / kg in male
   > 32 ml / kg in female
2: Arterial O₂ saturation
   > 92%
3: Splenomegaly

Category B:
1: Leukocytosis
   ≥ 12000 /µl
2: Thrombocytosis
   ≥ 400,000 /µl
3: Serum B₁₂ binding capacity
   > 2200 pg/ml
4: Serum vitamin B₁₂
   > 900 pg/ml
Presence of all three criteria in category A or the presence of criteria A₁ + A₂ and any two criteria in category B constitutes the diagnosis of polycythemia vera.

**Modified PVSG Criteria**

Recently the original criteria proposed by the PVSG have come under review. This was necessitated by recent advances in our understanding of the pathophysiology of polycythemia vera. These include cytogenetic abnormalities and in vitro growth pattern of erythroid progenitors suggesting biclonality of the erythroid progenitors. Incorporating these two characteristics, the criteria which have now been proposed for the diagnosis of polycythemia vera are:

A: Definitive criteria

1. Red cell mass > 25% of the predicted value.
2. No evidence of hypoxia (arterial oxygen saturation > 92%).
3. Palpable splenomegaly.
4. Abnormal marrow karyotype.

B: Supportive criteria

1. Platelet count > 400 x 10⁹ / L
2. Neutrophil count > 10 x 10⁹ / L
3. Ultrasonically demonstrable splenomegaly
4. Characteristic BFU.E growth response to EPO (or reduced serum EPO level).

Diagnosis of polycythemia vera is established in the presence of:

I. Definitive criteria 1 + 2 + 3 OR
II. Definitive criteria 1 + 2 + 4 OR
III. Definitive criteria 1 + 2 + any two supportive criteria.

According to the modified criteria it is mandatory to have access to the predicted values of the red cell mass (RCM) based upon age, sex, height and weight of the individual.

Whereas most of the parameters are easy to establish, the crucial parameter i.e. an increase in the red cell mass calls for radio tracer studies which are not available in many of the clinical laboratories. Such cases will have to be referred to a reference laboratory which is set up for this purpose.

**Secondary Polycythemias**

Secondary polycythemias are a group of disorders characterized by an increase in the peripheral as well as total body hematocrit in response to increased erythropoietin production in a vast majority of cases. A number of hematological as well as non-hematological disorders produce secondary polycythemia; these include:

- Pulmonary disorders
- Cyanotic heart disease
- High altitude living (> 2000 M)
- High O₂ affinity hemoglobins
- Tumor-associated hyper-erythropoietinemia
- Cobalt-induced histotoxic anoxia

Of these disorders, tumor-associated hypererythropoietinemia is the only condition in which oxygen delivery to the tissues is normal. All other causes of secondary polycythemias are
associated with variable degree of tissue hypoxia. The common effector which stimulates erythropoiesis and increases total body hematocrit is the increased production of erythropoietin.

**Relative Polycythemia**

Relative polycythemia is characterised by an increase in the red cell count, high hemoglobin and high hematocrit in the peripheral blood due primarily to a reduction in the circulating plasma volume. These disorders may therefore be viewed as hemoconcentration rather than polycythemias. In the bone marrow there is no erythroid hyperplasia and bone marrow cellularity, distribution of fat spaces and ratio between hematopoietic and non-hematopoietic marrow remain normal.

Relative polycythemia is caused by a reduction in the plasma volume while total body hematocrit remains unchanged. Normal red cell mass is 'suspended' in a smaller plasma volume resulting in hemo-concentration. This situation arises when large amount of water is lost from the circulation. Some of these conditions are:

**External fluid loss:**
- Severe vomiting and / or diarrhea as in cholera, other forms of gastro-enteritis and hyperemesis gravidarum.
- Polyuria as in diabetes mellitus, diabetes insipidus and injudicious use of diuretics.
- Excessive sweating coupled with water deprivation as in deserts and strenuous labor in arid conditions.

**Internal plasma sequestration**

Internal plasma sequestration also known as idiopathic hemoconcentration is a poorly defined entity which has been included under polycythemia because of an increase in the venous hematocrit.

In this condition hemoglobin, hematocrit and red cell count in the peripheral blood are increased. Total body hematocrit is normal while plasma volume is decreased as is the total blood volume. There are no features of any associated cause of erythrocytosis and there is no evidence of external fluid loss. Internal plasma sequestration includes two vaguely defined entities; these are:

- Stress polycythemia
- Smoker's polycythemia

Figure 19.5 lists the conditions that are commonly associated with relative polycythemia.
Myelofibrosis with myeloid metaplasia belongs to the group of chronic myeloproliferative disorders, characterized by the classic triad of fibrosis of the marrow, extramedullary hematopoiesis, and leukoerythroblastic blood picture. Myelofibrosis with myeloid metaplasia is also known as idiopathic myelofibrosis, agnogenic myeloid metaplasia, myelosclerosis, osteosclerosis, chronic erythroblastosis, aleukemic myelosis, and chronic or primary myelofibrosis.

As the name implies, etiology of MMM is unknown. Exposure to ionizing radiation, benzene, toluene, arsenic, fluorine, conditions associated with abnormal immunologic status are some of the most likely factors associated with the pathogenesis of MMM.

Pathophysiology
Myelofibrosis is a clonal stem cell disorder in which fibrosis occurs as secondary non-neoplastic process. Myelofibrosis is related to the accumulation of increased marrow collagen due to the proliferation of fibroblasts. Abnormal and dysplastic megakaryocytes in marrow result in the excessive release of platelet-derived growth factor (PDGF), transforming growth factor β and vascular endothelial growth factor (VEGF) in the bone marrow. These factors stimulate the proliferation and growth of fibroblasts and collagen deposition.

Clinical Features
Myelofibrosis with myeloid metaplasia is a chronic progressive disorder and patients may remain asymptomatic for years. Splenomegaly is the most significant clinical finding. As the disease progresses spleen enlarges. Mechanism of splenomegaly includes extramedullary hematopoiesis, fibrosis and congestion from increased blood flow through the celiac axis. Another reason of splenomegaly may be the pooling of red cells up to two thirds of the red cell mass that is detained in transit through the splenic cords. Patients present with left or mid-abdominal fullness and distension. Massive splenomegaly may impede urine excretion.

![Figure 19.5: Causes of 'relative polycythemia'.](image-url)
Signs and symptoms of anemia i.e. weakness, pallor, lethargy and dyspnea on exertion are common. Anemia occurs as a result of bone marrow failure, ineffective and/or dyserythropoiesis, pooling of red cells in the spleen and hemolysis due to hypersplenism. Ineffective erythropoiesis is associated with marked erythroid hyperplasia.

Bleeding manifestations due to thrombocytopenia or thrombocytosis, platelet functional defects and coagulation abnormalities include petechiae, ecchymoses, gastrointestinal or urogenital bleeding.

**Laboratory Findings**

**Hematological**

Patients with MMM usually present with normocytic and normochromic anemia that worsens with the progression of the myelofibrosis. Patient with bleeding diathesis may develop microcytic and hypochromic anemia. As the disease progresses peripheral blood morphology changes from normocytic red cells to leukoerythroblastic blood picture. Characteristic findings are the presence of large number of tear drop cells, nucleated red cells and immature granulocytes. Red cells passage through narrow sinusoids of the fibrotic bone marrow and spleen leads to the formation of tear drop shape.

Leukocyte count is variable in MMM that may be normal, increased or decreased. Progression of the disease leads to leukopenia with the presence of immature myeloid cells. It must be clear at this juncture, that the number of immature myeloid cells remains less than seen in patients with acute myeloid leukemia. Eosinophilia and basophilia may be observed in many patients. LAP score may be normal or increased.

Platelet count may be normal, increased or decreased. At diagnosis, platelet count is usually increased. Thrombocytopenia is evident as the disease progresses. Large platelets and even fragments of megakaryocytes may be seen in the peripheral blood smear.

![Figure 19.6: Leukoerythroblastic blood picture with marked teardrop cells](image)

**Bone marrow**

Bone marrow aspiration usually results in dry tape. In early stages of the disease, pan hyperplasia is evident with uneven fibrosis. With the passage of time, hematopoietic tissues are replaced by reticulin fibers and collagen deposition. Megakaryocytes are increased in number mainly in clumps with dysplastic changes.

Bone marrow biopsy shows qualitative and quantitative abnormalities in all types of cells with tri-lineage hyperplasia, fibroblastic proliferation, atypical osteocytes and prominent
megakaryocytes in cohesive clusters. In later stages increased reticulin fiber density is demonstrated by positive silver stain.

Histological course of myelofibrosis with myeloid metaplasia comprise of cellular phase, fibrotic phase, sclerotic phase and osteosclerotic phase. Features of each phase are given below;

- **Cellular phase**
  - Diffusely hyperplastic; normal erythropoiesis and granulopoiesis
  - Megakaryocytes may predominate
  - Reticulin +

- **Fibrotic phase**
  - Megakaryocytes still predominant; decreasing numbers of other hemopoietic cells
  - Altered sinus architecture
  - Reticulin ++
  - Collagen +

- **Sclerotic phase (myelosclerosis)**
  - Grossly disturbed architecture
  - Markedly reduced hematopoiesis
  - Megakaryocyte clusters
  - Fibroblasts +++
  - Reticulin +++
  - Collagen +++

- **Osteosclerotic phase (osteomyelosclerosis)**
  - Fibroblasts +++
  - Collage +++
  - Osteoblasts and osteocyte proliferation with bone formation

![Figure 19.7: Normal marrow (stained with reticulin stain)](image)

![Figure 19.8: Increased reticulin fibers (stained with reticulin stain)](image)
Hemostatic
Platelet aggregation studies show abnormal results. Response to adrenaline is abnormal, while other agonists i.e. ADP, collagen and thrombin show variable response. Prolonged prothrombin time, activated partial thromboplastin time and thrombin time are found in many patients. Elevated levels of fibrin degradation products (FDPs) and reduced levels of factors V and VIII indicates disseminated intravascular coagulation in patients with MMM.

Biochemical
Uric acid and LDH are usually increased. Liver function test also shows elevated liver enzymes. Serum levels of vitamin B₁₂ are increased.

Cytogenetics
Chromosome abnormalities reported in cases of MMM include deletions of the long arm of chromosome 13 (13q⁻), 20q⁻, translocation (1;13) and partial trisomy of 1q.

Differential Diagnosis
Myelofibrosis with myeloid metaplasia shall be distinguished from other chronic myeloproliferative disorders and fibrosis secondary to infiltrative disorders. Polycythemia Vera Study Group (PVSG) has set the following criteria for the differentiation and diagnosis of MMM:

- Splenomegaly
- Fibrosis involving more than one third of the sectional area of an adequate bone marrow biopsy specimen
- Leukoerythroblastic blood picture
- Absence of increased red cell mass
- Absence of Philadelphia chromosome
- Exclusion of systemic disorders
- A diagnosis of osteomyelosclerosis requiring the presence of sclerotic changes detected radiologically in axial skeleton long bones

ESSENTIAL THROMBOCYTHEMIA
Essential thrombocythemia (ET) (synonyms: idiopathic thrombocythemia, primary thrombocythemia, primary hemorrhagic thrombocythemia) is a myeloproliferative disorder characterized by increased platelet count in the peripheral blood and megakaryocytic hyperplasia in the bone marrow. This condition is usually associated with abnormal platelet function. Platelet count of more than 600 X 10⁹/L is the hallmark of essential thrombocythemia. However, other causes of raised platelet count shall be excluded.
Etiology of thrombocythemia is unknown. Patients with ET do not exhibit Philadelphia chromosome or BCR-ABL1. JAK2 (Val617Phe) mutation was found to be positive in 4% of patients with ET.

Clinical Features
Bleeding manifestations
Patients are usually asymptomatic at diagnosis. Bleeding manifestations are usually mild i.e. epistaxis and ecchymoses. Life-threatening hemorrhage may occur in cases of accidental trauma or surgery. Hemorrhage has been attributed to several mechanisms, that include;

- Platelet functional abnormalities
- Thrombosis
- Consumption of coagulation factors
- Excessive production of prostacyclin (PGI₂) by endothelial cells
Thrombosis
Thrombosis is caused by intravascular clumping of hyper active platelets. Micro vascular obstructions are generally found in ET however, large vessels may also be occluded that result in myocardial infarction and stroke.

Neurologic manifestations
These include visual disturbances, headaches, paresthesia, dizziness, transient ischemic attacks and rarely seizures.

Others
Other clinical findings include recurrent abortions, fetal growth retardation, pruritus, gout and priapism. Splenomegaly is found in half of the patients with ET. Splenic atrophy occurs in up to 20% of patients with ET.

Laboratory Findings
Hematological
Platelet count is always raised (600-2,500 x 10^9/L). Platelets may be morphologically normal or may show anisocytosis. Platelet anisocytosis correlates with increased platelet distribution width (PDW). Platelet anisocytosis may include giant, bizarre platelets (megathrombocytes), microthrombocytes, platelet aggregates, abnormally granulated platelets and fragments of the cytoplasm of megakaryocytes.

Mild normocytic and normochromic anemia may be present in some patients with ET. Bleeding leads to iron deficiency anemia in patients with ET that results in decreased MCV, MCH, MCHC and microcytic and hypochromic red cells.

Leukocyte count may be normal or increased. Leukocytosis is usually neutrophilic. Mild eosinophilia and/or basophilia may occasionally be seen. LAP score is variable but most commonly is normal.

Bone marrow
Bone marrow shows megakaryocytic hyperplasia in patients with ET. Erythroid and myeloid hyperplasia may also be seen. Megakaryocytes may be present in clusters. Megakaryocytes are typically larger than normal with abundant cytoplasm and may be dysplastic in appearance. Increased nuclear lobulation of megakaryocytes and mitotic forms are increased.

Perl’s stain usually demonstrates normal iron stores or may be slightly decreased in cases of chronic hemorrhage. Reticulin is often increased in patients with ET.

Platelet function tests
Platelet function studies show significant abnormalities. Abnormal platelet aggregation to epinephrine, collagen and adenosine di phosphate (ADP) are quite frequent. Reduced level of platelet factor 3 (PF3), reduced platelet adhesion, low protein S levels, and nucleotide storage pool defects have all been reported in association with ET. Despite all of these identified abnormalities, there is poor correlation with any of these findings and the incidence of clinical thrombo-hemorrhagic manifestations.

Biochemical
Serum cobalamin and unsaturated cobalamin binding capacity are increased in patients with ET. Uric acid, LDH and acid phosphatase are also elevated.
Diagnostic criteria for ET proposed by the PVSG
- Platelet count in excess of 600 x 10^9/L (and generally> 1,000 X 10^9/L)
- Megakaryocytic hyperplasia
- Absence of identifiable causes of reactive thrombocytosis
- Absence of the Philadelphia chromosome
- Hemoglobin no higher than 13 g/dL or normal red cell mass
- Absence of significant marrow fibrosis
- Presence of stainable iron in marrow or failure of iron trial

World Health Organization criteria for essential thrombocythemia

Positive Criteria
- Sustained platelet count ≥600X10^3/μl
- Bone marrow biopsy showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes

Criteria of Exclusion
- No evidence of polycythemia vera
- Normal red cell mass or hemoglobin <18.5 g/dl in men, 16.5 g/dl in women
- Stainable iron in marrow, normal serum ferritin or normal mean corpuscular volume

If the former condition is not met, failure of iron trial to increase red cell mass or hemoglobin levels to the PV range
- No evidence of chronic myeloid leukemia
- No Philadelphia chromosome and no BCR/ABL fusion gene
- No evidence of chronic idiopathic myelofibrosis
- Collagen fibrosis absent
- Reticulin fibrosis minimal or absent
- No evidence of myelodysplastic syndrome
- No del(5q), t(3;3)(q21;q26), inv(3)(q21q26)
- No significant granulocytic dysplasia, few, if any, micromegakaryocytes
- No evidence that thrombocytosis is reactive due to:
  - Underlying inflammation or infection
  - Underlying neoplasm
  - Prior splenectomy

Differential Diagnosis
ET must be differentiated from reactive thrombocytosis, other chronic myeloproliferative disorders with associated thrombocytosis and myelodysplastic syndromes (MDSs) in which the platelet count is markedly raised.

Suggested further reading


PLASMA CELL DYSCRASIAS
These are a group of malignant disorders characterized by an autonomous proliferation of plasma cells. Their malignant potential shows a wide spectrum both clinically and pathologically. Disorders included in this group are:

- Classical multiple myeloma
- Clinically manifest myeloma
- Smoldering myeloma*
- Solitary plasmacytoma
- Extramedullary plasmacytoma
- Heavy chain diseases
  - $\alpha$ heavy chain disease
  - $\gamma$ heavy chain disease
  - $\mu$ heavy chain disease
- Light chain disease
- Waldenstrom’s macroglobulinemia
- Primary amyloidosis
- Osteosclerotic myeloma
- POEMS (Polyneuropathy (P), organomegaly (O), endocrinopathy (E), monoclonal gammopathy (M), skin changes (S))
- MGUS (monoclonal gammopathy of uncertain significance)

* Smoldering myeloma is diagnosed on the basis of the following triad:
  - Serum M protein $\geq 3$ g/dl
  - 10% clonal plasma cells in the bone marrow
  - No myeloma related symptoms

MULTIPLE MYELOMA
Multiple myeloma is a clonal disorder characterized by increased proliferation of abnormal plasma cells and presence of high levels of monoclonal antibodies in the blood and urine and. Multiple myeloma is a rare disorder found in old age.

Etiology
It is proposed that environmental factors play a vital role in the development of multiple myeloma. Individuals (i.e. workers at nuclear power plants, radium watch dial painters and radiologists) exposed to radiation in the workplace are prone to the development of multiple myeloma. Chronic and abnormal stimulation of the immune system is also suspected as a cause of myeloma.

Pathophysiology and Clinical Manifestations
Pathophysiology of multiple myeloma is interplay of the following three components;
- Increased proliferation of plasma cells
- Excessive production of monoclonal antibodies
- Bone resorption

**Increased proliferation of plasma cells**
Activated B lymphocytes develop into plasma cells. Genetic aberration may occur in the transformation of B cells that develop into plasmablast. These mutated plasmablasts start relentless proliferation in the bone marrow and also in other parts of the body resulting in their accumulation known as plasmacytoma. Plasmacytoma replace the normal hematopoietic tissues that results in anemia, neutropenia and thrombocytopenia.

Expanding plasmacytoma in the bone marrow usually destruct the cortex of the bone and cause pain. Bone pains are the most consistent finding in patient with multiple myeloma. These plasmacytoma may also result in the compression of nerves especially of vertebrae. Metastasis of plasmacytoma and their proliferation may occur leading to their accumulation in the nasopharynx, paranasal sinuses, liver, spleen, skin, kidneys, and gastrointestinal tract.

**Excessive production of monoclonal antibodies**
Clonal proliferation of plasma cells results in the increased production of antibodies known as monoclonal antibodies. These malignant plasmacytoma also synthesize light chains and/or heavy chains. Excessive antibody production may lead to change the physiological milieu and result in a condition known as hyperviscosity syndrome. Hyperviscosity slows down the flow of blood in the micro vessels of brain, heart and other organs. Patients usually present with headache, confusion, blurred vision, chest tightness and numbness of the fingertips.

Low levels of normal antibodies may be found in multiple myeloma due to the suppressed function of B cells. Hypogammaglobulinemia predisposes increases the susceptibility to bacterial infections.

Light (κ or λ) chains produced in excessive amount are excreted from the body through urine. Sustained excretion of these light chains in the urine damages the kidneys. These light chains excreted in the urine are named Bence-Jones proteins after the physicians who first noted this unusual property. These light chains may be deposited in heart, liver, spleen, gastrointestinal tract, kidneys and skin. Accumulation of these light chains in organs is known as amyloidosis.

**Bone resorption**
Plasma cells induce the secretion of osteoclast activating factor (OAF) and IL-6. These cytokines develop lytic bone lesions by bone resorption leading to hypercalcemia, bone pains and neurological compression syndrome. Hypercalcemia leads to constipation, intestinal changes, muscle weakness and neurological complications.

Clinical manifestations in multiple are summarized in the hexagon shown in figure 20.1:

![Figure 20.1: Clinical manifestations of multiple myeloma](image-url)
Lytic bone lesions result in the thinning of the cortex of bones that leads to bone pains and fractures. Excretion of large amount of calcium, light chains and hyperviscosity syndrome are the factors of renal failure in patients with multiple myeloma.

Plasmacytoma replaces the normal hematopoietic tissues resulting in anemia, thrombocytopenia and neutropenia. Anemia results in fatigue, shortness of breath, and rapid heart rate. Thrombocytopenia may lead to bleeding manifestations. Neutropenia causes overwhelming bacterial infections.

**Laboratory Findings**

**Hematological**

Complete blood counts show normocytic and normochromic anemia with marked rouleaux formation. Marked rouleaux formation is caused by hypergammaglobulinemia. In advanced stages of the disease plasma cells may be seen in the peripheral blood. A bluish background tinge may be observed by gross examination of the Leishman's stained slide. Erythrocyte sedimentation rate is characteristically increased in multiple myeloma due to hypergammaglobulinemia.

**Bone marrow**

Bone marrow aspiration shows increased number of plasma cells (>20%) usually found in sheets. These plasma cells show morphological changes i.e. bi-nucleated plasma cells, plasmablasts and flame cells. Flame cells are characteristically found in IgA secreting type of multiple myeloma. Bone marrow biopsy is performed to evaluate the extent of plasma cell infiltration of the bone marrow.

![Figure 20.2: Plasma cells in multiple myeloma](image)

**Biochemical**

Biochemical parameters that are characteristically increased in multiple myeloma are serum urea, creatinine, calcium, LDH, β microglobulin and C-reactive protein (CRP). Albumin levels show decreased levels while alkaline phosphatase remains normal. Protein electrophoresis and immunoelectrophoresis

Protein electrophoresis shows a characteristic peak of monoclonal immunoglobulins known as monoclonal spike or M-spike. For the characterization of the type of antibodies, light chains and heavy chain immunoelectrophoresis is performed.

**Radiological**

Radiological findings are very characteristic and show the following changes:
- Multiple lytic lesions without marginal sclerosis
- Diffuse osteoporosis
- Osteosclerosis circumcised (skull)
- Localized punched-out lesions
- Pathological fractures
- No bone abnormalities (20%)

**Cytogenetics**
Cytogenetic abnormalities in patients with multiple myeloma include translocations \{t(11;14), t(14;16)\}, monosomy 13, deletion of 17p and gains of 1q and aneuploidy.

**Suggested further reading**
THROMBOCYTOPENIA

Numerical reduction in the number of platelets in the peripheral blood is called thrombocytopenia. Normal platelet count is 150-450x10³/µl. Although any reduction below the lower limit of normal is thrombocytopenia, conventionally platelet count below 100X10³/µl is viewed as clinically significant thrombocytopenia.

There are a number of mechanisms that produce thrombocytopenia. In order to appreciate the role of these mechanisms it is imperative to review the platelet kinetics.

Platelets are normally produced in the bone marrow. Their parent cells are the megakaryocytes that are derived from megakaryoblasts through the intermediate stage of pro-megakaryocytes.

After their release from the bone marrow into the peripheral blood, platelets remain in the circulation for a period of 7-10 days. During their passage through the spleen physiological intra-splenic platelet retention takes place and nearly 30% of the platelets are retained in the spleen. This implies that for every 100 platelets that enter the splenic artery only 70 platelets are retrieved from the splenic vein. This is a physiological phenomenon.

Under pathological conditions platelet retention increases proportionate to the size and the vascularity of the spleen. In massively enlarged spleens as much as 60-70% of the platelets may be retained. Conversely if the spleen is removed from a healthy individual as a result of trauma or for some other reason, the platelet count increases by about 25-30% above the pre-splenectomy level.

As mentioned earlier, the cardinal function of the platelets is to secure primary hemostasis at the level of microvasculature. Under physiological conditions small microthrombi are constantly being formed and removed from the circulation by the fibrinolytic system. In addition, micro-tears in the capillaries and venules are a constant happening. It is the function of the platelets to seal those tears and prevent any leakage of blood from the micro vessels. Since hemostasis in larger vessels is not a function of the platelets, large vessel bleeds are not caused by a reduction in the platelet count in the peripheral blood.

As a corollary to the above preamble, causes of thrombocytopenia fall into three groups:

- Hypoproliferation of the megakaryocytes
- Increased consumption of the platelets in the peripheral blood
- Hypersplenism
- Any combination of the above
Hypoproliferative thrombocytopenia
This is usually a part of marrow hypoplasia irrespective of the cause thereof. Some of the common causes of marrow hypoproliferation are:

- Aplastic anemia
- Megaloblastic anemias
- Subleukemic acute leukemia
- Myelodysplasias

Peripheral thrombocytopenia due to hypoproliferation of the bone marrow is usually a part of pancytopenia or bicytopenia (which may later progress to pancytopenia).

Hypersplenism
A better and a more descriptive term for hypersplenism is splenic sequestration or splenic pooling. Splenic sequestration is directly proportional to the size of the spleen. Massively enlarged spleens may trap as many as 60-70% of the platelets that pass through them. Vasularity of the spleen also determines the number of the platelets that are retained. Large spleens that are less vascular and more fibrous as in amyloidosis and Gaucher's disease retain fewer platelets than vascular spleens as in cirrhosis and congestive splenomegaly.

Increased peripheral consumption
There are two important groups of disorders that cause increased peripheral consumption / destruction of the platelets; there are:

- Immunological
- Non immunological

Immunologically mediated consumptive thrombocytopenia is seen in:
  - Systemic lupus erythematosus
  - Chronic lymphocytic leukemia
  - Heparin administration
  - Malaria
  - HIV-AIDS
  - Other viral illnesses
  - Post-transfusion purpura (PTP)
  - Drug – administration

Other causes of isolated thrombocytopenia are less common; some of these are:
  - Thrombotic thrombocytopenic purpura
  - Disseminated intravascular coagulation
  - Massive transfusion with stored blood (dilutional thrombocytopenia)
  - Hemolytic uremic syndrome

Clinical Features
Clinical manifestations of thrombocytopenia can be summarized in one word; "bleeding". These are dictated by the site, amount and rate of bleeding. A massive hemorrhage in the brain due to acute leukemia may be fatal while nose bleed is more of a nuisance and bleeding in the skin is more of a cosmetic concern. The most frequent site of bleeding, as anticipated from the physiological role of platelets, is from the mucosal surfaces where microvessels are in abundance and less protected as in the nose, mouth, uterus and gastro-intestinal tract. Also because of the size of the vessels, the amount of blood lost is not of critical magnitude and severe anemia is unlikely to be a sequelae thereof. It also does not pose any serious threat to life except in the
brain because of its strategic location. Similarly hemorrhage in the eye may seriously impair the vision. Barring these few exceptions, bleeding from the microvessels due to thrombocytopenia is not a threat to life. Bleeding in the urinary tract may be the number of platelets with the severity of distressing as a result of the formation of a ureteric clot and the resultant renal colic.

Another factor which is of paramount importance is the mechanism of bleeding. Correlation between the number of platelets and propensity to bleed is to a large extent determined by the cause of thrombocytopenia. Platelets from patients with ITP function much better than those from patients with acute leukemia or megaloblastic anemia. This is because marrow megakaryocytes in ITP are younger and well granulated. They produce platelets that are also hyperfunctional. Platelets in patients with acute leukemia and myelodysplasia are the progeny of sick megakaryocytes that are hypogranulated. These platelets may be hypogranular and manifest certain structural and functional defects.

Laboratory Findings

Since the numerical reduction in the platelet count is at the heart of the clinical symptomatology, it is imperative to elucidate the underlying mechanism of thrombocytopenia. Investigations for isolated thrombocytopenia are rather limited and are mainly determined by the age of the patient and the onset and the historical scenario. In a vast majority of cases, thrombocytopenia of sudden onset is due to peripheral consumption on an immunological basis.

Other causes of thrombocytopenia are usually associated with anemia and / or leukopenia. Investigations that are more likely to provide fruitful information are:

- Examination of the bone marrow smear and biopsy.
- Immunological tests for the detection and characterization of antiplatelet antibodies

Immunological tests that are now available to establish the cause of thrombocytopenia include demonstration of antibodies against platelet antigens GPIIb/IIIa complex and GPIb. PLA-1gG antibodies, though a measure of an immunological phenomenon, are less specific and non-diagnostic.

IMMUNE THROMBOCYTOPENIC PURPURA

Immune thrombocytopenic purpura (ITP) previously called as idiopathic thrombocytopenic purpura, as a confession of unawareness of the pathogenesis of the disease), is a scaring disease at the time of presentation. The title of the disease embodies its three cardinal features i.e. an immunological disease, thrombocytopenia and presence of purpura.

In the past, when immunological insight into various medical ailments was less this disease, for the sake of convenience and for want of understanding was called idiopathic. Now that it is better understood and immunological basis of thrombocytopenia has been established, the disease has been renamed as immune thrombocytopenic purpura. It is fortuitous that the alphabet ‘i’ stands not only for idiopathic but also for immune!

Pathophysiology

Immune thrombocytopenic purpura, as the name suggests, has an immunologic basis. In many cases the underlying cause of immunologic aberration is not known. In a small number of cases an association can be established between the causative agent and the anti-platelet antibodies in the patient’s serum. Cases in which the cause remains elusive are called primary ITP while those in which the cause is either established or is strongly suggested are called secondary ITP. This classification of ITP is shown in Figure 21.1.
Conditions which are associated with secondary ITP are:

- Systemic lupus erythematosus
- Chronic lymphocytic leukemia
- Viral infections
- Drugs

Antibodies which appear in the serum of patients with ITP show specificity against one or the other platelet antigens. Of these, the most vividly expressed antigen system is the platelet A₁ (PLA₁) system of antigens. Antibodies in most cases of ITP show specificity against this system.

**Types of ITP**

Clinically ITP exists in two forms; acute ITP and chronic ITP. Although no age is immune, acute ITP is commonly encountered in children while chronic ITP usually afflicts adults. Acute ITP can become chronic ITP if thrombocytopenia remains resistant to appropriate treatment with steroids for six months. Chronic ITP on the other hand may arise de novo as low grade thrombocytopenia, in many cases it is the transformation of acute ITP into chronic ITP. Acute and chronic ITP differ from other in a number of ways as shown in table 21.1.

<table>
<thead>
<tr>
<th>Features</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Usually children</td>
<td>Usually adults</td>
</tr>
<tr>
<td>Sex</td>
<td>F = M</td>
<td>Female &gt; M</td>
</tr>
<tr>
<td>Platelet count</td>
<td>5 - 10 x 10³/µl</td>
<td>50 - 60 x 10³/µl</td>
</tr>
<tr>
<td>Preceding illness</td>
<td>+ +</td>
<td>±</td>
</tr>
<tr>
<td>Onset</td>
<td>dramatic</td>
<td>Insidious</td>
</tr>
<tr>
<td>Response treatment</td>
<td>excellent</td>
<td>Fair</td>
</tr>
<tr>
<td>Course</td>
<td>Self-limiting</td>
<td>Chronic, relapsing</td>
</tr>
</tbody>
</table>

**Table 21.1: Acute vs chronic ITP.**

**Clinical Features**

There is only one symptom of thrombocytopenia i.e. bleeding. This is almost invariably microvascular bleed either from the mucosal lining or in the skin. Mucosal bleeding may occur from any of the mucosal surfaces of the body, nasal, gingival, oral, endometrium, urethra and gastrointestinal tract. Some patients present with more dramatic and rather frightening subconjunctival bleeding. Hemorrhage in the deeper tissue can occur but are distinctly less
common than expected. A more serious bleeding is the intracranial bleed which in practice is fortunately less common than feared.

Clinical features of ITP are dictated by three factors:
- Causative factor if any
- Severity of thrombocytopenia
- Site of bleeding

In secondary immune thrombocytopenia which usually accompanies SLE & CLL, the clinical manifestations include features of the underlying disease. This may aggravate thrombocytopenia through other mechanisms including marrow suppression, marrow invasion and splenic pooling.

Laboratory Findings

Hematological

The cardinal abnormality lies in the hematological domain and is characterized by a variable degree of thrombocytopenia. It is not usual to encounter platelet count as low as 5,000/µl. Hemoglobin and white cell count are usually normal unless there is an associated infection or blood loss. In children there is some degree of lymphocytosis but in adults the differential as well as the total white cell count is normal.

In practice, it may be safely stated that the hemogram in uncomplicated ITP is usually normal except for the reduced platelet count. In chronic ITP with bleeding there may be an associated anemia of iron deficiency type. In this instance, red cells will be microcytic and hypochromic and serum iron profile will be reminiscent of iron deficiency.

Bone marrow

It is customary to perform bone marrow examination in ITP. Bone marrow aspirates from almost all cases of ITP show the following features;
- Cellularity is normal or it is slightly increased
- Megakaryocytes are increased in number
- Megakaryocytes are younger in appearance
- Surface of the megakaryocytes is smooth compared to that of normal megakaryocytes which have rough surface because of the cytoplasmic projections which represent the future platelets
- Megakaryocytes in ITP are smooth in outline, this at one time, was attributed to the non-production of the platelets.
- Eosinophils are increased in number
- Lymphoplasmacytic cells are also increased in many cases

Immunological

Detection of anti-platelet antibodies is rather difficult and in many cases is an unrewarding task. A number of immunological procedures have been devised; some of these include:
- Antiplatelet antibodies test
- Direct coomb’s test
- Indirect coomb’s test
None of these have given satisfactory result and they are all fraught with considerable difficulties. An inherent difficulty in performing immunological tests with patient’s platelets is the difficulty in obtaining enough platelets from these patients because in most cases of chronic ITP, the platelet count is low while in acute ITP the platelet count is extremely low. It is for this reason that in most cases of ITP indirect tests performed on are patient’s serum.

THROMBOCYTOSIS

Any increase beyond the upper limit of normal is called thrombocytosis. Platelet count up to 500X10³/µl is not considered to be significant and is usually not a manifestation of any overt thrombopoietic over activity in the bone marrow. Counts above 600X10³/µl are significantly increased and are encountered in a number of hematological and non-hematological disorders as shown in Figure 21.2.

**Figure 21.2: Causes of thrombocytosis**

![Thrombocytosis Diagram]

Platelet count over 800X10³/µl with morphological changes in the platelets in conjunction with mild polymorphonuclear leukocytosis is almost diagnostic of essential thrombocythemia which is a member of the myeloproliferative disorders.

Splenectomy is associated with thrombocytosis; the platelet count is usually around 500 X10³/µl. There are no significant morphological abnormalities of the platelets though some large platelets may be present. Intra-erythrocytic inclusions (Howell-Jolly bodies and iron granules) are commonly encountered.

Iron deficiency anemia is sometimes accompanied by mild thrombocytosis. Platelets, in this condition are usually small in size (microthrombocytes) and clumping of the platelets in the peripheral blood smear is uncommon.

Systemic malignancy is the commonest cause of reactive thrombocytosis. Almost 30% of the cases of thrombocytosis are due to systemic malignant disorders. It may be pointed out here that thrombocytosis in malignancy may develop irrespective of the bone marrow metastases.

Inflammatory bowel disease (ulcerative colitis and Crohn's disease) may be accompanied by some degree of thrombocytosis. Another characteristic hematological abnormality in these disorders is an increase in the basophil count in the peripheral blood.
Contrary to the common belief bleeding does not cause thrombocytopenia due to loss of platelets from the circulation. Hemorrhage is actually a potent stimulus for thrombopoiesis hence platelet count in hemorrhage is usually increased. Thrombocytosis in this scenario is however mild and transient and is associated with the appearance of large platelets in the peripheral blood.

**BERNARD-SOULIER SYNDROME**

Bernard-Soulier syndrome (BSS) is a rare autosomal recessive disorder characterized by prolonged bleeding time, thrombocytopenia and large platelets. It is associated with a defect of platelet glycoprotein IIb-IX-V complex.

GPIb-IX-V complex binds with vWF and thrombin. GPIb-IX-V also regulates the platelet shape and reactivity. Abnormality of GPIb-IX-V leads to the failure of attachment of platelets with the vWF and consequently to the exposed collagen. This leads to bleeding manifestations. Symptoms of the disease include gingival bleeds, epistaxis, purpura, and menorrhagia.

Laboratory findings show prolonged bleeding time and mild to moderately decreased platelet count. Clotting time remains normal. Peripheral smear shows large platelets. Bone marrow cytology shows normal megakaryocytes.

Platelet aggregation studies show normal response with ADP epinephrine and collagen. Aggregation with thrombin is reduced while ristocetin induced platelet aggregation is absent. Addition of normal plasma fails to correct the ristocetin induced platelet aggregation which differentiates Bernard-Soulier syndrome and von Willibrand disease. Flow cytometry for platelets receptors GPIb-IX-V confirms the diagnosis of Bernard-Soulier syndrome.

**GLANZMANN'S THROMBASTHENIA**

Glanzmann's thrombasthenia is a rare autosomal recessive disorder characterized by absent or deficient membrane glycoprotein IIb-IIIa (GPIIb-IIIa) complex, prolonged bleeding time and normal platelet count.

GPIIb-IIIa mediates aggregation of activated platelets by binding fibrinogen, vWf and fibronectin. Absence or defects of GPIIb-IIIa lead to the failure of clot retraction; a major event of platelet aggregation.

Bleeding manifestations are variable that ranges from minor bruising to fatal hemorrhages. Symptoms include bruising, epistaxis, spontaneous gingival bleeding, prolonged bleeding from minor cuts and menorrhagia.

Salient laboratory findings include normal platelet count, prolonged bleeding time, absent aggregation platelet with ADP collagen, thrombin and epinephrine. Clot retraction is absent.

**Suggested further reading**


HEMOPHILIA

Hemophilias are a group of coagulation factors deficiency characterized by hemorrhagic diathesis. Primary defect lies in qualitative or quantitative abnormalities in the coagulation factors. There are essentially three coagulation factors, the deficiency of which causes hemophilia. These are factor VIII, IX and XI. Based upon the deficient factor, hemophilias are named as shown in table 22.1.

<table>
<thead>
<tr>
<th>Deficient factor</th>
<th>Hemophilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII</td>
<td>A</td>
</tr>
<tr>
<td>IX</td>
<td>B</td>
</tr>
<tr>
<td>XI</td>
<td>C</td>
</tr>
</tbody>
</table>

Table 22.1: Types of hemophilia

Factor V deficiency was, in the past, known as Para hemophilia but this terminology has gone into oblivion in recent years.

Hemophilia A

Also known as classical hemophilia is the result of a congenital defect of factor VIII. The defect is almost always a quantitative reduction in the amount of factor VIII in the plasma.

Since coagulation factors are present in the plasma, all laboratory diagnostic procedures and therapeutic interventions are based on plasma and/or plasma fractions.

Inheritance

Hemophilia A is a genetic disorder caused by mutation of F8 gene present on X-chromosome at position 28 (Xq28ter). The disease is therefore a prototype of X-linked or sex linked disorders. Because of its gene location, hemophilia A manifests a characteristic inheritance pattern as illustrated in the following marital combinations.

- Normal female and a hemophilic male: In this mating all female children will be the carriers of this disease (heterozygous) and all male children shall be normal.
- Carrier female and a normal male: In this marriage, 50% of the girls shall be normal while the other 50% shall be the carriers of hemophilia. 50% of the boys shall have hemophilia A while the other 50% shall be normal.
- Carrier female and a hemophilic male: In this set up, 50% of the girls will be hemophilic while the other 50% shall be the carriers. This marriage shall not produce any normal female children. 50% of the boys shall be hemophilic while the other 50% shall be normal.
- Hemophilic female and hemophilic male: In this marital set up all children shall have hemophilia; no normal children will be born.
Review of the four pedigrees listed above indicates that situation III and IV shall result in the birth of female hemophiliacs, a disease which is traditionally considered to be a male-oriented disease.

**Clinical Features**

Hemophilia, being a hemorrhagic disorder is characterized by bleeding episodes. Clinical picture is determined by the site, severity and the complications of bleeding. Other factors which contribute towards its clinical features are the transfusion transmitted diseases.

**Clinical features due to bleeding**

Bleeding from the umbilical stump and after circumcision are the earliest pointers of the existence of hemorrhagic disorders in the new born. As the child starts to walk, trauma to the joints and soft tissues becomes a prominent cause of soft tissue and joint bleeding. Mucosal bleeding, though it does occur, is somewhat less common (cf. platelet defects). Gastrointestinal bleeding and genito-urinary bleeding is not infrequent. Intracranial bleeding is more common than in platelet disorders and is a terminal event in a number of hemophiliics. Recurrent hemarthroses cause bone resorption, fibrosis, ankylosis and pseudo-tumor formation, particularly in the iliac bones.

**Transfusion-related features**

Transfusion of whole blood and fresh frozen plasma were the order of the day in the early days in the management of hemophilia. Because of lack of awareness of transmission of AIDS and hepatitis viruses, majority of the hemophiliacs became infected with these viruses resulting in full blown clinical manifestation of AIDS, Hepatitis B and C. Subsequent clinical evolution culminated in hepatic cirrhosis and hepatocellular carcinoma.

Since hemophiliacs generally do not receive whole blood or non-plasmatic constituents, there is no risk of transfusion siderosis and cell-sensitization. Previously encountered volume over-load due to transfusion of plasma has been obviated by the use of cryoprecipitate and purified anti-hemophilic globulin (AHG). With the availability of human AHG, the previously encountered hypersensitivity reaction to bovine and porcine products has also been eliminated.

**Clinical severity of hemophilia A**

Hemophilia A, on the basis of clinical severity is classified as mild, moderate and severe subsets. Clinical symptomatology correlates well with the level of factor VIII in plasma as shown in table 22.2 and 22.3.

<table>
<thead>
<tr>
<th>Factor VIII (%)</th>
<th>Subset</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>Severe</td>
<td>Frequent spontaneous bleeding from early childhood</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crippling joints, deformities</td>
</tr>
<tr>
<td>1-5</td>
<td>Moderate</td>
<td>Post-traumatic bleeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Occasional spontaneous bleeding</td>
</tr>
<tr>
<td>5-20%</td>
<td>Mild</td>
<td>Post traumatic bleeding only</td>
</tr>
</tbody>
</table>

*Table 22.2: Clinical classification of hemophilia A*
Clinical features

<table>
<thead>
<tr>
<th>% Factor VIII</th>
<th>Severe</th>
<th>Moderate</th>
<th>Mild</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td></td>
<td>1-5</td>
<td>5-20</td>
</tr>
<tr>
<td>Spontaneous bleeding</td>
<td>+</td>
<td>±</td>
<td>_</td>
</tr>
<tr>
<td>Bleeding after minor trauma</td>
<td>++</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Bleeding after major trauma</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Joint deformities</td>
<td>++ +</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Beneficial effect of DDAVP</td>
<td>_</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 22.3: Clinical features and its association with the deficiency of factor VIII

Laboratory Findings

Being a plasmatic defect, there are usually no significant abnormalities in the blood counts or in the blood cell morphology. As expected the most significant abnormalities include:

- Prolonged APTT (activated partial thromboplastin time)
- Normal PT (prothrombin time)
- Normal T.T (thrombin time)
- Normal BT (bleeding time)
- Prolonged APTT corrected by the addition of adsorbed plasma but not by the aged serum
- Reduced factor VIII pro-coagulant activity as determined by immulogical assays

Carrier detection and antenatal diagnosis

Availability of PCR technology and refinement in chorion villous sampling has made it possible not only to establish the status of factor VIII but also to determine the severity of its deficiency in the fetus by the 12th week of gestation. Analysis of the fetal cord blood obtained from the umbilical vein at 16-20 weeks of gestation also helps in the antenatal diagnosis of hemophilia.

VON WILLEBRAND DISEASE (VWD)

vWD is an autosomal disease with variable expression. Gene for the synthesis of von Willebrand factor (vWF) is located on chromosome 12. The genetic defect may be gene deletion, point mutation or both mechanisms may be operative. vWF is a large protein with a molecular weight of 300 kDa. This protein quickly polymerizes to form macromolecules of sizes up to 1000 kDaltons. vWF has two functional sites; on one end it has a receptor for platelets which helps binds the platelets to the damaged endothelium; thus it acts as a bridge between these two hemostatic surfaces. At the other end of the molecule vWF binds also acts as a carrier protein for factor VIII. As it becomes bound with vWF factor, it circulates in plasma in a 'protected form' and is immune to premature and non specific degradation.

Clinical Features

Clinical manifestations of vWD are determined by a number of factors; some of these are:

- Quantitative reduction in vWF
- Qualitative abnormality in vWF
- Decreased levels of factor VIII
- Platelet count
Clinical manifestations are characterized primarily by bleeding episodes. Unlike hemophilia A and B, these episodes are mostly mucosal in origin. Deep seated hematomas and hemarthrosis are less frequent than in classical hemophilia A or B. Epistaxis, gum bleeding, metro-menorrhagia, purpuric rashes, bruises and post-partum hemorrhage are some of the common presenting features. Excessive bleeding at the onset of menarche may pose an alarming situation for the patient. Intracranial hemorrhage which is seen in 10% of the patients with classical hemophilia of (hemophilia A) and Christmas disease (hemophilia B) is not a feature of vWD.

**Laboratory Findings**
These are generally confined to hematological abnormalities. There are no biochemical or radiological abnormalities of any significance. Hematological abnormalities usually relate to:

- Complete blood counts
- Coagulation profile
- Platelet function studies

**Complete blood counts**
Because of the metro-menorrhagia, women tend to develop microcytic and hypochromic anemia due to iron deficiency.

In the event that iron deficiency due to excessive and repeated blood loss, the typical picture of microcytic and hypochromic red cells appear in the blood smear. Total and differential leukocyte count and platelet count are unremarkable. Bone marrow examination is not recommended in vWD.

**Coagulation profile**
The only abnormality in coagulation screen is prolonged APTT which is correctable with adsorbed plasma. Prolongation of APTT is in parallel with the quantitative and qualitative abnormalities of vWF. Bleeding time is almost invariably prolonged as a result of the disease and also sometimes due to associated thrombocytopenia.

Salient abnormalities which are commonly encountered in the coagulation screen are:

- Decreased factor VIII level
- Decreased vWF
- Prolonged APTT

**Platelet aggregation studies**
The only two conditions which have been associated with abnormal ristocetin response are the vWD and Bernard-Soulier’s disease which is extremely uncommon. Therefore in practice an abnormal platelet aggregation with ristocetin amounts to confirmation of vWD.

**Christmas Disease**
It was in 1952 that hemophilia B was first diagnosed as a discrete entity. Christmas disease or hemophilia B is clinically indistinguishable from classical hemophilia or hemophilia A. Both are sex-linked diseases and both are characterized by coagulation abnormalities which manifest as bleeding episodes characterized primarily by hemathroses, soft tissue hematoma and internal bleeding. Both are commonly encountered in males while females characteristically serve as carriers of this disease.
DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation (DIC), also known as defibrinated syndrome, consumption coagulopathy, is a hemorrhagic disorder characterized by the uncontrolled, inappropriate and widespread activation of platelets, coagulation proteins and fibrinolytic system. This activated triad of hemostatic system results in the simultaneous bleeding diathesis and microvascular thrombosis.

Etiology

The process of hemostatic system activation in DIC may be localized or diffuse depending on stimulus. The triggering stimuli may be tissue factor or cellular material (e.g. tumors, trauma, obstetrics accidents, bacterial endotoxins, antigen antibody complexes i.e. mismatch transfusion). Following are the causes of DIC according to its types:

Acute
- Obstetrical accidents; abruptio placentae, amniotic fluid embolism, abortion
- Surgery of the heart and lungs
- Hemolytic transfusion reaction
- Septicemia especially gram negative and meningococcal
- Pulmonary embolism
- Snake bite
- Hypersensitivity reaction
- Heart stroke

Chronic
- Disseminated or localized carcinoma
- Septicemia
- Acute leukemia
- Fetal death (in utero)
- Purpura fulminans
- Giant hemangioma

Pathophysiology

DIC may occur when the normal compensatory mechanisms of hemostasis have been overcome. The triggering mechanism includes:

- Activation of the extrinsic pathway of the coagulation cascade resulting in the release of excessive amount of tissue thromboplastin
- Activation of intrinsic pathway of coagulation
- Direct activation of factor X or prothrombin

Increased activation of platelets and coagulation proteins by triggering factor(s) results in the clot formation within the vessels. These aggregated platelets cause partial blockage of microcirculation. Fibrin-platelet plug acts as a fine sieve. When red cells pass through this mesh they are distorted and fragmented resulting in intravascular hemolysis. Simultaneously, fibrinolytic system is activated that result in the generation of free plasmin. Plasmin degrades circulatory fibrinogen as well as preformed cross-linked fibrin. The progressive degradation of cross-linked fibrin yields D, E, X, Y and D–dimers.

Raised levels of circulating fibrin complexes and fibrin degradation products (FDPs) inhibit the action of thrombin and also inhibit platelet function by nonspecifically binding to the
platelet membrane. Free plasmin also nonspecifically cleaves variety of peptide with arginine-lysine bonds that includes factor V and VIII and the first component of complement.

Combination of coagulation factors deficiency, thrombocytopenia, impaired platelet function and inhibitory action of raised FDPs causes generalized and continued widespread bleeding tendency.

**Clinical Features**
Disseminated intravascular coagulation may be clinically manifested as acute or chronic. Acute DIC present severe bleeding with sudden onset. Chronic DIC exists for long period of time and may have either mild or no clinical symptoms.

Patients with DIC may present with either bleeding or thrombosis. Bleeding is the most common clinical presentation. Bleeding may be localized or generalized. Localized may be prolonged bleeding from venipuncture sites, excessive bleeding at the site of operation, uterine bleeding at site of placental detachment.

Generalized bleeding manifestations include ecchymosis, hematomas, GIT bleeding and hematuria. Obstetrical and surgical cases may present with serious catastrophic bleeding in pregnancy. Abruptio placentae is the most common cause of bleeding due to DIC in pregnancy. Bleeding may be localized to the placental site initially, it may be concealed retroplacental hemorrhage which later becomes manifest as per vaginal bleeding.

![Pathological basis of clinical manifestations of DIC](image)

**Figure 21.3: Pathological basis of clinical manifestations of DIC**

Thrombosis obstructs the microvasculature and cause tissue anoxia, infarcts of the heart, kidney, brain and liver. Renal failure caused by small vessel occlusion with fibrin deposits may occur in postpartum or post-surgical patients and as a complication of septicaemia. Microangiopathic hemolytic anemia may occur with complicated disseminated carcinoma. Occasionally, venous or arterial thrombosis may occur.
**Laboratory Findings**

DIC cannot be diagnosed by a single laboratory test nor is there any set of test specific for DIC. A set of screening tests may help establish the diagnosis of DIC that include platelet count, PT, APTT, TT, FDPs and D-dimers. Following are the laboratory features of DIC;

- Platelet count (<100 x 10^3/µl) decreased
- Prothrombin time (PT) prolonged
- Prolonged activated partial thromboplastin time (APTT) prolonged
- Prolonged thrombin time (TT) prolonged
- Hypofibrinogenemia (fibrinogen level less than 1 g/L)
- FDPs levels Raised
- Levels of factor II, V and VIII
- D-dimer levels raised
- Plasminogen levels decreased
- Antithrombin-III levels decreased
- Peripheral blood counts may show normocytic and normochromic anemia with marked schistocytosis (fragmented cells).

**Suggested further reading**

MANAGEMENT AND TREATMENT OF HEMATOLOGICAL DISORDERS

Treatment of hematological disorders like that of other systemic diseases revolves around three domains as shown in the triangle in figure 23.1.

A clear understanding of this triangular approach shall make therapeutics logical, systematic and result oriented. Human machinery can react only in a limited way to any ‘mishaps’ in the body. It is therefore imperative to appreciate the body’s response and to suggest appropriate remedies to ameliorate the situation.

As shown in the triangle the therapeutic modalities can be trifurcated according to the objective of the treatment. This is described in some detail below as this constitutes the basis of therapy and provides a rational, firm and all inclusive approach to the treatment of any medical ailment.

General somatic symptoms

These are a group of clinical features that most often distressing to the patient and bring them to the care of medical profession. These are not necessarily system targeted. Some of the common non-specific general somatic symptoms at the time of initial hospital/ office visit are:

- Pain
- Headache
- General weakness
- Fever
- Anorexia
- Somnolence
- Insomnia
- Anorexia
- Nausea
- Vertigo
- Mouth ulcers
- Abdominal pain
- ‘Gasses’
Of these features, pain is the most common complaint that brings the patient to the doctor. A careful review of these symptoms shall testify that almost none of them is organ specific nor is there any specific therapy for them. All that is needed is some symptomatic care in the form of pain killers, antipyretics, sedatives and some multi vitamin preparation. In many cases these symptoms ameliorate and patients get better and no further treatment is required. Some household remedies and a visit to the family physician are usually ‘curative’.

**System oriented symptomatology**

This group of clinical symptomatology is the result of the involvement of organs and systems and is a product of some underlying disease process.

For the sake of clarity and brevity features due to the involvement of organs are listed in table 23.1. It may however be pointed out that this is not a water-tight situation as the involvement of one system is likely to affect other systems. In other words mono system diseases are likely to become multi organ/ multisystem disease.

From the foregoing it may be appreciated that whereas certain symptoms are more specifically organ oriented (jaundice in liver and blood diseases), hepatosplenomegaly and lymphadenopathy (RE system) and diarrhea and vomiting (GIT). There are large number of shared symptoms that are encountered in diseases of many organs (shortness of breath, ankle edema).

<table>
<thead>
<tr>
<th>Organ/ system</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastro intestinal</td>
<td>Anorexia, nausea, vomiting, abdominal pain, abdominal distention, diarrhea, constipation</td>
</tr>
<tr>
<td>Liver</td>
<td>Jaundice, fever, pain, nausea, vomiting, anorexia, abdominal distention, ascites, ankle edema</td>
</tr>
<tr>
<td>Blood</td>
<td>Bleeding, infections, weakness, palpitations, dyspnea</td>
</tr>
<tr>
<td>CVS</td>
<td>Dyspnea, ankle edema, chest pain, cyanosis, cough</td>
</tr>
<tr>
<td>Lungs</td>
<td>Cough, expectoration, chest pain, cyanosis, hemopty and dyspnea</td>
</tr>
<tr>
<td>Kidneys</td>
<td>Polyuria, oligouria, ankle edema, SOB</td>
</tr>
<tr>
<td>Musculo skeletal</td>
<td>Muscle pain, joint pain and swelling, redness, heat, weakness, fever</td>
</tr>
<tr>
<td>RES</td>
<td>Enlargement of lymph nodes, spleen and liver, fever, weight loss</td>
</tr>
<tr>
<td>ENT</td>
<td>Headaches, nasal stuffiness, sore throat, dysphagia, dyspnea and throat pain</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>Metromenorrhagia, abdominal pain, abdominal swelling</td>
</tr>
</tbody>
</table>

**Table 23.1: Systems and their disorders**

It is therefore suggested that before prescribing any treatment based upon the above symptomatology, it is imperative to make every effort to ascertain the exact cause and the anatomic localization of the disease. This can be assisted by certain basic laboratory tests. These include CBC, blood sugar, X ray chest, UCE and urine D/R. It must be re-emphasized that the laboratory tests are not to be done ‘as a battery of routine tests’. They should be based on rational judgment and after ascertaining the domain of the underlying disease.
Specific disease therapy

Once the diagnosis is established and the supportive care has been administered and the patients have improved symptomatically, definitive treatment may be instituted. Hematological disorders are more streamlined in terms of medical treatment.

In the hematopoietic system there are three cell lines. They may be involved one, two or all three of them. All clinical features of the diseases of hematopoietic system are due to involvement of these lines either singly, or in group of two or in some cases all three cell lines may be affected.

Hematological disorders that arise out of numerical aberrations in hematopoietic system are listed in table 23.2.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Aberration</th>
<th>Clinical disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>Increased</td>
<td>Polycythemia</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>Anemia</td>
</tr>
<tr>
<td>WBC</td>
<td>Increased</td>
<td>Leukocytosis</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>Leukopenia</td>
</tr>
<tr>
<td>Platelets</td>
<td>Increased</td>
<td>Thrombocytosis</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>Thrombocytopenia</td>
</tr>
</tbody>
</table>

Table 23.2: Numerical aberrations of blood cells
IRON DEFICIENCY ANEMIA

Iron deficiency anemia is the most common medical ailment that afflicts Man. It is estimated that nearly 50% of the world’s female population is depleted of iron stores. Of this, almost 20% have significant iron deficiency anemia; it is more prevalent in the developing countries for a number of reasons.

Iron is one of the micronutrients with a precarious state of homeostasis. This is caused by limited iron stores, obligatory iron losses from the body, tightly regulated iron absorption from the gut, poor intake of iron, hookworm infestation, increased physiological demands as in pregnancy, menstruation, lactation in females (vulnerable gender group) and increased demand during rapid growth in young children and adolescents (vulnerable age groups).

Pathogenesis

Conditions that predispose to latent or overt iron deficiency are;
- Increased demand
- Increased iron loss
- Malabsorption
- Iron utilization defects

Genesis of clinical spectrum of iron deficiency anemia

In a young healthy adult, there are sufficient stores to meet daily needs of iron. It is estimated that in the face of total ‘embargo’ on external supply of iron in any form, the hematological parameters in adult males can be sustained for a period of up to 6-8 years. Conditions associated with increased demand reduce this period.

Clinical Features

For the sake of simplicity and clarity, the clinical features of iron deficiency anemia are described under three headings:

Features due to low hemoglobin

These are the outcome of reduced oxygen supply to all organs of the body. Tissue hypoxia causes a wide range of non-specific symptoms. Since oxygen is the ‘fuel’ for all body functions, it is neither unexpected nor is it surprising that somatic symptoms are a common feature of anemia.

Features due to iron deficiency

Glossitis, angular stomatitis, pyroglossia (burning of tongue) dysphagia, koilonychia and PICA (desire to eat clay, wall paint, ice cubes, raw flour and uncooked rice) are common. These are most likely due to some metabolic changes at the cellular level. They all disappear as body’s iron stores are replenished.

Third set of clinical manifestations are a reflection of the causative disorder. These may include metromenorrhagia, black stools (peptic ulcer disease) hemorrhoids and other bleeding disorders.
It may be emphasized at this juncture that the clinical features of iron deficiency anemia blossom over many years; it is never an acute episode. The 'latent phase' is determined by the duration, the severity and the underlying cause of iron deficiency.

**Laboratory Findings**

Investigations of iron deficiency anemia can be pursued according to a triangular approach. Hematological parameters include CBC (particularly Hb, MCV, MCH, MCHC and RDW) and bone marrow examination (in most cases the role of bone marrow examination is of little importance in the diagnosis of iron deficiency anemia).

The only hematological investigation required for the documentation of anemia is the level of hemoglobin; no other information is required to document anemia.

Investigations to document the causative pathological process

These are dictated by the personal, social, nutritional and gestational history, occupation, melena, dyspepsia, analgesic abuse etc. A close differential diagnosis for iron deficiency anemia includes:

- Thalassemia minor
- Anemia of chronic disorders
- Lead intoxication

**Treatment**

The only treatment of iron deficiency anemia is to increase the intake of iron. This can be achieved by two means:-

- Increased intake of dietary items that are rich in iron e.g. apples, dates, liver, spinach and other natural food items
- Medicinal iron
  - Oral: Tablets, capsules, syrups and drops
  - Parenteral: Intramuscular and intravenous iron preparations; fractional dose infusion and total dose infusion

- Blood transfusion

**Oral iron therapy**

Over decades, the mainstay of treatment of iron deficiency anemia has been the oral iron supplementation. This is a safe, economical and effective therapeutic modality. It is available in various forms i.e. drops, syrups, tablets and capsules.

Until a few years ago, only the inorganic iron salts were available for oral administration. More recently slow release iron preparation and also sucrose based iron preparations have become available. A brief account of these preparations is given below:

- For children palatable iron drops and syrups are available. Their disadvantage is that they cause black discoloration of the teeth. This can be prevented by rinsing the mouth a few times with water after ingestion of liquid iron preparations. These preparations are bottled as pure iron preparations like Fer-in-sol or as part of multivitamin preparations like ViDaylin-M.
- Inorganic preparations contain iron in the ferrous state. This is because the absorption of inorganic ferric iron is considerably less.
To enhance the absorption of elemental iron, it is recommended to administer iron preparations on empty stomach to get the maximum benefit of the availability of gastric hydrochloric acid. Many clinicians prescribe 500 mg of vitamin C as an adjunct to oral iron therapy; this enhances iron absorption.

Measures that are sometimes suggested by some physicians to counter GI intolerance to oral iron are the use of antacids, H₂ receptor blockers and taking iron preparations with milk. A careful review of these measures suggests that these measures, though sometimes helpful in countering the adverse effects of iron therapy, are counterproductive to therapeutic response as they inhibit the absorption of iron.

Some of the time tested measures to overcome GI intolerance to iron are as follows:

- Change the medicinal preparation; some patients show preference to selective iron preparations; ferrous fumerate, ferrous sulphate, ferrous gluconate or ferrous succinate.
- Reduce the dose; a good way is to start with one tablet a day and then prescribe the full complement if this is well tolerated. Treatment may be continued with smaller doses over a longer period. After all, some treatment is better than no treatment.
- Another useful modification to oral iron therapy is to administer iron approximately 1½ hour after meals. This is the time when there is still some food in the stomach (to prevent gastric irritation) while gastric pH has started to fall (to facilitate iron absorption). This modification is well accepted by many patients and the therapeutic response is also not appreciably reduced.

In the absence of any complicating factors (persistent bleeding, malabsorption etc.) nutritional iron deficiency anemia when treated with appropriate amount of iron by mouth usually registers an increase in hemoglobin by 1½ – 2 g/dl after 3 weeks of therapy. This is considered to be a satisfactory response. It can also be monitored by the reticulocyte count after the initiation of therapy.

Before discontinuing oral iron therapy in favor of parenteral iron, it is important to ascertain the cause of therapeutic failure. Further investigations may un-earth some hitherto unsuspected cause of persistent anemia. Some of the causes of lack of response to oral iron therapy are non-compliance, inadequate dose, malabsorption, wrong diagnosis, continued blood loss and co-morbid illnesses.

In such cases further investigations are warranted to determine the cause of therapeutic failure.

**Parenteral iron therapy**

Iron can be administered orally as well as parenterally. Whatever be the route, an absolute indication for its use is iron deficiency state or iron deficiency anemia. The only other justification is its prophylactic use in pregnancy. This is because a vast majority of pregnant females have either iron deficiency state (no iron reserves) or iron deficiency anemia. Cost of each pregnancy to the mother in terms of iron loss is 350 mg. Another non sideropenic indication for iron therapy is anemia of rheumatoid arthritis and other chronic infections / degenerative disorders.

Whereas the preferred route of iron administration is oral, there are situations where oral therapy is not recommended and parenteral iron therapy is the only choice. Some of these conditions are:
Gastrointestinal intolerance to iron
Ulcerating lesions in the GI tract
Selective malabsorption of iron
Chronic hemoglobinurias
Intractable blood loss
Non-compliance

Oral iron administration and parenteral iron therapy have the following features in common:

- Erythropoietic response in the form of reticulocytosis starts on the 3rd day of therapy; it does not start any sooner with parenteral iron.
- Reticulocytosis reaches its peak at about the 10th day and the maximum reticulocyte response is also similar as is the rate of rise in hemoglobin. This implies that hematological response to parenteral iron is neither accelerated nor it is enhanced. This is contrary to the general belief that parenteral preparations are therapeutically superior to oral medication. The only edge that parenteral iron therapy has over oral administration is the certainty of iron replacement in the desired amount and ensured replenishing of body's iron stores.

Since parenteral administration of iron may sometimes produce serious adverse reactions i.e. anaphylaxis, it is mandatory that the first injection be preceded by test dose. It should be given by the same route as the subsequent rout of therapy i.e. intramuscular for intramuscular injection and intravenous for intravenous infusion. It must not be given as subcutaneous or intradermal injection as this may cause unsightly staining and pain at the site of the test dose. It may at times cause sloughing and necrosis of the tissues. It is also prudent to give the first injection in a hospital setting to be able to face any eventuality.

Once the test dose is safely administered, subsequent iron therapy can be given without any risk of serious ill effects. Test dose is not required for subsequent iron treatment.

**Intramuscular iron therapy**

The only preparation available at present for intramuscular use is jectosol/jectosol plus. It is supplied in dark colored ampoules. Salient features of jectosol plus are:

- **Composition**
  - Iron sorbitol citric acid : 75 mg
  - Vitamin B₁₂ : 75 μg
  - Folic acid : 750 μg
  - Volume : 1.5 ml
- **Amount of iron/ml** : 50 mg
- **Amount of iron/ampoule** : 75 mg
- **Site of injection** : Intragluteal

**Intramuscular iron therapy; technique for the test dose**

A proper way to give intramuscular test dose is as follows:
- Take a 1 ml syringe and draw 0.1 ml of jectosol solution in it.
- Replace its needle with a longer needle from a 10 ml syringe.
- Replace its needle with a longer needle from a 10 ml syringe.
- Clean the upper and outer quadrant of the gluteal region.
- Draw the skin to one side, keep it drawn and inject the test dose into the chosen site. Remove the needle and release the stretch.
• Wait for 10-15 minutes and watch for any untoward reactions. Serious adverse effects are unusual; however a rare case may cause scare because of cardiovascular collapse.

• Take another syringe with a long needle and inject the rest of the iron solution in the other gluteal region if no adverse reactions are observed with the test dose.

Total dose of jectosol to be given is determined by the body weight, the age and the sex of the patient and the level of hemoglobin at the time of presentation. Recommended dose is 1.5 mg of iron/Kg body weight to a maximum of 100 mg of iron/ injection.

In practice, 1 ampoule of jectosol plus (75 mg of elemental iron) is well tolerated when given twice a week. Total dose to be administered is shown in Table 24.1. It is based on the assumption that 180 mg of iron increases the hemoglobin by 1 g/dl in an adult male of 60 kg provided that there is no ongoing blood loss.

<table>
<thead>
<tr>
<th>Initial Hb g/dl</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of injections</td>
<td>24</td>
<td>22</td>
<td>20</td>
<td>17</td>
<td>14</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Amount of iron in mg</td>
<td>1800</td>
<td>1650</td>
<td>1500</td>
<td>1275</td>
<td>1050</td>
<td>900</td>
<td>750</td>
</tr>
<tr>
<td>180 mg of elemental iron = 1 g Hb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 24.1: Total dose of jectosol plus in a 60 kg adult male; target hemoglobin being 15 g/dl

Intravenous iron therapy

Intravenous preparations are marketed as 5 ml glass ampoules containing iron. The brown colored solution contains 20 mg of iron-sucrose solution. Each ampoule thus contains 100 mg of elemental iron.

Intravenous iron therapy; technique:

• With a medium size cannula give a clean venous prick.
• Connect the cannula with a bottle containing 100 ml of normal saline; make sure that there is no swelling at the venipuncture site.
• Add 2 ampoules of intravenous iron preparation to the saline bottle and mix gently. Do not add iron solution to the saline bottle before ensuring that saline drip is running well and there is no swelling at the venipuncture site.
• Run the diluted iron solution fast so as to fill the tubing of the infusion set with the light brown solution of intravenous iron preparation.
• As the tubing of the infusion set is filled with the iron solution, slow down the rate of infusion to 10 drops per minute as the test dose. Maintain this rate of infusion for 15 minutes and watch for any untoward reactions.
• If no adverse reaction is noted, the rest of the solution is infused at 30-40 drops per minute so as to finish the infusion in 60-90 minutes. Do not give test dose subcutaneously or intramuscularly.

This procedure applies to the very first infusion of intravenous iron in a patient who had never received intravenous iron before. First infusion should preferably be given in a hospital.
setting. All subsequent injections and all subsequent courses of treatment may be given in an office setting.

For the very first injection of intravenous iron only one ampoule should be added to 50 ml of normal saline so as to prepare an infusate which contains 2 mg of the intravenous iron preparation per ml. This is to reduce the expense for the test dose if the patient develops untoward reactions and the test dose infusion has to be discarded. For all subsequent infusions two injections of intravenous iron preparation can be added to 100 ml normal saline and infused over one hour period.

A new intravenous sucrose based preparation has been marketed for total dose infusion; it has not yet achieved any significant acceptance.

It is recommended that more than two ampoules of intravenous iron preparation should not be infused in one session. Total dose infusion of intravenous iron is not recommended by the manufacturer.

Intravenous administration of iron in children below 3 years of age is not recommended. For older children the dose is to be calculated according to the body weight, level of hemoglobin at the time of presentation and the target hemoglobin.
MACROCYTIC ANEMIAS

Macrocytic anemias are a group of anaemias characterized by the presence of larger than normal red cells in the peripheral blood. Size of the red cells expressed as mean cell volume (MCV) is usually greater than 100fl (normal 80-96 fl; one femtoliter = 10^{-15}L). These are the least common of the three morphological subtypes of anemias and account for 3-5% of all anemias. Taking into consideration the shape of the macrocytes in the peripheral blood, these are sub classified as round macrocytes and oval macrocytes.

Round macrocytes represent a nonspecific change in the size and shape of the red cells and are seen in disorders like hypothyroidism, liver disease, lipid abnormalities and in all those conditions characterized by high reticulocyte count. Oval macrocytes on the other hand are a highly specific and diagnostic group of mature red cells in the peripheral blood. They are invariably associated with some degree of anemia which is called oval macrocytic anemias. This morphological appearance in the peripheral blood is reflected in the form of abnormal red cells precursors called megaloblasts (normal red cell precursors are called normoblasts). The subsequent presentation is restricted to oval macrocytic anemias; round macrocytes shall be mentioned any further.

Etiology

Oval macrocytes, as mentioned above, are the product of an abnormal erythropoiesis (megaloblastic erythropoiesis) which results from deficiency of vitamin B_{12} (cobalam) and/or folic acid. These are the essential nutrients for the synthesis of DNA in all cells of the body; these are collectively called maturation principles.

Causes of cobalamin deficiency

- Nutritional; this is an uncommon cause of cobalamin deficiency; it is encountered almost exclusively in vegans (strict vegetarians)
- Gastro-duodenal: Gastrectomy, atrophic gastritis, Pernicious anemia, fish tape worm infestation
- Intestinal: Vitamin B_{12} is specifically absorbed in the terminal two feet of ileum which has receptors for intrinsic factor. Hence all those conditions characterized by ileo-cecal disease (tuberculosis, Chron’s disease, intestinal lymphoma, amyloidosis and ileal resection are commonly associated with malabsorption of vitamin B_{12} with consequent oval macrocytic anemias. Gastro-colic fistulae are also associated with oval macrocytic anemia as a result of bypassing the site of absorption. Some of the congenital disorders like transcobalamin II deficiency, absence of intrinsic factor and intrinsic factor receptors deficiency are also associated with vitamin B_{12} deficiency and oval macrocytic anemia.

Causes of folic acid deficiency

Folic acid is present in the green leafy vegetables, liver and kidneys (the only two animal sources). Causes of folic acid deficiency are:

- Inadequate dietary intake
- Intestinal malabsorption
- Increased demand: Pregnancy, lactation, rapid growth, hemolytic anemias, myeloproliferative disorders, malignant disorders, chronic diseases

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Liver disease
Drugs, anticoagulants, salazopyrin

Clinical Findings

There are four systems that are characteristically and most severely affected in cobalamin/folic acid deficiency; these are hematopoietic, gastrointestinal, neurological and gonadal systems. It may be pointed out that these are the systems in which DNA synthesis is very active and cellular turnover is very high. Clinical manifestations are a result of anemia (tissue hypoxia) and thrombocytopenia (bleeding tendency). Leukopenia is usually of mild degree and propensity to develop common infections is not common. An important feature of B12 deficiency (particularly seen in pernicious anemia) is the damage to the spinal cord tract (postero lateral sclerosis or s/ac combined degeneration of the cord) and an organic brain syndrome called megaloblastic mania.

Whereas all bodily systems are affected, gastro intestine tract, including oral mucosal skin, gonads and bone marrow are mostly severely affected. This is because adequate supply of vitamin B12 and folic acid is essential for normal maturation of DNA. Organs listed above are all characterized by rapid DNA turnover and high mitotic rate. It is for these reasons that these organs are most vulnerable to the deficiency of folic acid and B12.

Symptoms of B12 deficiency anemias

- Those due to anemia and thrombocytopenia
- Gastrointestinal abnormalities, glossitis, angular stomatitis
- Neurological defects
  - Postero-lateral sclerosis also known as sub-acute combined degeneration of the spinal cord
  - Neural tube defects (anecephaly, spina bifida and encephalocele) in folate deficiency

Laboratory Findings

Laboratory manifestations of vitamin B12 deficiency are listed below;

Hematological

- Anemia, thrombocytopenia, hypersegmented neutrophils. Hypersegmentation of neutrophils is defined as more than 5% of the neutrophils containing more than 5 lobes in the peripheral blood smear
- Megaloblastic erythropoiesis, giant bands and giant metamyelocytes in the bone marrow
- Increased LDH, increased serum bilirubin, decreased serum uric acid

Treatment of cobalamin deficiency with or without anemia

Most common causes of cobalamin deficiency are gastrointestinal conditions leading to its malabsorption. Nutritional deficiency, increased demand for vitamin B12 and increased loss from the body uncommonly produce significant cobalamin deficiency to result in clinically manifest anemia. Because of the absorption defects, oral cobalamin therapy is ineffective (except when administered in mega doses). The only scientific way to treat cobalamin deficiency is the parenteral administration to side track any absorption defects and to ensure that appropriate amount of vitamin B12 has been administered.
There are many complex protocols for the administration of vitamin B₁₂; these are probably unnecessary. The important point is to give enough of vitamin B₁₂ over a prolonged period (perhaps for life).

Once daily intravenous injections for the first two weeks and then 1 ampoule every week for eight weeks is a simple, effective and practical therapeutic regime. Maintenance therapy of 500µg at cobalamin once a month may be given for life. Since vitamin B₁₂ is water soluble, it cannot be over stored. Any excess B₁₂ is promptly excreted in the urine.

One important point needs be emphasized at this juncture. Cobalamin is only indicated for the treatment of those disorders in which cobalamin deficiency has been documented. Unfortunately many practicing physicians prescribe it for such vague symptoms as general weakness, malaise, tingling, numbness, paraesthesias and also as a general tonic. This practice should be discouraged as it is neither scientific nor is it likely to benefit the patients except for some psychological support. Whereas there are no risks to indiscriminate use of parenteral cobalamin and the cost is also not prohibitive, there is however a real risk of transmitting hepatitis through the use of contaminated syringes.

Hematological response starts on the 3rd or the 4th day when reticulocytes start to appear in the peripheral blood. Reticulocyte response reaches its plateau by the 10th day. This is usually maintained till the peripheral blood hemoglobin reaches normal level which takes about 3-4 weeks after the start of treatment.

**Treatment of folate deficiency**

There are a number of hematological and non-hematological disorders that produce folic acid deficiency anemia. Folic acid (as opposed to cobalamin therapy) is almost always given orally. This mode of therapy is therapeutically effective where indications exist. Parenteral folic acid is used less commonly and that too under highly specialized circumstances. Folic acid preparations used for parenteral propose is folinic acid. In this substituted derivative of folic acid, a formyl radical is added to the 5th nitrogen atom in the molecule of folic acid. Rationale behind the use of folinic acid is that malignant cells are unable to utilize it for the purpose of DNA synthesis while normal cells can use it to synthesize normal DNA. There is only one specific indication for the use of folinic acid i.e. after high dose methotrextate therapy for leukemias and lymphomas. It is given orally or intravenously starting 18 hours after methotrexate infusions usually eight doses are given six hours apart in a dose of 10 mg every 6 hour X 8 doses.

Lately an oral form of folinic acid has been marketed. Whatever be its rationale and therapeutics benefits, it certainly has increased the cost of folate supplementation. Considering the physiology of folic acid and it’s interconversion in the body, it seems unlikely that oral folinic acid has any edge over the traditional folic acid tablets.
ACUTE MYELOID LEUKEMIA

What classifies acute leukemia as acute is the presence of more than 20% blast cells in the bone marrow. This is the gold standard for the diagnosis of acute leukemia; without fulfilling this criterion, the diagnosis is untenable. All other parameters by themselves are weak criteria but in the presence of infiltration of the bone marrow by more than 20% of blasts cells, these weak criteria provide firm basis for the diagnosis. The tetrad shown in the square constitutes an irrefutable basis for the diagnosis of acute leukemia.

Based on the type of blasts in the peripheral blood/marrow, acute leukemias are dicotomized into two major groups;

- Acute myeloid leukemia
- Acute lymphoblastic leukemia

This division is important therapeutically and prognostically as drugs that are used in acute myeloid leukemia are completely different from those used for acute lymphoblastic leukemia. Therapeutic strategies are also polar apart while the clinical presentation and disease progression have their characteristic patterns. Diagnostic avenues that help in making this distinction are;

- Morphology of the blast cells
- Immunophenotyping
- Cytogenetics
- Molecular analysis

Clinical Features

Clinical features of acute myeloid leukemia are generally stereotyped, their genesis is explained on the pathophysiology illustrated in figure 26.2.
Laboratory Findings

- Complete blood counts
- Marrow aspiration
- Marrow biopsy
- Cytochemistry of blast cells
- Flow cytometry for immunological markers
- Molecular genetics
- Cytogenetics
- Serum uric acid
- LDH

Treatment

Definitive (disease oriented) treatment must be preceded by symptomatic and supportive care. Particular attention should be paid to general somatic features i.e. fever, dehydration, body aches, infections, mouth ulcers, joint pains, anorexia, insomnia, headache and bleeding tendencies.

Once the patient becomes asymptomatic, chemotherapy is given according to the plan dictated by the objectives of chemotherapy i.e. palliative/curative. Drugs that have established their role in the treatment of AML are:

- Cytosine arabinoside
- Daunorubicin
- Idarubicin
- Mitoxantrone
- Etoposide

These drugs are administered in various combinations and dosage schedules. This domain is outside the scope of this book and also beyond the realms of the readers. These highly specialized aspects are best left to the experts who are well versed with chemotherapy of AML.
Course and prognosis

Up until a decade ago, the outcome of AML was uniformly disappointing particularly in the elderly (it has still not improved to any satisfactory level). In the younger age (3rd and 4th decade), the prognosis has improved and those with good cytogenetic markers and stem cell transplantation can now expect long term survival.

Prognostic markers in AML include the age of the patient, white cell count (blast cell count) at the time of presentation and genetic markers, response to initial induction therapy and minimum residual disease.

A commonly practiced and internationally recommended treatment plan for younger patients (30-50 years of age) is as follows:

Induction phase

Drugs that have shown for induction of remission efficacy are:

- Cytosine arabinoside
- Daunorubicin
- Thioguanine or etoposide

Successful remission induction is followed by the first consolidation phase using the same drugs (schedule and dosage) according to the protocol to be followed.

Consolidation phase II

At the end of the completion of the first consolidation phase while the patient is still in remission, the second consolidation phase drugs are administered. These include m-Amsa, etoposide and cytosine arabinoside.

Second consolidation phase is aimed at gaining additional ground in the battle against this disease.

Completion of the second successful consolidation phase is followed by autologous/ allogeneic stem cell transplantation if a genetically compatible donor is available. In the event that this were not possible, the treatment aimed at further consolidation is continued using mitoxantrone, idarubicin, cytosine arabinoside and anti CD33 antibodies. Despite all this arduous efforts, the prognosis is still guarded; overall 5 years disease free survival in still extremely unsatisfactory. This is particularly so for patients who are over 70 years of age; only 10% of these patients can expect long term remission (not cure).
ACUTE LYMPHOBLASTIC LEUKEMIA

The title embodies three features;

- biological behavior of this disease is characterized by the triad; rapid onset, rapid progression and rapid demise (if left untreated)
- the proliferating cells in the blood and marrow are lymphoblasts
- it is a clonal disease

It is most commonly encountered in young children between the ages of 2-6 years. Also it is the most common malignant disorder in children. The disease is also not uncommon past the age of 40 years. There is no sex predisposition in ALL of B cell type while T cell ALL is more commonly seen in males.

Clinical features

Pathogenesis of the clinicopathological features in ALL are summarized in the pentagon in figure 27.1.

![Figure 27.1: Clinical manifestations of ALL](image)

*Meningeal syndrome; headache, nausea, vomiting, blurring of vision, diplopia,

Bone marrow failure

![Figure 27.2: Basis of laboratory manifestations of ALL](image)
Laboratory Features
Hematological

- Anemia
- Thrombocytopenia
- Leukocytosis (neutropenia)
- Blast cells

Bone marrow
> 20% blast cells

Biochemical
- Increased uric acid
- Increased LDH
- Increased serum calcium

Cytochemistry

Cytogenetics
Flow cytometry
Radiological; chest X ray and CT chest

Treatment
Before specific therapy is given, it is imperative to improve the general condition of the patient, treat the infection, attend to oral hygiene and alleviate bone pains (dactylitis).
Chemotherapeutic drugs for the treatment of ALL

A large number of drugs are administered according to highly complex protocols using a wide range of drugs. The four well established phases of treatment of ALL are;

- Induction phase
- Consolidation phase
- Cranial radiation with or without intrathecal methotrexate
- Maintenance therapy
- Late intensification
- Maintenance therapy

Drugs that are used for comprehensive treatment of ALL (with the intent of cure) are administered in the form of various protocols comprised of a number of drugs and administered according to predesigned schema.
Some of the commonly used drugs for the treatment of ALL are listed below;

- Vincristine
- L-asparaginase
- Dexamethasone
- Doxorubicin
- Cytosine arabinoside
- Thioguanine
- Mercaptopurine
- Cyclophosphamide
- Methotrexate

Stem cell transplantation is sometimes carried out in certain selective cases.
It may be pointed out that some of these drugs used in ALL are also useful for the treatment of AML. Poor prognostic indicators of ALL are:

- Age less than 1 year at the time of presentation
- High initial leukocyte count
- High initial blast cell count
- Male children
- Slow initial response
- Persistence of MRD (minimal residual disease)
- T cell lineage
- CNS disease at presentation
- Philadelphia chromosome positivity
- Hypodiploidy (total number of chromosome less than 46; usually 44)

The list is by no means complete. Other variables at the time of presentation and during the course of the treatment may affect the prognosis.

Almost 25% of the children relapse after initial apparent complete cure. With additional treatment a significant number of these children achieve long term cure. Almost 75% of the children below the age of six with good prognostic markers are cured of the disease.
CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia is a clonal (malignant) disorder of pluripotent hematopoietic stem cell and their precursors. The disease starts in the bone marrow, remains dormant for sometimes and then breaks through into peripheral blood with subsequent involvement of other organ system including the liver and the spleen.

Clinical Features

The disease starts in the bone marrow and after a variable period, usually 2 years, it appears in the peripheral blood. This period is called the latent phase of CML. During this period it is not possible to diagnose, predict or even suspect CML. Once it appears in the blood, the diagnosis of the disease becomes easy.

Clinical manifestations are usually well recognized and well appreciated, these are summarized in the hexagon in figure 28.1.

It may be appreciated that in early years of the disease there usually are no symptoms or signs and the diagnosis in some cases is purely incidental as a result of complete blood examination for insurance and visa purpose or as a part of the annual physical check-up.

Uncontrolled myeloproliferation produce a wide variety of laboratory abnormalities as shown in figure 28.2.

Biological behavior of chronic myeloid leukemia

Chronic myeloid leukemia faithfully passes through 4 phases;

- Latent phase
- Chronic phase
- Accelerated phase
- Blast transformation

Latent phase is undetectable by any available modern diagnostic tools but its existence cannot be denied. It is estimated that this phase lasts for 18-24 months.

If untreated, the chronic phase lasts for 24-36 months with considerable mortality. Accelerated phase, if untreated extends over a limited period of few months and evolves into blast transformation. Blast transformation, which is the terminal phase, usually leads to the death of the patient in few weeks.
Treatment

CML is a malignant disease; left untreated it is fatal within a period of 1-3 years. Availability of effective treatment promises cure in a large number of cases. Drugs that have proven efficacy in this disease are:

- Conventional chemotherapy
  - Busulphan
  - Hydroxyurea
  - α-interferon
- Tyrosine kinase inhibitors
  - Imatinib (Glivec)
  - Dasatinib
  - Nilotinib
- Stem cell transplantation

Conventional chemotherapy has the advantage of symptomatic improvement by controlling myeloid hyperproliferation in the bone marrow. It is also relatively inexpensive and affordable. None of these drugs promise cure nor is there any effect on Philadelphia chromosome positivity nor is there reversion of BCR-ABL gene translocation.

Tyrosine kinase inhibitors have the following advantages over the conventional chemotherapy:

- Produce symptomatic improvement
- Cause prolongation of disease-free survival
- Eliminate Ph chromosome (not in all patients)
- Eliminate the genetically mutated clone (not in all patients)

As mentioned above, diagnosis of CML makes it imperative to start chemotherapy in one form or the other with the object of symptomatic improvement and control of myeloproliferation leading to permanent cure.

Whereas the available drugs are well known and their adverse effects and benefits are also defined, comprehensive care of patients with CML does not stop at administering one or the
other drug. Like the treatment of other malignant disorders, treatment of CML is also an art with long term planning aiming ultimately at cure of the disease. This task cannot be entrusted to the novice. It is therefore best to transfer the care of these patients to the centres with expertise, experience and facilities to offer the patients the best possible help that the medical profession can offer.
CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia, as conventionally understood, is a neoplasm of B lymphocytes (there is also a T cell CLL and a large granular cell CLL). It is a member of a much larger group of chronic lymphoid leukemias; the ensuring description is confined to CLL of B cell type.

This is a disease of the elderly, most of the cases are encountered in the 5th to the 7th decade with a male to female ratio of 2:1. The disease is characterized by persistent lymphocytosis in the peripheral blood and bone marrow with enlargement of lymphoid organs i.e. spleen, lymph nodes and other aggregates of lymphoid cells, Waldeyer’s ring, Peyer’s patches and skin.

For the diagnosis to be established, it is important that monoclonal B cells in the peripheral blood should be greater than 5000/µl.

Clinical Features

Clinical manifestations of CLL are dictated by two important features;

- Accumulation of lymphocytes in the blood and lymphoid organs
- Hypogammaglobulinemia as a result of impaired immunoglobulin synthesis

Commonly encountered clinical spectrum of CLL is illustrated in the hexagon in figure 29.1.

Laboratory Findings

Laboratory manifestations of CLL are usually precise, discrete and easily documented by simple, inexpensive and routine laboratory procedures. These are summarized in the hexagon in figure 29.2.
Treatment

These patients who are symptomatic with clinically manifest disease need treatment. Besides the usual symptomatic and supportive care, these patients are candidates for chemotherapy which may be administrated with the intent of symptomatic and hematological relief or it may be given with object of cure. As mentioned in the section on treatment of CML chemotherapy is the domain of those who are well versed with this mode of its demonstration. Also it should be offered in the centres with adequate support facilities.

Drugs that are available for the treatment of B cell CLL are listed below;

- Chlorambucil
- Corticosteroids
- Cyclophosphamide
- Bendamustine
- Fludarabine
- Lenlidomide
- Rituximab (anti CD20)

These drugs are usually administered in combinations in the form protocols; their mention is beyond the scope of this presentation.

Chlorambucil with or without corticosteroids provides safe, effective ad affordable treatment which however is non-curative therapeutic modality.

Some of the drugs listed above are highly expensive, have serious adverse effects and need professional expertise beyond the realms of the family physician and the general internists. Other therapeutic modalities and supportive care that may be required in individual patient includes;

- Immunoglobulin therapy
- Radiotherapy
- Splenectomy
- Stem cell transplantation

**Biological behavior of CLL**

B cell CLL is usually an indolent disease. Many a times, the diagnosis is made on routine examination of peripheral blood. Incidentally diagnosed CLL usually follows a slowly progressive course or produces minimum symptoms for many months or years. These patients need no treatment, only periodic follow up of peripheral blood counts (every 1 to 3 months) is all that is required. There are a few clinicopathological features that suggest a more aggressive behavior and increased incidence of complications. These are illustrated in the pentagon in figure 29.3.

![Figure 29.3: Clinicopathological features of CLL](image-url)
In addition there are certain technically advanced immunological, cytogenetic and cytokinetic markers that herald poor prognosis. These are listed in the pentagon in figure 29.4.

Figure 29.4: Prognostic factors in CLL

Many patients usually follow an asymptomatic though relentlessly progressive downhill course. These patients usually have favorable indicators (Binet stage A, Rai stage 0-1, females, nodular lymphoid infiltration in the bone marrow and normal LDH) at the time of diagnosis. Rai classification and Binet staging are listed in table 29.1 and 29.2.

Rai system of staging of CLL

This is a clinicopathological system of staging which takes into account two hematological parameters i.e. hemoglobin and platelet count in the peripheral blood and organomegaly. According to this staging system, there are 4 stages of CLL which are important in the prognosis of the disease. These are listed in table 29.1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Absolute lymphocyte count in the peripheral blood</th>
<th>Enlarged lymph nodes</th>
<th>Enlarged liver and/or spleen</th>
<th>Hb &lt;10g/dl</th>
<th>Platelet count &lt;100,000/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;15,000/µl</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>&gt;15,000/µl</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>&gt;15,000/µl</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>III</td>
<td>&gt;15,000/µl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IV</td>
<td>&gt;15,000/µl</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 29.1: Rai staging of CLL
<table>
<thead>
<tr>
<th>Stage</th>
<th>Organ enlargement</th>
<th>Hemoglobin</th>
<th>Platelet count</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0,1 or 2 areas</td>
<td>Not considered</td>
<td>Not considered</td>
</tr>
<tr>
<td>B</td>
<td>3,4, or 5 areas</td>
<td>&gt;10g/dl</td>
<td>≥100,000/µl</td>
</tr>
<tr>
<td>C</td>
<td>Not considered</td>
<td>&lt;10g/dl</td>
<td>&lt;100,000/µl</td>
</tr>
</tbody>
</table>

Areas: Superficial nodes > 1cm, spleen and liver

**Table 29.2: Binet classification (International working Party Classification)**

**Complications of CLL**
- Recurrent bacterial infections
- Viral illness; herpes zoster
- Auto immune hemolytic anemia (10%)
- Auto immune thrombocytopenia (10%)
- Aggressive malignant lymphoma (Richter’s lymphoma)
MULTIPLE MYELOMA

Multiple myeloma is a member of plasma cell dyscrasias. It is a clonal disorder characterized by relentless proliferation of plasma cell and excessive production of immunoglobulins.

Clinical Features

There are three complications that pose threat to the life of the patients with multiple myeloma. These are hypercalcemic crises, coma paraproteinemia and spinal cord compression. Every physician must be aware of their clinical features and must have sufficient expertise in their management. Whereas timely institution of appropriate treatment can save the life of these patients while inappropriate or delayed treatment can kill the patient.

Hypercalcemic crisis

Hypercalcemic crisis are characterized by mental confusion, dehydration, polyuria, polydypsia, hypotension and marked muscular weakness. Laboratory features include hypercalcemia (>12 mg/dl), hypophosphatemia, increased blood urea and creatinine, hyperkalemia with hyponatremia.

Many drugs are available that can lower serum calcium level rapidly. The most important, highly effective, least expensive and readily available is saline infusion, intravenous Lasix and intravenous corticosteroids. Up to 5 liter of normal saline with I/V lasix to correct dehydration and lower serum calcium and correct hyponatremia improve the clinical condition and laboratory parameters. Bisphosphonate in the form of Pamidronate (Aredia) if available is an extremely effective and important adjunctive therapy. 60 mg of Pamidronate in 500 ml of normal saline can be given in 4 hours. It lowers serum calcium rapidly by removing calcium from the blood and depositing it in the bones. With effective saline diuresis and Pamidronate infusion serum calcium falls to safe level (<10mg/dl) in less than 12 hours.

A word of caution is in order at this stage. Since multiple myeloma is a disease that is mostly encountered in patients over 50-60 years of age, many male patients may have enlarged prostate, this may be a bar to effective saline diuresis. It is therefore advisable to pass and retain Foley's catheter in these patients to ensure unobstructed urinary flow.

Hyperviscosity syndrome

Hyperviscosity syndrome is the other life threatening situation that patients with multiple myeloma are afflicted with (10% of all cases). It is seen in patients who have IgA myeloma and also in patients who produce IgG3 immunoglobulins. Other conditions in which hyperviscosity develops are Waldenstrom's macroglobulinemia and cryoglobulinemia. This syndrome usually arises when plasma viscosity exceeds 4 units (normal plasma viscosity is 1.8 with respect to the viscosity of water which is considered as the reference standard with a value of 1). Clinical severity due to hyperviscosity usually parallels the plasma viscosity.

Clinical manifestations range from mild headache, vertigo and blurring of vision to full-fledged coma. Prompt plasmapheresis or plasma exchange are life saving measures. If untreated the patients may die in a comatose state (coma paraproteinemia).
Compression of spinal cord
Third life threatening complication of plasma cell myeloma is the spinal cord compression. Compression in the dorsal spine produces paraplegia and disturbances of the organic reflexes. Compression collapse of cervical vertebrae is more serious and causes quadriplegia, abnormalities of organic reflexes and a serious threat to cardiopulmonary centers. Immediate intravenous methyl prednisolone and radiotherapy to the spine and emergency surgical intervention for decompression of the cord is mandatory. Treatment given with utmost urgency may save the life and disability of the patient. Basis of clinicopathological manifestations of multiple myeloma are shown in figure 30.1.

![Figure 30.1: Clinical manifestations of multiple myeloma](image)

Laboratory Findings
Basis of laboratory features in multiple myeloma are summarized in figure 30.2.

![Figure 30.2: Clinicopathological basis of laboratory manifestations in multiple myeloma](image)

Hematological
- CBC: normocytic, normochromic anemia
- ESR: markedly increased
- Bone marrow aspiration: > 20% monoclonal plasma cells
- Bone marrow trephine biopsy
Immunological
- Increased free light chains in the serum
- Decreased level of normal serum immunoglobulin
- Immunoglobulin quantitation and immunofixation for paraproteins

Biochemical
- Increased serum urea and creatinine
- Increased serum uric acid
- Hypoalbuminemia
- Urinary Bence Jones proteinuria
- Increased serum calcium
- Normal serum alkaline phosphatase
- Increased serum β microglobulin

Radiological
- Multiple lytic lesions without marginal sclerosis
- Diffuse osteoporosis
- Osteosclerosis circumscripta (skull)
- Localized punched-out lesions
- Pathological fractures
- No bone abnormalities (20%)

Diagnosis
Diagnosis of multiple myeloma is made on the basis of;
- Monoclonal immunoglobulin peak in the serum or urine
- Marrow plasmacytosis
- Related tissue damage, popularly known as CRAB
  - Increased serum calcium
  - Renal disease
  - Anemia
  - Bone disease

Treatment
Story of chemotherapy of multiple myeloma is an example of medical profession’s frustrations and disappointments that it experienced in the long journey of 60 years to produce an effective, affordable, safe and curative therapeutic regimen.

Not only the potentially harmful and toxic drugs have failed to achieve the desired results, they actually harmed the patients and caused serious complications which hastened the demise of these patient. It started in 1965 with the introduction of Alexanian regimen comprised of melphalan and prednisone with a survival rate of three years. A large number of drugs and many complicated protocols were subsequently designed, experimented and tried over 60 years with considerable morbidity, expense and hardships. At the end of the day, the median survival in multiple myeloma is still approximately three years.

Chemotherapy for multiple myeloma (like that of other malignant disorders) should not be administered by those who are not fully conversant with cancer chemotherapy nor should it be offered in centers where supportive care of the highest quality is not available.

Before chemotherapy is administered, it is important to improve the general condition of the patient. Supportive care in patients with multiple myeloma includes treatment of:
anemia with packed red cell transfusion. Erythropoietin is a useful adjunct for the treatment of anemia in these patients
- any bleeding tendencies due to thrombocytopenia
- infections with appropriate antibiotics after appropriate blood examination and cultures
- bone pains with bisphosphonates, local radiotherapy and analgesics
- azotemia
- dehydration and azotemia

**Drugs used for the treatment of multiple myeloma**

Listed below are some of the drugs that have been tried over the past many years in an attempt to achieve therapeutic breakthrough but without any satisfactory outcome. The list is by no means complete. The last word is that the original Alexanian regimen is still the best compromise.

Drugs used for the treatment of multiple myeloma (and other plasma cell dyscrasias) are:
- Melphalan
- Glucocorticoid
- Adriamycin
- Vincristine
- Cyclophosphamide
- Bisphosphonates
- Interferon α
- Thalidomide
- G-CSF
- Lenalidomide
- Bortezomib

**Alexanian regimen**
- Tablet melphalan 2 mg; 5 tablets daily 4 days.
- Tablet prednisone 5 mg; 3 tablets 4 times daily X 4 days

Treatment is stopped after 4 days and the rest period (for the marrow to recover) is observed for 5 weeks. Six such cycles are repeated over nine months period. Treatment is stopped and the patients are followed for hematological response and improvement in non-hematological parameters.

The other effective and popular, though more toxic, protocol is the VAD protocol. It comprises of three drugs i.e. vincristine (V), adriamycin (A) and dexamethasone (D).

**Course and prognosis**

Multiple myeloma, if untreated is a rapidly fatal disease, median survival is usually less than one year. The short life is punctuated by unbearable pain, bone fractures, recurrent incapacitating infections and episodes of bleeding sometimes in critical area. The three emergency situations hypercalcemic crises, hyperviscosity induced coma paraproteinemia and spinal cord compression hasten the demise if prompt treatment is not instituted.

Clinicopathological features which are associated with poor prognosis are:
- Tumor mass >1.2X10^{12}/m^2
- Extent of marrow plasmacytosis >50%
- Hemoglobin < 8.5 g/dl
Plasma cell labeling index >2%
LDH > twice normal
β-microglobulin >4mg/dl
Serum albumin <3.5 mg/dl
CRP > 4mg/dl
Hypercalcemia >12mg/dl
IgG > 7g/dl
IgA >5g/dl
Bence Jones protein in urine > 12g/dl
Increased number of circulating clonal plasma cells

**Durie-Salmon staging of multiple myeloma**

**Stage III**
- High tumor mass as reflected by:
  - Hemoglobin <8.5g/dl
  - Serum calcium >12mg/dl
  - Serum / urinary myeloma protein
  - IgG >7g/dl
  - IgA >5g/dl
  - Bence Jones protein >12g/24hrs
  - More than 3 lytic lesions on X ray

**Stage I**
- Low tumor mass
  - Hemoglobin >10.5g/dl
  - Normal serum calcium
  - Low serum myeloma protein
    - IgG <5g/dl
    - IgA <3g/dl
    - Bence Jones protein <4g/24hrs
  - No bone lesions or osteoporosis

**Stage II**
- Laboratory abnormalities between stage I and III

Durie-Salmon classification and serum β microglobulin levels and serum albumin have important bearing on the clinical behavior of the disease. Serum β microglobulin greater than 5.5 µg/l, serum albumin less than 3.5g/dl and Durie-Salmon stage III indicate poor prognosis. Similarly increased number of circulating clonal plasma cells heralds poor prognosis.

Despite all efforts at producing an effective curative combination of drugs the prognosis is still bleak and the median survival with best possible drug combinations and supportive care is still only 3-5 years. This has improved somewhat with the introduction of autologous stem cell transplantation.

Consolation that the medical profession can claim in the treatment of patients with multiple myeloma is that the life of these patients has become more comfortable and somewhat more productive. With therapeutic approaches that include the use of monoclonal antibodies and the drugs to undo the basic genetic aberration it is expected that there will soon be a breakthrough in the disappointing prognosis of multiple myeloma.

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POLYCYTHEMIA RUBRA VERA

The term polycythemia rubra vera (PRV) literally mean ‘a true increase in the number of red cells’. It is a member of a group of disorders called myeloproliferative disorders; a term initially coined by an English hematologist William Dameshek in 1956. It is also called primary proliferative polycythemia.

This disease is characterized by an autonomous proliferation of predominately the red cells series in the bone marrow with variable participation of granulocytes, megakaryocytes and fibroblasts resulting in a morphological appearance of panmyelosis (excessive proliferation of all three hematopoietic cells lines) in the bone marrow. This is reflected as pancytosis in the peripheral blood. There is a marked increase in the level of hemoglobin with slight increase in the granulocytes (neutrophils, eosinophils and basophils) and a variable increase in the platelet count.

Etiology

Polycythemia vera is a malignant disorder of hematopoietic stem cells with no known etiology but some suspected predisposing factors.

Age and sex

This disease is more common in middle age and older individuals. Males and females are equally affected.

Clinical Features

Polycythemia rubra vera is an uncommon disease with a prevalence rate of king pin of all. Clinical manifestations is an increased red cell mass due to a relentless over production of the red cells; all other clinical and laboratory manifestations are a reflection of this critical abnormality.

Clinical manifestations in PRV are produced by interplay of a number of biological abnormalities arising from the basic pathology characterized by an autonomous proliferation of myeloid stem cells. These are illustrated in the hexagon in figure 31.1.

![Autonomous myeloproliferation](image)

Hypermetabolism

Hyperviscosity

Hypertension

Figure 31.1: Pathological basis of clinical manifestations of PRV
Some of the common clinico-pathological features that develop as a result of these mechanisms are:

- Headache, vertigo, blurred vision, suffusion of conjunctiva and ruddy cyanosis of the face, nose, ear lobes and finger tips (acrocyanosis) are a result of vascular dilatation to accommodate the extra red cell mass (hematocrit). This compounded by the hyper viscosity causes slowing of the circulation. Stasis predisposes to increased oxygen extraction by the tissues, extravasation of water into the tissues resulting in acrocyanosis and thrombohemorrhagic complications in the eyes, brain portal vein, renal vein, hepatic vein and peripheral vessels, both arterial and venous.
- A common complaint of weakness, burning sensation over the face and body and weight loss are manifestations of hypermetabolic state because of the exuberant proliferation of the myeloid stem cells and their progenitors.
- Enlargement of spleen is a common feature of polycythemia rubra vera; it is seen in almost 50% of the patients. Hepatomegaly, though somewhat less common is also frequently encountered in this disease. Lymph node enlargement is uncommon. This is not unexpected because PRV is a myeloproliferative disorder which does not affect the lymphoid stem cell. Liver and spleen being the myeloid organ (these were the blood forming organs in the intrauterine life) become involved in the myeloproliferative process while lymph nodes being the lymphoid organs are spared.
- Hypertension is another common and important component of PRV. This most likely is an outcome of hypervolemia, vascular damage, renal vascular disease (as part of vasculopathy of PRV) and hyperuricemia. Hypertension is not only an important clinical feature; it is also an important cause of morbidity and mortality in PRV.
- An important biochemical abnormality which takes its own toll in the life of these patients is hyperuricemia. Increased amount of uric acid is produced as a result of destruction of the vast number of nuclei which are extruded from the nucleated red cells as they mature into erythrocyte in the bone marrow. Hyperuricemia cause gouty arthritis, nephropathy (urate stones), renal damage and hypertension.
- Yet another biochemical abnormality is an increased production of histamine from the basophils which are also the partners in the proliferative process. These patients are more prone to peptic ulcer disease. They also complain of itching after taking a warm bath. This is caused by dilatation of the already over distended vessels stimulating the pruritus associated nerve endings and compounded by excessive release of histamine from the tissue mast cells.

Besides polycythemia rubra vera there are other conditions that many produce polycythemia.

This type of polycythemia is called secondary polycythemia or secondary erythrocytosis. Some of the common acquired causes of secondary polycythemia are:

- Arterial hypoxia PO2<92%
  - High altitude livin, right to left intra cardiac shunt, Fallot's tetralogy, transposition of great vessels, chronic obstructive pulmonary disease
- Renal diseases
  - Renal artery stenosis, hydronephrosis, renal cyst, post renal transplant, polycystic kidneys
- EPO-producing neoplasia
- Cerebellum hemangioma
- Hepatocellular carcinoma
- Renal cell cancer
- Leiomyoma
- Androgen therapy
- High O\textsubscript{2} affinity hemoglobins (rare)

(This list is by no means complete)

**Laboratory Findings**

Polycythemia rubra vera is accompanied by a set of highly characteristic laboratory abnormalities; these are listed in the hexagon in fig 31.2.

![Figure 31.2: Laboratory features of PRV](image)

*colonies forming units; erythroid.

**Burts forming units; eruthroid.

**Diagnosis of polycythemia rubra vera**

Current criteria for the diagnosis of polycythemia rubra vera is based on the presence or absence of JAK2 mutation. According to this classification polycythemia rubra vera is initially grouped into:

1. JAK2 positive polycythemia vera
2. JAK2 negative polycythemia

In JAK2 positive PRV, it is mandatory to fulfill both of the following criteria:

A1. High Hct > 0.52 in male and > 0.48 in female

OR

Red cells mass > 25% of the predicted value

A2. Mutations in JAK2

In JAK2 negative polycythemia, the criteria are more diverse. They are arranged in 2 groups i.e. group A (A1-A5) and group B (B1-B5). These are listed below:

A2: Increased red cell mass

>25% above the predicted value in either gender

OR

High hematocrit > 0.52 in men and > 0.48 in women

A3: No demonstrable JAK2 mutation
A4: No cause of secondary erythrocytosis
A5: presence of an acquired genetic abnormality (nor BCR-ABL translocation)

B1: Platelet count  >450x10^9/L
B2: Neutrophil count
   In non-smokers  >10x10^9/L
   In smokers  >12.5x10^9/L
B3: Ultrasonically enlarged spleen
B4: Endogenous erythroid colonies or low serum erythropoietin

Diagnosis of polycythemia rubra vera becomes established in the presence of A1+A2+A3+ either another OR any two B criteria.

**Treatment**

Diagnosis of PRV makes it imperative that the patients must receive some form of treatment to either slow down or stop the excessive myeloproliferation. This is because if left untreated, the disease is rapidly fatal, most patients die within 3 years.

Appropriate treatment not only curtails morbidity, it also prevents complications and prolongs life. Therapeutic modalities that are available for the treatment of polycythemia rubra vera are:

- Venesection
- Hydroxyurea
- Busulfan
- P32
- α-interferon
- Aspirin
- Allopurinol

Therapeutic modality of choice is determined primarily by the age of the patient, concomitant co-morbid disorders and pregnancy. These are highlighted in table 31.1.

<table>
<thead>
<tr>
<th>Therapeutic modalities</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venesection</td>
<td>Under 40 years of age</td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
</tr>
<tr>
<td></td>
<td>Life threatening high hematocrit</td>
</tr>
<tr>
<td></td>
<td>Mild disease</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>Older patients</td>
</tr>
<tr>
<td></td>
<td>Thrombocytosis</td>
</tr>
<tr>
<td></td>
<td>Progressive splenomegaly</td>
</tr>
<tr>
<td></td>
<td>Hypermetabolism</td>
</tr>
<tr>
<td>P32</td>
<td>Older patients</td>
</tr>
<tr>
<td>α-interferon</td>
<td>Less than 40 years of age</td>
</tr>
<tr>
<td>Aspirin</td>
<td>For all ages</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>For all ages</td>
</tr>
</tbody>
</table>

Table 31.1: Therapeutic modalities in PRV
Course and prognosis

Polycythemia rubra vera, being a malignant disease, leads to the death of the patient within 3 years if no treatment is given. Cause of death is mostly related to vascular catastrophies (CVA, myocardial infarction and thrombohemorrhagic episode).

Treatment in any form has drastically improved the outlook for these patients with a median survival between 10 years and 16 years. Whereas administration of chemotherapeutic agents has morbidity and mortality, it has added new dimensions in the complications and natural history of the disease. Nearly 30% of the patients develop myelofibrosis while another 5% develop acute myeloid leukemia. $P_{32}$ and Busulphan administration has high association with leukemogenesis. Phlebotomy alone is not associated with any these complications. Availability of inhibitors of JAK2 mutation hopefully shall be more effective, safer and devoid of leukomogenesis potential.
THROMBOCYTOPENIA

Platelets (thrombocytes) are the smallest of the three hematopoietic elements in blood. Like the red cells and the granulocytes these too are produced in the bone marrow. Stem cells that produce red cells and granulocytes also produce the precursors of platelets called megakaryocytes. Platelets are produced through an orderly process of multiplication and maturation. As the platelets are released from the bone marrow they circulate in the blood for 7-10 days (life span of platelets). About 20-30% of the platelets released from the marrow are sequestered in the spleen (this has an important bearing in the pathogenesis of thrombocytopenia). Platelets, as they circulate, are used up for repairing the damaged endothelial lining; they also participate in the micro-thrombotic episodes under physiological and pathological conditions.

Normal platelet count irrespective of the age and gender is 150-450X10³/µl. thrombocytopenia is defined as a reduction in the number of platelets below the lower limit of normal (150X10³/µl).

For the sake of clinical application it seem appropriate to know the severity of thrombocytopenia as the clinical features of thrombocytopenia become severe with the degree of thrombocytopenia. Causes of thrombocytopenia can be summarized as shown in figure 32.1.

[Diagram: Causes of thrombocytopenia]

- Decreased production
- Increased destruction
- Increased consumption
- Splenic pooling
- Combination

Figure 32.1: Causes of thrombocytopenia

Decreased production is most commonly due to primary or secondary bone marrow failure, marrow infiltration, acute leukemias, megaloblastic anemias and a host of other conditions that afflict the bone marrow.

Increased consumption implies excessive consumption of platelets in intravascular thrombotic disorders as in DIC, hemangiomas, aneurysm etc.

Excessive destruction of platelets is classically seen in immune thrombocytopenia where antibodies directed against platelet specific antigens coat the platelets that are destroyed by the RES.
As mentioned above, almost 20-25% of the platelets that are released from the bone marrow are retained by the spleen. Percentage of retention increases as the size of the spleen enlarges; very large spleens as in malaria, Gaucher’s disease and cirrhosis may retain up to 60% of the platelets released from the bone marrow. Certain diseases like chronic lymphocytic leukemia and cirrhosis of the liver produce thrombocytopenia through more than one mechanism.

**Clinical Features**

Bleeding from mucosal surfaces and the skin is the only symptom produced by thrombocytopenia. Clinical symptomatology is dictated by a number of factors; these are:

- Platelet count
- Cause of thrombocytopenia
- Trauma
- Co-morbid

Effect of platelet count on propensity to bleed is summarized in table 32.1.

<table>
<thead>
<tr>
<th>Platelet count X10³/µl</th>
<th>Bleeding after major trauma</th>
<th>Bleeding after minor trauma</th>
<th>Spontaneous bleeding</th>
<th>Intracranial bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;150</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>100-150</td>
<td>±</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>70-100</td>
<td>+</td>
<td>±</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>50-70</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>30-50</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>&lt;30</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 32.1: Bleeding tendency in thrombocytopenia**

Cause of thrombocytopenia has an important bearing on the propensity to bleed. Platelets that are produced in ‘sick’ bone marrows as in acute leukemias, post chemotherapy and radiations etc. are also ‘sick’ platelets, hemostatically they are weak and bleeding does occur at platelet count at which ‘healthy’ platelets do not permit bleeding. In immune thrombocytopenia (ITP) where platelets production is accelerated and younger platelets ‘flood’ the peripheral blood, there is very little tendency to bleed at a platelet count where even normally functioning platelets are accompanied by bleeding. This is explained in table 32.2.

<table>
<thead>
<tr>
<th>Platelet count 40X10³/µl</th>
<th>Bleeding tendencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal platelets</td>
<td>+</td>
</tr>
<tr>
<td>Leukemic platelets</td>
<td>++</td>
</tr>
<tr>
<td>ITP</td>
<td>−</td>
</tr>
</tbody>
</table>

**Table 32.2: Comparative analysis of bleeding tendency at platelet count of 40 X10³/µl**
Bleeding takes place from the nose, gums, skin, kidneys and uterus (metrorrhgia). Severity of bleeding is determined by the number of platelets and the mechanism of thrombocytopenia.

Platelet count between 10-20 X10^3/µl entails a serious risk of intra cerebral hemorrhage. Other signs and symptoms are dictated by the site and the amount of bleeding.

**Laboratory Findings**

Whereas it is easy to establish the diagnosis of thrombocytopenia, it is even more important to decipher the cause of thrombocytopenia. Some of the investigations that are recommended for the patients with thrombocytopenia are:

- Complete blood count and erythrocyte sedimentation rate
- X ray chest
- Ultrasound of abdomen for size of spleen and liver
- Bleeding time

**Treatment**

Symptomatic treatment to combat the effects of thrombocytopenia;

Drugs used for the treatment of thrombocytopenia

Treatment of thrombocytopenia follows the same cardinal principle as are applicable to other therapeutic domains. These include;

- Symptomatic supportive care for somatic complaints of specific and non-specific nature
- Disease oriented drugs
- Treatment of complications

For care of somatic features like fever, body aches, anorexia, vomiting, abdominal pain etc. the usual on-the counter drugs usually suffice. One specific symptom of thrombocytopenia is the bleeding which needs attention.

For superficial bleeding cold compresses and pressure bandage usually suffice. Nose bleed, gum bleed and menorrhagic bleeding may require the services of ENT surgeon, dental surgeon and gynecologist. Internal organ bleeding (brain, GIT, intrapulmonary) pose critical problems that require urgent treatment in specialize centers. Injection transamin is available as an emergency measure, its role is however limited. Therapy directed to treat the cause of thrombocytopenia is a domain on its own and it should be entrusted to the general physicians and the subject specialists.

Drugs that are available for the treatment of ITP are;

- Glucocorticoids
- Anti D (Rhogam) immunoglobulin
- High dose intravenous immunoglobulins
- Imuran
- Rituximab
- Eletrombopag
- Romiplostim (TPO receptor agonist)
- Platelet transfusion
- Splenectomy
Suggested further reading
16. Hallek M, Fingerle-Rowson G, Fink AM et al. (2008) Immunotherapy with fludarabine (F), cyclophosphamide (C), and rituximab (R) (FCR) versus fludarabine and cyclophosphamide (FC) improves response rates and progression-free survival (PFS) of previously untreated patients (pts) with advanced chronic lymphocytic leukemia (CLL) [Abstract]. Blood 112: 325.


CHAPTER - 33

LABORATORY HEMATOLOGY

SAHLI’S METHOD FOR HEMOGLOBIN ESTIMATION

Hemoglobin is the most abundant protein which is present in the blood. Chemically it contains a protein globin to which a non-protein prosthetic group, heme is attached. Hemoglobin molecule is a tetramer; each sub unit consists of one globin chain attached to one heme group. Hence one molecule of hemoglobin contains 4 molecules of globin and 4 molecules of heme. Each hemoglobin molecule can bind upto 4 molecules of oxygen.

Principle

Hemoglobin present in a specified volume of blood is converted into acid hematin by the action of hydrochloric acid which is a brown pigment. Distilled water is added to dilute the color of acid hematin, till the color of the solution becomes as that of the standard. Hemoglobin concentration is read directly from the tube in gm/dl or as percentage of the standard.

Requirements

- Hemometer or Hemoglobinometer
- Test tubes
- Blood collection tray
- Reagents
  - N/10 HCl
  - Methylated spirit

Procedure

- Arrange all reagents and glass wares on the table.
- With the help of a disposable syringe, collect venous blood sample and put it in a test tube containing anticoagulant (EDTA) to prevent clotting of blood.
- With the help of a dropper take N/10 HCl in Sahli’s tube upto the mark 10.
- Add 0.02 ml or 20 μl of anticoagulated blood sample in Sahli’s tube and mix it with the help of the gas rod.
- Wait for 4-5 minutes, to allow the reaction to complete i.e. formation of acid hematin.
- A blackish brown solution will be formed.
- Start diluting it with distilled until the color in the tube matches to that of the standard tainted glass.
- Compare the colour of solution and note the hemoglobin concentration directly form graduated Sahli’s tube.

Result

Hemoglobin concentration in blood by Sahil’s method is ______ gm/dl

Normal value

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult male</td>
<td>14 – 16 gm/dl</td>
</tr>
<tr>
<td>Adult female</td>
<td>12 – 14 gm/dl</td>
</tr>
<tr>
<td>New born</td>
<td>18 – 22 gm/dl</td>
</tr>
</tbody>
</table>
Sources of error
- Discard first drop of blood from the prick as it contains tissue fluid.
- During filling of Sahil’s pipette, air should not be sucked into the pipette as this will decrease the volume of the blood in the pipette.
- Any delay in transferring of blood will cause clotting of blood in the pipette.
- Accurate amount of HCl should be taken.
- Wipe out the extra blood on the sahli’s pipette before transferring it to graduated tube.

Clinical significance
A decrease in hemoglobin concentration in blood below lower normal value is a sign of anemia and increase in hemoglobin concentration on the other hand signifies polycythemia. An increase in hemoglobin concentration can occur due to hemoconcentration (loss of body fluid e.g. in severe diarrhea, vomiting) reduced oxygen supply (e.g. congenital heart disease, emphysema). A decrease or increase in hemoglobin concentration must be reported as it is a sign of disease requiring further investigation.

ERYTHROCYTES SEDIMENTATION RATE (ESR) BY WESTERGREN’S METHOD
Erythrocyte sedimentation rate (ESR) may be defined as the distance traveled by the red cells in a well-mixed anticoagulated sample of venous blood under specified conditions in a fixed period of one hour.

When blood is added Westergren’s tube after the addition of an anticoagulant red cells gradually settle down leaving a column of clear plasma at the top. The height of this column of plasma after exactly 1 hour is reported as ESR in mm in the first hour. Rate of setting of red cells is influenced by a number of factors. Primarily it depends on the density gradient between red cells and plasma.

ESR is greatly enhanced by those conditions which increase the density of the red cells. This is achieved through rouleaux formation, which is characterized by the piling up of red cells on top of one another like a pile of coins. This enhances the speed of sedimentation of red cells.

Principle
Well mixed whole blood, containing 3.8% trisodium citrate as an anticoagulant, is placed in a calibrated Westergren’s tube and is allowed to stand vertically undisturbed for exactly 1 hour. The distance in millimeters through red cells settle during this period is reported as ESR.

Requirements
- Westergren’s calibrated tube
- Westergren’s tube holding rack
- Disposable syringes (3cc)
- Cotton swabs
- Tourniquet
- Timer
- Trisodium citrate 3.8% (as an anticoagulant and diluent)
- Methylated spirit
- Sample tubes

Procedure
- Take 0.4 of 3.8% sodium citrate solution in a small glass tube. This serves as an anticoagulant as well as the diluent.
- Observing the usual precautions and following an aseptic technique, draw approximately 2 ml of blood in a syringe.
- Add 1.6 ml of blood immediately from the syringe to the tube to which sodium citrate was added so that the ratio of whole blood to the diluent is 4:1.
- Mix blood with the anticoagulant by rotation the tube gently.
- With the help of a syringe which is attached to the upped end of a Westergren’s tube through a rubber connector fill the tube with anticoagulated blood up to “O” mark.

**DO NOT SUCK BLOOD UP THE WESTERGREN TUBE WITH MOUTH**
(Make sure that air bubbles do not enter the tube).
- After filling the tube with blood to zero mark, place the Westergren’s tube in the stand. Make certain that the lower end of the tube is placed securely at the center of the rubber pad on the lower strip of the stand. This is to ensure that the blood does not leak out.
- Disconnect the rubber tubing along with the syringe from the Westergren’s tube and place it underneath a metal clip at the upper end of the stand. The tube should be absolutely vertical and the top of the column of blood must be at “O” mark. Also there should not be any air bubbles at the top of the column of blood.
- Let the tube stand undisturbed for an hour at room temperature.
- Note the height of the column of plasma after an hour. This is the ESR in mm.

**Results**
ESR of a given sample of blood by Wistergren’s method is ______ mm/1st hr.

**Normal values**
ERS is not a static value, it varies with age and is also different for the two sexes. It is also influenced by such physiological events as menstruation, pregnancy and exercise etc.

<table>
<thead>
<tr>
<th>Category</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult male</td>
<td>5 – 15 mm / 1st hour.</td>
</tr>
<tr>
<td>Adult female</td>
<td>10 – 20 mm / 1st hour.</td>
</tr>
</tbody>
</table>

**Precautions**
- All apparatus must be clean and dry.
- No air bubble should enter the tube while filling it with blood because it makes difficult to read the upper level of the plasma column.
- Westergren’s tube should be placed absolutely vertical; inclination of ever a few degree increases the ESR.
- Blood and anticoagulant should be mixed in a ratio of 4:1, over dilution increases the ESR.
- Westergren’s tube should be set up away from all vibrating surfaces, as vibrations interfere with rouleaux formation and affects the rate of red cell sedimentation.

**Significance of ESR**
- Children normally have a lower ESR than adults.
- In adults over 60 years, ESR is frequently higher than in young adults.
- ESR usually reflects qualitative as well as quantitative changes in plasma proteins which are encountered in many acute and chronic infections, malignancies and degenerative diseases.
- ESR may be used to gauge the progress of certain diseases such as tuberculosis, collagen vascular diseases and rheumatic fever.
**Wintrobe’s method**

**Requirements**

Ethylene diamine tetra acetic acid 1-2 mg/ml. Wintrobe’s tube (it is a straight tube 11 cm in length, 2.5 um in diameter).

**Procedure**

- Collect the blood in EDTA.
- With the help of pastauer pipette, fill the Wintrobe’s tube up to 0 mark.
- Keep it in the rack in the vertical position for 1 hour.
- After 1 hour note the column of the plasma and report the reading.

**Results**

Erythrocyte sedimentation rate of a given sample of blood by Wintrobe method is ________ mm/1st hr.

**Normal values**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>4-12 mm 1st hour.</td>
</tr>
<tr>
<td>Female</td>
<td>5-18 mm 1st hour.</td>
</tr>
</tbody>
</table>

**Precautions**

- Dilution should be proper.
- The tube shall be filled properly and there shall be no air bubbles in the tube.
- The tube should be placed in a vertical position, away from heat and vibration.
- Reading shall be noted exactly after 1 hour.
- Mouth suction is strictly prohibited.
- The sample shall be used within 1 hour.
- Hemolysed blood shall not be used.

**Note**

- ESR of children should be carried out by Wintrobe’s method because less amount of blood is required.
- Blood collected in EDTA can be processed for Westergren’s method by making 1.5 dilution (Ration 1:4) with normal saline.
- Note the color of the plasma because it gives valuable information about the sample:
  - Normal color of the plasma is amber.
  - Pink colored plasma is an indication of intravascular hemolysis.
  - Brown colored plasma is an indication of methemoglobinemia.
  - Yellow colored plasma is an indication of bilirubin in the plasma (jaundice).
Comparison of the two methods
ERS is usually determined by two methods, Westergren’s and Wintrobe’s methods. Some important differences between these methods are listed in table 33.1.

<table>
<thead>
<tr>
<th>Features</th>
<th>Westergren’s Method</th>
<th>Wintrobe’s Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of the tube</td>
<td>Westergren’s tube</td>
<td>Wintrobe’s tube</td>
</tr>
<tr>
<td>Characteristics of the tube</td>
<td>30 cm (300 mm) long open at both ends. -Upper 10 cm are not marked -Lower 20 cm are marked</td>
<td>12 cm (120 mm) long. Open at one end and closed at the other end -Upper 2 cm are not marked -Lower 10 cm are marked</td>
</tr>
<tr>
<td>Calibration</td>
<td>From above downwards only</td>
<td>In both directions.</td>
</tr>
<tr>
<td>Range of Calibration</td>
<td>From 0 to 200 mm</td>
<td>From 0 to 10 for ESR from above to downwards. From 10 to 0 for PCV From below to upwards</td>
</tr>
<tr>
<td>Internal diameter of the tube</td>
<td>2.5 mm</td>
<td>2.5 mm</td>
</tr>
<tr>
<td>Amount of blood required</td>
<td>1.6 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Height of the blood column</td>
<td>200 mm</td>
<td>100 mm</td>
</tr>
<tr>
<td>Anticoagulant</td>
<td>0.4 ml of 3.8% sodium citrate</td>
<td>2-3 mg/ml of EDTA Powder</td>
</tr>
<tr>
<td>Method of filling the tube</td>
<td>With a suction device</td>
<td>With a Pasteur pipette</td>
</tr>
<tr>
<td>Significance of the method</td>
<td>Only ESR can be determined</td>
<td>Both PCV &amp; ESR can be determined</td>
</tr>
</tbody>
</table>

Table 33.1: Comparison of Wistergen’s and Wintrob’s method of ESR

PACKED CELL VOLUME
When whole blood is centrifuged in a tube (hematocrit tube) red cells are packed together at the bottom of the tube by the centrifugal force as these are heavier than plasma.

Packed cell volume (PCV) represents the volume of the cells per unit volume of whole blood and is expressed as percentage. Even when the red cells are fully packed, about 2% of plasma remains trapped in among the cells. This percentage is more if the red cells are abnormal in shape (e.g. Spherocytosis).

If it is not possible to obtain blood from vein, microhematocrit method is employed. Thus the percentage volume of the blood occupied by RBCs is called hematocrit (HCT) or packed cell volume (PCV).

PCV can be used as a simple screening test for anemia. There are two methods available for the measurement of PCV directly:

- Wintrobe’s method
- Capillary method
Wintorbe’s method (macrohematocrit method)

Principle

When anticoagulated blood is centrifuged at a standard speed for specific period of time, red cells being heavier than other blood cells are packed at the bottom of the tube and reading is made directly from Wintrobe tube in Wintrobe’s method and in case of capillary method from readers chart.

Requirements

- Wintrobe’s tube
- Pasteur pipette
- Centrifuge machine (hematocrit centrifuge)
- Blood collection tray
- Anticoagulants used: Dried heparin, EDTA, double oxalate

Procedure

- Collect venous blood and anticoagulate it by EDTA at a concentration of 1.5 mg/ml or by heparin at a concentration of 15 – 20 IU/ml.
- Mix the blood carefully by repeated inversions.
- Fill the hematocrit tube (Wintrobe’s tube) at once to 100 mm mark by means of pasteur pipette.
- Centrifuge the tube at 2000–2300g for 30 minutes.

Interpretation

Height of the column of red cells is taken as the PCV. (Above the red cells not included in the figure for PCV, will be seen a creamy layer of leukocytes termed as “Buffy coat”.

Results

PCV / HCT by Macrohematocrit method is ____________.

Capillary method (microhematocrit method)

Principle

Anticoagulated blood is centrifuged in a sealed capillary tube. The volume of packed red cells and percentage of the whole blood (level of plasma) are determined by a special hematocrit reader.

Requirements

- Capillary tube
- Hematocrit centrifuge
- Plastascene
- Blood collection tray

Procedure

- Fill the capillary tube with the blood sample directly from sample containing tube.
- Seal the one side of the capillary tube with plastascene.
- Centrifuge the capillary tube for five minutes at 5000 RPM.
- Make the reading with the help of readers chart.

Result

Hematocrit of the given sample by capillary method is __________
Normal values

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td>41.9 – 48.7%</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>35.4 – 42.0%</td>
</tr>
</tbody>
</table>

Precautions

- Amount of EDTA should be appropriate because excess EDTA will shrink the red cells thereby effect the result as PCV depends upon red cell size and shape.
- If the blood sample is kept for 6 hours it will cause swelling of red cells. ATP will be utilized and sodium will move inside the red cell.
- Capillary tube should be smooth.
- Centrifuge speed should be checked by tachometer.
- Air bubbles should be avoided.

Clinical significance

Changes in PCV affect the maximum transport of the oxygen. Low PCV decreases the maximum content of oxygen in a volume of blood due to the less hemoglobin. Whereas high PCV decreases the maximum cardiac output because of increase viscosity.

High PCV is seen in:
- Polycythemia
- Dehydration
- Burn

Low PCV is seen in:
- Anemia
- Pregnancy
- Females

HEMOCYTOMETER (NEUBAUER'S CHAMBER)

Although the automated cell counters are widely used these days in many of the established laboratories, the standard techniques are still based on the use of the hemocytometer (Neubauer's Chamber) to count the blood cells.

Requirements

- Hemocytometer (Improved Neubauer Chamber)
- Microscope with low and high power lenses

Procedure

- Examine the hemocytometer carefully. It is a thick glass plate which has four vertical grooves in the central area. The central platform is divided into two equal halves by a transverse groove. A square is so marked on each half that the two preparations may be set up at the same time for observations.
- Each of these squares possesses a counting chamber having an area of 9 mm and is divided into nine squares, each measuring 1 mm.
- With a very fine technique these squares are further sub divided and the details can only be observed clearly by using the microscope.
- Under the low power it is observed that the four corner squares are divided into 16 smaller squares which are used for counting white blood cells (WBCs) called leukocytes.
- The central 1 mm is visible with its details under the high power lens and is divided into 25 small squares each measuring 1/25 mm and every small square is further sub divided into
16 smallest squares. The 1/25mm squares are bounded by double lines and are used for counting Red Blood Cells (RBC’s).

- Height of the central platform is 0.1mm less than rest of the slide. When a cover slip is placed covering central platform and fluid containing RBC’s or WBC’s is seen under it, depth of the fluid film is 0.1mm.

Precautions

- Handle the hemocytometer carefully as it is made of glass.
- Clean the hemocytometer only with distilled water, avoid chemicals as they alter the lining scales.
- Be careful to avoid a hasty contact of the microscope lens over the chamber while focusing which may break the cover slip or even the hemocytometer.

Figure 33.2: Neubauer chamber
RED BLOOD CELL COUNT

Red blood cell is one of the important formed elements of blood concerned in the exchange of gases during respiration. These are non-nucleated biconcave discoid shape being 7.2 micrometer in diameter.

It contains hemoglobin in the form of an important powerful blood buffer. These can change their shape remarkably when pass through much smaller size of capillaries. These have normal life span of about 120 days.

Requirements
- Hayem’s solution
- Neubauer chamber
- Blood collection tray
- Hemocytometer
- Microscope
- RBC diluted pipette / Thomas pipette

Preparation of Hayem’s solution
- Sodium Chloride (NaCl) : 0.5 gm
- Sodium sulphate (Na₂SO₄) : 2.5 gm
- Mercuric chloride (HgCl₂) : 0.25 gm
- Distilled water : 100 ml

Collection of sample
For blood counts the anticoagulant of choice is EDTA as this anticoagulant preserves the morphology of the cellular elements. Take fresh EDTA mixed blood with a fixed ratio of blood and anticoagulant.

Procedure
- Arrange all reagents and instruments.
- Take 4 ml of Hayem’s solution in a test tube.
- Add 20µl/0.02ml of blood in a tube containing diluting fluid. Mix it well (dilution= 1:200).
- Leave it for 5 minutes.
- Charge the Neubauer chamber with the sample with the help of a capillary tube.
- Perform manual counting of RBC’s under the microscope.

Counting the erythrocytes
- Place the counting chamber on the table which should be free from dust particles and cover the ruled area with a hemocytometer cover slip.
- Charge the counting chamber with the help of a capillary tube. Avoid over running of solution.
- Allow to stand for 3-5 minutes for the RBCs to settle.
- Locate the central large square which is divided into 25 medium sized squares. Each medium sized square is further divided into 16 small squares.
- Count the number of erythrocytes in 5 medium sized squares (80 small squares) using 40X objective lens.
Fig2: Magnified version of the central large square.

Calculations
Total No. of cells in 80 smallest squares = ______

Total No. of cells in 1 smallest squares = \( \frac{80}{80} = _____ \) cell.

Volume of 1 small square = \( \frac{1}{4000} \) mm\(^3\)

Thus, there are ______ cells in \( \frac{1}{4000} \) mm\(^3\) × 4000 cells in diluted blood.

Dilution = 1:200

Cells (in diluted blood) × 200 = \( \mu l \) of undiluted blood.

Result
Red cells in a given sample of blood are ______ \( \mu l \).

Normal values
- Male : 4.5 – 5.5 Millions / \( \mu l \)
- Female : 3.9 – 4.5 Millions / \( \mu l \)
Precautions
- Diluting fluid should not be contaminated with blood cells.
- Counting chamber and cover glass shall be kept free from dust and dried blood.

Clinical significance
Decrease in the number of circulating erythrocytes indicates anemia and increase number of erythrocytes indicates the possibility of polycythemia. Red cell count is also required for the calculation of indices.

TOTAL LEUKOCYTE COUNT (TLC)
White blood cells are the mobile units and one of the important formed elements of the body’s protective system that provide defense mechanism against infectious agents. WBC’s must be present in sufficient number to carry out this function properly. Thus counting of total number of WBC’s is an important clinical measurement which helps establish the capacity of blood for performing their different functions.
Principle

Blood is diluted with acetic acid solution which removes the red cells by lysis and also darkens the nuclei of the white cells. This makes the counting of the white cells easy. By knowing the volume of the fluid examined and the dilution, the number of WBC’s in the undiluted blood can be calculated.

Requirements

- Turk’s solution (White cell diluting fluid)
- Hemocytometer
- Microscope
- Graduated pipette

Preparation of Turk’s solution

- Glacial acetic acid : 2 ml
- Aqueous methylene blue 0.3% : 10 drops
- Distilled water : 100 ml

Note: **Methylene blue solution is prepared by dissolving 0.3 g of methylene blue in 10 ml of distilled water. Filter the solution before adding it to the acid solution.**

Procedure

- Pipette 0.38ml of diluting into a test tube using 1ml graduated pipette.
- With the help of a 20 μl Sahli’s pipette drawn well-mixed anticoagulated venous blood up to the blue mark.
- Wipe the tip of the pipette with a tissue paper and add blood into the test tube containing the diluting fluid. Mix for 2 minutes to ensure complete lysis of the red cells. When 0.02ml (20μl) of blood is added to 0.38ml of the diluent, the final dilution of the mixture is 1:20.
- Fix a cover slip on the hemocytometer.
- Fill the counting chamber in the same way as for RBC’s count, place the counting chamber on the stage of the microscope and wait for 5 minutes for the WBC to settle.
- Count WBC under low power objective in 4 primary (or large squares) one on each corner of the counting area.

Calculation

\[
\text{Total No. of cells in 64 small squares} = \text{__________}
\]
\[
\text{Total No. of cells in 1 small square} = \text{__________} = \text{__________}
\]

\[
\text{Volume of 1 small square} = \frac{1}{60} \text{ mm}^3
\]

Thus, there are _______ cells in _______ mm

\[
\text{_______} \times 160 = \text{_______ cells in diluted blood.}
\]

\[
\text{Dilution} = 1:20
\]

\[
\text{_______ cells (in diluted blood) } \times 20 = \frac{\text{_______}}{\mu l \text{ of undiluted blood}}.
\]

262
Result
Total leukocyte count of a given sample of blood is ______________ / μl

Normal value
Male and Female : 4000 – 11000 / μl

Clinical significance
Total leukocyte count provides a useful piece of information for the diagnosis of several pathological conditions such as:

TLC is increased in : Infections, leukemias and MDS
TLC is decreased in : Pancytopenia, malaria and typhoid fever

Precautions
- Diluting fluid should not be contaminated with blood cells.
- Keep the counting chamber and glass cover free from dust.
- Once the counting chamber is filled, complete the counting as early as possible before the fluid begins to dry up and air bubbles enter the chamber.
- Blood and diluting fluid should be taken exactly to the required mark in the pipette.
- Before charging the chamber, blow out the fluid from the stem of the pipette.

PLATELET COUNT
Platelets are the smallest cells of the blood being 2-5 μm in diameter. These are granulated bodies and are non-nucleated. Megakaryocytes in the bone marrow form platelets by pinching off the bits of cytoplasm and extruding them into the circulation. Platelets are important on the formation of hemostatic plug and their deficiency i.e. thrombocytopenia causes a bleeding disorders. Platelets are also important because they produce many important substances like ADP, calcium, serotonin, 5-HT etc.

Principle
Blood diluted with ammonium oxalate solution (platelet diluting fluid) in RBC pipette or Sahli’s pipette. Neubauer chamber is then charged and kept in a moist chamber. The cells are counted in the specific chamber of red cells and the cells are expressed in / ul of blood.

Requirements
- Disposable lancets
- Spirit swabs
- Improved Neubauer chamber
- Sahli’s pipette
- Diluting fluid

1% ammonium oxalate is recommended. It is prepared by dissolving 1g dried ammonium oxalate in 100 ml of distilled water. It hemolyse red cells but leaves the platelets unaltered.

Procedure
- Take 0.38ml of % ammonium oxalate in a test tube.
- Add 0.02ml of blood sample in a tube containing ammonium oxalate. This will make dilution of 1:20.
- Mix well and place it at room temperature for 5–10 minutes for the settling of platelets.
- Fix the Neubauer chamber on the stage of microscope and focus the counting area of the chamber under low power objective lens.
• Charge the Neubauer chamber and count the number of platelets in five primary squares.
• Calculate the results according to the given formula.

Calculation
Total No. of cells in 80 smallest squares = ______
Total No. of cells in 1 smallest squares = \( \frac{80}{1} \) = ______ cell.

Volume of 1 small square = \( \frac{1}{4000} \) mm

Thus, there are ______ cells in \( \frac{1}{4000} \) mm

\times 4000 = ______ cells in diluted blood.

Dilution = 1:20

Cells (in diluted blood) \times 20 = ______ μl of undiluted blood.

Result
Platelets count of a given sample of blood is ______ / μl

Normal range
Male and female : 150–450X10\(^3\)/μl

Precautions
• Platelets count must be done within two hours. Delay causes disintegration and clumping of the platelets.
• Debris and dust are the most important sources of error as they are easily mistaken for platelets.
• Keep the glass-ware clean and filter the diluting fluid before use.

Clinical significance
Increase platelet count (thrombocytosis) is found in polycythemia vera, chronic myeloid leukemia, following splenectomy and other clinical conditions.

Decreased platelet count (thrombocytopenia) occurs in aplastic anemia, acute leukemia, megaloblastic anemia etc. Thrombocytopenia is often associated with prolonged bleeding and poor clot retraction.

MAKING A PERIPHERAL BLOOD SMEAR
Examination of the peripheral blood smear is the key investigation in the diagnosis of many hematological disorders. This is an economical and rapid means to diagnose many hematological and some non-hematological systemic ailments.

Requirements
• Glass slides
• Blood sample
• Capillary tube
• Spreader

Procedure
• Take a new or clean glass slide.
• Mix the blood sample well by gentle inversion of the tube.
• Gently invert the tube to an angle of 30° and hold the capillary tube in it.
• If the slides are to be prepared from direct skin puncture technique then directly aspirate the blood in the capillary tube after pricking.
• Under the capillary action the blood will be automatically aspirated in the capillary tube.
• Place a drop of blood on the slide from the capillary tube.
• Place the spreader on the front side of the drop of blood.
• Take the blood to the back side by spreader; it will help in equal distribution of blood with the edge of the spreader.
• Move the spreader in forward direction to make a smear.
• Keep the slide over hot plate for drying.

Microscopy

For microscopic examination of the blood film, divide the film in the three parts:

Head

Overcrowded areas, RBC’s overlap discrete RBC’s are not visible, white blood cells are shrunken and they cannot be differentiated from each other.

Tail

Only a few cells are present in this area; ample space is available, white blood cells are shrunken and they cannot be differentiated from each other.

RBC’s are placed far apart and appear totally hemoglobinized, (i.e. even distribution of Hb across the cell surface. False target cells may appear.

Body

This can be focused by looking at the proper field where RBCs are separately placed with a little overlapping. Such areas are easily in the body of the blood smear.

For leukocyte morphology the same area is also eminently suited. Leukocyte differential count is carried out in a strip which is perpendicular to the length of the slides by going up and down the slides but not sideways as this likely to give an inaccurate differential count. If one vertical strip does not contain 100 cells, then the next strip may be counted again by going upwards or downwards but not sideways.

STAINING A PERIPHERAL BLOOD SMEAR WITH LEISHMAN’S STAIN

Romanowsky’s stains are universally employed for staining blood and bone marrow smears in order to study their morphology and also for the differential leukocyte count. These stains also help in the morphological identification of various parasites and inclusions.

Principle

Romanowsky’s stains are polychrome stains which incorporate basic stain (methylene blue) and acidic stain (eosin) in their composition. Basic components of the cells stain with eosin while the acidic components take up methylene blue to produce shades of purple or blue. Neutral components of the cells probably take up both dyes, albeit very faintly. Methanol which is present in the stain acts as a solvent as well as a fixative.

Leishman’s stain

Leishman’s stain is the most commonly used Romanowsky’s stain in clinical laboratories. Hence its preparation, storage and used are described in detail.

Requirements

• Leishman’s stain
• Glass slides
• Microscope
• Staining racks
• Conical flask: 250ml
• pH meter flask: 1000 ml
• pH paper

Reagents
• Leishman’s powder : 0.2 g
• Methanol; acetone free : 100 ml
• Disodium hydrogen phosphate (Na₂HPO₄·2H₂O) : 3.76 g
• Potassium dihydrogen phosphate anhydrous (KH₂PO₄) : 2.10 g
• Distilled water : 1000 ml

Preparation of Leishman’s stain
• Take a conical flask of 250 ml volume and place a few glass beads in it.
• Rinse the flask a few times with acetone free methanol.
• Weigh 0.2 g of Leishman’s powder and transfer it to the flask.
• Add 50 ml of acetone free methanol.
• Warm the flask to 50°C with occasional shaking. When the stain is almost completely dissolved, adjust the volume to 100 ml by adding some more acetone free methanol.
• Filter the stain; it is ready for use. The stain can be stored at room temperature for an indefinite period.

Note
• Quality of the stain improves on standing. For better results it is recommended that the stain be used 1-2 weeks after it is prepared.
• In some laboratories dilute Leishman’s Stain is used. It is prepared by mixing one part of the Stock Stain (described above) to two parts of distilled water to produce 1:3 dilution of the stain.
• Do not store the dilute stain. Prepare enough for one day only.
• Preparation of buffered water
• Weigh 3.76 g of disodium hydrogen phosphate and 2.1 g of potassium dihydrogen phosphate and place them in a 1000 ml volumetric flask.
• Add sufficient amount of water to dissolve the salt. Bring the volume to 1000 ml mark with distilled water.
• Check the pH with a pH meter. It should be between 7.0 and 7.2. If pH meter is not available, a narrow range pH paper can also be used satisfactorily.

Note
If the pH is less than 7.0 add a small quantity of disodium hydrogen phosphate powder with constant stirring to ensure complete dissolution. Continue the process till pH is between 7.0 and 7.2. Conversely, if the pH is above 7.2 add potassium dihydrogen phosphate powder and bring the pH to the prescribed range.

Store it in refrigerator. It can be stored for an indefinite period if pH is maintained and molds do not grow in it.

Sample collection
EDTA anticoagulated blood or blood from skin puncture can be used to prepare blood smears.
Staining procedure

Undiluted Leishman’s stain:

- Cover the slides with Leishman’s stain for 2 minutes: this also ensures fixation of the smear due to the presence of methanol.
- Add buffered water twice the volume of the stain on the slide and gently with the stain of a horizontally held Pasteur pipette. Allow the staining to continue for another 5 minutes.
- Wash the stain off in a stream of buffered water till it acquires pink color.
- Remove the stain from the back of the slide, air dry and examine under the microscope.

Diluted Leishman’s stain

- Fix the smears in methanol for five minutes.
- Cover the smears with diluted Leishman’s stain for 10 minutes.
- Wash off the stain with buffered water.
- Add fresh buffered water for 2-3 minutes for differential staining of blood cells.

Interpretation

Scan the film and select an area where red cells barely touch each other. Study the morphology of the cells in this part of the blood smear. Staining characteristics of various blood cells as they appear under the light microscope are given in table 4.2.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Blue</td>
<td>Pale pink</td>
<td>Fine mauve</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Blue</td>
<td>Faint pink</td>
<td>Red orange red</td>
</tr>
<tr>
<td>Basophils</td>
<td>Blue</td>
<td>Pale pink</td>
<td>Blue – black</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Violet</td>
<td>Pale pink</td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>Blue</td>
<td>Grey blue</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>-</td>
<td>Dark pink</td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>Mauve</td>
<td></td>
</tr>
</tbody>
</table>

Table 33.4: Staining characteristics of blood cells stained with Leishman’s stain

Significance

Leishman’s stain is the most commonly used stain in laboratories. It used for the differential staining of the cytoplasm and the granules of the leukocytes.

Precautions

- For Leishman’s stain the pH must be carefully monitored as it affects the quality of the staining reaction.
- Keep the slides horizontal so that the stain covers them evenly and uniformly.
- Washing in the buffered water must be adequate to ensure proper color development and differentiation.
- Do not make blood smears from samples which are more than 4 hours old as the granulocytes start to show degenerative changes.
DIFFERENTIAL LEUKOCYTE COUNT (DLC)

Differential leukocyte count is the percent distribution of various white cells in the peripheral blood as determined from the examination of blood smear stained with Romanowsky’s stains (Leishman’s, Wright’s etc).

Requirements
- Leishman’s stain
- Buffer
- Water bath
- Glass slides
- Microscope

Collection of sample
- EDTA anticoagulated blood
- Blood from skin puncture (non-anticoagulated) may also be used.

Three major steps are required in the differential leukocyte count:
- Preparation of peripheral blood smear
- Staining of the smears with Romanowsky’s stain
- Microscopic examination of the stained smear

Procedure
- Make properly spread blood film for accurate identification of white cells.
- Stained the slides with any of the familiar Romanowsky’ stains. Leishman’s stain is most commonly used in the clinical laboratories.
- Dry the slides.
- Observe them under the microscope by using 40X objective lens.
- Differential leukocyte count is carried out in a strip which is perpendicular to the length of the slides by going up and down the slides but not sideways as this likely to give an inaccurate differential count. If one vertical strip does not contain 100 cells, then the net strip may be counted again by going upwards or downwards but not sideways.
- Calculate the percentage of each type of leucocytes.

Result

Differential leukocyte count of the given sample of blood is:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Neutrophil</td>
<td>_______ %</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>_______ %</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>_______ %</td>
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<tr>
<td>Monocyte</td>
<td>_______ %</td>
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</tbody>
</table>

Normal differential leukocyte count in an adult is shown in table 33.5:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Normal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>40 – 75</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>25 – 40</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1 – 5</td>
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<tr>
<td>Monocyte</td>
<td>2 – 6</td>
</tr>
<tr>
<td>Basophils</td>
<td>0 – 1</td>
</tr>
</tbody>
</table>

Table 33.5: Normal differential leukocyte count
Differential leukocyte count (DLC)

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Absolute leukocyte count

Absolute leukocyte count has an important significance in diagnosing various blood disorders like lymphocytosis, eosinophilia, monocytosis etc.

Absolute leukocyte count: Total leukocyte count x percentage observed.

Clinical significance

Differential count is vital for the diagnosis of a number of hematological disorders involving either red cells or white cells. Its primary use to identify changes in the percent distribution of white cells which may be related to specific type of disorders like infection (bacterial, viral or parasitic) or leukemias (myelogenous, lymphocytic and monocytic). Rise in specific white cells such as neutrophilia, eosinophilia, lymphocytosis and monocytosis or their fall such as neutopenia and eosinopenia are based on the differential count.

Precautions

- Smear should be prepared properly.
- Smear must be stained properly.
- Differential count must be carried out on an area between the body and tail where the cells appear in a monolayer using oil immersion magnification.
- Move the slide up and down to an angle about 90 degree during counting.

**RETICULOCYTE COUNT**

This is a simple test to evaluate the bone marrow activity and the rate of red cell production. Because of the variations in the rate of reticulocyte maturation and their release, the reticulocyte count does not always reflect the absolute erythroid activity. However, the ease with which reticulocytes can be counted serially in the same individual makes reticulocyte count a practical way to evaluate changes in the rate of cell production.
Principle

Reticulocytes are juvenile red cells. They contain remnants of ribosomes and ribonucleic acid which take up certain basic dyes such as azure B, brilliant cresyl blue or new methylene blue and form precipitates which appear as granules and filaments. Films are prepared and cells containing precipitates are counted.

Requirements

- Test tubes
- Pasteur pipettes with teats
- Heparinized capillary tubes (Optional)
- Glass slides
- Microscope
- Water bath
- Watch

Reagents

In most clinical laboratories the stains which are employed for reticulocyte count are the new methylene blue and the brilliant cresyl blue. Both are equally reliable and the staining principles for both of them are the same.

New Methylene Blue (NMB)

- New methylene blue : 0.5 g
- Sodium chloride pure grade : 0.7 g
- Sodium oxalate : 0.13 g
- Deionized water : 100 ml

Take 0.7g sodium chloride 0.13g sodium oxalate and 0.5g new methylene blue; mix well in 100ml of deionized water. Filter it before use. Store at 4°C in a refrigerator. Discard after 4 months.

Brilliant cresyl blue (BCB)

- BCB selt : 1.0 g
- Sodium chloride (0.9%) : 80 ml
- Sodium citrate (3.2% or 3.8%) : 20 ml

Mix 80ml of 0.9 sodium chloride solution and 20ml of 3.2% (anhydrous) or 3.8% (hydrated) sodium citrate solution. Add 1.0g of BCB salt; shake well and filter. Store in a refrigerator. The stain is stable for an indefinite period.

Sample collection

Take fresh EDTA mixed blood or heparinized capillary blood and test within 2 to 3 hours. Because ribosomes tend to disaggregate on storage, samples which are processed after a delay of 6 hours or more have a lower reticulocyte count as compared to those which are processed soon after collection. Also older samples show artifacts and are not recommended for reticulocyte count.

Procedure

- Add equal volumes of freshly filtered dye solution and blood sample in a test tube.
- Mix well and incubate at 37°C for 15 minutes.
- Resuspend red cells by frequent but gentle shaking during incubation.
• Make films on glass slides and air dry them. Examine under the microscope using an oil immersion lens without fixing staining.

Reticulocyte counting

Count the number of cells containing any blue granules or reticulum among 1000 red cells (including the reticulocytes). For an accurate count choose area where cells do not overlap. Areas where red cells are in clumps and areas of rouleaux formation are not suitable for reticulocyte count.

Calculation of results

Reticulocyte count can be reported either as percentage (number of reticulocytes per 1000 red cells) or as reticulocyte index or in absolute number per liter of blood.

• Percent reticulocyte count (PRC) This can be calculated as follows:

\[
\text{Reticulocyte count \%} = \frac{\text{No. of reticulocyte counted} \times 100}{\text{No. of RBC including reticulocytes}}
\]

• Absolute reticulocyte count: (ARC)

\[
\text{Absolute reticulocyte count/liter} = \frac{\% \text{ reticulocyte count} \times \text{ RBC count/\mu l}}{100}
\]

Since red cell count is now readily available through automated blood analyzers, it is recommended to calculate and report reticulocytes in absolute number rather than as percentage.

Clinical significance

Counting of reticulocytes in a peripheral blood is a simple and direct way to assess the effective rate of red cell production by the bone marrow. When the bone marrow is very active (e.g. in hemolytic anemia or active blood loss) their number increases. This is known as reticulocytosis and in case of aplastic anemia, the reticulocyte count decreases.

BLEEDING TIME

Bleeding starts if the skin of the fingertip is pricked with a needle; it continues for some time before it finally stops. Time interval between puncturing of the skin and the arrest of bleeding is called bleeding time. Bleeding time is a measure of the number and the function of the platelets and the integrity of the microvasculature.

Principle

A standard prick/incision is made in the skin and the time required for the bleeding to stop is noted.

There are two methods for the determination of bleeding time:

• Duke’s method
• Ivy’s Method
In Duke’s method a prick is given on the fingertip or the ear lobe. In children heel is the preferred site for this purpose. In Ivy’s method two incisions are made on the forearm and the average of the bleeding time of the two incisions is reported as the bleeding time.

**Duke’s method**

Most of the laboratories determine bleeding time by this method as it is easy to perform and requires only a minimum of the equipment.

**Apparatus and reagents**
- Disposable blood lancets
- Circular filter paper or blotting paper
- Stop watch
- Cotton swabs
- Methylated spirit or 70% ethanol

**Procedure**
- Ask the patient to be seated comfortably.
- Brief him about the procedure in simple words and in his own language
- Select the middle finger of the left hand for the prick (other fingers can also be used in case the middle finger cannot be pricked).
- Clean the fingertip thoroughly with a spirit swab.
- Allow the skin to dry because on wet skin the blood spreads and the drop will not form.
- Remove the paper wrapping of a sterilized disposable blood lancet.
- Hold the lancet firmly in the hand by its body.
- Bring the lancet near the fingertip and puncture it quickly to a depth of about 3 mm. To prick the ear lobe place a glass side behind the ear lobe and hold it firmly. This provides a stable base for the prick.
- Start the stop watch as soon as possible as blood flows out freely from the puncture site.
- Touch the edge of the filter paper with drop of blood after 15 seconds. DO NOT WIPE THE BLOOD.
- Repeat the procedure after every 15 seconds; use a separate area of the filter paper on each occasion.
- Continue with the procedure till no blood stain is detected on the filter paper, stop the watch immediately.
- Express the bleeding time in minute and seconds.

**Results**
Normal range for bleeding time by Duke’s method is 2-6 minutes. Values between 6 and 10 minutes are viewed as borderline while bleeding beyond 10 minutes is distinctly abnormal.

**Interpretation**

Bleeding time (BT) is prolonged in:
- Thrombocytopenia

In this condition bleeding time is prolonged when platelet count falls below 60x10³/µl as exemplified by immune thrombocytopenic purpura (ITP), aplastic anemia etc.

Platelet function defects

Some of the conditions that are characterized by platelet function defects are von-Willebrand’s disease, thrombasthenia, aspirin ingestion etc.
WHOLE BLOOD CLOTTING TIME

Whole blood clotting time is an insensitive test for assessing the efficacy of hemostasis. Earlier when better and more precise procedures were not available this test furnished some insight into the problems of coagulation. With the availability of more precise and sensitive tests, whole blood clotting time test has been relegated to a position of low priority in most clinical laboratories. Its role in monitoring heparin therapy has also been superseded by APTT and other tests. Nevertheless whole blood clotting time is still performed and furnishes useful informations like the clot size, clot retraction and clot lysis if the clot is periodically examined after clotting has taken place.

There are two methods that are in vogue for determining clotting time in vitro:

- Capillary method
- Tube method (Lee & White method)

Capillary method
This method is used if venous blood cannot be obtained. It is an insensitive method and at present it is only a replica of the past.

Apparatus
- Stop watch
- Cotton swabs
- Capillary tubes
- Disposable blood lancets
- Methylated spirit or 70% ethanol

Principle
Formation of fibrin threads is noted by breaking the capillary tube at regular intervals.
Time taken from the beginning of the skin bleeding to the first appearance of a fibrin thread in the capillary tube is the clotting time.

Procedure
- Ask the patient to be seated comfortably.
- Brief the patient about the procedure.
- Select the left hand for a deep skin puncture.
- Clean the tip of the middle finger with a spirit swab.
- Allow the skin to dry for at least one minute.
- Remove the wrapping of a disposable blood lancet: do not touch the tip of the lancet.
- Hold the lancet in your right hand in such a way that the thumb is on one side while the index finger is on the other side of the body of the lancet. This ensures a firm grip on the lancet.
- Bring the tip of the lancet near the fingertips and puncture the skin to a depth of 3 mm; wipe off the first ‘show’ of blood.
- Start the stop watch as soon as blood begins to ooze from the fingertip freely and rapidly.
- Touch the tip of a capillary tube with the drop of blood. The tube will fill by capillary action. Make sure that no air bubble enters the tube during its filling. After the capillary is completely filled, wait for 2 minutes and then break off a small piece of the tube 1 to 2 cm from one end.
• Repeat this step every 30 seconds till a fibrin thread appears between the broken ends when they are moved apart.
• Stop the watch and note the time from the appearance of blood over the fingertip to the formation of the first fibrin thread in the capillary tube.

Precautions
• Warm or massage the finger to be pricked before the procedure; this is to improve the blood flow.
• Avoid squeezing the puncture site to obtain blood as tissue fluid will mix with blood and shorten the clotting time.
• Capillary tube should be placed adjacent to the drop of blood; at an angle so that the distal end of the capillary tube is at a lower level than the proximal end. This will facilitate the filling of the capillary tube by gravity.
• Avoid air bubbles in the capillary tube because they reduce the quantity of blood.
• Always fill 2 or 3 capillary tubes at the same time as clotting time may be prolonged and more than one tube may have to be broken before the blood finally clots.

Lee and white clotting time or tube method:

This is a more reliable and sensitive method than the capillary method for the determination of clotting time. It however requires a little more elaborate apparatus for venipuncture including water bath for controlled temperature.

Requirements
• Disposable plastic syringes
• Glass tubes 12x75mm
• Water bath at 37°C
• Spirit swabs
• Tourniquet
• Stop watch

Procedure
• Place three glass tubes in a rack in a water bath at 37°C.
• Collect over 3 ml of venous blood by venepuncture which must be done neatly and quickly. Start the stop watch as soon as the blood enters the syringe.
• Remove the needle from the syringe and dispense 1 ml of blood to each of the three glass tubes.
• After 3 minutes remove the first tube from the water bath and tilt it gently through an angle of 45° to see if blood has clotted. Return the tube to the water bath if there is no clot. Examine this tube at 30 seconds intervals for the formation of a clot. When blood clots, the tube can be tilted through an angle of 90° without spilling its contents.
• Examine the second tube as soon as the blood clots in the first tube.
• In the second tube blood will clot soon after the clot appears in the first tube.
• Repeat the same procedure with the third tube.
• Stop the watch and note the time when the blood clots in the third tube.

Clotting time is the time which lapses between the entries of blood in the syringe to the formation of the clot in the third tube.

Normal value = 4-11 minutes

Precautions
• Temperature of the water bath must be maintained at 37°C.
• Glass tubes should be dry, clean and of the same size.
• Glass tubes should be gently tilted without agitating the blood excessively.
• Venipuncture should be neat and clean.
• Tourniquet should not be applied tightly.
Precautions
- If venipuncture is not clean, some tissue fluid may enter the syringe with the blood sample and shorten the clotting time.
- Vigorous agitation of blood during tilting may shorten the clotting time.
- Technical factors that may alter clotting time include:
  - Size of the tube
  - Cleanliness of the tube
  - Volume of blood in the tube
  - Temperature of the water bath

Clinical significance
Coagulation of blood has physiological as well as pathological significance. Deficiency of clotting factors may be suspected by determining the clotting time which is prolonged in many diseases e.g. afibrinogenemia, liver disease, obstructive jaundice and congenital deficiency of clotting factors.

Clotting time is prolonged in hemophilia but is normal in thrombocytopenia. It is also prolonged during treatment with anticoagulants like heparin and warfarin.

It may of interest to note that clotting time is normal in factor VII deficiency. This is because in vitro coagulation is dependent on contact factor activation.

ABO GROUPING
Cell grouping
Principle
Red cells are reacted with known antisera (anti A, anti B and anti AB). Agglutination indicates the presence of corresponding antigens on the surface of the red cells.

Tube method
Sample: 3-5 ml clotted blood or EDTA anticoagulated blood

Material
- Round bottom glass tubes
- Pasture pipettes
- Isotonic saline (0.9%)
- Centrifuge
- Blood grouping antisera (anti-A, anti-B and anti-AB)

Procedure
- Wash the red blood cells to be tested 3-4 times with isotonic saline.
- Prepare 5% red cells suspension in saline.
- Label the test tubes as Anti-A, Anti-B and Anti-AB.
- To the labeled test tubes, place one drop of Anti-A, Anti-B and Anti-AB sera.
- Mix all tubes well and centrifuge for 15-30 seconds at approximately 3,500 rpm.
- Gently disperse cell button and inspect for the presence of agglutination/hemolysis using a well-lighted background and record result.
- Incubate all negative tests at room temperature for 10 minutes.
- Mix well centrifuge and observe for agglutination and/or hemolysis.
- Record the results.
- All negative results should be examined microscopically.
Tile method
This method is although satisfactory but is less sensitive than the tube method.
Sample: 3-5 ml Clotted blood.

Requirements
- Opaque white tile permanently ruled out
- Pasture pipettes
- Mixing sticks
- Blood grouping antisera for cell grouping (anti-A, anti-B and anti-AB)

Procedure
- Wash the red blood cells to be tested 3-4 times with isotonic saline.
- Prepare 35% - 45% suspension of red cells.
- Place one drop of each sera Anti-A, Anti-B, Anti-AB to appropriately labeled tile.
- Add one drop of cell suspension of red cells to be tested to each drop of reagent.
- Using separate clean applicator sticks mix each cell reagent mixture over an area of approximately 20-40mm.
- Slowly rotate the tile and observe for macroscopic agglutination for a period not to exceed 2 minutes.
- Record the results.

Serum grouping
Principle
Serum is reacted with known A cells, B cells and O cells. Agglutination indicates the presence of corresponding antibodies in the reacted serum.
Sample: 3-5 ml Clotted blood.
Tube method
Sample: 3-5 ml clotted blood or EDTA anticoagulated blood.

Requirements
- Round bottom glass tubes 4 (75mm x 12mm)
- Pasture pipettes
- Isotonic saline 0.9%
- Centrifuge
- Known A cells, B cells and O cells

Procedure
- Wash the known red blood cells 3-4 times with of isotonic saline.
- Prepare 5% red cells suspension in saline.
- Label the test A cells, B cells, O cells and auto control.
- To tubes labeled as A cells, B cells, O cells and auto control add 2 drops of serum.
- Add one drop of suspension of A1 cells, B cells, and O cells to appropriate tubes containing serum.
- Mix all tubes well and centrifuge for 15-30 seconds at approximately 3,500 rpm.
- Gently disperse cell button and inspect for presence of hemolysis/agglutination, using a well-lighted background and record the results.
- Incubate all negative tests at room temperature for 10 minutes.
- Mix well, centrifuge and observe for agglutination and /or hemolysis.
- Record the results.
- All negative results should be examined microscopically.
Tile method
Requirements
- Opaque white tile permanently ruled out
- Pasture pipettes
- Mixing sticks
- Stop watch or timer
- Known A cells, B cells and O cells

Procedure
- Wash the known red blood cells 3-4 times with isotonic saline.
- Prepare 35% - 45% suspension of red cells.
- Add 2 drops of patient serum to columns labeled as A cells, B cells and O cells.
- Add one drop of 35-45% suspension of known A cells, B cells and O cells to the respective columns.
- Using separate clean applicator sticks mix each cell reagent mixture over an area of approximately 20-40mm.
- Slowly rotate the tile and observe for macroscopic agglutination for a period not to exceed 2 minutes.
- Record the results.

<table>
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<th>Anti B</th>
<th>Anti AB</th>
<th>A1 Cells</th>
<th>B Cells</th>
<th>O Cells</th>
<th>Auto Control</th>
<th>Results</th>
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<td>Bombay</td>
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</tbody>
</table>

Table 33.6: Interpretation of results of ABO grouping
{+} Positive  = Cells are agglutinated, {-} Negative = Cells are not agglutinated

Rh TYPING
Rh positive and Rh negative refers to the presence or absence of the D antigen on the red cells. The principle of detection of D antigen on red cell is the same as that for ABO antigens.

Tile method
Sample: 3-5 ml clotted blood or EDTA anticoagulated blood

Material
- Opaque white tile permanently ruled out
- Pasteur pipettes
- Mixing sticks
- Anti-D

Procedure
- Place one drop of Anti-D on a labeled tile.
- Add 2 drops of 35-45% suspension of red cells in saline to be tested.
- Mix with clean stick over a circle area of approximately 20-40 diameter.
• Gently rotate the tile and observe for agglutination within 2 minutes.
• Record the results.
• In order to detect weak forms of the D antigen, Du test should be performed on all samples that give negative or doubtful positive reaction by tile method.

Tube method
• Prepare 5% suspension of RBC's to be tested in isotonic saline.
• Place one drop of anti-D in a labeled test.
• Add one drop of the 5% suspension of RBC's to be tested in the tube.
• Mix the contents of the tubes thoroughly.
• Centrifuge the tube at 3500rpm for 15-30 seconds.
• Gently resuspend each cell button from the bottom of the tube and observe macroscopically for agglutination.
• Record the results.
• Check negative results under the microscope.
• If agglutination is < 2+, perform D\textsuperscript{u} test.

DIRECT ANTIGLOBULIN TEST (DAT)
Direct antiglobulin test is used to demonstrate whether or not red cells have been coated with antibody in vivo.

Requirements
• Sample
• Antihumangammaglobulin
• Centrifuge

Procedure
• Place one drop of 5% saline suspension of red cells to be tested in two test tubes.
• Labeled as DAT negative blank.
• Wash the red cells four times in large volume of saline. Care should be taken for adequate removal of the supernatant after each wash.
• Add 2 drops of anti-human globulin and two drops of normal saline to the labeled tubes and mix the contents of the tube thoroughly.
• Centrifuge the tube for 15-30 seconds at 3500rpm. Gently resuspend the cells button and examine for agglutination.

Note: The manner in which the red cells are dislodged from the bottom of the tube is of great importance. The tube should be held at angel shaken gently until all cells are dislodged, then tilted gently back and forth until an even suspension of cells or agglutination is observed.

• Record the results.
• All negative reactions should be examined microscopically. Confirm the validity of all negative or weakly positive reactions with IgG sensitized red cells.

Results
• Positive DAT: Agglutination of red cells at the antiglobulin phase
• Negative DAT: No agglutination of red cells at the antiglobulin phase

Applications
• Hemolytic disease of newborn (newborn)
• Hemolytic transfusion reactions
• Autoimmune hemolytic anemia

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• Drug induced immune hemolytic anemia
• Hyper gammaglobulinemia

**INDIRECT ANTIGLOBULIN TEST (IAT)**

The IAT is used for the detection of antibodies that may cause red cells sensitization in vitro. If both IgG antibody and the corresponding antigen are present in the serum, incubation will cause the antibody to attach to the specific antigenic receptors on the red cell. After washing, to remove the excess antibody in the serum, the addition of antiglobulin serum will link the IgG molecules on neighboring red cells producing agglutination.

**Procedure**

- Place 2-3 drops of the serum in a labeled test tube.
- Add one drop of 5% of suspension of donor red cells.
- Mix the content of the tube thoroughly and incubate at 37°C for 45 minutes.
- Centrifuge at 3500 rpm for 15-30 seconds.
- Examine supernatants for hemolysis gently re-suspend cells button and examine for agglutination.
- Record the results.
- Wash the cells four times with normal saline being careful to decant completely after each wash.
- Add two drops of antihumangammaglobulin to the tube. Mix the contents of the tube thoroughly and centrifuge. Observe for agglutination under the microscope.
- Record the results.
- Confirm the validity of negative or weakly positive results with IgG sensitized red cells.

**Results**

- Positive Antiglobulin test: Agglutination of the red cells at the antiglobulin phase.
- Negative Antiglobulin test: No agglutination of red cells at the antiglobulin phase.

**Applications**

- Antibody detection
- Antibody identification
- Antibody titration
- Red cell phenotyping D\textsuperscript{u}
- Hemolytic disease of newborn (mother)

**Suggested further reading**

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Dedicated to

All undergraduate medical students
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