16MBU411 **IMMUNOLOGY – PRACTICAL** **(4H – 2C)**

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 6 Hours

SCOPE

Introducing the science of immunology and to study various types of immune systems their classification structure and mechanism of immune activation.

OBJECTIVES

- To strengthen the knowledge of students in immunodiagnostics.
- To learn the latest trends in immunology.
- Rapid diagnosis and Immune reaction.

EXPERIMENTS

1. Identification of human blood groups.
2. Perform Total Leukocyte Count of the given blood sample.
3. Perform Differential Leukocyte Count of the given blood sample.
4. Separate serum and plasma from the blood sample (demonstration).
5. Perform immunodiffusion by Ouchterlony method.
6. Perform DOT ELISA.
7. Perform immunoelectrophoresis.

SUGGESTED READINGS

1. Goldsby RA, Kindt TJ, Osborne BA. (2007). Kuby's Immunology. 6th edition, W.H. Freeman and Company, New York.
2. Delves P, Martin S, Burton D, Roitt IM. (2006). Roitt's Essential Immunology. 11th edition, Wiley- Blackwell Scientific Publication, Oxford.
3. Murphy K, Travers P, Walport M. (2008). Janeway's Immunobiology. 7th edition, Garland Science Publishers, New York.
4. Abbas AK, Lichtman AH, Pillai S. (2007). Cellular and Molecular Immunology. 6th edition, Saunders Publication, Philadelphia.
5. Peakman M, and Vergani D. (2009). Basic and Clinical Immunology. 2nd edition Churchill Livingstone Publishers, Edinberg.
6. Richard C and Geiffrey S. (2009). Immunology. 6th edition. Wiley Blackwell Publication.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021.

16MBU411
IMMUNOLOGY – PRACTICAL

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Expt 1: Identification of blood group

Objective:

To understand the basic concept of Blood Grouping.

Principle:

It was in 1901, that Austrian-American immunologist and pathologist **Karl Landsteiner** discovered human blood groups. Karl Landsteiner's work helps to determine blood groups and thus opened a way for blood transfusions which can be carried out safely. He was awarded the **Nobel Prize in Physiology or Medicine** in 1930 for this discovery.

Death of the patient was the result in most cases before 1900, when blood transfusion was attempted. Blood transfusion was made much safer by the discovery of blood groups, as blood of the same ABO group could be chosen for each patient. However, there were still many cases of unexplained blood transfusion reactions. Biologists still went in search of these unexplained questions.

In 1902, the fourth main type, AB was found by Decastrello and Sturli. It was the observations of Levine and Stetson in 1939, and Landsteiner and Weiner in 1940, that laid the foundations of our knowledge about the remaining major blood group- the Rhesus system. Once reliable tests for Rhesus grouping had been established, transfusion reactions became rare! For this discovery Landsteiner was awarded the Nobel Prize in Physiology or Medicine in 1930.

The Components of Blood

The circulatory system distributes about 4-6 liters of blood to the adult human body. The blood mainly has 2 portions: **the Plasma** and**the Blood Cells**. Plasma is mainly composed of water, but contains different types of proteins and other chemicals such as: enzymes, glucose, fat particles, salts ,hormones , antibodies etc. It constitutes about 60% of the blood. Blood cells can be observed under a microscope on staining. The formation of blood cells occur in the bone marrow by the 'Hematopoietic stem cells'. They can be divided into 3 basic cell types:

Erythrocytes- Red Blood Cells (RBC):

As the name suggests, these red coloured cells give blood its red colour. (The word erythrocyte is from erythro-Gk.meaning red and Latin-cytos meaning cell.) 1 ml of blood contains approximately 5 million RBCs! The proportion of blood occupied by red blood cells is referred to as the hematocrit, and is normally about 45%. Mature RBCs are biconcave in shape, lack a Nucleus and many other organelles. They circulate in the system for about 120 days, carrying out their job, i.e., to supply oxygen.
This function is carried out by them most efficiently. since they are rich in an Iron-containing biomolecule called haemoglobin. Haemoglobin has high affinity for oxygen, thus binds to it and is transported from the alveoli (in lungs) to every part of the body. There is constant replenishment of RBCs in order to remove old cells that break down. (This process is carried out

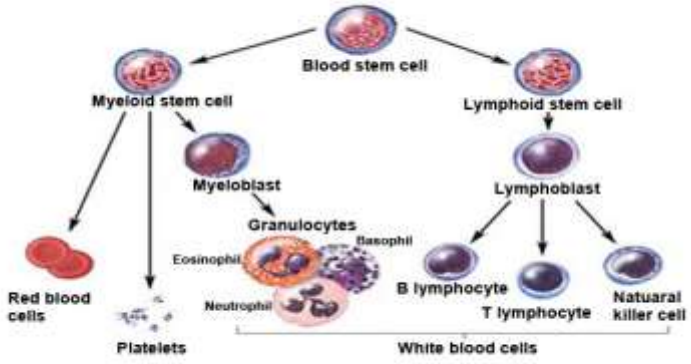
in organs like the liver, also producing by-products like bile pigments.) Millions of cels are released into the bloodstream from the bone marrow each day.

Leukocytes- White Blood Cells:

These cells are key players in our immune system. They are of different types such as neutrophils, lymphocytes, eosinophils, monocytes, basophils. Each of them have a variety of functions in our immunity. 1 ml of blood of an adult human contains about 4,000-11,000 leukocytes. Basically, they destroy and remove old or aberrant cells and clear cellular debris, as well as attack foreign substances and infectious agents (pathogenic entities).

Thrombocytes- Platelets:

The coagulation or blood clotting process is taken care of by them. They act on clotting proteins like Fibrinogen, converting it into Fibrin. They create a mesh onto which RBCs collect and form a clot. This prevents excessive blood loss and also checks the entry of pathogens into the body. 1 ml of blood of an adult human contains about 200,000-500,000 platelets.



The observations that led to the discovery of blood groups:

At times, it was observed that mixing blood from two individuals led to blood clumping or agglutination. Later it was understood that the agglutinated red cells can clog blood vessels and stop the circulation of the blood to various parts of the body. The agglutinated red blood cells also crack and their contents leak out in the body.

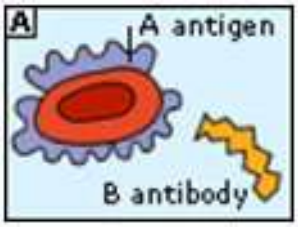
The RBCs contain haemoglobin which becomes toxic when outside the cell. This must have been the phenomena that occurred in the blood transfusion cases that ended up with fatality of the patient at the receiving end. Karl Landsteiner discovered that blood clumping was an immunological reaction which occurs when the receiver of a blood transfusion has antibodies against the donor blood cells! People learned that, compatibility of blood groups needed to be

checked before anything else was done. If they are not, the red blood cells from the donated blood will agglutinate. This can have fatal consequences for the patient.

ABO blood grouping system:

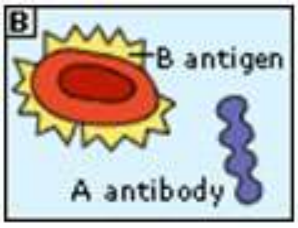
According to the AB0 blood group system there are four different kinds of blood groups: **A, B, AB and O** (null).

Blood group A



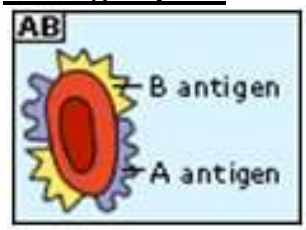
Blood group A: If you belong to the blood group A, you have A antigen on the surface of your red blood cells and B antibodies in your blood plasma

Blood group B



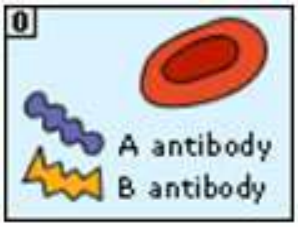
Blood group B: If you belong to the blood group B, you have B antigen on the surface of your red blood cells and A antibodies in your blood plasma

Blood group AB



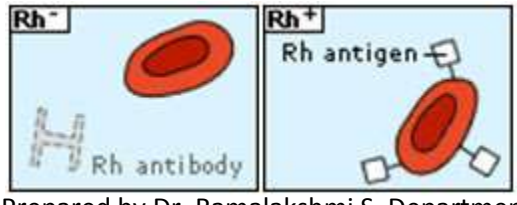
Blood group AB: If you belong to the blood group AB, you have both A and B antigens on the surface of your red blood cells but, neither A nor B antibodies in your blood plasma

Blood group O



Blood group O: If you belong to the blood group O (null), you have neither A nor B antigens on the surface of your red blood cells, but you have both A and B antibodies in your blood plasma

Rh factor

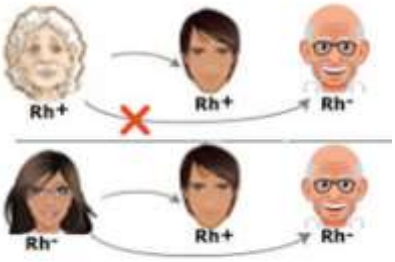


Rh (Rhesus) factor is found on the RBC's surface in most people. Like A and B, this is also an antigen and those who have it are called Rh+. Those who lack the antigen on the surface of RBCs are called Rh-. A person

with Rh- blood does not have Rh antibodies naturally in the blood plasma. But a person with Rh- blood can develop Rh antibodies in the blood plasma if he or she receives blood from a person with Rh+ blood, whose Rh antigens can trigger the production of Rh antibodies (as the immune system is triggered by the presence of an unknown antigen in the system). A person with Rh+ blood can receive blood from a person with Rh- blood without any problems.

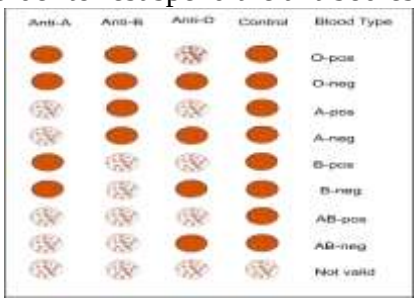
Inheritance of Blood Groups:

Blood groups for each individual are determined by genes or alleles (small packets of information in cells contained in the DNA) which are inherited from both parents. Genes for the Rh-ve and O groups from one parent are masked (i.e., they are recessive) by the presence of Rh+ve and A or B genes from the other parent. That is, O and Rh negative genes only produce an effect when there is a "double dose" of such genes, i.e., one from each parent (homozygous condition). Thus, people who are apparently A or B Rh+ve may also carry genes for the O and Rh-ve blood groups which can be inherited by their children.



Principle behind blood tests: ***Blood clumping or Agglutination observation.***

Compatibility between the blood groups of donor and recipient determines the success of a blood transfusion. The AB0 and Rh blood groups are looked at while conducting the test. In a diagnostic lab, Monoclonal antibodies are available for A, B and Rh antigen. Monoclonal antibody against Antigen A (also called Anti-A), comes in a small bottles with droppers; the monoclonal suspension being BLUE in colour. Anti-B comes in YELLOW colour. Anti-D (monoclonal antibody against Rh) is colourless. All the colour codes are universal standards. When the monoclonal antibodies are added one by one to wells that contain the test sample (blood from patient), if the RBCs in that particular sample carry the corresponding Antigen, clumps can be observed in the corresponding wells. A drop of blood is left without adding any of the antibodies; it is used as a control in the experiment. The monoclonal antibody bottles should be stored in a refrigerator. It is recommended to tilt the bottle a couple of times before use in order to resuspend the antibodies that have settled at the bottom of the bottle.



Blood Group	Antigens	Antibodies	Can Donate To	Can Receive from
A Rh+	A and Rh	B	A Rh+ AB Rh+	A Rh+ A Rh- O Rh+ O Rh-
A Rh-	A	B (Can develop Rh antibodies)	A Rh+ A Rh- AB Rh+ AB Rh-	A Rh- O Rh-
B Rh+	B and Rh	A	B Rh+ AB Rh+	B Rh+ B Rh- O Rh+ O Rh-
B Rh-	B	A (Can develop Rh antibodies)	B Rh+ B Rh- AB Rh+ AB Rh-	B Rh- O Rh-
O Rh+	Rh	A and B	O Rh+ A Rh+ B Rh+ AB Rh+	O Rh+ O Rh-
O Rh-	None	A and B (Can develop Rh antibodies)	AB Rh+ AB Rh- A Rh+ A Rh- B Rh+ B Rh- O Rh+ O Rh-	O Rh-
AB Rh+	A, B and Rh	None	AB Rh+	AB Rh+ AB Rh – A Rh+ A Rh- B Rh+ B Rh- O Rh+ O Rh-
AB Rh-	A and B	None (Can develop Rh antibodies)	AB Rh+ AB Rh-	AB Rh- A Rh- B Rh- O Rh-

Expt 2: Total Leukocyte Count (TLC)

Aim:

to perform Total leukocyte count of the given blood sample.

Introduction:

Total leukocyte count (TLC) refers to the number of white blood cells in 1 µl of blood (or in 1 liter of blood if the result is expressed in SI units). There are two methods for estimation of TLC:

- Manual or microscopic method
- Automated method

A differential leukocyte count should always be performed along with TLC to obtain the absolute cell counts.

The purpose of carrying out TLC is to detect increase or decrease in the total number of white cells in blood, i.e. leukocytosis or leukopenia respectively. TLC is carried out in the investigation of infections, any fever, hematologic disorders, malignancy, and for follow-up of chemotherapy or radiotherapy.

Principle

A sample of whole blood is mixed with a diluent, which lyses red cells and stains nuclei of white blood cells. White blood cells are counted in a hemocytometer counting chamber under the microscope and the result is expressed as total number of leukocytes per µl of blood or per liter of blood.

Equipment

(1) Hemocytometer or counting chamber with coverglass: The recommended hemocytometer is one with improved Neubauer rulings and metallized surface. There are two ruled areas on the surface of the chamber. Each ruled area is 3 mm × 3 mm in size and consists of 9 large squares with each large square measuring 1 mm × 1 mm. When the special thick coverglass is placed over the ruled area, the volume occupied by the diluted blood in each large square is 0.1 ml. In the improved Neubauer chamber, the central large square is divided into 25 squares, each of which is further subdivided into 16 small squares. A group of 16 small squares is separated by closely ruled triple lines. Metallized surface makes background rulings and cells easily visible. The 4 large corner squares are used for counting leukocytes, while the central large square is used for counting platelets and red blood cells. Only special coverglass, which is intended for use with hemocytometer, should be used. It should be thick and optically flat. When the special coverglass is placed on the surface of the chamber, a volumetric chamber with constant depth and volume throughout its entire area is formed. Ordinary cover slips should never be employed since they do not provide constant depth to the underlying chamber due to bowing.

When the special cover glass is placed over the ruled area of the chamber and pressed, Newton's rings (colored refraction or rainbow colored rings) appear between the two glass surfaces; their formation indicates the correct placement of the cover glass.

(2) Pipette calibrated to deliver 20 µl (0.02 ml, 20 cmm): WBC bulb pipettes, which have a bulb for dilution and mixing (Thoma pipettes) are no longer recommended. This is because blood and diluting fluid cannot be mixed adequately inside the bulb of the pipette. Bulb pipettes are also difficult to calibrate, costly, and charging of counting chamber is difficult. Tips of pipettes often chip easily and unnecessarily small volume of blood needs to be used.

(3)Graduated pipette, 1 ml.
(4)Pasteur pipette
(5) Test tube (75 × 12 mm).

Reagent

WBC diluting fluid (Turk's fluid) consists of a weak acid solution (which hemolyzes red cells) and gentian violet (which stains leucocyte nuclei deep violet). Diluting fluid also suspends and disperses the cells and facilitates counting. Its composition is as follows:

- Acetic acid, glacial 2 ml
- Gentian violet, 1% aqueous 1 ml
- Distilled water to make 100 ml

Specimen

EDTA anticoagulated venous blood or blood obtained by skin puncture is used. (Heparin should not be used since it causes leukocyte clumping). While collecting capillary blood from the finger, excess squeezing should be avoided so as not to dilute blood with tissuefluid.

Method

(1) Dilution of blood: Take 0.38 ml of diluting fluid in a test tube. To this, add exactly 20 µl of blood and mix. This produces 1:20 dilution. Alternatively, 0.1 ml of blood can be added to 1.9 ml of diluting fluid to get the same dilution.
(2) Charging the counting chamber: Place a coverglass over the hemocytometer. Draw some of the diluted blood in a Pasteur pipette. Holding the Pasteur pipette at an angle of 45° and placing its tip between the coverglass and the chamber, fill one of the ruled areas of the hemocytometer with the sample. The sample should cover the entire ruled area, should not contain air bubbles, and should not flow into the side channels. Allow 2 minutes for settling of cells.
(3) Counting the cells: Place the charged hemocytometer on the microscope stage. With the illumination reduced to give sufficient contrast, bring the rulings and the white cells under the focus of the low power objective (× 10). White cells appear as small black dots. Count the number of white cells in four large corner squares. (To reduce the error of distribution, counting of cells in all the nine squares is preferable). To correct for the random distribution of cells lying on the margins of the square, cells which are touching the left hand lines or upper lines of the square are included in the count, while cells touching the lower and right margins are excluded.

(a) Calculation of TLC:

$$\text{TLC}/\mu l = \frac{Nw \times Cd \times Cv}{NLS}$$

$$= \frac{Nw \times 20 \times 10}{4}$$

$$= Nw \times 50$$

Where Nw is the number of WBCs counted, Cd is the correction of dilution, Cv is the correction of volume and NLS is the number of large squares counted.
(b) TLC/L = Number of WBCs counted × 50 × 10_6 (10_6 is the correction factor to convert count in 1 µl to count in 1 liter). Example: If 200 WBCs are counted in 4 large squares, TLC/µl will be 10,000/µl and TLC/liter will be 10.0 × 10_9/liter.

If TLC is more than 50,000/ml, then dilution of blood should be increased to 1:40 to increase the accuracy of the result.

If TLC is less than 2,000/ml then lesser dilution should be used.

Expression of TLC: Conventionally, TLC is expressed as cells/µl or cells/cmm or cells/mm^3. In SI units, TLC is expressed as cells × 10_9/liter. Conversion factors for conventional to SI units is 0.001 and SI to conventional units is 1000.

Correction of TLC for nucleated red cells: The diluting fluid does not lyse nucleated red cells or erythroblasts. Therefore, they are counted as leukocytes in hemocytometer. If erythroblasts are markedly increased in the blood sample, overestimation of TLC can occur. To avoid this if erythroblasts are greater than 10 per 100 leukocytes as seen on blood film, TLC should be corrected for nucleated red cells by the following formula:

$$C_{TLC} = \frac{TLC \times 100}{N_{RBC} + 100}$$

Where C_{TLC} is the Corrected TLC/µl, TLC is the Total Leukocyte Count and N_{RBC} is the Nucleated RBCs per 100 WBCs.

REFERENCE RANGES

- Adults:4000-11,000/µl
- At birth: 10,000-26000/µl
- 1 year: 6,000-16,000/µl
- 6-12 years: 5,000-13,000/µl
- Pregnancy: up to 15,000/µl

CRITICAL VALUES

TLC < 2000/µl or > 50000/µl.

Expt 3: DIFFERENTIAL LEUKOCYTE COUNT (DLC)

Aim:

To perform differential leukocyte count (DLC) of the given blood sample.

Introduction:

The leukocytes are also called white blood corpuscles (WBC) and formally known as white cells of the blood but these are not white colour, these are colourless. The white blood cells are protect our body against any diseases by fighting with infections (bacterial, viral, protozoan, parasitic etc.), antigens and also against malignancy. These are two types; Granulocytes (Neutrophil, Eosinophil and Basophile) and Agranulocytes (Monocyte and Lymphocyte). After staining the blood film with Leishman stain, the blood smear examine in the microscope under oil immersion objective (100X). Under oil immersion objective the leukocytes are seen as follows:

Morphology and function of leukocytes

Neutrophil

Neutrophils are round shape, 10 – 15µ in diameter. The cell contains cytoplasm and nucleus. The nucleus shows variable numbers of lobes, 2 – 7 lobes hence called polymorphonuclear leukocytes. The nucleus stain purple blue and the chromatin are coarse and ropy. The cytoplasm contains two types of granules – Primary granules and Secondary granules. When the cell stained with Leishman stain, only secondary granules are stained and these are violet colour granules, which are amphophilic. The cytoplasm takes pink colour.

Neutrophils are called first line defenses as they move first to fight the invading micro-organisms (bacteria etc.). Neutrophil with their enzymatic armory are superb killers. The activated neutrophils engulf the bacteria (phagocytosis) and released different enzymes into the phagocytic vesicles, which killed the bacteria and then digest it.

Eosinophil

Eosinophils are round shaped, 10 – 15µ in diameter. The cell contain cytoplasm packed with coarse brick red colour granules which takes acidic stain (eosin) hence called eosinophil. And the nucleus of the cell consists of 2 – 3 lobes. The nucleus stains purple blue colour and chromatin is course and ropy.

Eosinophil contains a Major Basic Protein (MBP) which damages the larvae of the parasites. There is one eosinophilic cation protein which probably neutralizes heparin anticoagulant. They have a property that prevents anaphylaxis (anti allergic action). They are motile and phagocytic. Chemotoxis is also shown by the eosinophils.

Basophile

Basophils are round shape, 10 – 15µ in diameter occasionally seen in the blood smear from a healthy person. The cell contain 'bilobed' or 'S' shaped nucleus stains purple blue and chromatin is coarse and ropy. The cytoplasm of the cell packed with course blue basophilic granules. On an average all granules are of equal size, which obscured the nucleus.

Basophils release the histamine resulting in immediate hypersensitivity reaction and also have role in inflammation. They contain heparin, protease and other mediators of inflammation. They are motile and phagocytic.

Monocyte

Monocytes are large, round, 10 – 20µ in diameter The cell contains a large kidney shaped, light purple blue stained nucleus and pale blue colour cytoplasm contain no granules. Some time few fine purple granules and vacuoles are seen in the cytoplasm.

The monokines secreted by monocytes stimulated T-cells, take part in inflammation, act as pyrogen and stimulate formation of acute phase proteins. They converted into macrophases and phagocytosis the microorganism, dead tissues etc.

Lymphocyte

In the blood circulation two types of lymphocyte are found, these are small lymphocyte and large lymphocyte. The small lymphocytes are believed to be the resting phase and when active they become large.

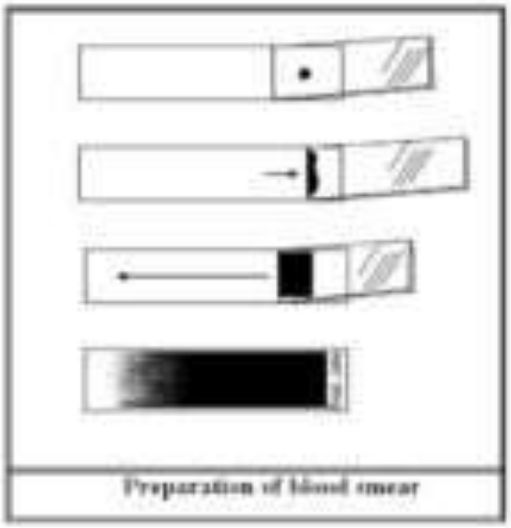
- **Small Lymphocyte :**

Small lymphocytes are small; round shaped, 8 – 10µ in diameter agranulocyte. The cell contains a large nucleus that occupied almost the total cell and scanty cytoplasm is seen. The cell contain cytoplasm takes blue colour and nucleus stains deep blue colour.

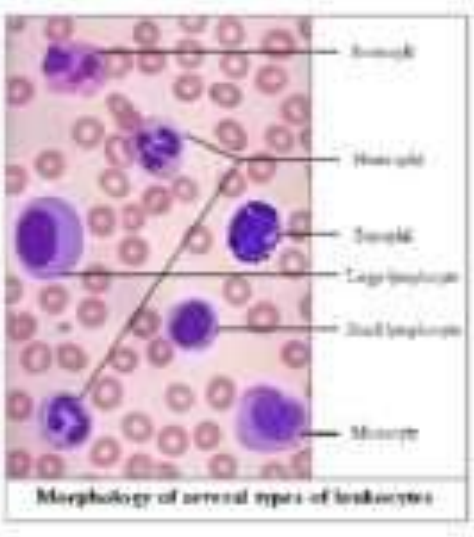
- **Large Lymphocyte :**

Large lymphocytes are round shaped, 10 – 18µ in diameter agranulocyte. They contain a round shaped deep blue nucleus with course and ropy chromatin and abundant clear blue cytoplasm with out any granules.

They are involver in the very important defense mechanism, called immunity. The B-lymphocytes are responsible for Humoral immunity and T-lymphocytes are responsible for Cell mediated immunity.



Preparation of blood smear



Morphology of several types of leukocytes

Clinical significance of DLC

Differential Leukocyte Count is useful to identify changes in the distribution of white blood cells which may be related to specific type of disorders. It also gives idea regarding the severity of the disease and the degree of response of the body.

Neutrophilia

Increase in the percentages of neutrophils in the blood is called neutrophilia.

Causes :

All the physiological causes that produces leukocytosis give rise to neutrophilia.

Physiological

- Exercise.

- Pregnancy.
- Neonatal period.
- Exposure to cold etc.

Drugs

- Epinephrine.
- Steroids etc.

Pathological

- Infection with pyogenic organisms.
- Non-infective inflammations.
- Myocardial infarction, Pulmonary embolism, Haemorrhage.
- Trauma and following surgery.
- Uremia, Hepatic come, Chemicals.
- Leukaemia, Neoplasm.

Neutropenia

Decrease in neutrophils below normal in the blood is called neutropenia.

Causes :

- Starvation and debility.
- Toxaemia in old people.
- Infections like typhoid, measles, malaria, kala-azar, hepatitis, influenza etc.
- Hyperspenism.
- Bone marrow failure : Aplastic anaemia, Leukaemia, Megaloblastic anaemia etc.
- Drugs : Sulphonamides, Diuretics etc.
- Chemotherapy etc.

Lymphocytosis

Increase in the percentages of lymphocytes in the blood is called lymphocytosis.

Causes :

Absolute

- Tuberculosis, brucellosis, syphilis, pertussis, toxoplasmosis.
- Mumps, rubella, infectious mononucleosis.
- Leukaemia.
- Thyrotoxicosis.

Relative

- All causes of neutropenia.
- Infective hepatitis.
- Convalescence from acute infections.
- Infants with infections.
- Malnutrition and avitaminosis.

Lymphocytopenia

Decrease in lymphocytes below normal in the blood is called lymphocytopenia.

Causes :

- Severe bone marrow failure.
- Immunosuppressive drugs.
- Hodgkin's disease.
- Irradiation.
- Viral infection like HIV.

Eosinophilia

Increase percentage of eosinophil present in the blood is called eosinophilia.

Causes :

Allergic

- High fever.
- Urticaria.
- Bronchial asthma.
- Food sensitivity.

Parasitic infections

- Hook worm.
- Hydatid cyst.
- Amebiosis.
- Filariasis etc.

Collagen disease : Periarteritis nodosa.

Recovery from acute infection.

Skin disease : Psoriasis, Pemphigus, Drug rash etc.

Monocytosis

Increase percentage of monocytes present in the blood is called monocytosis.

Causes :

- Chronic bacterial infection.
- Protozoan diseases.
- Hodgkin's disease.
- Chronic neutropenia.
- Monocytic or myelomonocytic leukemia.

Basophilia

Increase percentage of basophils present in the blood is called basophilia.

Causes :

- Chronic myeloid leukemia.
- Polycythaemia Vera.

NORMAL VALUES

Neutrophils : 40 – 75 % (mean : 57 %)
Band Formed : 2 – 6 % (mean : 3 %)
Segmented : 50 – 70 % (mean : 54 %)
Eosinophils : 1 – 4 % (mean : 2 %)
Basophils : 0 – 1 %
Lymphocytes : 20 – 45 % (mean : 37 %)
Monocytes : 2 – 8 % (mean : 6 %)

PRINCIPLE OF DLC

The polychromic staining solution (Leishman stain) contains methylene blue and eosin. These basic and acidic dyes induce multiple colours when applied to cells. Methanol acts a fixative and also as a solvent. The fixative does not make them adhere to the glass slide. The basic components of white blood cells (cytoplasm) are stained by acidic dye and they are describing as eosinophilic or acidophilic. The acidic components (nucleus & nucleic acid) of the cells take blue to purple shades by basic dye and they are called basophilic. The neutral components of the cell are stained by the dyes.

SPECIMEN FOR DLC

EDTA anticoagulated venous blood or free flowing capillary blood.

REQUIREMENTS FOR DLC

1. Microscopic slide and a glass Spreader slide.
2. Cedar wood oil (immersion oil)
3. Leishman stain
    - Leishman powder : 0.15 gm
    - Acetone free methanol : 1.0 liter

Preparation of stain : 0.15 gm of Leishman powder is dissolved in 100 ml of Acetone free methanol and the mixture is warmed at 50°C for 15 minutes. It is then filtrated and dye is ripened by keeping the filtrate in incubator at 37°C for 7 days. The stain is routinely used for staining the blood smear.

4. Buffer solution (P^H : 7.0)
    -
        -
            - Sodium Dihydrogen Phosphate (NaH_2PO_4) : 3.76 gm
            - Potassium Dihydrogen Phosphate (KH_2PO_4) : 2.10 gm
            - Distilled water : 1.0 liter
5. Staining rack.
6. Cotton and Tissue paper.
7. Pipette
8. Timer
9. Microscope with 10X and 100X objectives.
10. Cell counter.

PROCEDURE OF DLC

1. A thin blood film is prepared by spreading a drop of well mixed blood evenly on a clean and dry glass slide and dries the smear at room temperature. Adequate drying is essential to preserve the quality of the blood film.
2. Write the Identification number on the slide by using a lead pencil or a Marker pen.
3. Dried blood film prepared slide is placed on the staining rack.
4. Covered the smear with the Leishman staining solution by adding 10 – 15 drops on the smear by using a Pasteur pipette. Wait for exactly 1½ minutes.
5. Added equal amount of buffer solution on the slide. Mixed the reaction mixture adequately by blowing the contain on it through a pipette. Wait for 10 minutes.
6. Washed the smear by using running tap water.
7. Stand the slide in the draining rack or on the laboratory counter to dry the smear.
8. Examine the stained smear under the low power objective (10X) in the microscope for screening purpose and Chooses the proper portion of the smear. Placed one drop of Cedar wood oil (immersion oil) on the smear. Switch to the oil immersion objective (100X) and increase the light by opening the iris diaphragm. Examine the field by moving from one field to next field systematically. Record the types of leukocytes seen in each field. Count at least a total of 100 leukocytes and give the result as percentage (%) of the cells.

Expt 4: Separation of serum and plasma from blood sample experiment

Aim:

Effective Separation of blood products

Purpose:

To standardize separating procedures so that research samples will be uniform in quality The decision to collect anticoagulated (plasma, buffy coat, RBC) or coagulated (serum, clot) blood samples must be made prior to collection so that proper blood draw tubes will be used.

Serum (needs clot time)

A serum separator tube (SST, tiger top tube). Let the blood sit for 30 minutes to one hour at room temperature to clot before spinning and separating. A delay in centrifugation may have a detrimental effect on the sample quality and may result inaccurate results. Avoid hemolysis.

Separating plasma (time sensitive)

Tube with an anti coagulant eg: Edta (lavender top)sodium heparin (green top), sodium citrate (blue top) are used for separating Plasma You need to spin and separate within one hour of receiving the specimen (time sensitive)

Note: Universal Precautions must be used when working with blood. Use of personnel protective equipment is mandatory. Use of eye protection is mandatory unless blood tubes are being opened and serum/plasma/whole blood are being aliquoted inside a BL2 safety cabinet. *Keep blood on wet ice and process within one hour of blood draw

Separation of plasma

1) Blood will be collected into purple top EDTA tubes and centrifuged (2000 rpm) at 4 degrees centigrade for 20 minutes.

2) After centrifugation using clean pipette technique place 1.0ml of plasma into 1.5ml eppendorf tube labeled with tracking number and “plasma”

3) Freeze immediately at –80 degree freezer

Separation of Serum

1. A 10 ml tube of whole blood will be collected following standard procedures using a serum separator tube (SST, tiger top tube) from each patient.

2. Allow samples to clot for one hour at room temperature

3. Centrifuge for 10 minutes at approximately 1000g

4. Using clean pipette technique Aliquot 210ul of serum into labeled cryovials.

5. Immediately freeze vials of serum at –80-degree freezer

Results:

The given blood sample was processed to separate serum and plasma.

Expt 5: Perform immunodiffusion by ouchterlony method

AIM:

To learn the technique of Ouchterlony double diffusion.

PRINCIPLE:

Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. An antigen reacts with a specific antibody to form an antigen-antibody complex, the composition of which depends on the nature, concentration and proportion of the initial reactants. Immunodiffusion in gels are classified as single diffusion and double diffusion. In ouchterlony double diffusion, both antigen and antibody are allowed to diffuse into the gel. This assay is frequently used for comparing different antigen preparation. In this test, different antigen preparations, each containing single antigenic species are allowed to diffuse from separate wells against the antiserum. Depending on the similarity between the antigens, different geometrical patterns are produced between the antigen and antiserum wells. The patterns of lines form can be interpreted to determine whether the antigens are same or different as illustrated below

Pattern of identity: A The antibodies in the antiserum react with both the antigen resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens. i.e.) the two antigens are immunologically identical. Pattern of identity: B In the 'pattern of partial identity', the antibodies in the antiserum react more with one of the antigens than the other. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen. Pattern of identity: C In the 'pattern of non-identity', none of the antibodies in the antiserum react with antigenic determinants that may be present in both the antigen ie) the two antigens are immunologically unrelated as far as that antiserum is concerned.

MATERIALS REQUIRED: 1. Agarose 2. 10X assay buffer 3. Antiserum(A, B, C) 4. Test antigens 5. Glass plate 6. Gel punch with syringe 7. Template 8. Assay buffer: PBS

PROCEDURE:

1. 25 ml of 1.2% Agarose (0.3g/25ml) was prepared in 1X assay buffer and Agarose was dissolved completely by boiling.

2. The solutions were cooled at 50-60○c and pour 4ml/plate on to 5 grease free glass plates placed on a horizontal surface. The gel was allowed to set for 30minutes.

3. Wells were punched by keeping the glass plate on the template.

4. The wells were filled with 10 µl each of the antiserum and the corresponding antigens.

5. The glass plates were kept in a moist chamber overnight at 37○c.

6. After incubation, opaque precipitin lines between the antigen and antisera wells were observed.

RESULTS:

1. Reaction of identity: This occurs between identical antigenic determinants. The line of precipitation is given as a continuous arc.

2. Reaction of non-identity: When they do not contain any common antigenic determinant the two lines are found independently and were without any interaction.

3. Reaction of partial-identity: This has 2 components:

i. Those antigenic determinants which are common to both give a continuous line of identity.

ii. The unique determinant recognition is one of the antigen, in addition a line of non-identity, so that a spur is formed.

Expt 6: DOT-ELISA

Aim: To learn the technique of Dot ELISA for the detection of an antigen.

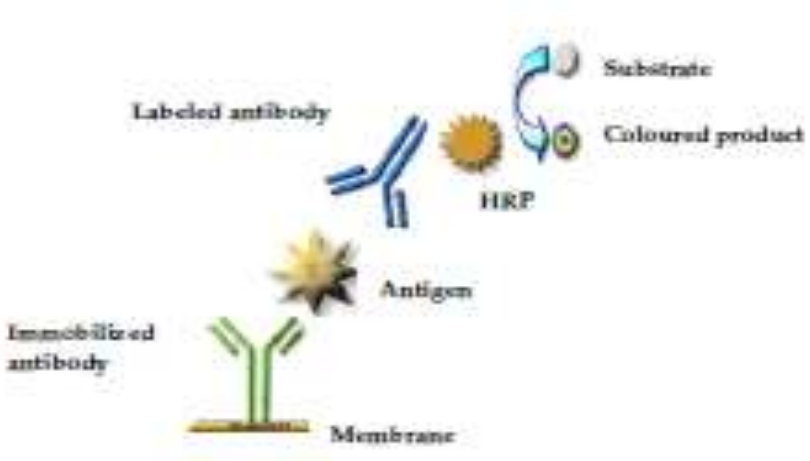
Introduction:

Enzyme linked immunosorbent assay or ELISA is a sensitive immunological technique to detect the presence of a specific antigen (Ag) or antibody (Ab) in a biological sample. It utilizes the dual properties of antibody molecules being specific in reactivity and their ability to be conjugated to active molecules such as enzymes. An enzyme conjugated with an antibody reacts with a chormogenic colourless substrate to generate a coloured reaction product. ELISA is extensively used for diagnostic purpose which utilizes the dual. It requires an immobilized antigen/antibody bound to a solid support (e.g. microtitre plate or membrane). There are different types of ELISAs for the detection of a protein of interest in a given sample. One of the most common ELISA is dot ELISA which can visually detect the presence of an antigen very quickly. The nitrocellulose dot technique was first developed for screening large number of hybridoma antibodies in 1983.

Principle:

There are various forms of ELISA for the detection of antigen or antibody based on antibody-antigen interactions. Dot ELISA, a qualitative ELISA test, can be performed very quickly with the end detection done visually. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA. In Dot-ELISA, small volumes of antibodies are immobilized on a protein binding membrane (Nitrocellulose) and the other antibody is linked to an enzyme Horse radish perxoidase (HRP). The test antigen at first reacts with the immobilized antibody and later with the enzyme-linked antibody. The amount of enzyme linked antibody bound is determined by incubating the strip with an appropriate substrate (Hydrogen peroxide, H_2O_2) and a chromogen [Tetramethylbenzidine (TMB)]. HRP acts on H_2O_2 to release nascent oxygen, which oxidizes TMB to TMB oxide, which gives, a blue coloured product. The latter precipitates onto the strip in the area of enzyme activity and appears as a coloured dot, hence the name Dot-ELISA. The results can be visualized in naked

eye. The enzyme activity is indicated by intensity of the dot, which is directly proportional to the antigen concentration.



Procedure:

1. Take 2 ml of 1X Assay Buffer in a test tube and add 2 µl of the test serum sample. Mix thoroughly by pipetting. Insert a Dot-ELISA strip into the tube.

2. Incubate the tube at room temperature for 20 minutes. Discard the solution.

3. Wash the strip two times by dipping it in 2 ml of 1X Assay Buffer for about 5 minutes each. Replace the buffer each time.

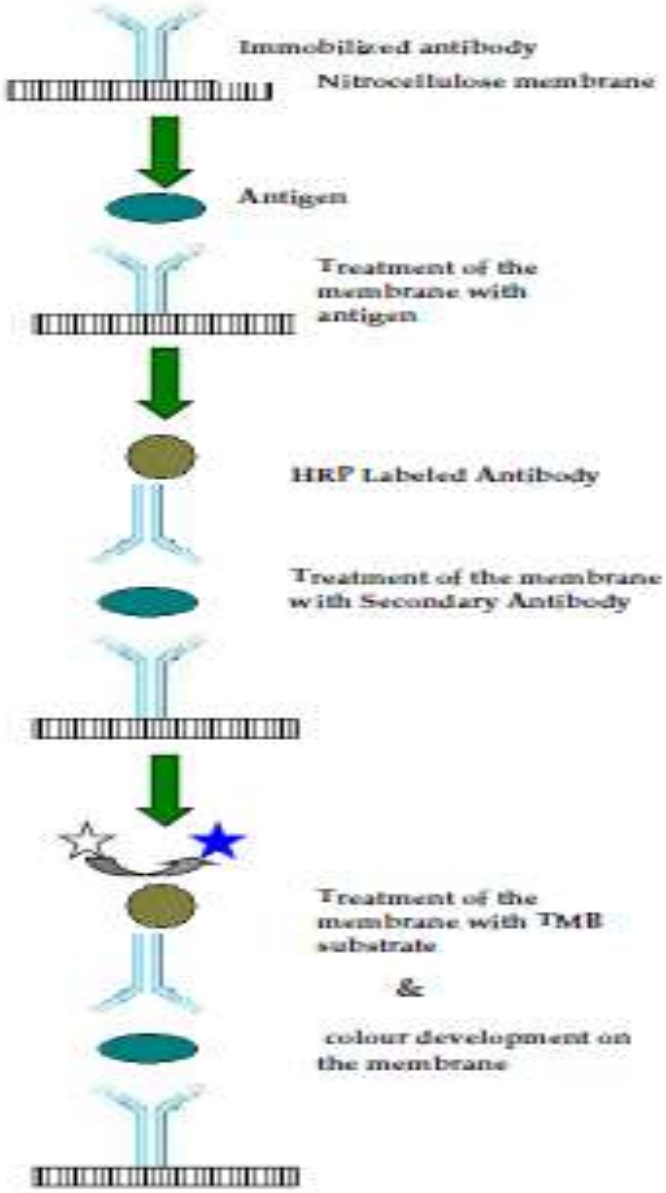
4. Take 2 ml of 1X Assay Buffer in a fresh test tube, add 2 µl of HRP conjugated antibody to it. Mix thoroughly by pipetting. Dip the ELISA strip into it and allow the reaction to take place for 20 minutes.

5. Wash the strip as in step # 3 for two times.

6. In a collection tube (provided in the kit) take 1.3 ml of TMB/H2O2 and dip the ELISA strip into this substrate solution.

7. Observe the strip after 5 - 10 minutes for the appearance of a blue spot.

8. Rinse the strip with distilled water.



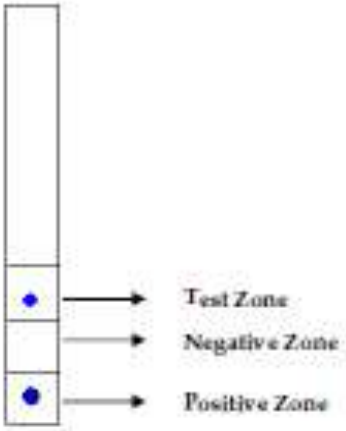
Interpretation and Results :

Spot in the positive control zone and no spot in the negative control zone indicates proper performance of test. In the negative control zone the immobilized antibody is not present and the region is blocked with an inert protein.

Therefore, there is no reaction when the reagents are added and no spot can be seen. In the test zone an antibody (specific to the test antigen, serum) is immobilized on it and then blocked with an inert protein.

The test serum binds to this region and the HRP-labeled antibody binds to serum which when reacts with substrate develops blue dot. In the positive control zone, the test serum binds to the immobilized antibody and the HRP labeled antibody binds to serum which when reacts with substrate develops blue dot.

Look for the appearance of the blue dot as shown below:



Record your observations as follows:

Zone	Spot
Positive Zone	
Negative Zone	
Test Zone	

Denote +ve : on appearance of a blue spot and -ve : on absence of a blue spot.

Expt 7: Immunoelectrophoresis

Aim:

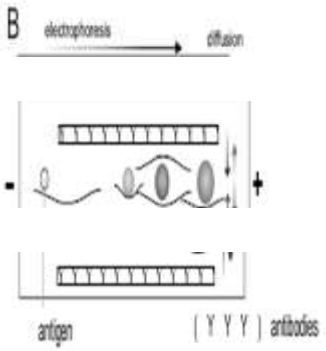
To learn the technique of Immunoelectrophoresis.

Introduction:

Immunoelectrophoresis is a powerful qualitative technique for the characterization of any antibody. In this method one antigen mixture is electrophoresed in an agarose gel that allows the separation of its different components based on their charge along the gel slide, followed by the lateral diffusion of the serum or monoclonal antibody within the gel. Antibodies specific to the antigens form white precipitation arcs which can be seen against a dark background. This technique is useful in determining the blood levels of three major immunoglobulins: IgM, IgG and IgA. The process combines antigen separation technique of electrophoresis and immunodiffusion of the separated antigen against an antibody. It is used extensively to check the presence, specificity and homogeneity of the antibodies and can detect relatively high antibody concentrations.

Principle:

In immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its constituents by charge. The antiserum containing the antibodies added into the troughs diffuses with a plane front to react with the antigens. Due to diffusion, density gradient of the antigen and antibody are obtained and at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed. They form a visible white complex which precipitates as arcs in the gel. The arc is closer to the trough at the point where the antigen is in highest concentration. The method is very specific and highly sensitive because distinct zones are formed. In this method it is important that the ratio between the quantities of antigen and antibody be proper (antibody titer).



Procedure:

1. Prepare 10 ml of 1.5% agarose (as given in important instructions).

2. Mark the side of the glass plate that will be towards negative electrode during electrophoresis.

3. Cool the solution to 55-60oC and pour 6 ml/plate on to grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4. Place the glass plate on the template provided.

5. Punch a well with the help of the gel puncher corresponding to the markings on the template at the negative end. Use gentle suction to avoid forming rugged wells.

6. Cut two troughs with the help of the gel cutter, but do not remove the gel from the troughs.

7. Add 10 µl of the antigen to the well and place the glass plate in the electrophoresis tank such that the antigen well is at the cathode/negative electrode.

8. Pour 1X Electrophoresis buffer into the electrophoresis tank such that it just covers the gel.

9. Electrophorese at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 cms from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

10. After electrophoresis, remove the gel from both the troughs and keep the plate at room temperature for 15min. Add 80 µl of antiserum A in one of the trough and antiserum B in the other.

11. Place the glass plate in a moist chamber and incubate overnight at 37oC. Observation and

Interpretation:

The formation of precipitin line indicates the presence of antibody specific to the antigen.

1. Homogeneity of the antiserum to the antigen is denoted by presence of a single continuous precipitin line

2. Heterogeneity of the antiserum to the antigen is denoted by presence of more than one precipitin line which not only gives an indication of the number of immunodominant epitopes, but also the non identical nature of such epitopes.

Results:

Observe for precipitin lines between antiserum troughs and the antigen well.