KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: II B.Sc., BIOCHEMISTRY COURSE NAME: IMMUNOLOGY
COURSE CODE: 16BCU413 (BATCH-2016-2019)

SYLLABUS

1. Isolation of lymphocytes from blood and macrophages from peritoneal cavity or spleen.

2. Purification of immunoglobulins.

3. Assays based on precipitation reactions
   Ouchterlony double diffusion (ODD) and
   Mancini radial immunodiffusion.

4. Assays based on agglutination reactions Blood typing (active) & passive agglutination.

5. Enzyme linked immune-sorbent assay (ELISA).

Ex No.1

Isolation of lymphocytes from blood and macrophages from peritoneal cavity or spleen.

This protocol describes isolation of live peripheral blood lymphocytes (PBL) for subsequent use in translational research. The procedure is compatible with modern molecular techniques (e.g. RNA & DNA, and protein isolation for genomics & proteomics) and use of live cells for subsequent in vitro experiments.

Method:

* 10 ml blood with EDTA as anticoagulant and 10 ml blood with heparin

* Separate lymphocytes and monocytes from other blood components using the
* Ficoll method:

* Dilute the blood with sterile PBS 1:1.

* Add 10 ml of Ficoll in a centrifuge tube, (the proportion between Ficoll and

blood should be 1/3 and 2/3, respectively)

* Carefully pour the diluted blood onto the ficoll solution (the blood must remain on top, do not mix).
* Centrifuge the tubes 20 min at 1600rpm (350g).
* Harvest the ring with white blood cells without touching the Ficoll using a sterile pipette tips.
* Dilute the white blood cells with PBS, then wash them twice in PBS.
* Resuspend the pellet in 1.5 ml FCS containing 10% DMSO. Put in 2 cryotubes. (For purposes not requiring live lymphocytes (e.g. DNA & RNA isolation) the pellet can be frozen directly)
* Mark Heparin or EDTA pretreatment on the label.
* Store PBL at -80°C.

Experiment 2

Purification of Immunogloulin

Antibody purification with Protein A

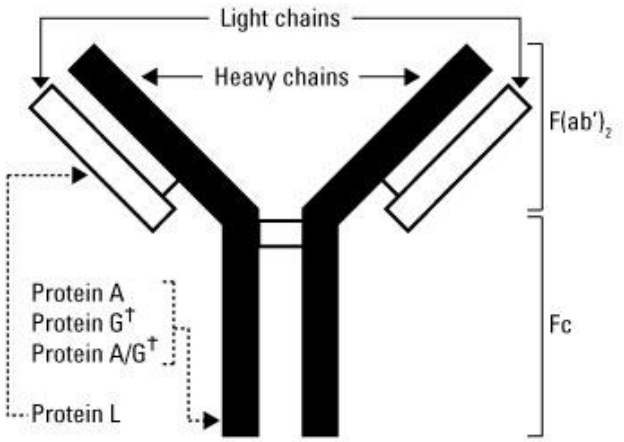
To accomplish antibody purification with Protein A, Protein G, Protein A/G or Protein L, they are covalently immobilized onto porous resins (such as beaded agarose) or magnetic beads. Because these proteins contain several antibody-binding domains, nearly every individual immobilized molecule, no matter its orientation maintains at least one functional and unhindered binding domain. Furthermore, because the proteins bind to antibodies at sites other than the antigen-binding domain, the immobilized forms of these proteins can be used in purification schemes, such as immunoprecipitation, in which antibody binding protein is used to purify an antigen from a sample by binding an antibody while it is bound to its antigen.

Protein A, G, A/G and L have different binding properties, which make each one suitable for different types of antibody targets (e.g., antibody subclass or animal species). It is important to realize that use of Protein A, G or L results in purification of general immunoglobulin from a crude sample. Depending on the sample source, antigen-specific antibody may account for only a small portion of the total immunoglobulin in the sample. For example, generally only 2–5% of total IgG in mouse serum is specific for the antigen used to immunize the animal.

Using a column of Protein A agarose resin and rabbit serum as the example, the general procedure for antibody purification with these ligands is as follows:

Bind: Add a clarified, physiologic-buffered (pH 7 to 8) sample of rabbit serum to the column and allow it to slowly pass through or mix with the Protein A resin to allow the IgG to bind to the immobilized ligand.

	Protein A
Species	*Staphylococcus aureus*
Human Pathology	Component of human body flora; cause of "Staph" infections
Native Size(s)	40 to 60kDa (variable numbers of repeated domains)
Binding Domains	5 for IgG (most common form)
Ig-binding Target	heavy chain constant region (Fc) of IgG (CH2-CH3 region)



Binding sites of antibody-binding proteins. Proteins used to immobilize antibodies to beaded support show specificity to different antibody domains. Protein A and G bind to the heavy chains of the antibody Fc region, while Protein L specifically binds the light chains of the two Fab regions of the F(ab')2 antibody fragment. † Protein G can also bind Fab fragments in certain conditions.

Wash: Add phosphate-buffered saline (PBS) and allow it to pass through the column to wash away nonbound serum components. Use a volume of wash buffer equivalent to 5 to 10 times the resin volume.

Elute: Add acidic elution buffer (e.g., 0.1M glycine-HCl, pH 2.8), and collect small fractions of solution that pass from the column. The low-pH condition dissociates the antibody from the immobilized Protein A, and the IgG is recovered in its purified state in one or several of the collected fractions.

Neutralize or exchange buffer: Use a protein assay or other means to identify and combine elution fractions that contain the purified antibody. Then add 1/10th volume of 1M Tris-HCl (pH 8.5) to neutralize the buffer. Alternatively, use a desalting column or dialysis to exchange the purified antibody into a more suitable buffer for long-term storage.

Experiment 3

Radial Immunodiffusion

Aim:

To study the immunodiffusion technique by Single Radial Immunodiffusion

Introduction:
Single Radial Immunodiffusion, also known as Mancini technique, is a quantitative immunodiffusion technique used to detect the concentration of antigen by measuring the diameter of the precipitin ring formed by the interaction of the antigen and the antibody at optimal concentration. In this method the antibody is incorporated into the agarose gel whereas the antigen diffuses into it in a radial pattern.

Principle:
Single Radial Immunodiffusion is used extensively for the quantitative estimation of antigen. Here the antigen-antibody reaction is made more sensitive by the addition of antiserum into the agarose gel and loading the antigen sample in the well. As the antigen diffuses into the agarose radially in all directions, it's concentration continuously falls until the equivalence point is reached at which the antigen concentration is in equal proportion to that of the antibody present in the agarose gel. At this point ring of precipitation ('precipitin ring') is formed around the well. The diameter of the precipitin ring is proportional to the concentration of antigen. With increasing concentration of antigen, precipitin rings with larger diameter are formed. The size of the precipitin rings depend on Antigen concentration in the sample well Antibody concentration in the agarose gel Size of the sample well Volume of the sample

Radial Immunodiffusion (Mancini)

Method

Ab incorporated in gel (anti-IgG)

Ag in a well(IgG, IgM)

Interpretation

Diameter of ring is proportional to the concentration

Quantitative

Ig levels ,C3,C4 conc.

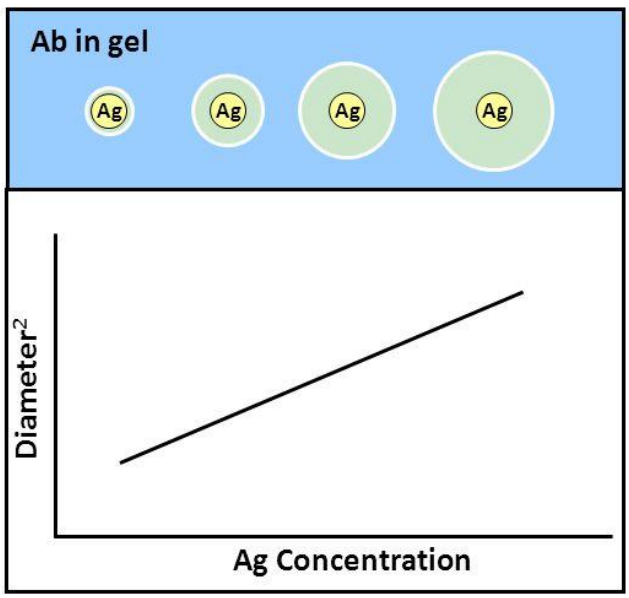


Fig 1: In Single Radial Immunodiffusion assay the diameter of the precipitin ring increases with increasing concentration of the antigen Thus, by having various concentrations of a standard antigen, standard curve can be obtained from which one can determine the amount of an antigen in an unknown sample. Thus, this is a quantitative test. If more than one ring appears in the test, more than one antigen/antibody reaction may have occurred. This could be due to a mixture of antigens or antibodies.

1.Prepare 10 ml of 1% agarose gel (as give in the important instructions). Take 6 ml of this gel solution in a clean test tube.

2. Allow the solution to cool down to 55-60oC and add 80 µl of antiserum to 6 ml of agarose solution. Mix well for uniform distribution of the antibody.

3.Pour agarose solution containing the antiserum on to a 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 wells with the help of gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming rugged wells.
6.Add 10 µl of the given standard antigen and test antigen samples to the wells.
7. Incubate the glass plate in a moist chamber overnight at 37oC.

Observation and Result:
Observe for precipitin rings surrounding the antigen wells (Fig 3). Mark the edges of the precipitin rings and measure the diameter of the rings as shown in table

Experiment 4

Assays based on agglutination

Blood Grouping

Aim:
To determine the blood group and Rh factor of an individual.

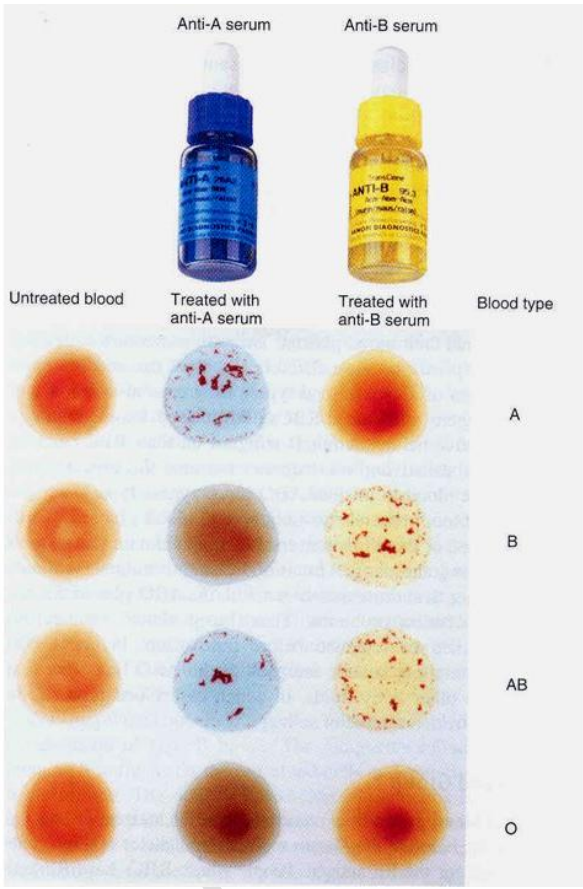
Introduction:
Blood grouping is the classification of blood based on the presence or absence of two inherited antigenic substances on the surface of red blood cells (RBCs). The ABO and Rh are the major, clinically significant and the most important of all the blood group systems.
The ABO blood group system was first discovered by Karl Landsteiner in 1900. The human ABO blood group system is divided into the following four major groups depending on the antigen present on the surface of their red blood cells:
1.“A” group
2.“B” group
3.“AB” group
4.“O” group

Antigens on the surface of Red Blood Cells	Antibodies in the Serum	ABO Blood Group	Genotype

A	Anti B	A	AA or AO
B	Anti A	B	BB or BO
A and B	Neither Anti A nor Anti B	AB	AB
Neither A nor B	Anti A, Anti B, Anti AB	O	OO

Principle:

The ABO and Rh blood grouping system is based on agglutination reaction. When red blood cells carrying one or both the antigens are exposed to the corresponding antibodies they interact with each other to form visible agglutination or clumping. The ABO blood gr oup antigens are O-linked glycoproteins in which the terminal sugar residues exposed at the cell surface of the red blood cells determine whether the antigen is A or B. Blood group A individuals have A antigens on RBCs and anti-B antibodies in serum. Similarly, blood group B individuals have B antigens on RBCs and anti-A antibodies in serum. Blood group AB individuals have both A and B antigens on RBCs and neither anti-A nor anti-B antibodies in serum. Whereas, blood group O individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum. The Rh antigens are transmembrane proteins in which the loops exposed on the surface of red blood cells interact with the corresponding antibodies.



Experiment 5
Enzyme-Linked ImmunoSorbent Assay (ELISA)

Description:

An enzyme-linked immunosorbent assay (ELISA) is used to detect the presence of an antigen in a sample. The antigen is immobilized to the well of a plate by adsorption, or captured with a bound, antigen-specific antibody. A detection antibody is then added forming a complex with the antigen, if present. The detection antibody can be covalently linked to an enzyme, or itself be detected by a secondary, enzyme linked antibody. Enzyme

substrate is then added to the wells producing a visible signal that is correlated with the amount of antigen and measured by a spectrophotometer.

Procedure:

- 100µl peptide (@4µg/ml) in coating buffer is added to individual wells of a microtiter plate. Incubate the plate for 2 hours at 37°C or overnight at 4°C.
- Remove the coating solution and wash the plate three times by filling the wells with 100 µl PBS-0.05%Tween20. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.
- Block the remaining protein-binding sites in the coated wells by adding 100µl blocking buffer, 3% skim milk in PBS per well. Incubate for 1 hour at RT with gentle shaking.
- Wash the plate three times with 100ul PBS-0.05% Tween 20.
- Add 50µl of diluted antibody to each well. Incubate the plate at 37℃ for an hour with gentle shaking.
- Wash the plate six times with 100ul PBS-0.05%Tween 20.
- Add 50µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Incubate at 37℃ for an hour.
- Wash the plate six times with 100ul PBS-0.05%Tween20.
- Prepare the substrate solution by mixing acetic acid, TMB and 0.03% H2O2 with the volume ratio of 4:1:5.
- Dispense 50µl of the substrate solution per well with a multichannel pipe. Incubate the plate at 37℃ in dark for 15-30mins.

* After sufficient color development, add 100µl of stop solution to the wells (if necessary).

* Read the absorbance (optical density at 450nm) of each well with a plate reader.

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4. Assays based on agglutination reactions Blood typing (active) & passive agglutination.

5. Enzyme linked immune-sorbent assay (ELISA).

1. What are the functions of lymphocytes?
2. What is leukocyte adhesion deficiency?
3. What is immunoglobulin?
4. Name the methods available to purify immunoglobulins
5. Which Ig is secretory in nature?
6. Which Ig can activate complement system on its own?
7. Which Ig can activate complement system upon dimerization?
8. Which Ig is involved in type 1 hypersensitivity?
9. What is precipitation?
10. What is agglutination?
11. Name the type of antigens
12. Who discovered single immuno diffusion?
13. What are the benefits of radial immunodiffusion?
14. What is the nature of blood group antigens
15. What is protein A?
16. What is ELISA
17. Name the macrophage present in CNS
18. What is the role of spleen in immunity?
19. What is double immunodiffusion?
20. What are the uses of double immunodiffusion?
21. What is HRP?

22. What do you interpret if you get X shape in double immunodiffusion
23. What do you interpret if you get Y shape in double immunodiffusion
24. What is particulate antigen?
25. Which immunity is responsible for handling intracellular pathogens?