Exercise 27
DIAGNOSTIC IMMUNOLOGY
(PRECIPITATION, AGGLUTINATION & ELISA)

Introduction

The science of **immunology** has yielded many techniques invaluable in the diagnosis of infectious disease as well as in the identification of microorganisms and other entities. Most of the diagnostic tools used involve interactions between **humoral antibodies** (**immunoglobulins**) and their homologous **antigens** (**antigenic determinants** or **epitopes**). The study of antigen-antibody interactions as they occur in vitro is known as **serology**. Since even the largest antibodies are too small to be observed microscopically, a variety of techniques have been developed to indicate their presence indirectly through their interactions with the antigenic determinant groups or epitopes that occur on cell surfaces, on viruses or as free molecules. The fact that antigen-antibody interactions are very specific is critical to their usefulness. In the clinical setting, detecting the presence of antibodies in the blood of infected individuals is of primary interest; however, since antigen-antibody interactions are complementary, laboratory techniques may also involve the use of known antibodies to detect antigens.

Although **serum** samples containing antibodies can be purchased from biological supply companies, their initial source is most likely animal (though antibodies are also produced by animal cells maintained in tissue cultures). The antigen used in association with the Ouchterlony test described below is **bovine serum albumin** (BSA), a protein normally found in the blood of cattle (about 55% of blood protein is albumin). When BSA is injected into animals that are not closely related to cattle (rabbits for example), those animals will produce **anti-BSA antibodies**. When this procedure is carried out under specific conditions, most of the antibody collected is **immunoglobulin gamma** (**IgG** or **gamma globulin**). This anti-BSA antibody can then be injected into other non-related animals (such as goats) and those animals will produce **anti-isotype antibodies** (antibodies against anti-BSA IgG). In the procedures that follow, the anti-BSA antibodies are sometimes referred to as **primary antibodies** (because they were produced against the original antigen). Antibodies produced against (in response to inoculation with) anti-BSA are referred to as anti-isotype or **secondary antibodies**.

During this exercise you will become familiar with some of the techniques most commonly used, or most important in diagnostic immunology. These include **precipitation reactions**, **agglutination reactions** and the **enzyme-linked immunosorbent assay** (ELISA).

I. PRECIPITATION REACTIONS:

Interaction between a soluble antigen and antibodies/immunoglobulins of the **isotypes gamma** (**IgG**) or **mega** (**IgM**) can result in the formation of an insoluble complex known as a **precipitate**. This is actually a lattice-like structure formed when divalent or polyvalent antibodies bind and cross-link two or more antigen molecules. Since the binding of antigens and antibodies is not permanent (does not involve covalent bonding) the concentration of antigen and antibody present will influence the degree to which precipitation occurs (either too much or too little antibody relative to antigen will interfere with precipitation). When concentrations allow for maximum bonding, precipitation reactions are often visible to the naked eye (within test tubes, within agar in petri dishes or in agar poured on glass slides). Several common immunological assays (which vary in their sensitivity) are based upon precipitation reactions.

Immunodiffusion - Ouchterlony Method

Precipitation reactions may be observed in Petri plates containing **agarose** gels, or in gels poured on the surfaces of glass slides. In the **Ouchterlony method**, antiserum is placed into a well in the center of an agarose gel and two or more samples of soluble antigen are placed into wells located around the periphery. Conversely, soluble antigen can be placed in the center well, and serum samples from a variety of sources can be placed into the peripheral wells. The antigens and antibodies will diffuse into the agarose until they interact. Where **homologous** molecules meet in equivalent concentration, **precipitation reactions** will occur. These are readily visible as opaque bands in the agarose somewhere between the central and peripheral wells. If two or more different antigen preparations are used, the pattern formed by the precipitin bands will indicate if or not these antigens share a common epitope (antigenic determinant). If two (or more) identical antigen samples are used in the peripheral wells, a single continuous line of precipitate will form between these and the central well. This pattern is called **identity**. If two (or more) unrelated antigen samples are used, independent precipitin lines will form between each antigen and the antibody in the central well, and these lines will cross. This pattern is referred to as **non-identity**. If the antigens used have one epitope in common, but one or the other has an additional unrelated epitope, a pattern called **partial identity** is observed. In this case, antibodies reacting with the common epitopes will form a continuous line while those reacting with the unrelated epitopes will form a "spur" to one side or the other of that line.

Materials:

Glass slides containing Ouchterlony agar with required wells (one per lab group)
Simulated bovine serum albumin (BSA - 1: 500 dilution)
Simulted goat or rabbit anti-BSA antibody
Other simulated antigen samples (type not specified)
10-100µl digital pipettes and tips

Procedure - Ouchterlony Method (Simulation):

1. Obtain a premade Ouchterlony gel and the specified reagents from your instructor.

2. Using a digital pipette and clean tips, fill the center well with 50µl of rabbit anti-BSA antibody. Fill each of the four surrounding wells with 50µl of a different one of the four antigens provided (W-Z). Be careful not to spill any of these liquid preparations onto the agar surface around the edges of the wells. Record the locations of the various antigens used.

3. Place the slide against a dark background (the lab bench surface works well) and allow diffusion to occur for 10-20 minutes. Check the slide for the formation of precipitate bands. These will appear as white lines in the agar, so will not be visible if your gel is resting on a white background (paper, kimwipe, etc.).

4. Assuming each of the precipitin bands indicates antibody-antigen interaction, determine which of the unknown antigen preparations contains Bovine Serum Albumin. Record your results.
   Note – Because this is a simulation, non-identity and partial identity bands cannot be observed.

II. AGGLUTINATION REACTIONS

The binding of antibody to antigens on cell surfaces can result in a clumping reaction called **agglutination**. This reaction is similar to precipitation in that the antibodies form "bridges" between the antigen particles and it is inhibited if the antibody is present in too low or too high a concentration. The effect of too much antibody relative to antigen sometimes causes a lack of agglutination referred to as a **prozone effect**. The binding of antibodies that do not induce agglutination (incomplete antibodies) will also interfere with agglutination. Agglutination reactions are routinely used in blood typing (**hemagglutination**), but can also be used to diagnose bacterial infections and to identify unknown bacteria in the laboratory (serological typing or **serotyping**).

Introduction to Human Blood Groups and Blood Typing:

Around 1900, a scientist by the name of **Karl Landsteiner** discovered that there are at least four different types or groups of human blood as determined by the presence or absence of specific **agglutinogens** (agglutinating antigens) or **epitopes** on the membrane surfaces of red blood cells (erythrocytes). The antigens typically involved are oligosaccharide chains attached to proteins (glycoproteins) or lipids (glycolipids) anchored to the cell membrane and designated as A and B. The four blood types/groups were identified as A, B, AB, and O (although the "O" may initially have been a zero). The type of agglutinogens present (and thus the blood type of the individual) is genetically determined, and is controlled by a single gene (the ABO gene) with three alternate forms or **alleles** often identified as A, B and O (with A and B being **codominant** while O is not expressed). These alleles can also be identified as I^A, I^B, and i, where the I stands for **isoagglutinogen**, and the lower case i is recessive (antigen is not expressed). The ABO gene encodes a type of enzyme called a **glycosyltransferase**, that modifies the carbohydrate content of a RBC surface antigen (agglutinogen) called antigen H or H antigen. The H antigen can be converted into either A antigen or B antigen by the activity of different glycoslytransferase enzymes (encoded by A or B alleles). The "O" allele is missing one nucleotide in an expressed region (exon) and this deletion causes a **frameshift** resulting in the loss of enzyme function (the H antigen cannot be modified). Individuals having RBCs with only A agglutinogens (genotype AA or AO, also I^AI^A or I^Ai) are said to have type A blood and in the United States, make up about 39% of the population. Those expressing only B agglutinogens (genotype BB or BO, also I^BI^B or I^Bi) have type B blood and account for about 12%. Persons with both A and B agglutinogens (genotype AB also I^AI^B) have type AB blood and occur with a frequency of about 4%. The last type (type O), which is characterized by the absence of A or B agglutinogens (genotype OO, also ii), is most common and accounts for about 45 % of the population.

Between 1900 and 1940 a great deal of research was done to uncover the presence of other antigens on human red blood cells. Finally, in 1940 Landsteiner and Wiener reported that rabbit sera containing antibodies reactive against the red blood cells of the Rhesus monkey would also agglutinate the red blood cells of humans. The human antigen involved was designated as the **rhesus factor**, or **Rh factor** (in respect to the Rhesus monkey) and has since been found to be quite complex. The formation of Rh antigens (transmembrane proteins) is controlled by two different genes, the *RHD* gene, which encodes RhD antigen (and variants), and the RHCE gene, which encodes Rh antigens designated as C, E, c, and e (and their variants). Over 30 antigenic variations have been identified, but the most clinically significant antigen is RhD. This is responsible for the **Rh-positive** condition and is found in 85% of people of European descent, 94% of people of African descent and 99% of people of Asian descent.

Determination of blood type is significant, because humans (and other animals) are capable of producing antibodies that can bind with the antigenic groups present on RBCs. These antibodies can cause **hemagglutination** (agglutination or clumping of RBCs) and initiation of **complement-mediated** RBC destruction. These antibodies, variously identified as **agglutinins**, **haemagglutinins** or **isohaemagglutinins,** are usually of the **isotype IgM**, but can also be **IgG**. Humans do not normally produce antibodies against their own antigens, but will produce antibodies against RBC antigens not recognized as "self", i.e., not their own. Circulating antibodies can bind with and bring about the destruction of any transfused RBCs recognized as "foreign", resulting in an **acute hemolytic transfusion reaction** (AHTR) that is potentially deadly.

Note – It is important to recognize that the ABO and Rh blood groups, though very important, are not the only ones (thirty-five different human blood group systems have been identified). Some people lack the ability to produce the H antigen described above, so can produce antibodies against A, B and H type agglutinogens. This rare "Bombay" phenotype (*hh*) would not be detected with the routine blood typing method described below, but could not safely receive O Rh-negative blood.

ABO blood typing is performed with **antisera**, i.e., blood serum samples containing a high titer of either anti-A or anti-B **agglutinins** (agglutinating antibodies) of the **isotype IgM**. These antibodies will bind very specifically to their homologous agglutinogens, and because they have more than one binding site can form "bridges" linking one cell to another. The RBCs that are thus linked will form clumps visible to the naked eye and are said to have undergone **hemagglutination** (hem = blood, agglutination = clumping).

IgM antibodies are usually **pentameric** (made up of five antibody units), and have ten antigen binding sites. They will agglutinate RBC more readily and at lower temperatures than will IgG antibodies; for this reason they are more commonly used for ABO typing. The test may be performed by either slide or tube method. In both cases, a drop of each kind of antiserum (A or B) is added to separate samples of red blood cells alone or suspended in a small quantity of saline solution. If agglutination occurs only in the suspension to which the anti-A serum is added, the blood is type A. If agglutination occurs only in the anti-B mixture the blood is type B. Agglutination in both samples indicates that the blood is type AB. The absence of agglutination indicates that the blood is type O.

Typing blood for the Rh factor can also be performed using both tube and slide methods, but certain differences in the techniques are involved. First of all, the antibodies in the typing sera are of the incomplete IgG type and will not agglutinate human red cells when they are diluted with saline. Therefore, it is necessary to use whole blood or to dilute the cells with serum albumin. Another difference is that the test is most effective if performed at higher temperatures (37^{o} C for tube test, 45^{o} C for slide test). Note - The ABO and Rh Blood group systems are only two of many blood group systems currently recognized, but are among the best known and most commonly utilized.

In this exercise you will observe **simulated hemagglutination** reactions, and will perform both ABO and Rh blood typing at room temperature. In a clinical setting, two separate slide methods may be used (for reasons stated above) but will not be demonstrated here.

Note - In order to eliminate the risks associated with handling human blood samples (potential exposure to HIV and/or HBV/HCV), students will not be permitted to use their own blood in any typing exercise conducted in this laboratory. The materials utilized are harmless (though messy) and students will not be required to wear gloves (a precaution mandatory when handling real blood).

Materials:

Simulated blood samples (blood from student volunteers is not acceptable)
Wax marker or glass marking pen
Clean glass slides
Simulated anti-A, anti-B and anti-D (Rh) typing sera
Applicators or toothpicks
Disposable pipettes

Procedure:

1. Select a container representing one of the subject individuals provided (dad, mom, son or daughter). Use the materials provided within the container to conduct the blood typing exercise. You will need to type the blood of all four, subject individuals, but should be careful to keep the materials within the various containers separate from one another. **Do not mix them up!**

2. Obtain four clean, glass slides, one for typing the blood of each individual, and place these on the lab bench.

3. Place three drops of blood on one slide such that they are evenly spaced and well separated from one another. To obtain the proper proportion of serum to blood, do not use a drop larger than three mm in diameter.

4. Add one drop of anti-D serum to the blood droplet on the right side, being careful not to contaminate the tip of the serum pipette with the material on the slide. Mix the blood/antiserum sample with a clean toothpick and watch for agglutination. Record your results.

5. Add one drop of anti-B serum to the middle blood droplet, and one drop of anti-A serum to the left blood droplet. Mix each sample with the toothpick and watch for agglutination. Record your results.

6. Obtain the appropriate materials and repeat the typing procedure for each of the other individuals provided (mother, father, daughter or son) and record your results.

7. Use the information obtained to determine the blood type of each subject being tested. Consider the genetics involved in ABO and Rh blood types and determine if or not your subjects represent a legitimate family group.

III. ROLE OF ABO AND Rh BLOOD GROUPS IN TRANSFUSIONS

Blood **transfusions** typically involve **packed cells** (mostly RBCs) rather than whole blood, so the antibody content of the donor's blood is not significant. The antibody content of the recipient's blood is very significant, because if the recipient has antibodies capable of binding antigens on the surfaces of transfused RBC, those cells will undergo **complement-mediated lysis**.

A person with type **AB Rh-positive** (AB+) blood will have the antigens (agglutinogens) A, B and D on their RBC's and would therefore not produce antibodies against any of these (baring autoimmune disorder). Such an individual is referred to as the "**universal recipient**", because they could potentially receive blood from any donor with a typical ABO and Rh blood group type.

A person with type **O Rh-negative** (O-) blood will not carry A, B, nor D antigens (agglutinogens), and could therefore donate blood to any recipient with a typical ABO and Rh blood group type. Such a person is designated as the "**universal donor**", because their blood cells (without agglutinogens on their surfaces) could not be bound by any of the antibodies likely to be present. A person with Rh-negative blood has the Rh-genotype (*dd*) and cannot make type D Rh antigen (there is no Rh-negative antigen).

III. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The **Enzyme-Linked Immunosorbent Assay** or **ELISA** involves a technique sometimes referred to as "sandwiching" and can be used to detect either antibody or antigen with a high degree of sensitivity. In this type of assay, antibody is made "visible" by being bound to an enzyme that can cleave a colorless substrate yielding a colored end product. (Two enzymes sometimes used for such assays are alkaline phosphatase and horseradish peroxidase.) The amount of antibody present is indicated by the amount of colored end product visible. Since enzymes do not bind permanently to their substrates, they will form more and more of the colored end product as long as substrate is available. This factor serves to amplify the effect of any antigen-antibody complexing, and makes the ELISA a highly sensitive test. (Note - Since even minute amounts of enzyme will eventually bring about a color change over time, it is necessary to stop the reactions when performing a quantitative enzyme-linked assay.) ELISAs are typically conducted in microtiter plates containing multiple wells and the results may be assessed visually or with a spectrophotometric plate reader.

To conduct an **indirect ELISA**, known antigen (in varying concentration) is bound to the inside surface of several microtiter wells. The **serum sample** to be tested (primary antibody) is then added to the wells and is allowed to react with the bound antigen. The wells are then washed (to remove excess antibody) and **anti-isotype antibody** (secondary antibody) conjugated to enzyme is added. This will bind with the primary antibody already bound to the antigen in the well. Finally the colorless substrate is added. In those wells containing enzyme, a colored product will be formed. The degree of color (concentration of end-product) present at a specified time indicates the amount of secondary antibody bound and therefore the amount of primary antibody present.

A technique sometimes referred to as the "Double Antibody Sandwich" may be used to detect or measure antigen. In this case, antibody (in varying dilutions) is first adsorbed to a microtiter plate. The solution containing the antigen to be assayed is then added. The plate is washed, and then enzyme-conjugated antibody is added. When the substrate is added, the degree of coloration will indicate the amount of antigen present.

An indirect ELISA is one method used for detecting serum antibodies being formed against the human immunodeficiency virus (HIV). Viral envelope and core proteins (now available via recombinant DNA techniques) are used as the antigens to be bound to the microtiter wells. Serum antibodies against HIV can generally be detected within 6 weeks of infection.

The procedures described below will be used to simulate an **indirect ELISA**, where serum samples are bound to antigen (pathogen components) present in the wells of a microtiter plate. In addition, students will be introduced to **Epidemiology**, the quantitative study of disease occurrence and factors influencing disease frequency and transmission. By simulating a population being exposed to a disease-causing agent, and subsequently producing antibodies against that agent, students will gain an appreciation for disease transmission and the analysis involved in determining a disease source (the index case, or patient zero).

Procedure A – Transmission phase – Interact with people outside your lab group.

1. Obtain a 1.5ml tube containing "body fluid" and label the cap with your initials.

2. At the instructor's signal, find one other person in the lab, and using a 1000μL pipette (blue tips) transfer 500μL of your body fluid into the other person's tube. This is to be a reciprocal exchange of body fluid, so be certain that both persons remove 500μL of their body fluid from their tubes before the exchange is made. After the exchange is completed each person will have 1000μL of body fluid in his or her tube.

3. Record the name of the person you have made contact with.

4. At the instructor's signal, find a second person in the laboratory and repeat the exchange process. Again record the name of your contact.

5. At the instructor's signal, find a third person in the laboratory and repeat the exchange process. Again record the name of your contact.

Procedure B – ELISA Test phase – Use one microtiter plate per lab section.

1. Using a yellow pipette and yellow tip, transfer 100μL of your body fluid (primary antibody) into each of three adjacent wells in a clean microtiter plate. Be sure to record the coordinates of your samples (column number and row letter).

2. One group will be selected to set up either a positive or negative control. If your group is selected, add 100μL of the control solution provided into three adjacent wells of the microtiter plate in the location indicated. Both positive and negative controls are required.

3. After all student samples have been added to the microtiter plate, allow it to incubate on the counter for five minutes.

4. Throw the liquid from the wells into a deep sink (side counter) by inverting the plate as it is being moved downward, and then stopping the downward motion abruptly.

5. Make certain all fluid has been removed from the wells by rapping the inverted plate sharply on a section of folded paper towel resting on the counter.

6. Rinse the wells with phosphate buffered saline plus tween 20 (PBS-tween) and again with phosphate buffered saline (PBS) as follows:

    a) Fill all the wells with PBS-tween and allow this to remain in the wells for several seconds while gently agitating the plate on the desktop (keep it level).
    b) Throw the liquid out of the wells into the sink and then rap the plate sharply on a piece of paper towel to remove any remaining liquid.
    c) Refill all the wells with PBS and allow this to remain for several seconds while gently agitating the plate on the desktop (keep it level).
    d) Throw the liquid out of the wells into the sink and then rap the plate sharply on a piece of paper towel to remove any remaining liquid.

7. Add three drops of antibody solution (secondary antibody) to each well containing a student sample or a control.

8. Allow this solution to remain in the plate for five minutes at room temperature, and then throw off the liquid into the sink. Rap the plate sharply on a section of paper towel to remove any excess liquid.

9. Rinse the plate as described in step 6 above.

10. Add three drops of color reagent solution to each well containing a student sample or a control. Allow the plate to incubate at room temperature for five minutes and then observe the results.

11. Move the microtiter plate to a light background so the colored reactions can be readily observed within the wells. Record the results obtained and determine if or not you are among those individuals infected with the disease-causing agent.

12. Record your condition (infected or not infected) on the board, as well as the names of the individuals you made contact with. Use this information to determine the pattern of disease transmission within your lab section.

Questions:

1. What is serology?

2. When applied to antibodies, what does isotype mean, and what is an anti-isotype?

3. What type of serological reaction is involved in the Ouchterlony test?

4. What are agglutinogens and how do they relate to blood type?

5. What are haemagglutinins (agglutinins or isohaemagglutinins)? Where are they found?

6. What types of agglutinogens would a person have if their blood type were A+? What type of haemagglutinins would this person produce?

7. What is antiserum and what was it used for in the blood typing exercise?

8. Hemagglutination due to IgM is useful as a diagnostic test, but what would happen to such cells inside the body, and why?

9. What is an ELISA, and what is this type of procedure used for?

10. Is an ELISA more or less sensitive than the Ouchterlony test, and why?

11. What is epidemiology?

Name _____ Lab Section _____

WORKSHEET

Exercise 27

Diagnostic Immunology

Goals: _____
_____
_____

Materials & Methods:
ABO and Rh blood typing was performed as described.

Data & Results:

Record locations of agglutination reactions observed for each individual.

	Agglutination observed (yes or no)?			
	Mother	Father	Daughter	Son
Anti-A				
Anti-B				
Anti-D				

Conclusions:

Record the **phenotype** (blood type) for each individual, both ABO blood group and Rh.

Mother _____ Father _____ Daughter _____ Son _____

Record the **genotypes** for the following individuals assuming the son is the natural offspring of this mother and father.

Mother _____ Father _____ Son _____

What are the possible **genotype** combinations for the daughter? _____

Which of these individuals are **homozygous** and which are **heterozygous** for the genes controlling ABO blood groups and Rh? _____
_____

What types of antibodies (anti-A, anti-B, anti-D) will each of these individuals carry within their bloodstream.
Mother _____
Father _____
Daughter _____
Son _____

Some important aspects of blood transfusion:

Blood transfusion typically involves the transfer of packed RBCs with little or no immune serum present. In order to prevent agglutination and the potentially lethal affects of activating the **complement cascade** on RBC surfaces throughout the body, donor and recipient blood types must be carefully monitored. Assuming ABO blood groups and Rh are the only factors influencing blood compatibility (which they are not), answer the following questions.

1. RBCs of what blood type could be transferred safely into recipients with any other blood type? _____ Note - individuals with this blood type are sometimes referred to as "universal donors". Why would these blood cells not stimulate agglutination reactions and complement activation?
   _____
   _____

2. Individuals with what blood type could theoretically receive blood from donors having any other blood type. _____ These individuals are sometimes referred to as "universal recipients". What aspect of the blood from these individuals prevents agglutination and the associated complement activation? _____
   _____

3. If the daughter from this family was injured in an automobile accident and required a blood transfusion, would it be safe for her to receive blood from her brother? _____ Explain why or why not. _____
   _____

4. If the son was injured in an automobile accident and required a blood transfusion, would it be safe for him to receive blood from his sister? _____ Explain why or why not.
   _____
   _____