2. Basic Immunologic Procedures

I. Introduction

A. General characteristics of antigen/antibody reactions.

1. **Sensitization** is the basic reaction of an antigen and antibody binding.

2. Factors that affect antigen/antibody reactions include:
   
   a. Concentration of the reactants
   b. Temperature
   c. Length of incubation
   d. pH of the test system

3. Classification of Antigen/Antibody reactions:

   a. Primary phenomenon (*sensitization*)

      1) Combination of single antibody to single antigen site.

      2) Tests to detect this reaction are technically difficult, complex, expensive, may require special equipment and are time consuming.

      3) Techniques utilized to detect this type of reaction include: immunofluorescence, radioimmunoassay and enzyme immunoassay.

   b. Secondary phenomenon

      1) Basic antigen/antibody reactions are taken a further step, forming cross links or **lattice formation** to create large molecules that are easily detectable.

      2) Methods used to detect these reactions are quick and easy to perform, less expensive, less time consuming and usually do not require special equipment.

      3) These methods are less specific, less sensitive and have more interferences.

      4) Techniques utilized to detect this type of reaction include precipitation, agglutination and complement fixation.

   c. Tertiary phenomenon

      1) Antigen/antibody reaction is not visible but is detected by the affect the reaction has on tissues or cells.
2) These types of reactions include: inflammation, phagocytosis, deposition of immune complexes, immune adherence, deposition of immune complexes, and chemotaxis.

3. The secondary phenomena is a method of choice for many serological tests to detect in-vitro presence of antigen or antibody.

   a. *Precipitation* involves combination of soluble antibody with soluble antigen to produce insoluble complexes.

   b. *Agglutination* is the process by which particulate antigen such as cells are aggregated to form large, visible aggregates if the specific antibody is present.

   c. *Complement fixation* is the triggering of the classical complement pathway due to combination of antigen with specific antibody.

B. Antigen-Antibody Binding

1. Primary union of antigen and antibody are depends on two characteristics, affinity and avidity.

2. *Affinity* is the initial force of attraction that an antibody for a specific antigenic epitope or determinant.

   a. As they come close together a chemical bond forms which is rather weak and can easily dissociate.

   b. How well the antibody fits to the shape of the antigen will determine the stability of the bond.

   c. Antibodies may react with antigens that are structurally similar to the original antigen and results in *cross reactivity*, the greater the similarity of the original antigen, the stronger the reaction.

   d. Antigens and antibodies with the perfect lock and key fit will have the strongest affinity.

3. *Avidity* is the sum of all attractive forces between an antigen and an antibody.

   a. It is the force that stabilizes the antigen-antibody reaction, keeping the molecules together.

   b. The stronger the chemical bonds which form between the antigen and antibody, the less likely that the reaction will reverse.


   a. Free reactants are in equilibrium with bound reactants.
b. The equilibrium constant represents the rates of antigen/antibody binding and dissociating.

c. Measures the “goodness of fit” of the reaction, as the avidity increases the rate of dissociation decreases.

C. Precipitation Curve (page 114)

1. Precipitation reactions are also dependent on the amount of antigen and antibody present in the test system.

2. *Prozone* phenomenon occurs when excess antibody is present.
   a. So much antibody is present that all antigen sites are coated and lattice formation cannot occur.
   b. Results in false negative reaction.

3. *Postzone* phenomenon occurs when excess antigen is present.
   a. There is so much antigen present that the antibody molecules bind in such a way that lattice formation cannot occur.
   b. The antibody may bind to antigen sites on two separate molecules, sensitization may occur, but the binding is not on enough adjacent molecules to form lattice.
   c. Results in a false negative reaction.

4. *Zone of equivalence* is when antigen and antibody are present in optimal proportions, sufficient antibodies can bind to antigens on adjacent molecules resulting in lattice formation.

II. Measurement of Precipitation By Light Scattering

A. Turbidimetry

1. Measures the turbidity or cloudiness of a solution.

2. Turbidity is caused by formation of antigen-antibody complexes resulting in the formation of a precipitate, the more antigen or antibody present, the greater the turbidity.

3. The tube is placed in the direct path of a light source, the more turbid the solution, the less light will pass through.

4. The amount of substance being quantitated (antigen or antibody) is calculated based on results obtained on standards and controls.

5. Very simple method but not very sensitive.
B. Nephelometry

1. Instead of measuring the decrease in light in turbidimetry, nephelometry measures the amount of light scattered by the antigen-antibody complexes.

2. The amount of light scattered is dependent on the number and the size of particles in the light beam.

3. Nephelometers measure light scattered at an angle, laser beams are the most accurate as the detect light deflected on a few degrees from the original light path.

4. Nephelometry is more sensitive as immunocomplexes tend to scatter light forward, which interferes with absorbance values.

5. In endpoint nephelometry the reaction is allowed to run to completion, but particles tend to fall out of solution and decrease light scatter.

6. Kinetic nephelometry measures the rate of increase in scattering immediately after the reagent is added, this rate is directly proportional to antigen or antibody concentration.

7. This technique is used to quantitate immunoglobulins, complement components, C-reactive protein, haptoglobin and other acute phase proteins.

III. Passive Immunodiffusion Techniques

A. Introduction

1. Antigen and antibody reactions occur in a gel, migrating towards each other and forming a detectable precipitate in the gel.

2. The rate of diffusion is affected by:

   a. Size of the particles 
   b. Temperature 
   c. Gel viscosity and hydration 
   d. Interaction of the reactants with the gel

3. Classified into 4 types:

   a. Single diffusion (one reactant moving) single dimension (up or down) 
   b. Single diffusion, double dimension (moving out radially from a well) 
   c. Double diffusion (both reactants moving) single dimension 
   d. Double diffusion, double dimension

B. Oudin Single Diffusion

1. Oudin first one to employ gels.
2. Antibody is added to agarose gel and placed in a tube, antigen is layered on top of the gel and will diffuse down into the gel.

3. If the antibody present reacts with the added antigen a precipitin band will form in the gel.

C. Radial Immunodiffusion-RID (single diffusion/double dimension)

1. Antibody is added to the gel and poured into a plate, wells are cut into the plate.

2. Antigen is added to the well and will diffuse out radially from the well.

3. If the antibody present is specific for the antigen added a ring of precipitate will form, the size of the ring is directly proportional to the concentration of the antigen.

4. Standards are run at the same time and a standard curve is created.

5. Two methods:
   a. Endpoint method allows the reaction to go to completion.
   b. Kinetic method employs measurements taken before the zone of equivalence is reached.

6. Technical sources of error:
   a. Overfilling or under filling the well.
   b. Spilling sample outside of the well.
   c. Nicking the well.
   d. Improper incubation time or temperature.

D. Ouchterlony Gel Diffusion (double diffusion/double dimension)

1. Ouchterlony Immunodiffusion is a method used for comparison of antigens.

2. Holes are cut in the agar, one central hole surrounded by other wells.

3. Antibody is added to the central well, antigens are added to the outer wells, the position of the bands formed between the antigens allows for comparison of the antigens to each other.

4. Three possibilities (page 117):
   a. Identity-the bands form an arc.
   b. Partial identity-fusion of 2 lines with a spur.
   c. Non-identity- pattern of lines which cross each other.
IV. Electrophoretic Techniques

A. Introduction

1. Immunodiffusion can be combined with electrical current to speed things up.

2. Electrophoresis is a technique which separates molecules according to differences in their electrical charge when they are exposed to an electric current.

3. A direct current is applied to the gel, the antigen and antibody migrate through the gel, as diffusion takes place precipitin bands are formed.

4. Can be applied both as a single or double diffusion method.

B. Rocket Immunoelectrophoresis (page 118)

1. Wells cut in a row in the agar mixed with antibody.

2. Antigen is placed in the wells, an electrical current applied, and precipitation begins.

3. As the concentration of antigen changes, there is dissolution and reformation of the precipitate at ever increasing intervals.

4. The end result is a precipitin line with a conical shape.

5. The height is measured and is directly proportional to the concentration of antigen.

6. If standards are run a standard curve is constructed and concentration determined.

7. Advantage over RID is results are obtained in a few hours.

8. Primarily used to quantitate immunoglobulins and to assay proteins whose concentrations are too low for nephelometry, but too high for RID.

C. Countercurrent Immunoelectrophoresis (CIEP) (page 119)

1. Double diffusion method in which antibody and antigen are placed in wells in a gel that are directly opposite from one another.

2. An electrical current is applied to induce migration, antigens migrate to the anode and antibody to the cathode.

3. A precipitin band will form where the two meet.

4. Useful as a rapid qualitative method when rapid results are necessary, as in diagnosis of bacterial meningitis.
D. Immunoelectrophoresis (IEP) (page 119)

1. A two step double diffusion technique.

2. Proteins are first electrophoresed to separate them.

3. A trough is cut in the gel parallel to the line of separation, antiserum is added to the trough and the gel is incubated overnight.

4. Double diffusion occurs as the antibody and separated proteins diffuse towards one another in the gel and form precipitin lines in the gel.

5. The lines can be compared in shape, intensity and location to that of normal serum control to detect abnormalities and is semiquantitative.

6. Excellent screening test to differentiate serum proteins and detect abnormalities such as in myelomas, Waldenstrom’s macroglobulinemia, malignant lymphomas and other lymphoproliferative disorders.

7. Can also identify immunodeficiencies.

E. Immunofixation Electrophoresis (IFE) (page 120)

1. Similar to IEP except that after electrophoresis is performed the antiserum is applied directly to the surface of the gel.

2. The antibody is impregnated into a cellulose acetate strip which is placed over the gel.

3. The antibody from the strip diffuses down into the gel and a precipitate will form if the specific antigen is present.

4. After washing the strip to remove extraneous proteins a stain is applied.

5. The best adaptation of this test is the Western Blot test to detect antibodies to the Human Immunodeficiency virus 1 (HIV-1).

   a. A mixture of HIV antigens is placed on a gel and electrophoresed to separate the components of the HIV antigen.
   b. The components are transferred to nitrocellulose paper.
   c. Patient serum is added to the paper and allowed to react.
   d. The strip is washed and stained to detect precipitin bands indicating the presence of antibodies to the HIV antigen.
   e. Must have several bands present to be positive.
F. Sources of Error in Electrophoresis

1. Applying current in wrong direction.
2. Incorrect buffer pH.
3. Incorrect timing.
4. Amount of current applied.

V. Labeled Immunoassays

A. Introduction

1. Need rapid, specific, sensitive assays.

2. Labeled immunoassays
   a. Some antigen/antibody reactions not detected by precipitation or agglutination.
   b. Measured indirectly using a labeled reactant.
   c. Referred to as receptor-ligand assays.
   d. **Ligand** is the substance to be measured and is defined as a molecule that binds to another molecule of a complementary configuration, usually it binds to the substance the test is trying to detect.
   e. The **receptor** is what binds the specific target molecule.

3. Sometimes referred to as a sandwich technique.
   a. Have a receptor of some kind that will bind to the antigen or antibody the system is trying to detect.
   b. If the substance is present it will bind and a washing process eliminates extraneous substances present.
   c. A labeled ligand is added which also has specificity for the substance being detected and results in a labeled product.
   d. This results in a sandwich, receptor, substance to be detected and ligand.
   e. Depending upon the ligand label a visible or detectable reaction will occur.
B. **Constituents of Labeled Assays**

1. Labels which may be used include: *fluorescent, radioactive, chemiluminescent and enzymes.*

2. The assay includes the use of:
   
   a. Labeled and non-labeled ligands
   b. Specific antibody
   c. Standards and calibrators
   d. Means of separating bound from free components
   e. Means of label detection.

3. Competitive binding
   
   a. A labeled ligand is mixed with the patient serum.
   b. It is added to the receptor.
   c. Competitive binding occurs in which patient sample and labeled ligand “compete” for antibody sites.
   d. If little or no antigen is present in the patient sera a strong positive occurs.
   e. If there is a lot of antigen in the patient sera, it will successfully bind in large quantities, causing a decrease in the binding of the labeled ligand, causing a decrease in color, radioactivity, etc.

4. Antibodies used in labeled immunoassay reactions must have high affinity.

5. **Standards** or **calibrators** are substances of **known concentration**.
   
   a. Standards are run at the same time as patient sample.
   b. Usually three standards are run, the results are graphed, and a standard curve is drawn.
   c. The concentration of the unknown patient sample can be determined from the standard curve.

6. Once a reaction has occurred there must be a way to remove unbound analytes from the reaction, this is done through a **separation method**.
   
   a. Can measure either bound ligand or free ligand remaining after separation method.
   b. Unreacted ligand can be removed by **adsorption** onto inert particles added to the system.
c. **Precipitation** of the antigen-antibody complexes.

d. Use a *second antibody* to precipitate out the antigen-antibody complexes.

e. **Solid phase** is the most popular method, antigen or antibody is physically attached to a tube, plate, etc, the substance being detected will bind, the excess is washed away.

f. In all procedures, the separation method is a limiting factor.

g. Separation procedure must be precise and reproducible.

7. Detection of the labeled analyte.

a. There must be an accurate system for detecting and measuring the labeled product produced.

b. For radioimmunoassay, radioactivity is measured.

c. For labels such as enzymes, fluorescence or chemiluminescence, changes in absorbancy on a spectrophotometer are used.

8. Quality control procedures must always be performed to ensure the accuracy of the results obtained.

a. *Blanks* are tubes filled with a clear solution or, if the solutions added have color, with the solution only, to determine the “background”, any substances present in the original solution must be calculated out.

b. *Controls* are substances with a known range of values and generally three levels are run: normal, high and low.

c. If controls do not give the expected values the results cannot be reported out.

C. **Radioimmunoassay** (RIA) Techniques (page 147)

1. Competitive Binding Assays

a. Uses radioactive substance as a label, usually I$^{125}$.

b. Antibody is bound to a tube or other solid matrix.

c. A measured amount of patient sample is added to a measured amount of radiolabeled analyte or ligand.

d. The antigen in the patient sample and the radiolabeled antigen compete for the binding to the antibody.
2. **Immunoradiometric Assay** (IRMA) (page 147)

   a. Excess labeled antibody is added to a tube with patient antigen.

   b. All patient antigen is bound by the antibody.

   c. Solid phase antigen is added which will then bind up all excess labeled antibody.

   d. The tube is spun and the solid phase antigen will go to the bottom, all antibody bound to patient antigen remains in solution.

   e. The radioactivity of the supernatant solution is determined.

   f. The count obtained is directly proportional to the amount of patient antigen present in the specimen.

   g. Advantages: faster reaction, increased sensitivity and specificity.

   h. Disadvantage: pure antigen and antibody are needed.

3. Procedures which utilize RIA are: human chorionic gonadotropin (HCG), follicle-stimulating hormone (FSH), gastrin, insulin, carcinoembryonic antigen (CEA), thyroxine, estrogens, androgens, IgE and erythropoietin.

4. Disadvantages of using RIA:

   a. Health hazard
   b. Disposal of radioactive waste
   c. Short shelf life
   d. Expensive equipment
D. Enzyme Immunoassay

1. Introduction

   a. **Advantages** of enzyme immunoassay:

   1) labels cheap and plentiful.
   2) labels have a long shelf life
   3) easily adapted to automation
   4) reaction measured using inexpensive equipment
   5) very sensitive
   6) no health hazards associated with reagents

   b. Enzymes chosen for us as labels according to the following:

   1) number of substrate molecules converted per molecule of enzyme
   2) purity
   3) sensitivity
   4) ease and speed of detection
   5) stability
   6) absence of interfering substances
   7) availability
   8) cost

   c. Typical enzymes used include:

   1) horseradish peroxidase - cheap, very popular, reacts with a number of chromogens
   2) glucose oxidase
   3) glucose-6-phosphate dehydrogenase - uses fluorimetric means
   4) alkaline phosphatase - expensive
   5) β-D-galactosidase

   d. The enzyme label is linked to antibody or ligand.

   e. Two classification of enzyme assays:

   1) **Heterogenous** requires a step to physically separate bound ligand from free.
   2) **Homogeneous** assays require no separation step.

3. Heterogenous Enzyme Immunoassays

   a. **Competitive Enzyme Linked Immunosorbent Assays (ELISA)**

   1) Enzyme labeled ligand competes with unlabeled patient ligand for binding sites on antibody molecules attached to solid phase.
2) After reacting, a washing step is performed.
3) Enzyme activity is determined.
4) Enzyme activity is inversely proportional the concentration of the test ligand.
5) Sensitivity in nanograms ($10^{-9}$ g/mL) can be achieved.

b. **Noncompetitive ELISA**

1) Referred to as indirect ELISA test.
2) Antigen is bound to solid phase, unlabeled patient antibody is added.
3) After incubation a wash step is performed and an enzyme labeled substrate is added.
4) The amount of enzyme label detected is directly proportional to the amount of antibody in the specimen.
5) Indirect ELISAs are very sensitive.
6) Disadvantage is there is more manipulation of the test.

c. **Immunoenzymometric Assay**

1) Non competitive ELISA
2) Detects unknown antigen by means of excess labeled antibody.
3) Antibody and patient sample allowed to react.
4) Antigen attached to solid phase, such as glass beads, added to test system.
5) Solid-phase antigen combines with unreacted labeled antibody.
6) Centrifuge tube and measure supernatant which contains antigen-antibody complexes.
7) This method requires less time, eliminates nonspecific reactivity and has greater sensitivity than other competitive ELISAs.

d. **Sandwich or Capture Assays**

1) Used with antigens having multiple epitopes.
2) Excess antibody attached to solid phase, allowed to combine with test sample.
3) After incubation, enzyme labeled antibody is added.

4) Second antibody may recognize same or different epitope than solid phase antibody.

5) Enzyme activity directly related to concentration of antigen.

6) Detects antigens present in low concentrations.

7) Suited to antigens with multiple epitopes.

8) Epitope must be unique to the organism and present in all strains.

9) Major use of this technique is for measurement of immunoglobulins, the immunoglobulin is the antigen and an anti-antibody is added to the test system.

10) Disadvantage: linear relationship does not exist, must prepare standard curve, test conditions must be carefully controlled.

4. **Homogeneous Enzyme Immunoassay**

   a. Reagent antigen is labeled with enzyme tag, patient sample is added.

   b. When antibody binds to antigen, stearic hindrance to the enzyme occurs which inactivates the enzyme.

   c. Antigen competes with enzyme labeled analyte for limited number of antibody binding sites, this is a competitive assay.

   d. Enzyme activity is directly proportional to concentration of patient antigen.

   e. No separation step necessary.

   f. Sensitivity of the procedure is determined by:

      1) detectability of enzymatic activity
      2) change in activity when antibody binds to antigen
      3) strength of antibody bond
      4) susceptibility of the assay to interferences such as cross reactivity.

   g. Sensitivity far less than heterogenous enzyme assays.

5. **Advantages of Enzyme Immunoassay**

   a. Sensitivity similar to RIA without health hazards and reagent disposal problems.

   b. Expensive equipment not necessary, spectrophotometers or notation of color change.
c. Most are fairly simple

6. Disadvantages
   a. Some samples may have natural inhibitors.
   b. Size of enzyme label limiting factor in designing some assays.
   c. Nonspecific protein binding may occur.
   d. Enzyme reactions very sensitive to temperature.

E. **Fluorescent Immunoassay**

1. Introduction
   a. Use fluorescent compounds called fluorophores or fluorochromes as markers.
   b. Markers have the ability to absorb energy and convert it into light.
   c. Fluorescent probe must exhibit high intensity to distinguish from background.
   d. Two compounds most frequently used are fluorescein and rhodamine.
      
      1) Fluorescein emits green color, it has high intensity and good photostability.
      
      2) Tetramethylrhodamine emits red light.
      
      3) Newer compounds include algae, porphyrins and chlorophylls.

   e. Fluorescent tags first used for localization of antigen in tissues.
      
      1) Immunofluorescent assay (IFA) is qualitative observation of fluorescent reaction using fluorescent scope for visualization.
      
      2) Can detect antigens in fixed cells or live cell suspensions.

3. Fluorescent Microscopy
   a. Uses light source that emits light of the appropriate wavelength necessary to excite fluorochrome used.
   b. The presence of a specific antigen is determined by the appearance of localized color against a black background.
c. Fluorescent staining

1) **Direct immunofluorescent** assay the antibody has a fluorescent tags, added directly to unknown antigen fixed to slide, after incubation and washing observe for fluorescence.

2) **Indirect immunofluorescent** assay react patient serum with known antigen attached to solid phase, wash, add anti-human antibody attached to fluorescent tag, wash, observe for fluorescence.

3. Heterogeneous Fluorescent Immunoassays

a. Fluorescent immunoassays (FIA) are classified as heterogeneous or homogeneous.

b. Heterogeneous assays require a separation step and include:

   1) indirect
   2) competitive
   3) sandwich
   4) capture

c. Same principle as EIA except fluorescent label is used.

d. Can detect either antigen or antibody.

e. Solid phase uses antigen or antibody attached to beads which may be magnetized.

f. When beads are utilized the reaction occurs, the tube centrifuged, supernatant discarded, and the beads observed for fluorescence.

g. Magnetized beads are placed on a magnetic surface to attract beads to the bottom of the tube.

h. Solid phase can also employ a dipstick coated with antigen or antibody, react with patient sample then add a labeled antibody.

5. Homogenous Assays

a. Just like EIA, no separation necessary, one incubations step and no wash step.

b. Usually involves competitive binding.

c. Direct relationship between amount of fluorescence and quantity of antigen in patient sample.

d. This technique suffers from lack of sensitivity.
6. Fluorescence Polarization Immunoassay (FPIA)
   a. Based on change in polarization of fluorescent light emitted from labeled molecule bound by antibody.
   b. When fluorophores are excited, emit partially polarized fluorescence.
   c. Antibody bound labeled antigens will emit more polarized light.
   d. Competitive binding.
   e. Degree of fluorescence inversely related to concentration of analyte.
   f. Technique limited to small molecules.
   g. Used most frequently for therapeutic drugs and hormones.
   h. Requires sophisticated instrumentation.

7. Advantages of fluorescent techniques:
   a. Methods fairly simple.
   b. No hazardous reagents to use or discard.
   c. Increased sensitivity over radiolabeled and enzyme reactions.

8. Disadvantages include:
   a. Fluorescent compounds are sensitive to environmental changes.
   b. Stability of labels
   c. Nonspecific binding can diminish signal.
   d. Bilirubin or hemoglobin can absorb the excitation or emission energy.
   e. Requires expensive dedicated instrumentation.

   a. Chemiluminescence is the production of light energy due to a chemical reaction.
   b. Most common substances used are: luminol, acridium esters and dioxetane phosphate.
   c. The substance is oxidized, usually with hydrogen peroxide and an enzyme for a catalyst.
d. Energy given off in the form of light.

e. Heterogeneous and homogenous assays available.

f. Disadvantages:

1) False results may be obtained if lack of precision in addition of hydrogen peroxide.
2) Some biologic materials cause quenching.
3) Need dedicated instrumentation.

g. Advantages:

1) reactive stability of label
2) speed of detection
3) sensitivity comparable to RIA and EIA
4) low cost