Lecture 5: Antigen-Immunoglobulin interactions, Experimental Systems, Monoclonal Antibodies.

Chapter 5, Benjamini et al.

5.1 Antibody-Antigen Affinity:

Antibodies form specific complexes with their antigen via non-covalent interactions. Nevertheless, the large number of van der Waals interactions, hydrogen bonds, electrostatic interactions and the hydrophobic effect that are possible within the epitope generally lead to very high affinities for protein-antibody interactions. Note that even with the high specificity of Antibody-antigen interactions it is possible to have **cross-reactivity**. That is more than one antigen can bind to a given antibody.

The only way to measure the affinity is to experimentally determine a binding curve. This involves measuring the amount of antibody-antigen complex as a function of the concentration of the free antigen. There are two common methods of performing this measurement:

i. Equilbrium dialysis (haptens and small antigens): Antibody is placed inside a dialysis bag and hapten is added to the outside of the bag. When equilibrium is reached the excess hapten inside the bag is due to the formation of antibody-antigen complexes.

ii. Surface Plasmon Resonance (Biacore): Antibodies are bound to a conductive surface, binding of antigen affects the electrical properties of conductive surface.

Analysis of Binding Data (See Biochemistry Lecture notes for Review):

The affinity, or association, constant for binding is: $K_A=k_1/k_{-1}$; where k_1 is the forward rate constant while k_{-1} is the reverse rate constant, the rate at which the antibody-antigen complex dissociates. Differences in binding affinities are usually due to differences in k_{-1}.

The fractional saturation, Y, is defined as:

$$Y = \frac{[AL]}{[A]+[AL]}$$

Where [AL] is the antibody (A) – antigen (L) complex, [A] is the free antibody. Using the definition of the dissociation constant, $K_A=[AL]/[A][L]$ to express [AL] in terms of [A], gives:

$$Y = \frac{K_A[A][L]}{[A]+K_A[A][L]} = \frac{K_A[L]}{1+K_A[L]}$$

The fractional saturation ranges from 0 ([L]=0) to 1 ([L] >>$1/K_A$). The K_A is 1/[L] that gives Y=0.5.

The dissociation constant is also used: $K_D=1/K_A$. The K_D is the ligand concentration that gives ½ fractional saturation. <u>You should be able to determine K_D from a binding curve</u>.

The K_A can be obtained directly from a binding curve. However, it is possible to linearize the above equation to give a linear equation:

$$\frac{Y}{[L]} = \left[\frac{-1}{K_D}\right]Y + \frac{1}{K_D}$$

Therefore a plot of Y/[L] versus Y should be straight line with a slope of $-1/K_D$.

The **avidity constant** of an antibody refers to the effect of multiple binding sites (e.g. in pentameric IgM). It is equal to the product of the individual binding constants (or equivalently, sum of binding free energies). Thus pentimeric antibodies have high avidity, even if they have low affinity.

5.2 Detection of Antibody-Antigen Complexes.

5.2.1 Precipitation Reactions: These methods rely on the ability of the antibody to form large crosslinked antibody-antigen complexes that precipitate out of solution. These methods are becoming less frequently used as solid-phase assays become more prevalent. A fixed amount of antigen is mixed with a set of serial dilutions of serum.

i. When antibody is in excess there is insufficient antigen to form an aggregate, i.e. small clusters of antigen-antibody complexes exist.

ii. When antigen is in excess, all antibodies are complexed to individual antigen molecules, so no aggregation or precipitation occurs.

iii. When antibody and antigen are about 1:1 (equivalence zone), efficient crosslinking and precipitation occurs.

The dilution of serum that gives the largest amount of precipitation is referred to as the **titer** of the serum.

These reactions can also occur in gels. Antigen and antibody are added to wells in an agar gel. As the two molecules diffuse towards each other they will sample different concentrations. A some location in the gel the concentration of both are just right for aggregation, leading to a precipitate in the gel. This method does not require any knowledge about the level of antibody in the serum.

5.2.2 Agglutination Reactions: These are similar to precipitation reactions, except that they involve the precipitation of cells. Usually, indirect crosslinking is used to form the aggregation. A secondary antibody is added that binds to the primary antibodies that have bound to their epitope on the surface of the cell.

This type of test is referred to as the indirect Coombs test. For example, the presence of anti-Rh antibodies in an Rh^- mother can be detected by incubating Rh^+ red blood cells with the serum form the mother, followed by the addition of antibodies (e.g. from a rabbit) that will bind to the human IgG and lead to precipitation.

5.2.3 Direct Binding Assays: In assays of these types, the antibody, or antigen are immobilized on a solid surface and the amount of bound antigen, or antibody, respectively is detected by radioactivity or by detection of color changes in the case of enzyme-linked detection schemes.

Western Blot: This is one of least quantitative measure, but is useful for detecting antigen in the presence of a complex mixture of proteins, or if the antibody available is polyclonal. A standard SDS gel is run to separate proteins according to size. These proteins are transferred to a membrane of nitrocellulose, forming an image of the gel. The gel is then washed with the primary antigens that will recognize the desired antigens. These antibodies binding to the immobilized and denatured protein. The membrane is treated with a secondary antibody that recognizes the constant region of the primary antibody. The secondary antibody is linked to alkaline phosphatase, horseradish peroxidase, or p-nitrophenyl phosphatase, all of which give colored products.

Radioimmunoassay: This is usually used to detect antigen. Antigen specific antibodies are immobilized on plastic or on beads and allowed to bind a known amount of radioactive antigen. The bound radioactivity is then measured. Serum (or other fluid), containing an unknown amount of the antigen (unlabeled of course) is added to the antibody and radioactive antigen. The amount of radioactivity bound to the antibody will decrease because the unlabeled material can compete for binding. The fractional saturation of antibody with radioactive antigen(L*) in the presence of unlabeled antigen (L) is given by the following:

$$Y = \frac{K_A[L^*]}{1 + K_A(L^* + L)}$$

ELISA (Enzyme-Linked Immunosorbent Assay):

Indirect ELISA (Detection of *Antibody*)

1. Antigen is bound to the bottom of a microtiter plate.
2. Specific antibody (primary antibody) is added that binds to the antigen.
3. A enzyme-linked secondary antibody is added
4. Amount of product produced is proportional to the amount of bound primary antibody.

Sandwich ELISA (Detection of *Antigen*)

1. Antigen specific antibody is bound to the bottom of the well.
2. Antigen in solution is added
3. Enzyme-coupled antibody that is specific for the antigen is added.
4. Product produced is proportional to the amount of bound antigen.

Immunofluorescence: Fluorescence is used to detect the presence of the complex.

- Primary antibody can be labeled with a fluorescent compound.
- Secondary antibody can be labeled with a fluorescent compound.
- Protein A, which binds to the constant region of IgG can be labeled with a fluorescent compound.

Fluorescence-Activated Cell Sorter

1. The bound fluorescence on the surface of cells can be detected.
2. Fluorescent cells can be separated from non-fluorescent cells
3. Using different antibodies with different fluorescent molecules allows the separation by two different cell surface markers.

Magnetic Cell Sorter

Similar to fluorescent cell sorter, except small magnetic beads are bound to the surface of the cell and these cells are separated from others using a magnet.

5.3 Generation of Monoclonal Antibodies

Animals (e.g. rabbits) are not the best source of antibodies for several reasons, here are some of the scientific ones:

- It is difficult to obtain large quantities of antibodies
- The antibodies will be polyclonal, they are a heterogeneous pool of antibodies that will recognize many different antigens.

Consequently, considerable effort was placed into developing cultured lines of B-cells that would secrete high levels of soluble immunoglobulins. Unfortunately, B-cells do not do well in cell culture and die with a few days. This problem was solved by fusing the B-cells with an immortal cell line (myeloma cells) to produce an immortal antibody producing cell line. The basic steps in this procedure are:

Inoculate a mouse with the antigen

- After an immune response is detected, harvest the plasma cells from the spleen
- Fuse the plasma cells with myeloma cells
- Select for hybridomas (plasma-spleen fusion)
- Select for stable chromosome number
- Screen for high-affinity/high-level antibody production.

The key tricks in this procedure were to obtain myeloma cells that did not secrete their own antibodies and to devise a selection scheme for hybridomas that selected against the myeloma cells. It is not necessary to select against the plasma cells from the spleen since they die anyhow.

Selection for hybirdomas:

- Nucleotides are produced by two pathways in mammalian cells:
- a de novo synthesis pathway and a salvage pathway.
- The de novo pathway can be inhibited using aminopterin, which inhibits the transfer of methyl groups from activated dihydrofolic acid.
- If the de novo pathway is blocked then cells need Hypoxanthine and Thymine as sources of purines and pyrimidines for the salvage pathway.
- Incorporation of hypoxanthine and thymine require the presence of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) in the cell.
- Myeloma cells are $HGPRT^-$ and cannot utilize hypoxanthine in the salvage pathway.
- Plasma cells are $HGPRT^+$ and can utilize hypoxanthine in the salvage pathway.

Therefore, growth of the cells on HAT media (hypoxanthine, aminopterin, thymidine) will select for immortal cells that have acquired the HGPRT gene via fusion with a plasma cell.

Uses of Monoclonal Antibodies:

- Protein purification
- Identification and isolation of cell sub-populations using fluorescence cell sorting.
- Tumor detection and imaging
- Tumor killing
- Diagnostic reagents.
- Catalytic antibodies
- .....etc

Humanized Monoclonal Antibodies:

When patients are treated with mouse monoclonal antibodies they rapidly develop antibodies against the constant region of the mouse (murine) antibody. There are at least three solutions to this problem:

1. Generate hybridomas from humans: it is more difficult to culture human cells and a ready source of B-cells is not particularly available.

2. Humanize murine antibodies: The hypervariable and variable regions from the mouse hybridomas are grafted onto human constant regions. This generally leads to a reduction in affinity.

3. Generate a antibody library using recombinant DNA techniques: This uses recombinant DNA techniques to generate considerable diversity in antibodies. In the case of murine antibodies, the genes encoding the variable regions of the heavy and light chain can be obtained by PCR of B-cell DNA and then these sequences can be incorporated into human imunoglobulin sequences.
4. Alternatively, the variable regions from human genomic DNA can be used. However, this approach will generate a population of antibodies with reduced diversity.