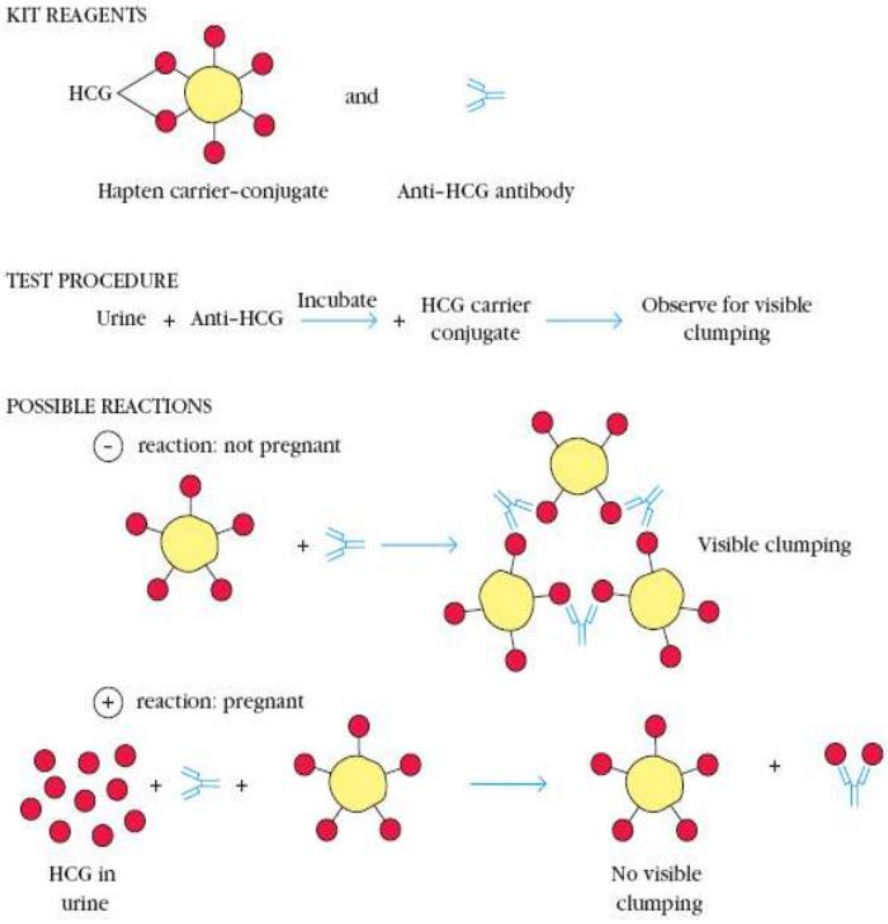
# IMMUNO TECHNOLOGY

## LECTURE 12: ANTIGENS, ANTIBODIES CONTINUED

### Passive Agglutination Is Useful with Soluble Antigens

In this technique, antigen-coated red blood cells are prepared by mixing a soluble antigen with red blood cells that have been treated with tannic acid or chromium chloride, both of which promote adsorption of the antigen to the surface of the cells. Serum containing antibody is serially diluted into microtiter plate wells, and the antigen-coated red blood cells are then added to each well; agglutination is assessed by the size of the characteristic spread pattern of agglutinated red blood cells on the bottom of the well, like the pattern seen in agglutination reactions.

### In Agglutination Inhibition, Absence of Agglutination Is Diagnostic of Antigen



# IMMUNO TECHNOLOGY

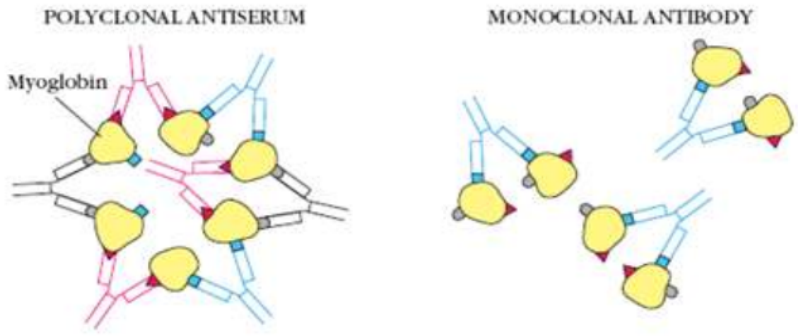
The original home pregnancy test kit employed hapten inhibition to determine the presence or absence of human chorionic gonadotropin (HCG). The original test kits used the presence or absence of visible clumping to determine whether HCG was present. If a woman was not pregnant, her urine would not contain HCG; in this case, the anti-HCG antibodies and HCG-carrier conjugate in the kit would react, producing visible clumping. If a woman was pregnant, the HCG in her urine would bind to the anti-HCG antibodies, thus inhibiting the subsequent binding of the antibody to the HCGcarrier conjugate. Because of this inhibition, no visible clumping occurred if a woman was pregnant. The kits currently on the market use ELISA-based assays.

**Precipitation Reactions**

Antibody and soluble antigen interacting in aqueous solution form a lattice that eventually develops into a visible precipitate. Antibodies that aggregate soluble antigens are called **precipitins.** Although formation of the soluble Ag-Ab complex occurs within minutes, formation of the visible precipitate occurs more slowly and often takes a day or two to reach completion. Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

- The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.
- The antigen must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.

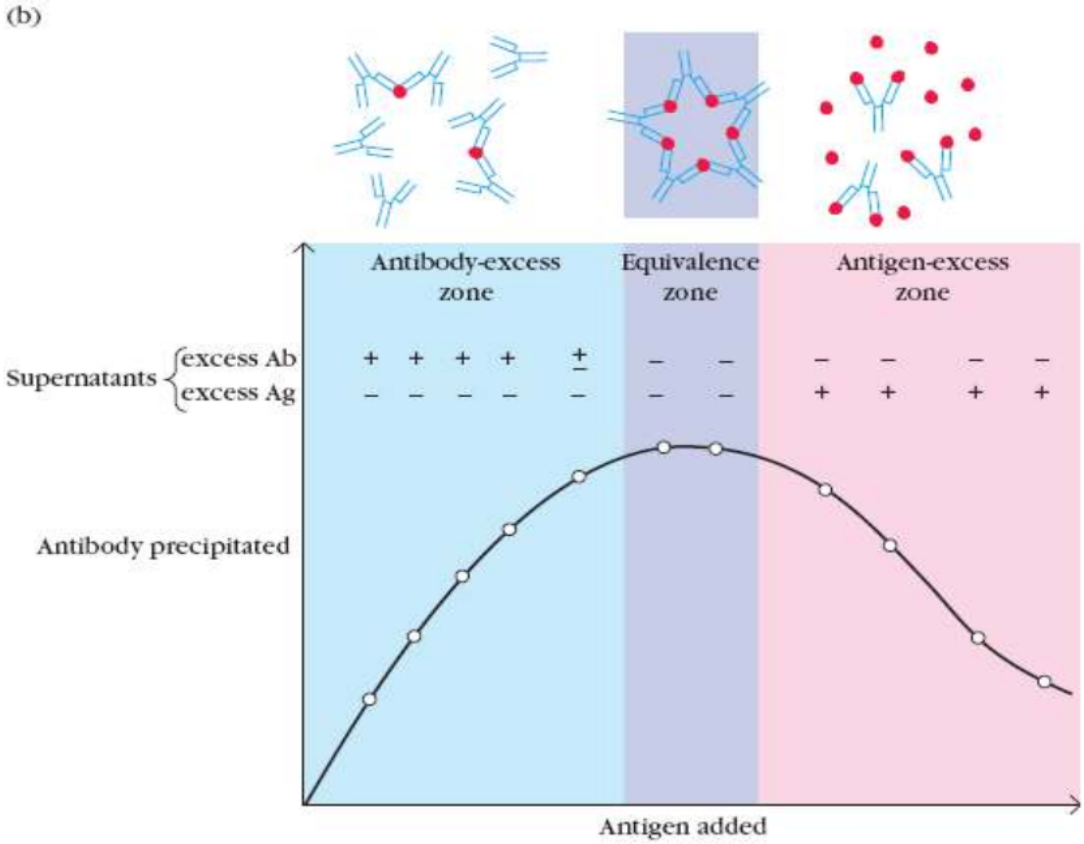
Experiments with myoglobin illustrate the requirement that protein antigens be bivalent or polyvalent for a precipitin reaction to occur. Myoglobin precipitates well with specific polyclonal antisera but fails to precipitate with a specific monoclonal antibody because it contains multiple, distinct epitopes but only a single copy of each epitope. Myoglobin thus can form a crosslinked lattice structure with polyclonal antisera but not with monoclonal antisera.




**Precipitation Reactions in Fluids Yield a Precipitin Curve**

A quantitative precipitation reaction can be performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. At one time this

method was used to measure the amount of antigen or antibody present in a sample of interest. After the precipitate forms, each tube is centrifuged to pellet the precipitate, the supernatant is poured off, and the amount of precipitate is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a precipitin curve.



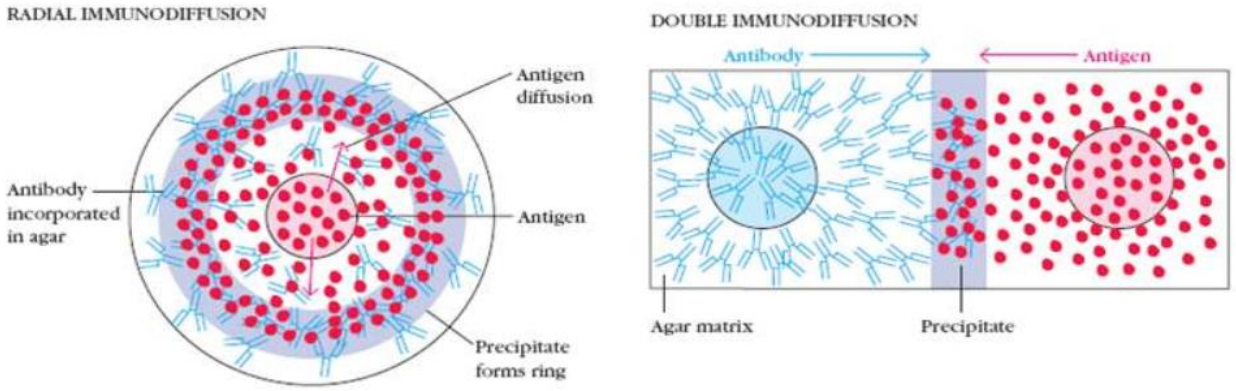

As Figure shows, excess of either antibody or antigen interferes with maximal precipitation, which occurs in the so-called **equivalence zone,** within which the ratio of antibody to antigen is optimal. As a large multimolecular lattice is formed at equivalence, the complex increases in size and precipitates out of solution. Under conditions of *antibody excess* or *antigen excess,* extensive lattices do not form and precipitation is inhibited. Although the quantitative precipitation reaction is seldom used experimentally today, the principles of antigen excess, antibody excess, and equivalence apply to many Ag-Ab reactions.

**Precipitation Reactions in Gels Yield Visible Precipitin Lines**

Immune precipitates can form not only in solution but also in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form. As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of *immunodiffusion*

*reactions* can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation. They are

1. **Radial immunodiffusion** (the Mancini method) and
2. **Double immunodiffusion** (the Ouchterlony method);




Both are carried out in a semisolid medium such as agar. In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5, upper panel). The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined. In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms.

**Immunoelectron Microscopy**

The fine specificity of antibodies has made them powerful tools for visualizing specific intracellular tissue components by **immunoelectron microscopy.** In this technique, an electron-dense label is either conjugated to the Fc portion of a specific antibody for direct staining or conjugated to an anti immunoglobulin reagent for indirect staining. A number of electron-dense labels have been employed, including *ferritin* and *colloidal gold.* Because the electron-dense label absorbs electrons, it can be visualized with the electron microscope as small black dots. In the case of immunogold labeling, different antibodies can be conjugated with gold particles of different sizes, allowing identification of several antigens within a cell by the different sizes of the electron-dense gold particles attached to the antibodies.

# IMMUNO TECHNOLOGY

**Immunoelectrophoresis Combines Electrophoresis and Double Immunodiffusion**

In **immunoelectrophoresis,** the antigen mixture is first electrophoresed to separate its components by charge. Troughs are then cut into the agar gel parallel to the direction of the electric field, and antiserum is added to the troughs. Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions (Figure 6-6a).

Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum. A sample of serum is electrophoresed, and the individual serum components are identified with antisera specific for a given protein or immunoglobulin class (Figure 6-6b). This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes, characteristic of certain immunodeficiency diseases. It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin.

A related *quantitative* technique, **rocket electrophoresis,** does permit measurement of antigen levels. In rocket electrophoresis**,** a negatively charged antigen is electrophoresed in a gel containing antibody. The precipitate formed between antigen and antibody has the shape of a rocket, the height of which is proportional to the concentration of antigen in the well. One limitation of rocket electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agar matrix. Some proteins, immunoglobulins for example, are not sufficiently charged to be quantitatively analyzed by rocket electrophoresis; nor is it possible to measure the amounts of several antigens in a mixture at the same time.

**Flow Cytometry and Fluorescence**

The fluorescent antibody techniques described are extremely valuable qualitative tools, but they do not give quantitative data. This shortcoming was remedied by development of the flow cytometer, which was designed to automate the analysis and separation of cells stained with fluorescent antibody. The flow cytometer uses a laser beam and light detector to count single intact cells in suspension (see Figure).

Every time a cell passes the laser beam, light is deflected from the detector, and this interruption of the laser signal is recorded. Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam.

The simplest form of the instrument counts each cell as it passes the laser beam and records the level of fluorescence he cell emits; an attached computer generates plots of the number of cells as the ordinate and their fluorescence intensity as the abscissa.

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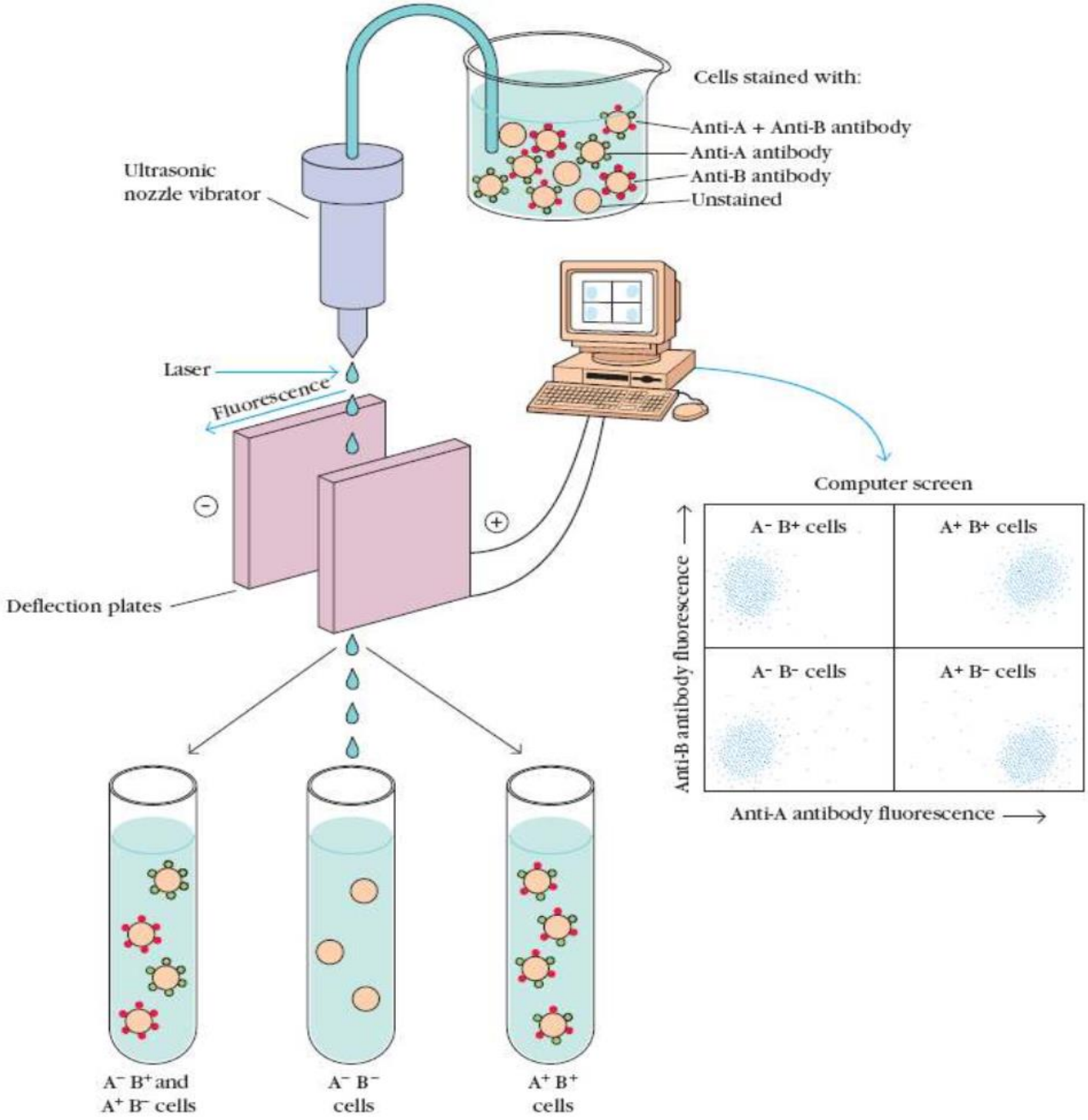


**Separation of fluorochrome-labeled cells with the flow cytometer**

In the example shown, a mixed cell population is stained with two antibodies, one specific for surface antigen A and the other specific for surface antigen B. The anti-A antibodies are labeled with fluorescein (green) and the anti-B antibodies with rhodamine (red). The stained cells are loaded into the sample chamber of the cytometer. The cells are expelled, one at a time, from a small vibrating nozzle that generates microdroplets, each containing no more than a single cell. As it leaves the nozzle, each droplet receives a small electrical charge, and the computer that controls the

flow cytometer can detect exactly when a drop generated by the nozzle passes through the beam of laser light that excites the fluorochrome.

The intensity of the fluorescence emitted by each droplet that contains a cell is monitored by a detector and displayed on a computer screen. Because the computer tracks the position of each droplet, it is possible to determine when a particular droplet will arrive between the deflection plates. By applying a momentary charge to the deflection plates when a droplet is passing between them, it is possible to deflect the path of a particular droplet into one or another collecting vessel. This allows the sorting of a population of cells into subpopulations having different profiles of surface markers.

In the computer display, each dot represents a cell. Cells that fall into the lower left-hand panel have background levels of fluorescence and are judged not to have reacted with either antibody anti-A or anti-B. Those that appear in the upper left panel reacted with anti-B but not anti-A, and those in the lower right panel reacted with anti-A but not anti-B. The upper right panel contains cells that react with both anti-A and anti-B. In the example shown here, the $A^-B^-$—and the $A^+B^+$— subpopulations have each been sorted into a separate tube. Staining with anti-A and anti-B fluorescent antibodies allow four subpopulations to be distinguished: $A^-B^-$, $A^+B^+$, $A^-B^+$, and $A^+B^-$.

**Hybridoma technology**

In somatic-cell hybridization, immunologists fuse normal B or T lymphocytes with tumor cells, obtaining hybrid cells, or heterokaryons, containing nuclei from both parent cells. Random loss of some chromosomes and subsequent cell proliferation yield a clone of cells that contain a single nucleus with chromosomes from each of the fused cells; such a clone is called a **hybridoma.**

Historically, cell fusion was promoted with Sendai virus, but now it is generally done with polyethylene glycol. Normal antigen-primed B cells can be fused with cancerous plasma cells, called **myeloma cells** (see Figure). The hybridoma thus formed continues to express the antibody genes of the normal B lymphocyte but is capable of unlimited growth, a characteristic of the myeloma cell. B-cell hybridomas that secrete antibody with a single antigenic specificity, called monoclonal antibody, in reference to its derivation from a single clone, have revolutionized not only immunology but biomedical research as well as the clinical laboratory.

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