RIA

One of the most sensitive techniques for detecting antigen or antibody is radioimmunoassay (RIA). In 1960, two endocrinologists, S. A. Berson and Rosalyn Yalow, designed an exquisitely sensitive technique to determine levels of insulin/anti-insulin complexes in diabetic patients. Their technique soon proved its value for measuring hormones, serum proteins, drugs, and vitamins at levels (0.001 microgram /ml or less) that were orders of magnitude lower than had previously been detectable. Their accomplishment was recognized in 1977 (several years after Berson's death), by the award of a Nobel Prize to Yalow.

The key to understanding the significance of this technological breakthrough is to realize that, prior to the development of the RIA, the concentrations of many biologically relevant proteins and hormones in body fluids were too low to detect by any known methods. Rather than representing merely an alteration in the sensitivity of established assays, the RIA therefore made it possible to measure substances that had hitherto been undetectable by any quantitative methodology.

Principle:

RIA involves competitive binding of radiolabeled antigen and unlabeled antigen to a high affinity antibody. The labeled antigen (The antigen is generally labeled with a gamma emitting isotope such as ^{125}I, but beta-emitting isotopes such as tritium (^{3}H) are also routinely used as labels) is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody molecule, and then the increasing amount of the test sample containing unlabeled antigen of unknown concentration are added. The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody. With increasing concentrations of unlabeled antigen, more labeled antigen will be displaced from the binding sites. The decrease in amount of radiolabeled antigen bound to specific antibody in the presence of test sample is measured in order to determine the amount of antigen present in the test sample.

The first step in setting up an RIA is to determine the amount of antibody needed to bind 50%-70% of a fixed quantity of radioactive antigen (Ag*) in the assay mixture. This ratio of antibody to Ag* is chosen such that the number of epitopes presented by the labeled antigen always exceeds the total number of antibody binding sites. This ensures that any unlabeled antigen added to the sample mixture will compete with radio labeled antigen for the limited supply of antibody. Even a small amount of unlabeled antigen added to the assay mixture of labeled antigen and antibody will cause a decrease in the amount of radioactive antigen bound, and this decrease will be proportional to the amount of unlabeled antigen added. To determine the amount of labeled antigen bound, the Ag-Ab complex is precipitated to separate it from free antigen, and the radioactivity in the precipitate is measured. A standard curve is generated using unlabeled antigen samples of known concentration (in place of the test sample), and from this plot the amount of antigen in the test mixture may be precisely determined.

Most RIAs still in use are based on the binding of antibody or antigen to a solid-phase support, such as the polystyrene or polyvinylchloride wells of a microtiter plate. The radioactive label that is most commonly used is ^{125}I, which binds to exposed tyrosine residues on proteins, with little effect on their overall structure.

Several methods have been developed for separating the bound antigen from the free antigen in RIA. One method involves precipitating Ag-Ab complex with a secondary anti-isotype antiserum. Another method makes use of the fact that protein A of *Staphylococcus aureus* has high affinity for IgG. For example, if the Ag-Ab complex contains rabbit IgG antibody, then goat anti-rabbit IgG will bind to the rabbit IgG and precipitate the complex in first case, while in the latter case complex can be precipitated by mixing with formalin -killed *S aureus*.After removal of the complex by either of these methods, the amount of free labeled antigen remaining in the supernatant can be measured in radiation counter; subtracting this value from the total amount of labeled antigen added yields the amount of labeled antigen bound.

Various solid-phase RIAs have been developed that make it easier to separate the Ag-Ab complex from the unbound antigen. In some cases, the antibody is covalently croslinked to sepharose beads. The amount of radiolabeled antigen bound to the beads can be measured after the beads have been centrifuged and washed. Alternatively, the antibody can be immobilized on polystyrene or polyvinylchloride wells and the amount of free labeled antigen in the supernatant can be determined in a radiation counter. In another approach, the antibody is immobilized on the walls of microtiter wells and the amount of bound antigen determined. This procedure is well suited for determining the concentration of a particular antigen in large number of samples. For example, a microtiter RIA has been widely used to screen for the presence of the hepatitis B virus.

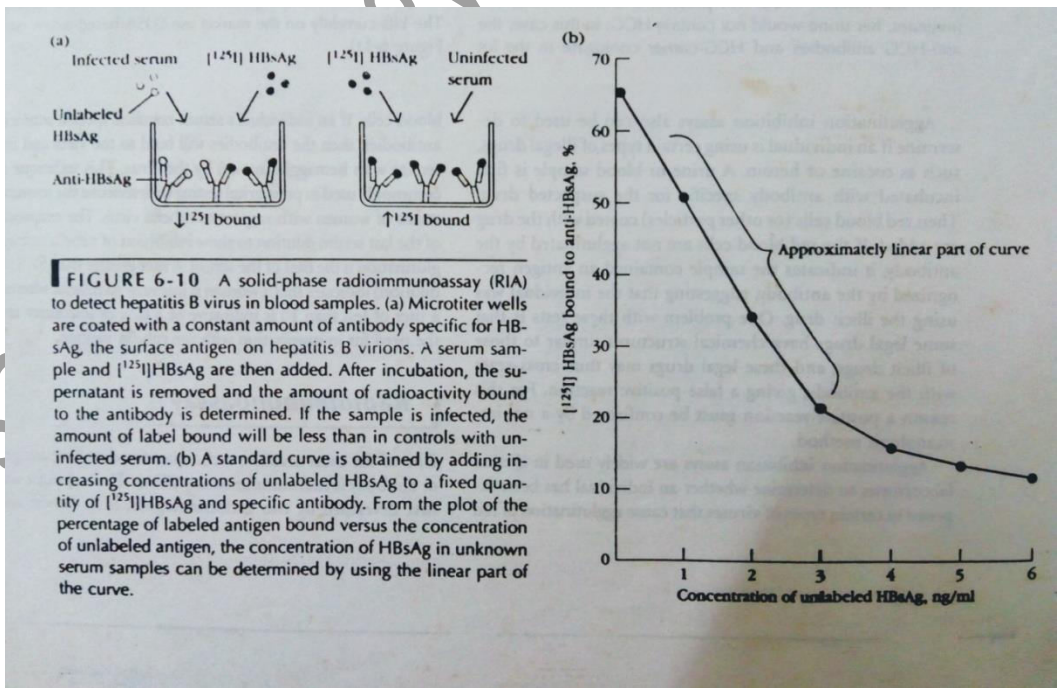

FIGURE 6-10 A solid-phase radioimmunoassay (RIA) to detect hepatitis B virus in blood samples. (a) Microtiter wells are coated with a constant amount of antibody specific for HBsAg, the surface antigen on hepatitis B virions. A serum sample and [^{125}I]HBsAg are then added. After incubation, the supernatant is removed and the amount of radioactivity bound to the antibody is determined. If the sample is infected, the amount of label bound will be less than in controls with uninfected serum. (b) A standard curve is obtained by adding increasing concentrations of unlabeled HBsAg to a fixed quantity of [^{125}I]HBsAg and specific antibody. From the plot of the percentage of labeled antigen bound versus the concentration of unlabeled antigen, the concentration of HBsAg in unknown serum samples can be determined by using the linear part of the curve.

The application of RIA can be understood by determining the concentration of a particular cytokine in the blood of a patient. First, the wells of a microtiter plate are coated with a constant amount of antibody specific for the cytokine. The surface of the plastic binds tightly to proteins that stick, essentially irreversibly, to the plastic surface. A known amount of radiolabeled cytokine is added to a set of control wells. A standard curve of unlabeled cytokines is then set up by adding increasing, known concentrations of cytokine to successive wells, along with the radiolabeled cytokine. As more unlabeled antigen competes with the labeled antigen, less and less radiolabeled cytokine will bind. After an incubation period, the amount of bound radiolabeled material is measured by washing off the unbound material and measuring the radioactivity in individual wells. An example of a standard curve generated in this way is shown in Figure. The measurement of the amount of cytokine in the experimental samples is accomplished by treating the unknown samples in exactly the same way as the standard curve. The investigator then compares the amount of radioactivity bound to the plate in the experimental wells with the radioactive signal obtained in the standard curve wells containing known amounts of unlabeled cytokine.

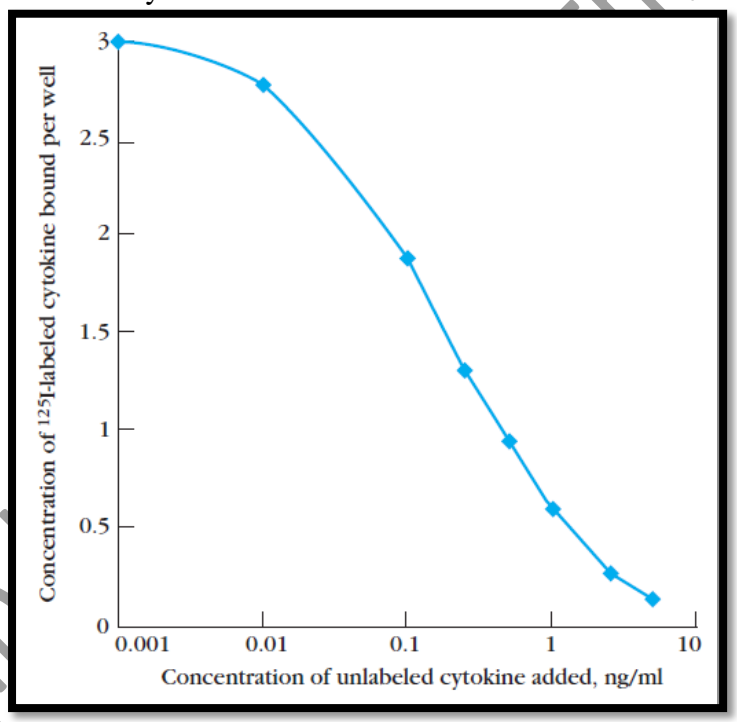

Fig: A competitive, solid-phase radioimmunoassay (RIA) to measure cytokine concentrations in serum.

Anticytokine antibody is used to coat an RIA plate. A standard curve is obtained by adding increasing concentrations of unlabeled cytokine to a constant amount of ^{125}I-labeled cytokine. The experimental sample is then added to duplicate or triplicate wells containing the same amount of labeled cytokine as that used for the standard curve. As the unlabeled cytokine outcompetes the labeled form for binding to the plate, the amount of radioactivity per well drops in a predictable fashion, shown here. The amount of cytokine in the experimental samples can then be measured by interpolation from the standard curve.

ELISA

Enzyme-linked immunosorbent assay, commonly known as **ELISA** are similar in principle to RIAs but, instead of using antibodies or antigens conjugated to radioisotopes, they use antibodies or antigens covalently bound to enzymes. The conjugated enzymes are selected on the basis of their ability to catalyze the conversion of a substrate (chromogenic substrate) into a colored, fluorescent, or chemiluminescent product. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and β-galactosidaseThese assays match the sensitivity of RIAs and have the advantage of being safer and, often, less costly.

A number of variations of the basic ELISA assay have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Each type of ELISA can be used qualitatively to detect the presence of antibody or antigen. Alternatively, a standard curve based on known concentrations of antibody or antigen can be prepared and from which the unknown concentration of a sample can be determined.

Indirect ELISA

Antibody can be detected, or its concentration determined with an *indirect ELISA* assay. Serum or some other sample containing primary antibody (Ab_1) is added to an antigen-coated microtiter well and allowed to react with the antigen attached to the well. After any free Ab_1 is washed away, the antibody bound to the antigen is detected by adding an enzyme-conjugated secondary antibody (Ab_2) that binds to Ab_1. Any free Ab_2 is again washed away, and a substrate for the enzyme is added. The amount of colored, fluorescent, or luminescent reaction product that forms is measured using a specialized plate reader and compared with the amount of product generated when the same set of reactions is performed using a standard curve of known Ab_1 concentrations. (A *direct* ELISA assay would detect the amount of antigen on the plate using enzyme coupled antibodies, and is rarely used.)

This version of ELISA is the method of choice to detect the presence of serum antibodies against *human immunodeficiency virus* (HIV), the causative agent of AIDS. In this assay, recombinant envelope and core proteins of HIV are adsorbed as solid-phase antigens to microtiter wells. Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins. Generally, serum antibodies to HIV can be detected by ELISA within 6 weeks of infection.

Sandwich ELISA

Antigen can be detected or measured by a sandwich ELISA. In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well. A sample containing unknown amounts of antigen is allowed to react with the immobilized antibody. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

A common variant on this assay uses a biotin-linked second antibody and then adds enzyme-linked avidin in an additional step (see below). Sandwich ELISAs have proven particularly useful for the measurement of soluble cytokine concentrations in tissue culture supernatants, as

well as in serum and body fluids. Note that, for this assay to work, the two antibodies used for the antigen immobilization (capture) and detection phases respectively must bind to different determinants (epitopes) on the antigen. Sandwich ELISAs therefore routinely use a pair of monoclonal antibodies specific for different regions on the antigen.

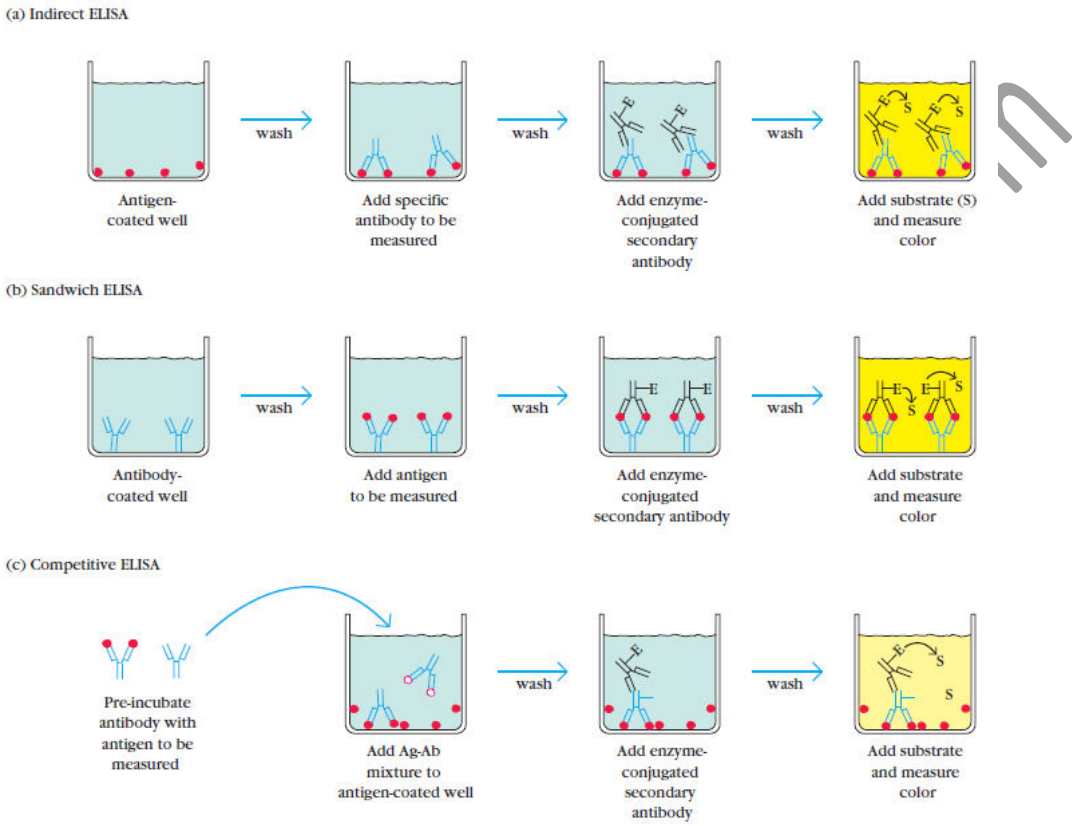

Fig: Variations in enzyme-linked immunosorbent assay (ELISA) technique allow determination of antibody or antigen.

Competitive ELISA

The competitive ELISA provides another extremely sensitive variation for measuring amounts of antigen. In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigen-coated microtiter well. The more antigens present in the initial solution-phase sample, the less free antibody will be available to bind to the antigen-coated well. After washing off the unbound antibody, an enzyme-conjugated secondary antibody (Ab_2) specific for the isotype of the primary antibody (Ab_1) can be added to determine the amount of primary antibody (Ab_1) bound to the well. In the competitive assay, the higher the concentration of antigen in the original sample, the lower the final signal (absorbance).