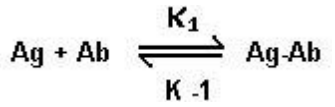
ANTIGEN ANTIBODY INTERACTIONS

ANTIGEN-ANTIBODY INTERACTIONS

- Antigen and antibody bind when they are specific to each other's.
- The non-covalent interactions that form the basis of antigen (Ag) and antibody (Ab) binding include hydrogen bonds, ionic bonds, hydrophobic interactions and vander Waal's interactions.
- These interactions are weaker when compared to covalent bonds and hence a large number of such interactions are required to form a strong Ag-Ab interaction.
- The combined strength of the non-covalent interactions between a single Ag binding site on an antibody and a single epitope is the *affinity* of the antibody for that epitope.
- The association between a binding site on an antibody with a monovalent Ag can be described by the equation:



  - K_1 - forward (association) rate constant
  - K_{-1} - reverse (dissociation) rate constant.
  - K_1/K_{-1} - association constant Ka, a measure of affinity.
- *Avidity* is the strength of Ag-Ab binding when multiple epitopes on an antigen interact with multiple binding sites of an antibody.
- The measurement of Ag-Ab interactions for diagnostic purposes is known as ***serology***.

ANTIGEN-ANTIBODY BINDING TESTS

Diagnostic serological techniques fall into three broad categories.

- Primary binding tests directly measure the binding of Ag to Ab.
- Secondary binding tests measure the results of Ag-Ab interactions in vitro. These are less sensitive but easier to perform.
- Tertiary binding tests measure the actual protective action of antibodies in an animal. Most complex of the tests.

INTRODUCTION

- Primary binding tests are performed by allowing Ag and Ab to combine and then measuring the amount of immune complexes formed.
- Radioisotopes, fluorescent dyes or enzymes are used as labels to identify one of the reactants.
  - Radio immuno assay (RIA)
  - Immunofluorescence assays
  - Enzyme immune assays (EIAs)
  - Immunoperoxidase test (IPT)
  - Western blotting (Immunoblotting)

RADIO IMMUNO ASSAY(RIA)

- It is one of the most sensitive techniques for detecting antigen or antibody. RIA was first developed in the 1960s by two endocrinologists S. A. Berson and Rosalyn Yalow to determine levels of insulin-anti insulin complexes in diabetics.
- RIA for antigen detection
  - Competitive RIA are based on the principle that unlabelled antigen will displace radio labeled antigen from immune complexes.
  - It is extremely sensitive and commonly used to detect trace amounts of drugs or antigen.
  - The antigen or drug is labeled with an isotope such as tritium, C-14 or I -125. When radio labeled antigen is mixed with its specific antibody it combines to form immune complexes that may be precipitated out of solution.
  - The radioactivity of the supernatant fluid is a measure of the amount of unbound antigen.
  - If unlabelled antigen is added to the mixture of labeled antigen and unbound antibody, it will compete with the labeled antigen for antibody binding sites.
  - As a result, some labeled antigen will be unable to bind antibodies and the radioactivity in the supernatant will increase.
  - If a standard curve is plotted using known amounts of unlabelled antigen, then the amount of antigen in a test sample can be measured by referring to this standard curve.

- RIA for antibody detection
    - In this technique antigen impregnated cellulose discs are immersed in test serum so that specific antibody binds to the antigen.
    - Unbound antibody is removed by washing and the disc is immersed in radiolabeled antiglobulin.
    - The antiglobulin binds to the disk only if antibodies have first bound to the antigen.
    - The amount of radioactivity bound to the disk is a measure of the level of antibody activity.
    - The cost of the equipment and the health hazard of working with radioisotopes argue against its use in small laboratories.
    - *The radio allergosorbant test* (RAST) is a primary binding test used to measure levels of specific IgE in allergic animals. Here the allergen is coated on a solid phase and IgE against that allergen only is detected. In radio immunosorbant test (RIST), which is used to detect serum levels of total IgE, anti IgE is coupled to a solid phase and is incubated in patient serum (figure).

IMMUNOFLUORESCENCE ASSAYS

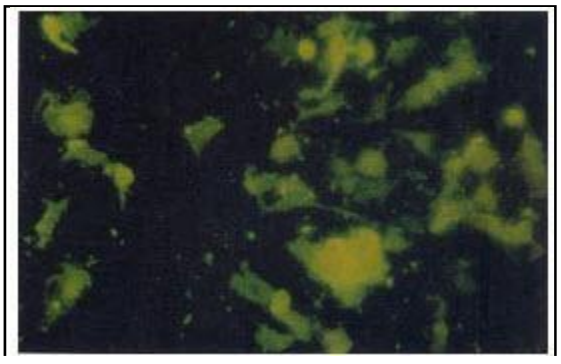
- Fluorescent dyes are commonly used as labels in primary binding tests. The most important and commonly used dye is FITC (fluorescein isothiocyanate). Rhodamine isothiocyanate is also used.
- FITC is a yellow compound that can be bound to antibodies without affecting their reactivity.
- When irradiated with UV light, FITC re- emits visible green/or yellow green light at 525 m m.
- Rhodamine emits deep red fluorescence (546 m m) and phycoerythrin emits red fluorescence.
- Three types of immunofluorescent assays are there,
    - Direct
    - Indirect
    - Sandwich

DIRECT FLUORESCENT ANTIBODY TESTS (DIRECT FAT)

- This test is used to identify the presence of antigen.
- The antibody against a specific antigen (bacteria/virus) is first labeled with FITC.
- A tissue or smear containing the organism is fixed to a glass slide and incubated with FITC labeled antibody and washed to remove unbound antibody.
- When viewed under UV light, the organisms that bind the labeled antibody fluoresce brightly.
- This technique can detect low levels of organisms in samples.
- ***Example:*** used for detection of Rabies virus in cold acetone fixed brain impression smears, for detecting viruses growing in tissue culture or in tissues from infected animals, Mycobacterium paratuberculosis in faeces etc.

INDIRECT FLUORESCENT TESTS (INDIRECT FAT)

- This test is used to detect antibodies in serum or to identify antigen in tissues or cell cultures.
- When testing for antibody, the antigen is allowed to bind on a solid surface (e.g. impression smear on a slide).
- This is incubated with a serum suspected of containing the antibody to that antigen, and the serum is washed off leaving only specific antibodies bound to antigen.
- These antibodies are visualized after incubating with FITC labeled antiglobulin.
- When the unbound antiglobulin is removed by washing and the slide examined under UV light, fluorescence indicates that antibody was present in the test serum.
- In the indirect method of detecting antigen, the material supposed to contain the antigen (smear, cell culture etc.) is incubated with the antibody specific for the antigen and after washing it is incubated with antiglobulin or anti-antibody which is tagged with FITC dye.
- After washing it is viewed under the fluorescence microscope to detect emission of fluorescence (the antibody added first is sometimes called *primary antibody* and that added next is called the *secondary antibody).*



FAT: Apple green fluroscence indicates positive reaction

- Advantages of the Indirect Test:
  - Each antibody molecule binding to antigen will itself bind several antiglobulin molecules, and the fluorescence become considerably brighter than in the direct test
  - By using antiglobulin specific for each immunoglobulin class, the class of the specific antibody present in the serum can be determined.

SANDWICH TEST

- It is a double layer procedure designed to visualize specific antibody.
- First, fix the cell with ethanol to prevent antibody being washed away during the test, and then treat with a solution of antigen.
- After washing, a fluorescein labeled antibody to the particular antigen would then be added to locate those cells which had specifically bound to the antigen.
- The name of the test derives from the fact that antigen is sandwiched between the antibodies present on the cell substrate and added as the second layer.

ENZYME IMMUNE ASSAYS(EIAS)

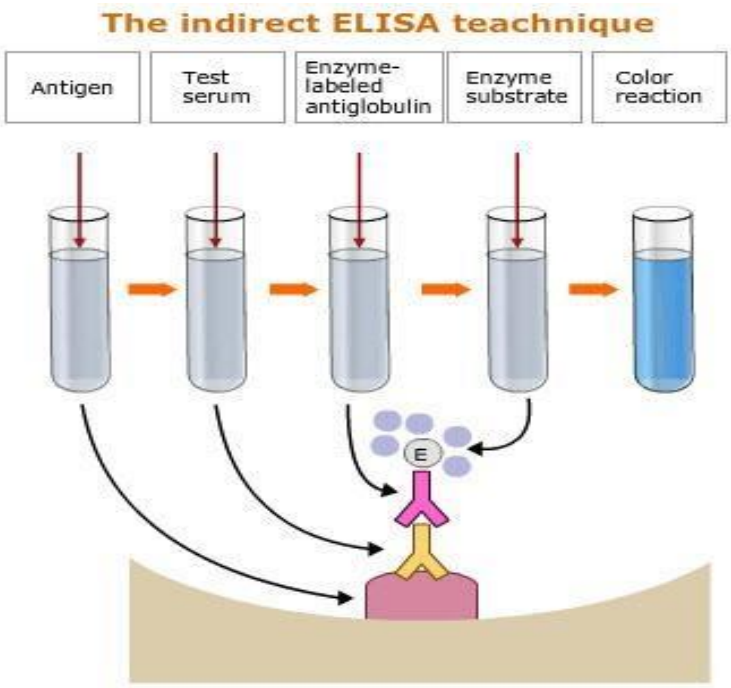
- EIAs, commonly called as ELISAs (enzyme linked immuno sorbant assays) are similar in principle to RIA but depend on an enzyme rather than a radioactive label.
- An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate.
- A number of enzymes have been employed for ELISA, including alkaline phosphatase, horse radish peroxidase and β-galactosidase. These assays match the sensitivity of RIA and have advantage of being safer and less costly.
- The various chromogenic substrates used are PNPP (para nitrophenyl phosphate) for alkaline phosphatase (conjugate), ONPG (O-nitro phenyl β – D – galactopyranoside) for β- galactosidase and OPD (ortho phenylene diamine), TMB (tetra methyl benzidine), ABTS (2,2'-Azino diethylbenzothiazoline sulfonic acid), 5 AS (5 - Amino salicylic acid) and DAB (diamino benzidine) for horse radish peroxidase. *DAB* forms an *insoluble* product.
- Different forms of ELISA are
  - Direct ELISA
  - Indirect ELISA
  - Sandwich ELISA
  - Competitive ELISA
  - Avidin biotin ELISA
  - Dot ELISA
  - Dipstick ELISA

DIRECT ELISA (To detect Antigen)

- Antigen is attached to the solid phase such as 96 well polystyrene plates. After washing enzyme labeled antibody is added.
- After incubation period and it is washed and substrate system is added.
- Development of colour is read using ELISA reader (figure).
- Reading is taken at a particular wave length depending on the substrate used.

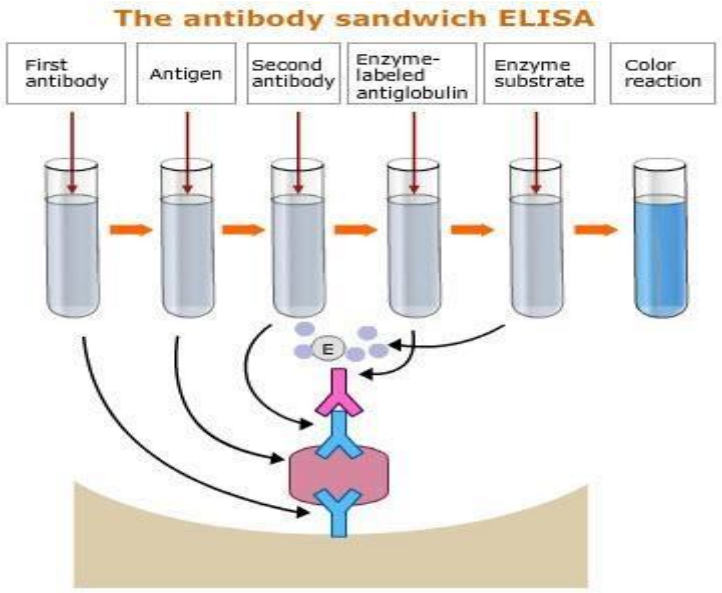
INDIRECT ELISA

- Antibody can be detected using indirect ELISA.
- Serum or some other test sample containing primary antibody is added to an antigen coated microtitre plate well.
- After an incubation period free primary antibody is removed by washing, the presence of antibody bound to the antigen is detected by adding an enzyme tagged secondary antibody or antispecies antibody or antiglobulin which binds to the primary antibody.
- After an incubation period any free secondary antibody is washed away and a substrate for the enzyme is added.
- The amount of colored reaction product formed is measured by specialized ELISA readers.



SANDWICH ELISA

- Antigen can be detected or measured by a sandwich ELISA. The antibody (capture antibody) is immobilized on amicrotitre plate well.
- A sample containing antigen is added and allowed to react with the immobilized antibody.
- After the well is washed, a second enzyme linked antibody (detecting antibody) specific for a different epitope on the antigen is added and allowed to react with the bound antigen.
- After any free secondary antibody is removed by washing, substrate is added and the colored reaction product is measured. This is the direct sandwich ELISA .
- In the double sandwich ELISA, the detecting antibody (specific antibody) can be from a different species than the captureantibody.
- The antispecies secondary antibody linked with enzyme bind to the detecting antibody and colour develops after substrateis added.



COMPETITIVE ELISA

- Competition ELISA imply that two reactants are trying to bind to a third.
- Proper competition assays involve the simultaneous addition of the two competitors.
- Competitive ELISA for detection of antibody
    - This is essentially the same as the indirect ELISA, except that a competing antibody (eg: antibody in serum to betested is added to the solid phase antigen either before or simultaneously with pretitrated specific antibody (eg: mouse monoclonal antibody).
    - The competing antibody must be from a different species from the pretitrated antibody since the antispecies conjugate must not react with both. If the competing antibody i.e. antibody in the test serum binds to antigen, itprevents the pretitrated antibody from binding to the antigen.
    - Then the antispecies conjugate directed against the pretitrated specific antibody is added.
    - Then after incubation and washing, the substrate is added and color reaction product is measured.
    - But in this test, presence of antibody in the test serum is denoted by decreased color production because this antibody prevents binding of the pretitrated antibody and we are using antispecies conjugate which detects onlybound pretitrated antibodies.

AVIDIN BIOTIN ELISA

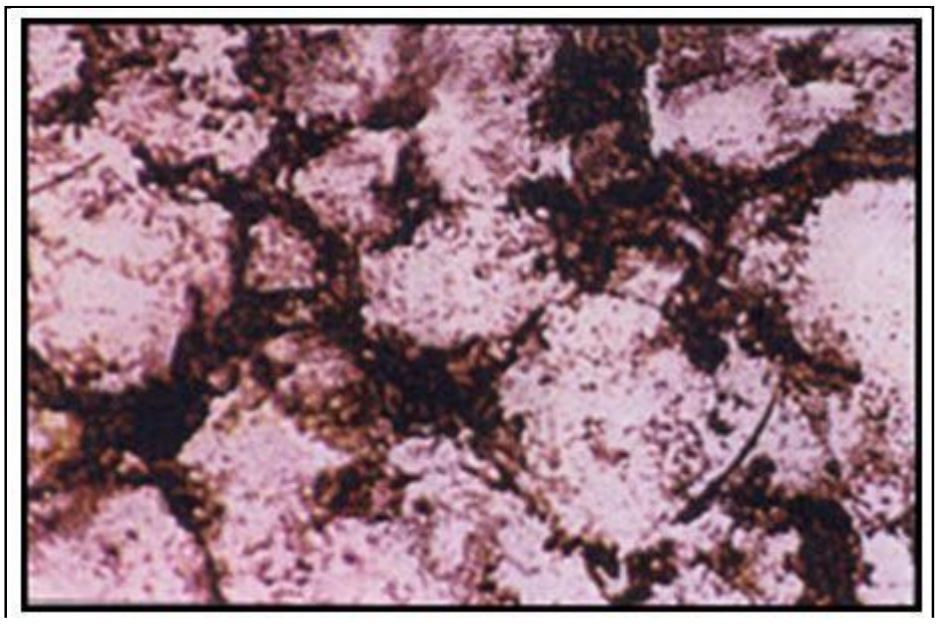
- Enzymes may inhibit antibody activity or lose enzymatic activity in the process of conjugating them to antiglobulin.
- One alternative is to use biotin and its specific binding protein avidin.
- Biotin can bind to protein (antibody) without affecting their biological activity.
- Avidin binds very strongly and specifically to biotin and may be conjugated with enzymes.

DOT ELISA

- Similar to the plate ELISAs, but the solid phase to which antigen is bound is the nitrocellulose membrane (usually) andthe chromogenic substrate used is DAB which forms an insoluble product.
- Hence in positive cases a brown dot is obtained at place of antigen deposition.
- The advantage of dot ELISA over other ELISAs is that the color reaction can be read visually without sophisticated ELISAreaders.
- The test is also easy to perform and can be done in the field.
- The results *i.e.* dots can be stored for retrospective analysis.

IMMUNOPEROXIDASE TEST (IPT)

- Used to detect antigen in tissue sections. Similar to immunofluorescence test or direct or indirect ELISA.
- Here enzyme labeled immunoglobulin is used and the chromogenic substrate is DAB.
- Positive reactions indicated by presence of brown deposit at site of antigen.



IPT - Brown colour in the cytoplasm indicates positive reaction

- Unlike in FAT, this deposition can be visualized by light microscopy and paraffine embedded and resin embedded sectionscan be used.
- Horse raddish peroxidase and alkaline phosphatase are the enzymes most often used.
- A disadvantage is that endogenous peroxidase present in the cells may produce false positives.

WESTERN BLOTTING (IMMUNOBLOTTING)

- Used to identify a specific protein in a complex mixture of proteins.
- It is named for its similarity to Southern blotting which detects DNA fragments and Northern blotting which detects mRNAs.
- In Western blotting, a protein mixture is electrophorectically separated on an SDS-polyacrylamide gel (SDS-PAGE).
- The protein bands are transferred to a nylon /nitrocellulose membrane passively or by electrophoresis and individualprotein bands are identified by flooding the membrane with radiolabeled or enzyme linked antibody specific for the protein of interest.
- The antigen-antibody complexes that are formed (on the band containing the protein recognized by the antibody) can be visualized by a variety of methods (figure).
- If a radiolabeled antibody is used, the position of the protein band can be determined by exposing the membrane to an Xray film (this is called *autoradiography).*
- But usually enzyme labeled antibodies are used rather than radiolabeled ones.
- After binding of the enzyme tagged antibody to protein band of the membrane, addition of a chromogenic substrate thatproduces a colored and insoluble product causes the appearance of a colored band at the site of the target antigen/protein(Western blotting can also be used to identify a specific antibody in a mixture).