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Syllabus:

1- ELECTROPHORESIS
2- IMMUNOFIXATION ELECTROPHORESIS.
3- FLUORESCENT IMMUNOASSAY
4- Cell Flow cytometry
5- Immunohistochemstry
6- MONOCLONAL ANTIBODY
7- Transplantation
8- CLINICAL HISTOCOMPATIBILITY TESTING

References:

1-Stevens,C.D.2010, Clinical immunology and Serology Alabortory prespective,3rded F.A Davis company.philadelphia.
2- Stevens,C.D.2021, Clinical immunology and Serology Alabortory prespective,5rded F.A Davis company.philadelphia

Lec (1) **Immunotechnology**

Prof. Dr. Ekhlass Noori Ali

Immunotechnology: it is an introduction to various techniques commonly used in diagnosing human disease or, rather, assays to evaluate the competence or incompetence of the immune system. Finally, it will serve as an introduction to the many new techniques emerging in the past several years that have widened our knowledge of the complex relationship of microbe–host interactions in human disease

ELECTROPHORESIS

Electrophoresis is the migration of charged solutes or particles in an electrical field. Using this principle, charged molecules can be made to move and different molecules can be separated if they have different velocities in an electrical field. The electrical field is applied to a solution with oppositely charged electrodes. Charged particles in this solution begin to migrate. Positively charged particles (cations) move to the negatively charged (−) electrode; negatively charged particles (anions)migrate to the positively charged (+) electrode (Fig. 1).

Serum proteins are often separated by electrophoresis.Serum electrophoresis results in the separation of proteins into five fractions using cellulose acetate as a support medium (Fig. 1). This separation is based on the rate of migration of these individual components in an electrical field.Electrophoresis is a versatile analytic technique. Immunoglobulins are separated by electrophoresis using agarose as a support medium. The immunologic applications of electrophoresis include identification of monoclonal proteins in serum or urine.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis (IEP) involves the electrophoresis of serum or urine followed by immunodiffusion.

Passive Immunodiffusion Procedures

Immunodiffusion is a laboratory method for the quantitative study of antibodies (e.g., radial immunodiffusion [RID]) and rocket electrophoresis or for identifying antigens (e.g.,Ouchterlony technique). Single diffusion preceded radial immunodiffusion. In the single diffusion procedure, antigen was layered on top of a gel medium and, as the antigen moved down into the gel, precipitation

occurred and migrated down a tube in proportion to the amount of antigen present. In radial immunodiffusion (RID) (Fig. 3) antibody is uniformly distributed in the gel medium

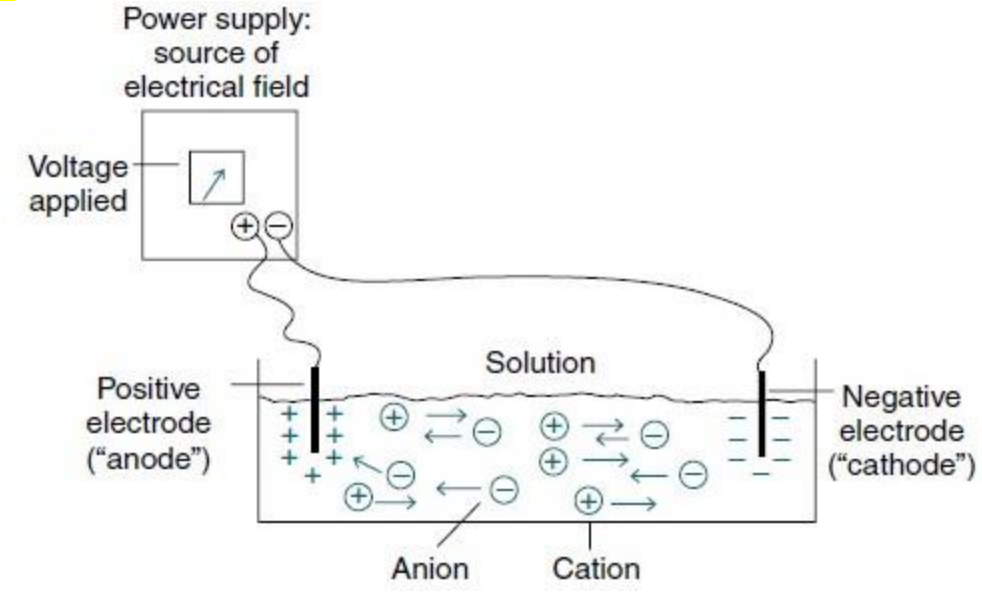


Fig1: **Application of electrical field to a solution of ions makes the ions move**

and antigen is added to a well cut into the gel. As the antigen diffuses from the well, the antigen antibody combination occurs in changing proportions until the zone of equivalence is reached and a stable lattice network is formed in the gel. The area of the visible ring is compared with standard concentrations of antigens. A variation of this principle is rocket immunoelectrophoresis (Fig. 4).The classic Ouchterlony double diffusion technique (Fig. 5). performed on a gel medium is used to detect the presence of antibodies and determine their specificity by visualization of lines of identity, or precipitin lines. The reaction of antigen-antibody combination occurs by means of diffusion.The size and position of precipitin bands provide information regarding equivalence or antibody excess. Proteins are differentiated not only by their electrophoretic mobility, but also by their diffusion coefficient and antibody specificity. Although double immunodiffusion produces a separate precipitation band for each antigen-antibody system in a mixture, it is often difficult to determine all the components in a complex mixture.

Principle

Immunoelectrophoresis is a combination of the techniques of electrophoresis and double immunodiffusion. IEP separates the antigen mixture by electrophoresis before performing immunodiffusion. In the first phase, electrophoresis, serum is placed in an appropriate medium (e.g., cellulose acetate or agarose)and then electrophoresed to separate its constituents according to their electrophoretic mobility—albumin; α1-, α2-,β-, and γ-globulin fractions After electrophoresis, in the second phase, immunodiffusion,the fractions are allowed to act as antigens and to interact with their corresponding antibodies. Antiserum (polyvalent or monovalent) is deposited in a trough cut into the gel to one side and parallel to the line of separated proteins. Incubation allows double immunodiffusion of the antigens and antibodies.

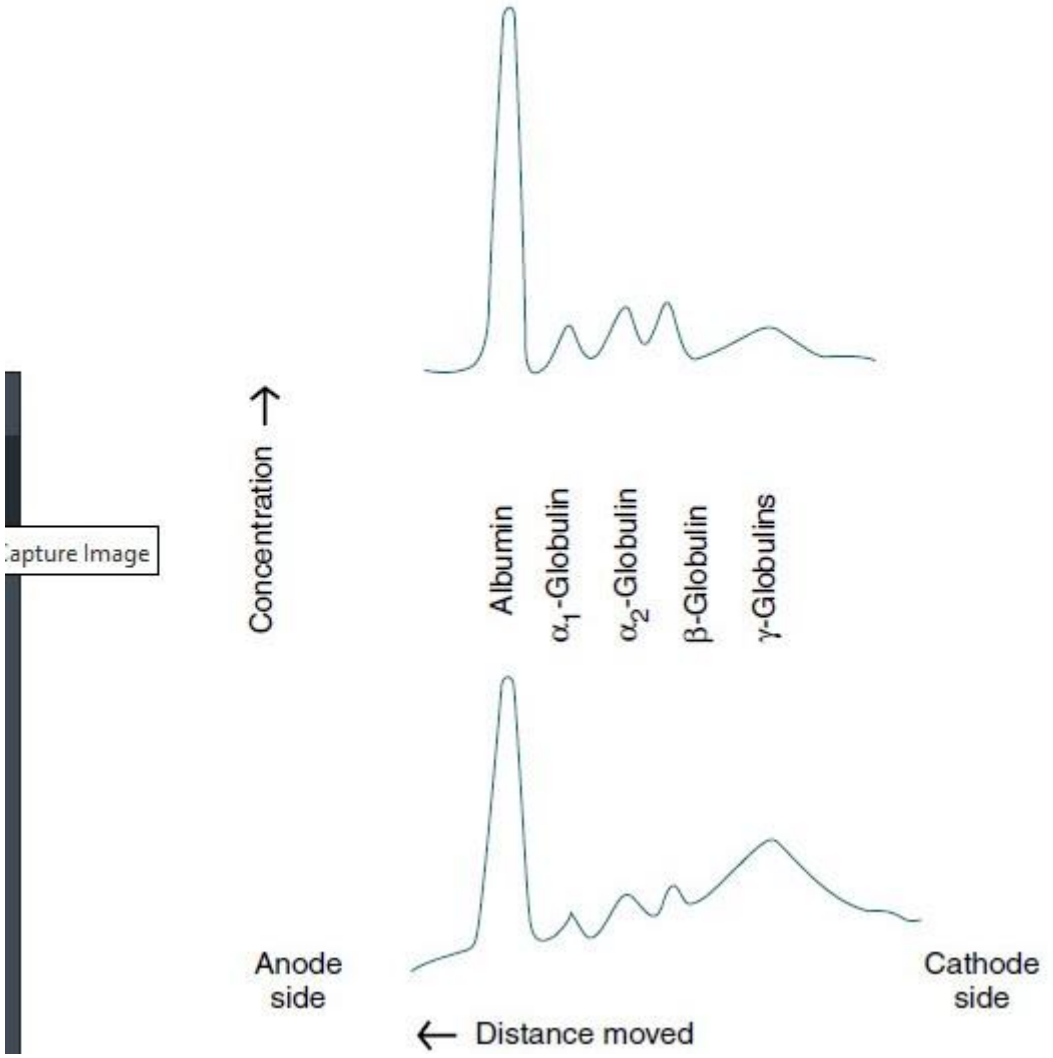


Fig2: **Example of the effect of disease (hepatic cirrhosis) on serum protein electrophoresis pattern.**

Each antiserum diffuses outward, perpendicular to the trough,and each serum protein diffuses outward from its point of electrophoresis.When a favorable antigen-to-antibody ratio exists (equivalence), the antigen-antibody complex becomes visible as precipitin lines or bands. Diffusion is halted by rinsing the plate in 0.85% saline. Unbound protein is washed from the agarose with saline and the **antigen-antibody precipitin arcs** are stained with a protein-sensitive stain.

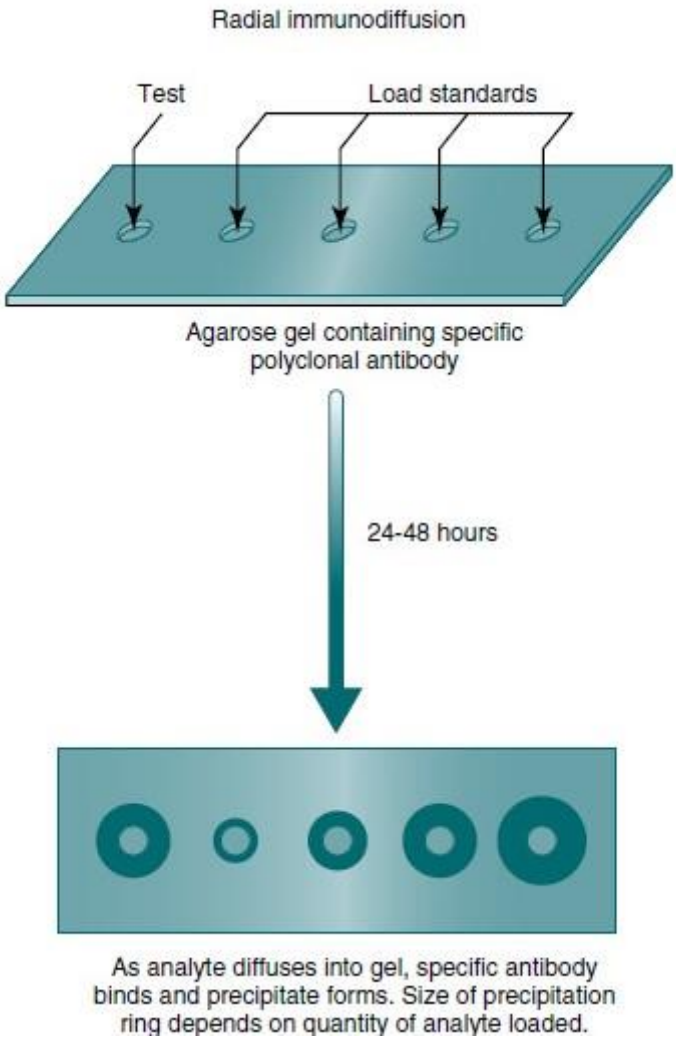


Fig3: **Measurement of immune-related proteins by a radial immune diffusion.**

Each line represents one specific protein (Fig. 6). Proteins are thus differentiated by their diffusion coefficient and antibody specificity as well as electrophoretic mobility. Antibody diffuses as a uniform band parallel to the antibody trough. If

the proteins are homogeneous or of like composition,the antigen diffuses in a circle and the antigen-antibody precipitation line resembles a segment, or arc, of a circle. If the antigen is heterogeneous or not uniform in composition, the antigen-antibody line assumes an elliptical shape. One arc of precipitation forms for each constituent in the antigen mixture.

This technique can be used to resolve the protein of normal serum into 25 to 40 distinct precipitation bands. The exact number depends on the strength and specificity of the antiserum used.

Normal Appearance of Precipitin Bands

Immuno precipitation bands should be of normal curvature,symmetry, length, position, intensity, and distance from the antigen well and antibody trough. In normal serum, immunoglobulin G (IgG), IgA, and IgM are present in sufficient concentrations of 10 mg/mL, 2 mg/mL, and 1 mg/mL, respectively,to produce precipitin lines. The normal concentrations of IgD and IgE are too low to be detected by IEP.A normal IgG precipitin band is elongated, elliptical,slightly curved, and clearly visible in undiluted serum and 1:10 diluted serum. An IgG band is located cathodic to the antigen well in the alpha (α) area of the electrophoretogram.

If monospecific serum is used, it is fused with a thin precipitin line positioned midway between the antigen well and antibody trough and extending into the beta (β) area. The IgM and IgA bands are visible in undiluted serum but disappear at a 1:10 dilution of serum. The IgA band is a flattened,thin arc, slightly cathodic to the well in the α-β position. The IgM line is a barely visible thin line, slightly cathodic to the antigen well

Clinical Applications

Immunoelectrophoresis is most often used to

1- determine qualitatively the elevation or deficiency of specific classes of immunoglobulins.Also,
2- IEP is a reliable and accurate method for detecting structural abnormalities 3-concentration changes in proteins. It is possible to identify the absence of a normal serum protein (e.g., congenital deficiency of complement component) or alterations in serum proteins. This method can be used to screen for circulating immune complexes, characterize **cryoglobulinemia** and **pyroglobulinemia,** and recognize and characterize antibody syndromes and the various **dysgammaglobulinemias.** The most common application of IEP is in the diagnosis of a **monoclonal gammopathy,**

Sources of Error

The **prozone phenomenon** is an incomplete precipitin reaction caused by antigen excess (antigen-to-antibody ratio too high). Prozoning should be suspected if a precipitin arc appears to run into a trough,

Abnormal Appearance of Precipitin Bands

The size and position of precipitin bands provide the same type of information regarding equivalence or antigen-antibody excess as **double immunodiffusion systems.** The position and shape of precipitin bands in the IEP assay of serum are relatively stable and reproducible; almost any deviation is abnormal . These abnormalities can be detected by evaluating the following features of the precipitin bands:

• Position of the band in relation to electrophoretically identified protein fractions
• Position of the band between the antigen well and antibody trough
• Distortion of the curvature or arc formation
• Thickening (density) and elongation of a band
• Shortening (inhibition), thinning, or doubling of a band

Position of Band

The precipitin band may be displaced compared with its normal position in the control serum because molecular charges in the abnormal protein may affect its speed of migration in the electrophoresis phase of IEP. A precipitin band may form a line of fusion or partial fusion with another protein, indicating the presence of proteins immunologically similar but electrophoretically distinct.A distinct abnormality in the position of the band is seen in cases of monoclonal IgA gammopathy. The monoclonal IgA band is closer to the antibody trough than normal IgA.

Polyvalent and Monovalent Antisera

Polyvalent antiserum confirms the presence or absence of major protein fractions. Monovalent antiserum for specific individual immunoglobulins identifies only the corresponding proteins.

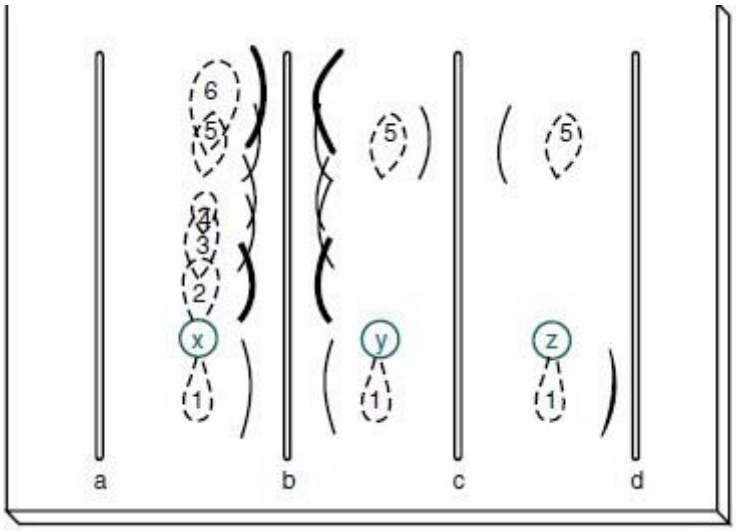


Fig4: **Configuration for immunoelectrophoresis.** Sample wells are punched in the agar-agarose, sample is applied, and electrophoresis is carried out to separate the proteins in the sample. Antiserum is loaded into the troughs and the gel is incubated in a moist chamber at 4° C (39° F) for 24 to 72 hours. Track *x* represents the shape of the protein zones after electrophoresis; tracks *y* and *z* show the reaction of proteins *5* and *1* with their specific antisera in troughs *c*
and *d*. Antiserum against proteins *1* through *6* is present in trough *b*.

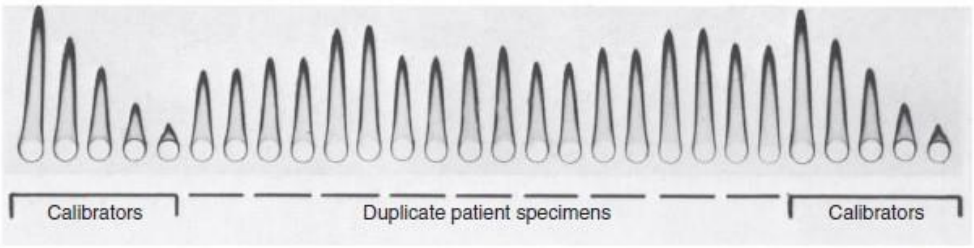


Fig5: **Rocket immunoelectrophoresis of human serum albumin.** Patient samples were applied in duplicate. Calibrators were placed at opposite ends of the plate.

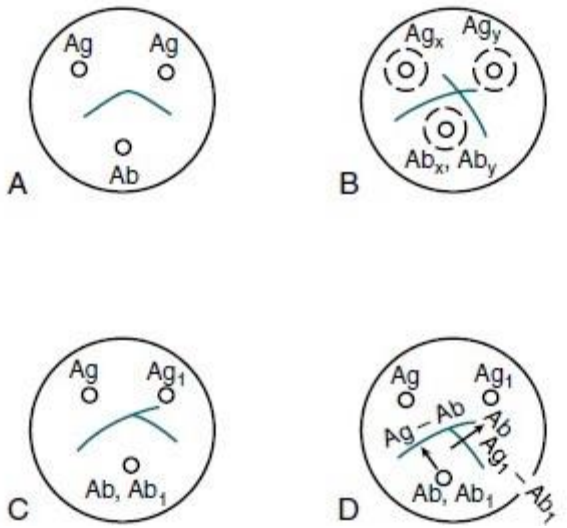


Fig6: **Double immunodiffusion in two dimensions by theOuchterlony technique. A,** Reaction of identity. **B,** Reaction of nonidentity.**C,** Reaction of partial identity. **D,** Scheme for spur formation.

Lec (2) **Immunotechnology**

Prof. Dr. Ekhlass N. Ali

IMMUNOFIXATION ELECTROPHORESIS

Immunofixation electrophoresis (IFE), or simply **immunofixation,**has replaced IEP in the evaluation of monoclonal gammopathies because of its rapidity and ease of interpretation.IFE is a two-stage procedure, agarose gel protein electrophoresis
and immunoprecipitation. The test specimen may be serum, urine, cerebrospinal fluid (CSF), or other body fluids.The primary use of IFE in clinical laboratories is for the characterization of monoclonal immunoglobulins.

Clinical Applications

Although IFE was first described in 1964, it was introduced as a procedure for the study of immunoglobulins in 1976. IEP and IFE are complementary techniques best used in the workup of a patient with a suspected monoclonal gammopathy. The laboratory protocol for ruling out monoclonal gammopathy should include high-resolution electrophoresis, IEP of both serum and urine, and a quantitative immunoglobulin assay. These procedures are usually sufficient to detect and characterize monoclonal proteins with a serum concentration of 1 g/dL or more.

The following three protein variables can be determined using IFE:

1. Antigenic specificity
2. Electrophoretic mobility
3. Quantity or ratio of test and control proteins

COMPARISON OF TECHNIQUES

IEP is technically simpler and less subject to antigen excess phenomenon than IFE. If high concentrations of monoclonal protein with IFE give no visible reactions, IEP is considered to be a better technique for typing large monoclonal gammopathies.

Immunofixation electrophoresis can be optimized to give greater sensitivity and resolution than IEP. IFE should be reserved for anomalous proteins, which are difficult to characterize by IEP. These include small bands, such as those exhibited
in the early stages of monoclonal gammopathies or L-chain disease, and any multiple, closely spaced bands. The results of IFE are easier to interpret than those of IEP because interpretation is based on examination of a precipitate pattern
directly analogous to routine electrophoresis; IFE does not depend on detecting slight deviations in the shape of a precipitin arc (Fig. 1; Table 1).

Table1: **Comparison of Immunoelectrophoresis and Immunofixation Electrophoresis .**

Feature	IEP	IFE
Ease of use	Easy	More complex
Sensitivity	Less sensitive	More sensitive
Monoclonal gammopathies	Better for typing large monoclonal gammopathies	Used for difficult to characterize anomalous proteins
Interpretation	Challenging	Easier

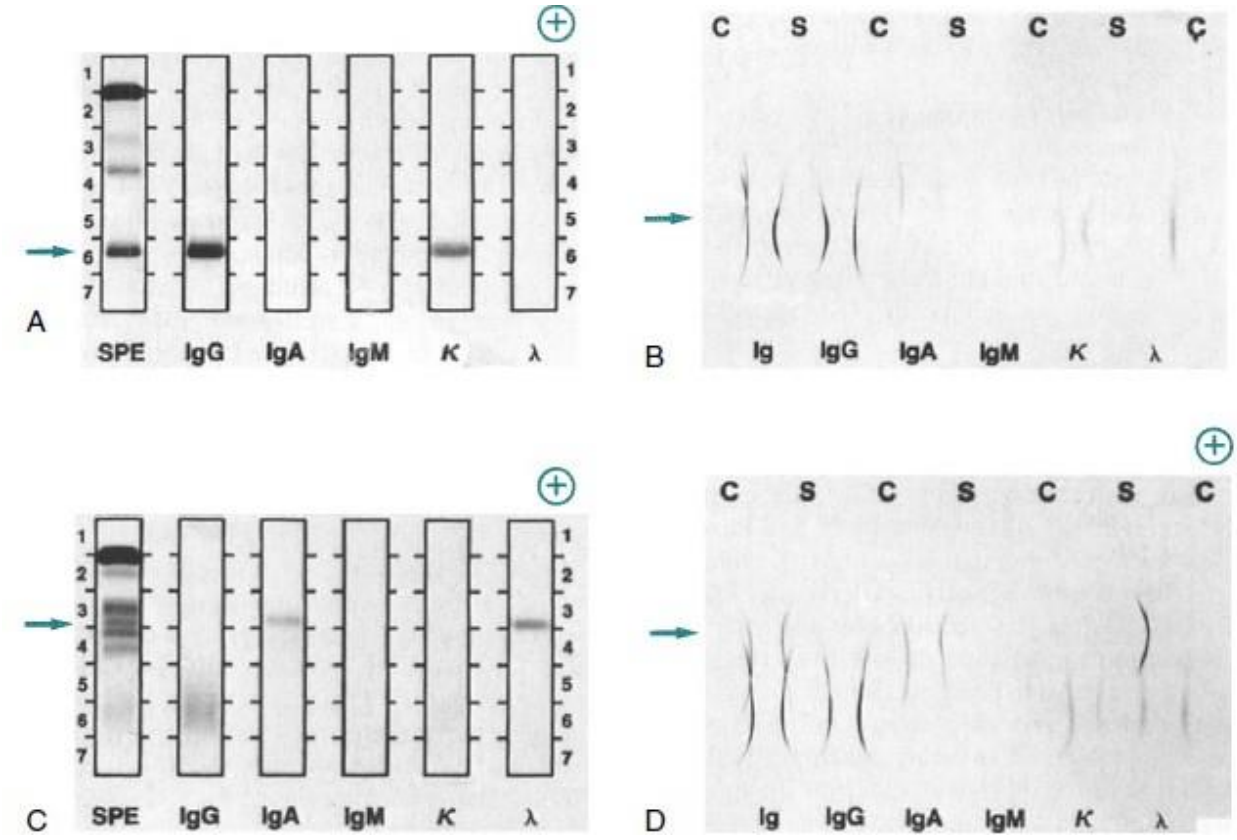


Fig1: **Comparison of immunofixation electrophoresis (IFE) and immunoelectrophoresis (IEP) for two patients with monoclonal gammopathies.**

A, Patient specimen with an IgG (κ) monoclonal protein, as identified by IFE. Note the position of the monoclonal protein *(arrow).* After electrophoresis, each track except serum protein electrophoresis (SPE) is reacted with its respective antiserum; then, all tracks are stained to visualize the respective protein bands. Immunoglobulins G, A, and M (IgG, IgA, IgM); kappa (κ); and lambda (λ)

indicate antiserum used on each track. **B,** Same specimen as in **A,** with proteins identified by IEP. Note the position of the monoclonal protein *(arrow).* Normal control (C) and patient sera (S) are alternated. After electrophoresis, antiserum is added to each trough, as indicated by the labels Ig, IgG, IgA, IgM, κ, and λ. The antisera react with separated proteins in the specimens to form precipitates in the shape of arcs. The IgG and κ arcs are shorter and thicker than
those in the normal control, showing the presence of the IgG (κ) monoclonal protein. The concentrations of IgA, IgM, and λ light chains also are reduced. **C,** Patient specimen with an IgA (λ) monoclonal protein identified by the IFE procedure, as described in **A. D,** Same specimen as in **C,**
with proteins identified by IEP, as described in **B.** The abnormal IgA and λ arcs for the patient specimen indicate an elevated concentration of amonoclonal IgA (λ) protein.

Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used type of CE because of its simplicity and versatility. As long as a molecule is charged, it can be separated by CZE.Also, CZE is simple to perform because the capillary is only filled with buffer. Separation occurs as solutes migrate at different velocities through the capillary. Another advantage of CZE is that it separates anions and cations in the same run, which is not done in other CE methods. However,CZE cannot separate neutral molecules.

Isotachophoresis define or talk about

Isotachophoresis (ITP) is a focusing technique based on the migration of the sample components between the leading and terminating electrolytes. Solutes with mobilities intermediate to those of the leading and terminating electrolytes stack into sharp focused zones. Although used as a mode of separation, transient ITP has been used primarily as a sample concentration technique.

Capillary Isoelectric Focusing

Capillary isoelectric focusing (CIEF) is a separation method that allows amphoteric molecules, such as proteins, to be separated by electrophoresis in a pH gradient generated between the cathode and anode. A solute will migrate to a point at which its net charge is zero. At the solute's isoelectric point (pI), migration stops, and the sample is focused into a tight zone. In CIEF, once a solute has focused at its pI,the zone is mobilized past the detector by either pressure or chemical means. CIEF is often employed in protein characterization as a mechanism to determine a protein's pI.

CAPILLARY ELECTROPHORESIS

In capillary electrophoresis (CE) the classic separation techniquesof zone electrophoresis, isotachophoresis, **isoelectric focusing,**and gel electrophoresis are performed in small-bore (10- to 100-µm), fused silica capillary tubes, 20 to 200 cm in length

The CE method is efficient, sensitive, and rapid. High electrical field strengths are used to separate molecules based on differences in charge, size, and ydrophobicity. Sample introductionis accomplished by immersing the end of the capillary into a samplevial and applying pressure, vacuum, or voltage.

Table2: Comparison of Traditional Capillary Electrophoresis and Microchip Capillary Electrophoresis

Feature	Conventional CE	Microchip CE
Separation channels	Mainly silica, single capillary or capillary array	Glass or polymer
Separation media	Buffers, gels, sieving polymers, microparticles	Buffers, sieving polymers, microparticles
Speed of analysis	Fast (typically minutes)	Very fast (typically seconds)
Integration	Difficult to connect capillaries	Easy to integrate multiple functions (e.g., PCR CE)
Potential for growth	Relatively mature	Emerging technology with potential for new designs and applications

Microchip CE was developed in the early 1990s. The advantages of microchip CE include high speed, reduced reagent consumption, integration analysis, and miniaturization. The applications of microchip CE are diverse and include immune

disorders. Conventional CE revolutionized DNA analysis and was vital to the Human Genome Project. Microchip CE is still in the early stages of development but has demonstrated distinct advantages compared with traditional CE(table2)

Principle

is intended for the identification of monoclonal gammopathies in serum, urine, or CSF using high-resolution protein electrophoresis and immunofixation.In the first step of the IFE procedure, a single specimen is applied to six different positions on an agarose plate and the proteins are separated according to their net charge by electrophoresis.In the second phase, monospecific antisera are applied to five of the electrophoresis patterns: IgG, IgA, IgM, and κ and γ antisera. A protein fixative solution is applied to the sixth pattern to produce a complete protein reference pattern. The plate is incubated for 10 minutes.If complementary antigen is present in the proper proportions in the test sample, antigen-antibody complexes form and

precipitate. The formation of a stable antigen-antibody precipitate fixes the protein in the gel. After fixation, the gel is washed in deproteinization solution (e.g., dilute NaCl) and nonprecipitated proteins are washed out of the agarose, leaving only the

antigen-antibody complex. The protein reference pattern and the antigen-antibody precipitation bands are stained with a protein-sensitive stain.

Western blot technique

proteins are separated electrophoretically, transferred to membranes, and identified through the use of labeled antibodies specific for the protein of interest (Fig. 3).

Western blot: technique detects antibodies to specific epitopes of antigen subspecies. Electrophoresis of antigenic material results in the separation of the antigen components by molecular weight (MW). Blotting the separated antigen to

nitrocellulose, retaining the electrophoretic position, and causing it to react with patient specimen will result in the binding of specific antibodies, if present, to each antigenic band. Electrophoresis of known MW standards allows for the determination of the MW of each antigenic band to which antibodies may be produced. These antibodies are then detected using EIA reactions that characterize antibody specificity.The Western blot technique is often used to confirm the

specificity of antibodies detected by enzyme-linked **immunosorbent** assay (ELISA) screening procedures.

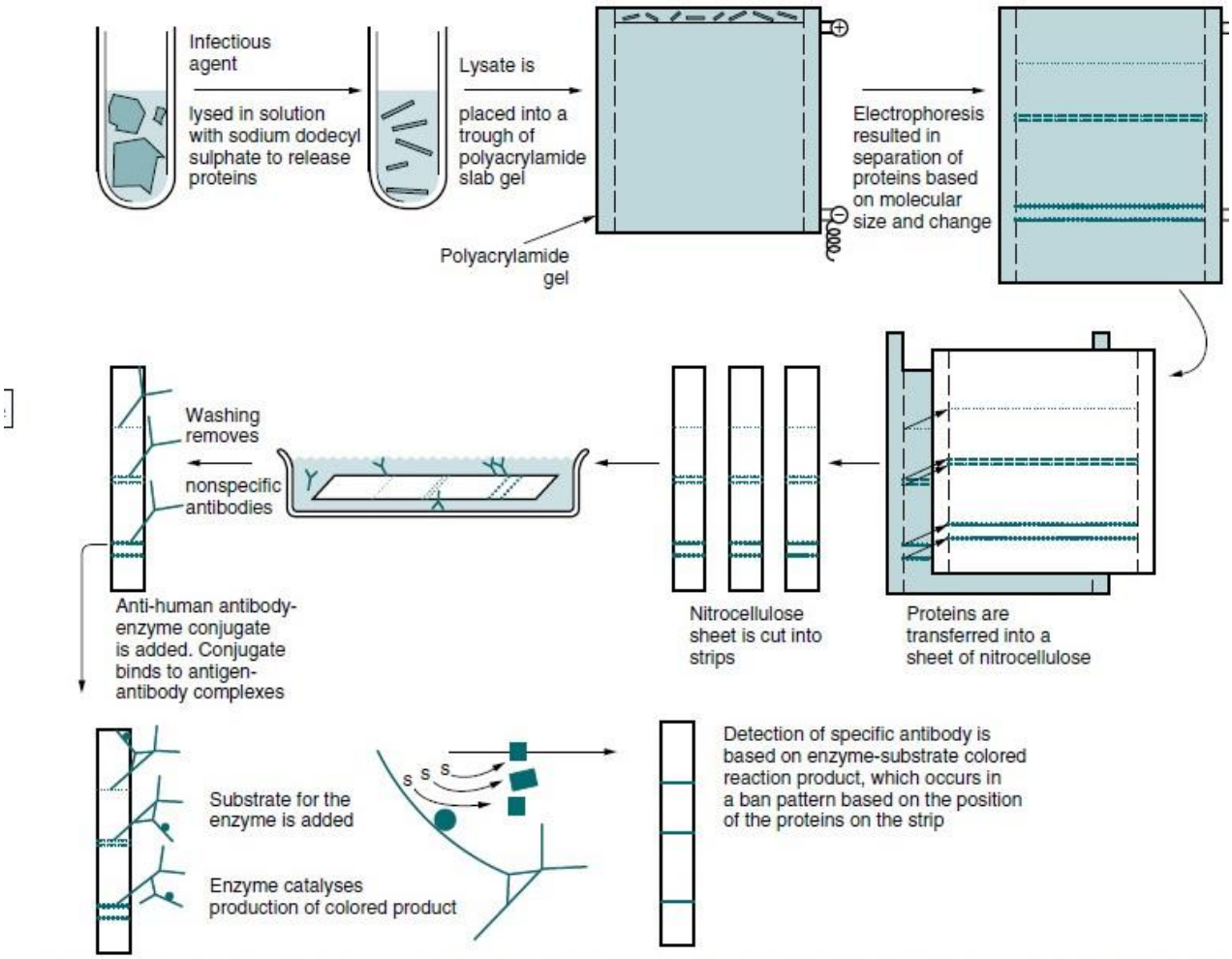


Fig3: **Western blot immunoassay**

Direct and Indirect Methods

Direct detection uses a labeled primary antibody. Because incubation with a secondary antibody is eliminated, this strategy is performed in less time than a classical Western blot. Additionally, background signal from secondary antibody cross reactivity is eliminated. Direct detection also enables probing for multiple targets simultaneously. Labeling a primary antibody, however, sometimes has an adverse effect on its immune reactivity, and even in the best of circumstances, a labeled primary antibody cannot provide signal amplification. Consequently, the direct method is generally less sensitive than indirect detection and is best used only when the target is relatively abundant. One option is biotinylating the primary antibody, which is an indirect method that both amplifies the signal and eliminates the secondary antibody. Labeling with

biotinylation reagents typically results in more than one biotin moiety per antibody molecule. Each biotin moiety is capable of interacting with an enzyme-conjugated avidin, streptavidin or Thermo Scientific Neutr Avidin Protein. These multiple enzymes catalyze the conversion of appropriate substrate to amplify the signal. Essentially, the avidin conjugate replaces a secondary antibody and its appropriate molar concentration is the same as if a secondary antibody were used.

Blocking buffer

Many different blocking reagents are available for Western blotting. Because no blocking reagent is appropriate for all systems, empirical testing is essential.

The system's antibodies or target. For example, using 5% non fat milk as a blocking reagent when using avidin/biotin systems results in high background because milk contains variable amounts of endogenous biotin, which binds the a vidin.

Western Blotting Protocol

1. Separate the proteins in the sample by gel electrophoresis.
2. Prepare the transfer buffer: Use Tris-glycine transfer buffer dissolved in 400 ml of ultrapure water plus 100 ml methanol (25 mM Tris, 192 mM glycine, pH 8.0, 20% methanol). Use and store the transfer buffer at 4°C.
3. Construct a gel "sandwich" (Figure 3) for wet transfer. For semi-dry transfer, prepare the sandwich in the same order between the anode and cathode.
4. Transfer proteins from the gel to a membrane. For wet transfer using a mini transfer apparatus designed for a 8 × 10 cm gel, transfer at 40 V for 90minutes keeping the buffer temperature at 4°C. For semi-dry transfer use 15 V for 90 minutes.
5. Remove the membrane and block nonspecific binding sites with a blocking buffer for 20-60 minutes at room temperature (RT) with shaking.
6. Incubate the blot with the primary antibody solution (see Table 2) containing 10%blocking solution with rocking for 1 hour.If desired, incubate the blot overnight a 2-8°C.
7. Wash the membrane three times for 5 minutes each with Tris-buffered saline (TBS), phosphate-buffered saline (PBS) or other physiological wash buffer containing 0.05% Tween-20. If using an enzyme-conjugated primary antibody, proceed to Step 10.

8. Incubate blot with the enzyme conjugate (see Table 3) containing 10% blocking solution for 1 hour with rocking at RT.
9. Wash the membrane five times for 5 minutes each in wash buffer to remove any non bound conjugate. It is crucial to thoroughly wash the membrane after incubation with the enzyme conjugate.
10. Prepare the substrate. Use a sufficient volume to ensure that the blot is completely wetted with substrate and the blot
does not become dry (0.1 ml/cm2).
11. Incubate the blot with substrate for 1 minute when using Pierce ECL or 5 minutes when using Super Signal Substrates.
12. Remove the blot from the substrate and place it in a plastic membrane protector. A plastic sheet protector works well,although plastic wrap also may be used. Remove all air bubbles between the blot and the surface of the membrane protector.
13. Image the blot using film or a cooled CCD camera.

Lec (3) **Immunotechnolgy**

Prof. Dr. Ekhlass N. Ali

FLUORESCENT IMMUNOASSAY

In 1941, Albert Coons demonstrated that antibodies could be labeled with molecules that fluoresce. These fluorescent compounds are called *fluorophores* or *fluorochromes.* They can absorb energy from an incident light source and convert that energy into light of a longer wavelength and lower energy as the excited electrons return to the ground state Fluorophores are typically organic molecules with a ring structure, and each has a characteristic optimum absorption range. The time interval between absorption of energy and emission of fluorescence is very short and can be measured in nanoseconds.

Ideally, a fluorescent probe should exhibit high intensity,which can be distinguished easily from background fluorescence. It should also be stable. The two compounds most often used are fluorescein and rhodamine, usually in the form of isothiocyanates, because these can be readily coupled with antigen or antibody. Fluorescein absorbs maximally at 490 to 495 nm and emits a green color at 517 to 520 nm. It has a high intensity, good photostability,and a high quantum yield. Tetramethylrhodamine absorbs at 550 nm and emits red light at 580 to 585 nm. Because their absorbance and emission patterns differ, fluorescein and rhodamine can be used together. Other compounds used are phycoerythrin, europium (β-naphthyl trifluoroacetone),and lucifer yellow VS.1,5Fluorescent tags or labels were first used for histochemical localization of antigen in tissues. This technique is called **immunofluorescent assay (IFA),** a term restricted to qualitative observations involving the use of a fluorescence microscope. In this manner, many types of antigens can be detected either in fixed tissue sections or in live cell suspensions with a high degree of sensitivity and specificity.The presence of a specific antigen is determined by the appearance of localized color against a dark background.

This method is used for rapid identification of microorganisms in cell culture or infected tissue, tumor-specific antigens on neoplastic tissue, transplantation antigens, and CD antigens on T and B cells through the use of cell flow cytometry.

Direct Immunofluorescent Assays

Fluorescent staining can be categorized as direct or indirect,depending on whether the original antibody has a fluorescent tag attached. In a

direct immunofluorescentassay,

1- antibody that is conjugated with a fluorescent tag is added directly to unknown antigen that is fixed to a microscope slide.
2- 2- After incubation and a wash step,
3- The slide is read using a fluorescence microscope.
4- Antigens are typically visualized as bright apple green or orange-yellow objects against a dark background. Direct immunofluorescent assay

is best suited to antigen detection in tissue or body fluids, while indirect assays can be used for both antigen and this method include *Legionella pneumophila*,*Pneumocysti scarinii*, *Chlamydia trachomatis*, and respiratory syncytial virus (RSV) **(Fig. 1)**.

Indirect Immunofluorescent Assays

Indirect immunofluorescent assays involve two steps,

1- The first of which is incubation of patient serum with a known antigen attached to a solid phase.
2- The slide is washed
3- And then an antihuman immunoglobulin containing a fluorescent tag is added. This combines with the first antibody to form a sandwich, which localizes the fluorescence. In this manner, one antibody conjugate can be used for many different types of reactions, eliminating the need for numerous purified, labeled reagent antibodies. Indirect assays result in increased staining, because multiple molecules can bind to each primary molecule, thus making this a more sensitive technique. Such assays are especially useful in antibody identification and have been used to detect treponema, antinuclear,chlamydial, and toxoplasma antibodies, as well as antibodies to such viruses such as herpes simplex, Epstein-Barr, and cytomegalovirus. **Figure 2** depicts the difference between the two techniques.Both techniques allow for a visual assessment of the adequacy of the specimen. This is especially helpful in testing for chlamydia and RSV antigens. Immunofluorescent assays in general.

Other Fluorescent Immunoassays

Quantitative fluorescent immunoassays (FIAs) can be classified as heterogeneous or homogeneous, corresponding to similar types of enzyme immunoassays. In this case,the label is fluorescent, and such a label can be applied to either antigen or antibody. Solid-phase heterogeneous fluorescent assays have been developed for the identification of antibodies to nuclear antigen, toxoplasma antigen ,rubella virus, and numerous other virus antigens.TORH, H= Hepatitis,

In addition ,fluorescent assays are used to detect such important biological compounds as cortisol, progesterone, and serum thyroxin (T4).

However, many of the newer developments in fluorescent immunoassay have been related to homogeneous immunoassays. Homogeneous FIA, just like the corresponding EIAs, requires no separation procedure, so it is rapid and simple to perform. There is only one incubation step and no wash step, and usually competitive binding is involved. The basis for this technique is the change that Occurs in the fluorescent label on antigen when it binds to specific antibody. Such changes may be related to wavelength emission, rotation freedom, polarity, or dielectric strength.

There is a direct relationship between the amount of fluorescence measured and the amount of antigen in the patient sample. As binding of patient antigen increases, binding of the fluorescent analyte decreases, and hence more fluorescence

Is observed.

Advantages and Disadvantages of Fluorescent Immunoassay

In principle, the use of fluorescence has the potential for high sensitivity and versatility. The methodology is fairly simple, and there is no need to deal with and dispose of hazardous substances. The main problem, however, has been separation of the signal on the label from auto fluorescence produced by different organic substances normally present in serum. Another difficulty encountered is the fact that nonspecific binding to substances in serum can cause quenching or diminishing of the signal and change the amount of fluorescence generated.

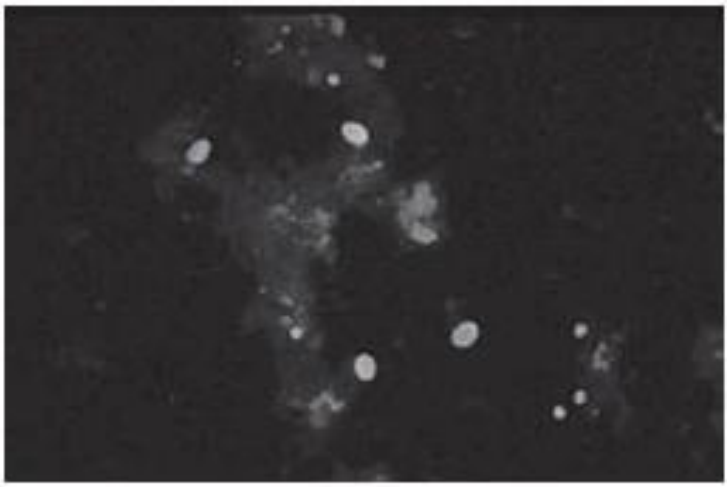


Fig1: Direct fluorescent antibody test for *Giardia* and *Cryptosporidium* in stool. Larger oval bodies are *Giardia lamblia* cysts, and the smaller round bodies are *Cryptosporidium sp.* cysts.

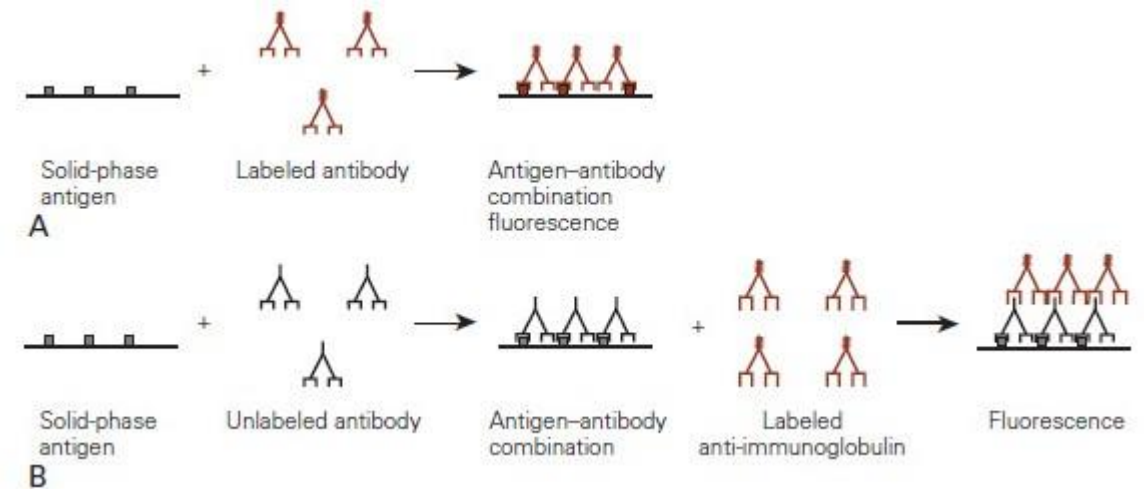


Fig2: Direct versus indirect immunofluorescent assays. *(A)* Direct fluorescent assay. Solidphase
antigen fixed to a microscope slide is incubated directly with a fluorescent-labeled antibody. The slide is washed to remove unbound antibody. If specific antigen is present in the patient sample,fluorescence will be observed.
(B) Indirect fluorescence. Patient antibody is reacted with specific antigen fixed to a microscope slide. A wash step is performed, and a labeled antihuman immunoglobulin is added. After a second wash step to remove any uncombined anti-immunoglobulin, fluorescence of the sample is determined. The amount of fluorescence is directly in proportion to the amount of patient antibody present.

Lec (4) Immunotechnolgy

Prof. Dr. Ekhlass N. Ali

Q/ Compare between Direct immunofluorescent assays& Indirect immunofluorescent assays

Direct immunofluorescent assays	Indirect immunofluorescent assays
involve antigen detection through a specific antibody that is labeled with a fluorescent tag	the original antibody is un labeled.Incubation with antigen is followed by addition of a second labeled anti-immunoglobulin that detects antigen–antibody complexes.

Cell Flow cytometry

Principle

The first true **flow cytometers** were developed in the 1960s,primarily for research purposes. However, in the early 1980s,physicians started seeing patients with a new mysterious disease characterized by a drop in circulating helper T cells,and flow cytometry was brought into the clinical laboratory. Since that time, flow cytometry has been routinely used to identify infection with HIV, and **immunophenotyping** cells—identifying their surface antigen expression—has become a major component of the workload in most clinical immunology laboratories.Cell flow cytometry is an automated system in which single cells in a fluid suspension are analyzed in terms of their intrinsic light-scattering characteristics and are simultaneously evaluated for extrinsic properties (i.e., the presence of specific surface proteins) using fluorescent labeled antibodies or probes. By using several different fluorochromes,cytometers can simultaneously measure multiple cellular properties.

Q/ Whats important of cell flow cytometry?

Answer By using several different fluorochromes,cytometers can simultaneously measure multiple cellular properties;1- Another significant advantage of flow cytometry is that because the flow rate of cells within the cytometer is so rapid, 2-thousands of cells can be analyzed in seconds, allowing for the accurate detection of very rare cells.

Q/The major components of a flow cytometer :include the fluidics, the **laser light source,** the optics and photodetectors, and a computer for data analysis and management

Q/ Define of the FLOW Cytometry ,whats the important?

Answer: Flow cytometry is a powerful tool to identify and enumerate various cell populations. It was first used in clinical laboratories to perform CD4_ T-cell counts in HIVinfected individuals. A flow cytometer measures multiple properties of cells suspended in a moving fluid medium. As each cell or particle passes single file through a laser light source, it produces a characteristic light pattern that is measured by multiple detectors for scattered light (forward and 90 degrees) and fluorescent emissions (if the cell is stained with a fluorochrome).

Q/ Compare between forward & Side scatter

Forward scatter	**Side scatter**
is a measure of cell size	determines a cell's internal complexity, or granularity.

Instrumentation

1-Fluidics

For cellular parameters to be accurately measured in the flow cytometer, it is crucial that cells pass through the laser one at a time in single file. Cells are processed into a suspension ,and the cytometer draws up the cell suspension and injects the sample inside a carrier stream of isotonic saline (sheath fluid) to form a laminar flow. The sample stream is constrained by the carrier stream and is thus hydronamically focused so that the cells pass single file through the intersection of the laser light source **(Fig. 1)**.

2- **Laser Light Source** Each cell is interrogated by a light source that typically consists of one or more small air-cooled lasers. The wavelength of monochromatic light emitted by the laser in turn dictates which fluorochromes can be used. A fluorochrome, or fluorescent molecule, is one that absorbs light across a spectrum of wavelengths and emits light of lower energy across a spectrum of longer wavelengths. Each fluorochrome has a distinctive spectral pattern of absorption (excitation) and emission.

Q/ WHY Not all fluorochromes can be used with all lasers?

Answer: because each fluorochrome has distinct spectral characteristics.Therefore, the choice of fluorochromes to be used in an assay depends on the light source used for excitation **(Table 1)**. Most clinical flow cytometers have at least one laser, typically argon, that emits at 488 nm.

Newer cytometers also have a second laser, helium-neon (He-Ne),that emits at 633 nm.Q/ whats the important? This allows more fluorochromes to be analyzed in a single tube at one time. As a result of a cell passing through the laser, light is scattered in many directions.

The amount and type of light scatter (LS) can provide valuable information about a cell's physical properties. Light at two specific angles is measured by the flow cytometer:

forward-angle light scatter (FSC), and orthogonal **right angle light scatter**, or 90-degree-angle light scatter (SSC).

Q/ What light source is used in most flow cytometers?

Table1: Fluorochromes communly used in clinical flow cytometry

EXCITATION WAVELENGTH	FLUOROCHROME OR DYE	EMISSION WAVELENGTH
488 nm (argon laser)	Fluorescein isothiocyanate (FITC)	530
	Phycoerythrin (PE)	580
	Propidium iodide (PI)	620
	Peridinin chlorophyll (PerCP)	670
633 nm (He-Ne laser)	Allophycocyanin (APC)	670
	Cy-5	670

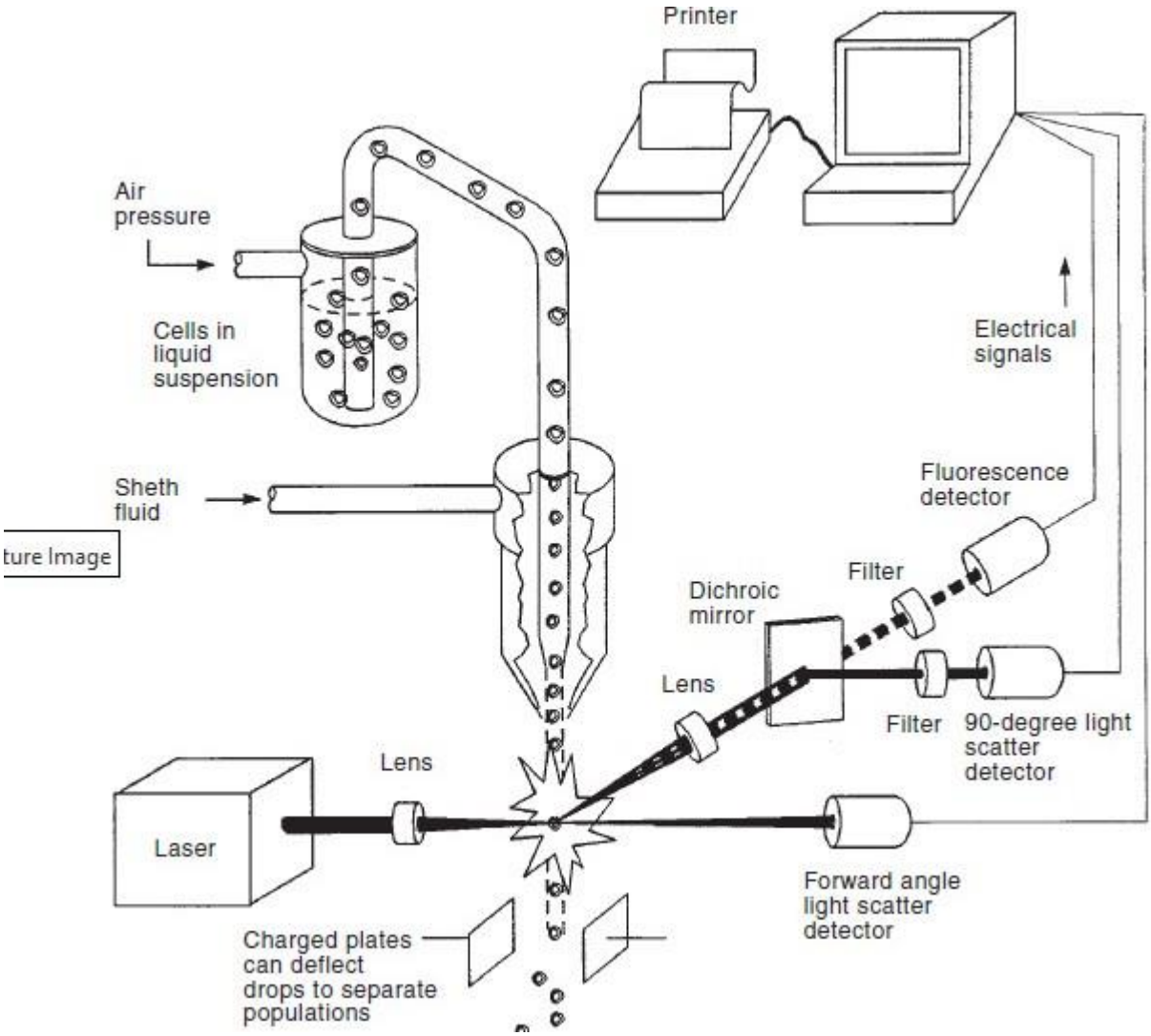


Fig1: Flow cytometry. Components of a laser-based flow cytometer include the fluidics system
for cell transportation, a laser for cell illumination, photo detectors for signal detection, and a computer- based management system .Both forward and 90-degree LS are measured, indicating cell size and type

FSC, or light scattered at less than 90 degrees, is considered an indicator of size, while the SSC signal is indicative of granularity or intracellular complexity of the cell. Thus , these two values, which are considered **intrinsic parameters,** can be used to characterize different cell types. If one looks at a sample of whole blood on a flow cytometer, where all the red blood cells have been lysed, the three major populations of white blood cells (lymphocytes, monocytes, and granulocytes) can be roughly differentiated from each other based solely on their intrinsic

parameters (FSC and SSC;**Fig. 2**).Unlike FSC and SSC, which represent light-scattering
properties that are intrinsic to the cell, **extrinsic parameters** require the addition of a fluorescent probe for their detection. Fluorescent labeled antibodies bound to the cell are interrogated by the laser. By using fluorescent molecules with various emission wavelengths, the laboratorian can simultaneously evaluate an individual cell for several extrinsic properties. The clinical utility of such multicolor analysis is enhanced when the fluorescent data are analyzed in conjunction with FSC and SSC

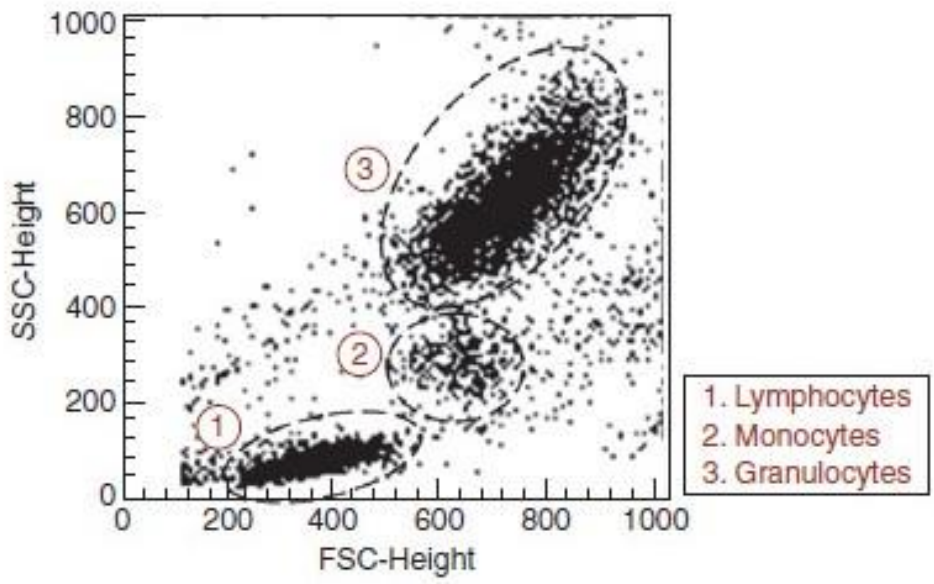


Fig2: Peripheral blood leukocyte analysis by simultaneous evaluation of forward-angle light scatter (FCS) and 90-degree LS (SSC). Based on the intrinsic characteristics of size (FSC) and granularity (SSC) only, the three main populations of white cells(lymphocytes, monocytes, and granulocytes) can be discriminated into individual populations.

3-Optics

The various signals (light scatter and fluorescence) generated by the cells' interaction with the laser are detected by photomultiplier tubes and detectors. The number of fluorochromes capable of being measured simultaneously is limited by the number of photodetectors in the flow cytometer.The specificity of each photodetector for a given band length of wavelengths is achieved by the arrangement of a series of optical filters that are designed to maximize collection

of light derived from a specific fluorochrome while minimizing collection of light from other fluorochromes used to stain the cells. The newer flow cytometers actually use fiber-optic cables to direct light to the detectors. Most clinical flow cytometers in use today are capable of three to six-color detection using one to two lasers.When fluorescent light reaches the photomultiplier tubes, it creates an electrical current that is converted into a voltage pulse. The voltage pulse is then converted (using various methods depending on the manufacturer) into a digital signal. The digital signals are proportional to the intensity of light detected. The intensity of these converted signals is measured on a relative scale that is generally set into 1 to 256 channels, from lowest energy level or pulse to the highest level.

Data Acquisition and Analysis

Once the intrinsic and extrinsic cellular properties of many cells (typically 10,000 to 20,000 "events" are collected for each sample) have been collected and the data digitalized, it is ready for analysis by the operator. Each parameter can be analyzed independently or in any combination. Graphics of the data can be represented in multiple ways. The first level of representation is the **single-parameter istogram,**

which plots a chosen parameter (generally fluorescence) on the x-axis versus the number of events on the y-axis, so only a single parameter is analyzed using this type of graph **(Fig3)**. The operator can then set a marker to differentiate between cells that have low levels of fluorescence (negative) from cells that have high levels of fluorescence (positive) for a particular fluorochrome labeled antibody.

The computer will then calculate the percentage of "negative" and "positive" events from the total number of events collected. The next level of representation is the bivariant histogram, or **dual-parameter dot plot,** where each dot represents an individual cell or event. Two parameters, one on each axis,are plotted against each other. Each parameter to be analyzed is determined by the operator. Using dual-parameter dot plots, the operator can then draw a "gate" around a population

of interest and analyze various parameters (extrinsic and intrinsic) of the cells contained within the gated region **(Fig. 4)**. This allows the operator to screen out debris and isolate subpopulations of cells of interest.

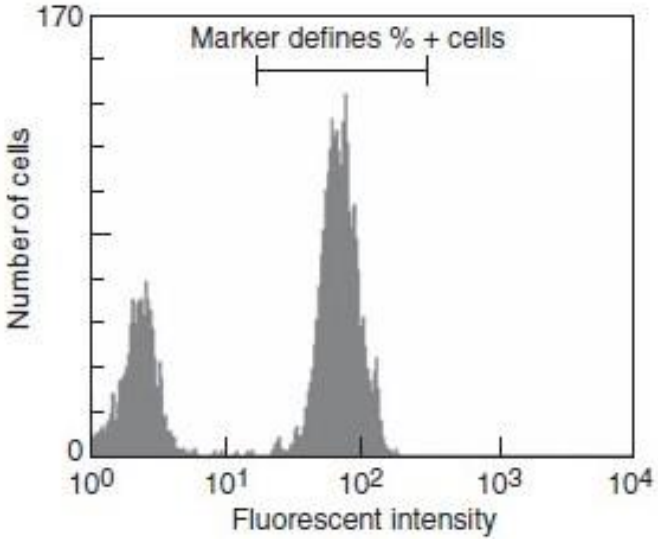


Fig3: Example of a single parameter flow histogram. The y-axis consists of the number of events. The x-axis is the parameter to be analyzed, which is chosen by the operator, usually an extrinsic parameter, such as a fluorescent labeled antibody. The operator can then set a marker to isolate the positive events. The computer will then calculate the percent positive events within the designated markers

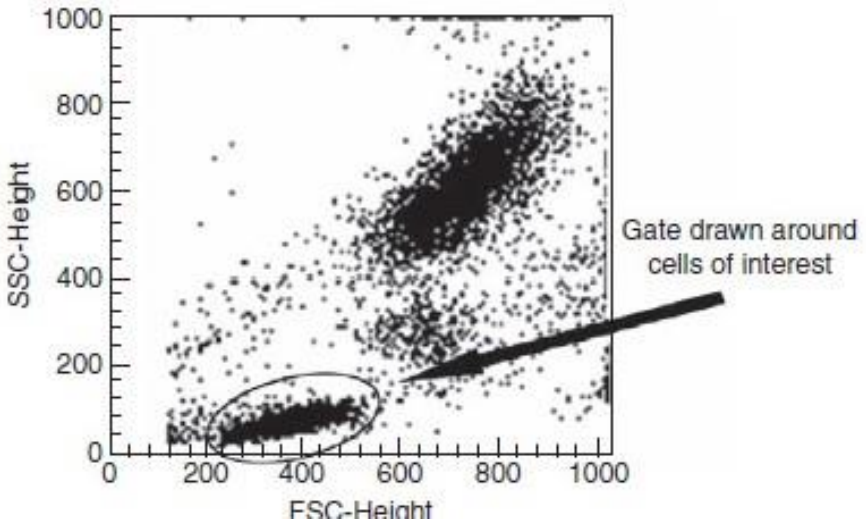


Fig4: A dual-parameter dot plot. Both parameters on the x- and y-axes are chosen by the operator. In this case, lysed whole blood is analyzed on FSC (x-axis) and SSC (y-axis). The operator then draws a "gate" or isolates the population of interest (e.g., lymphocytes)for further analysis.

characteristics of the gated population can be analyzed—that is, lymphocytes can be gated, and then the subpopulations of T cells (CD3_ and CD4_ or CD2_) and B cells (CD2_,CD19_) can be analyzed The absolute count of a particular cell type—for instance, CD4_ Tlymphocytes—is obtained by multiplying the absolute cell count of the population of interest (e.g., lymphocytes)
derived from a hematology analyzer by the percentage of the population of interest in the sample (CD3_ and CD4_lymphocytes).1,

Sample Preparation

Q/What type of biological sample is best suited for flow cytometric analysis? Samples commonly used for analysis include Answer: whole blood,bone marrow, and fluid aspirates.

Whole blood should be collected into ethylene di aminetetra acetic acid (EDTA), the anticoagulant of choice for samples processed within30 hours of collection. Heparin can also be used for whole blood and bone marrow and can provide improved stability in samples over 24 hours old. Blood should be stored at room temperature (20ºC to 25ºC) prior to processing and should be well mixed before being pipetted into staining tubes. Hemolyzed or clotted specimens should be rejected. Peripheral blood, bone marrow, and other samples with large numbers of red cells require erythrocyte removal to allow for efficient analysis of white cell s.Historically, density gradient centrifugation with Ficoll-Hypaque (Sigma, St. Louis, MO) was used to generate a cell suspension enriched for lymphocytes or blasts. However this method results in selective loss of some cell populations. Alternatively, there are numerous erythrocyte lysis techniques available, both commercial and noncommercial. Tissue specimens are best collected and transported in tissue culture medium (RPMI 1640) at either room temperature or 4ºC, if analysis will be delayed. The specimen is then disaggregated to form a single cell suspension, either by mechanical dissociation or by enzymatic digestion.Mechanical disaggregation, or “teasing,” is preferred and is accomplished by the use of either a scalpel and forceps, a needle and syringe, or wire mesh screen Antibodies are then added to the resulting cellular preparation and processed for analysis. The antibodies used are typically monoclonal, each with a different fluorescent tag.

Q/ Clinical Applications

Routine applications of flow cytometry in the clinical laboratory include 1-immunophenotyping of peripheral blood lymphocytes, enumeration of CD34_ stem cells in peripheral blood and bone marrow for use in stem cell transplantation,

and immune phenotypic characterization CD19 of acute leukemias, non-Hodgkin's lymphomas, and other lymphoproliferative disorders.Immunophenotyping by flow cytometry has become an important component of initial evaluation and subsequent post-therapeutic monitoring in leukemia and lympho mamanagement. Flow cytometric findings have been incur poratedinto current leukemia and lymphoma classifications ,beginning with the Revised European-American Lymphoma classification in 1994 and, more recently, in the proposed World Health Organization (WHO) classifications. One of the most important components of flow cytometric analysisis the stratification of hematopoietic malignancies by their lineage (i.e., B cell, T cell, or myeloid) and the degree of differentiation. Some of the more common cell differentiation antigens Immuno phenotyping by flow cytometry, in what ever capacity that it is used, is not possible without the use of

fluorescent-labeled monoclonal and polyclonal antibodies .Q/ Why**Monoclonal antibodies** specific for various surface antigens are preferable to using polyclonal antibodies.

Answer; The ability to produce monoclonal antibodies through hybridoma and recombinant DNA techniques has contributed greatly to the accuracy of flow cytometry and has widened its use.

Lymphocytes are identified using monoclonal antibodies directed against specific surface antigens. Reactions can be identified manually by employing a fluorescence microscope or by immune enzyme staining methods. However, flow cytometry is the most commonly used method for immunophenotyping of lymphocyte populations.

Lec (5) **Immunotechnology**

Prof. Dr .Ekhlass N. Ali

Immunohistochemstry

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of binding can be identified by direct labeling of the antibody or by use of a secondary labeling method.

Antigens

Antigens have two main properties. The first is immunogenicity, which is the ability to induce antibody formation. The second property is specific reactivity, which means that the antigen can react "with the antibody it caused to be produced. The reaction between an antigen and its antibody is one of the most specific in biology, and is the reason that immune histochemical reactions are more prec'ise than ordinary histochemical techniques.An antigen then. is a substance foreign to the host which stimulates formation of a specific antibody and which will react with the antibody produced. This reaction involves the formation of immune complexes comprised of several antigen and antibody molecules. These complexes may be come ver y I a I' gean d form precipitates which can be measured by various, techniques

Antibodies

An antibody is a serum protein that is formed in response to exposure to an antigen. and reacts specifically with that antigen to form immune complexes either in the body or in the laboratory. Antibody production is a response by the body to foreign material (an antigen), and is designed to rid the body of this invader Antibodies are contained in the gamma globulin fraction of serum, and are often called immunoglobulins (Ig). They can be divided into five classes based on their size. weight, strcture ,function, and other criteria. The classes are IgA(immunoglobulin A), IgD, IgE, IgG, and IgM. Antibody solutions utilized in immunohistochemical staining. contain mostly IgG type antibodies, with lesser amounts of the other classes

Antigen-Antibody binding

The amino acid side-chains of the variable domain of an antibody form a cavity which is complementary to a single type of antigen like a lock and key. The precise fit required explains the high degree of specificity seen in antigen antibody
interaction.

Affinity: is the 3 dimensional fit of the antibody to its specific antigen and is a measure of the binding strength between antigen and antibody.

Avidity: is the functional combined strength of an antibody with its antigen. An antibody against more than one epitope of an antigen will bind more strongly to it.

Antibody specificity: is the characteristic of an antibody to bind selectively to a single epitope or an antigen.

Sensitivity: is the relative amount of an antigen that a technique is able to detect

Antigen Detection

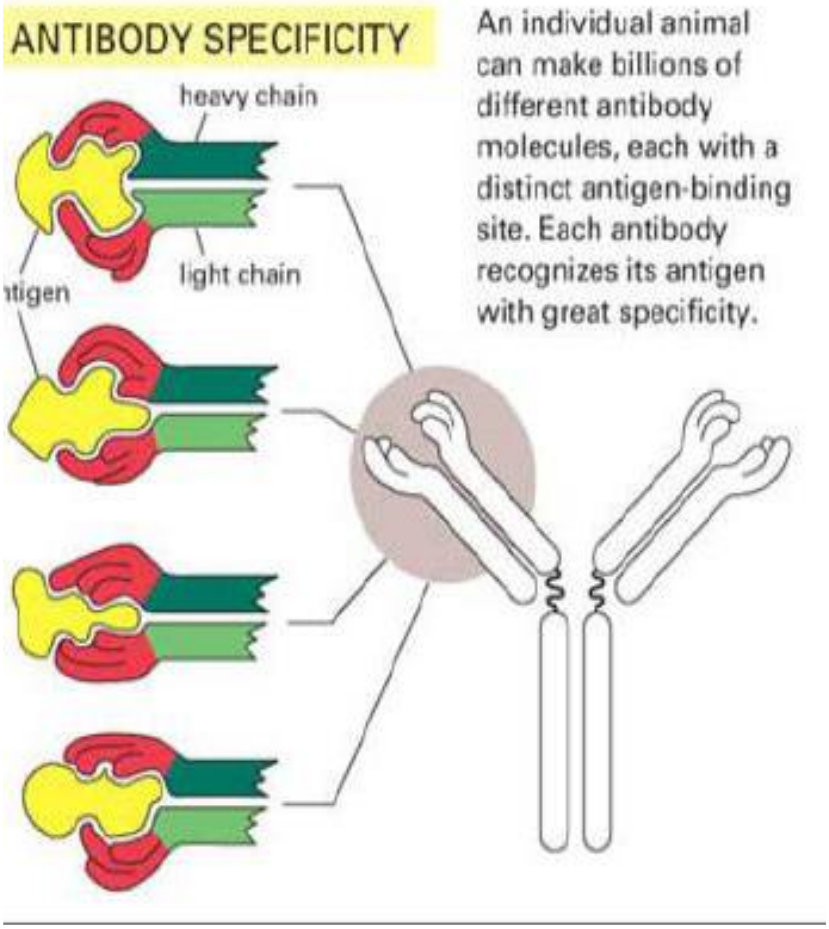
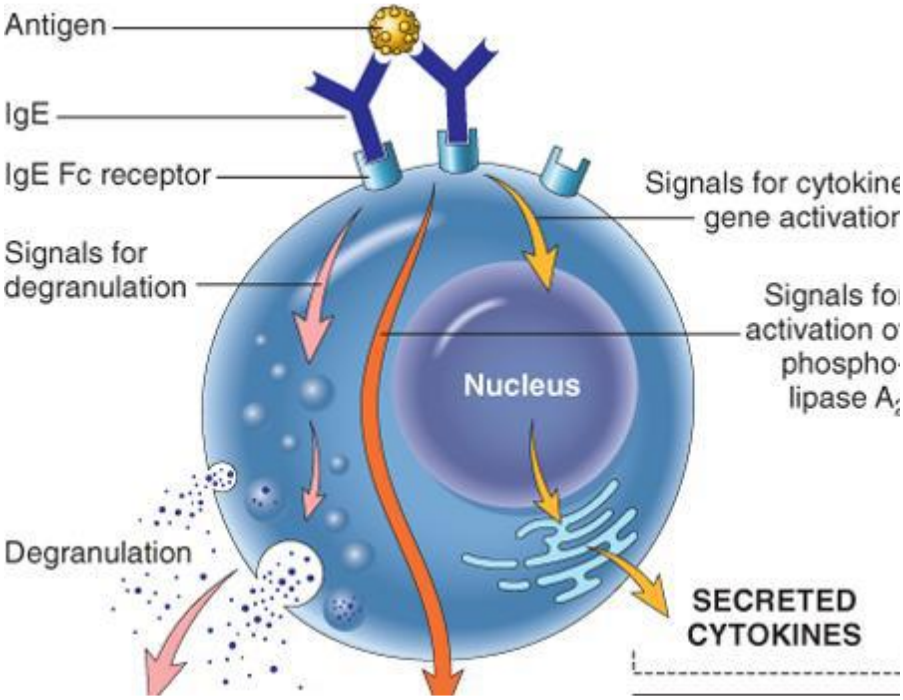
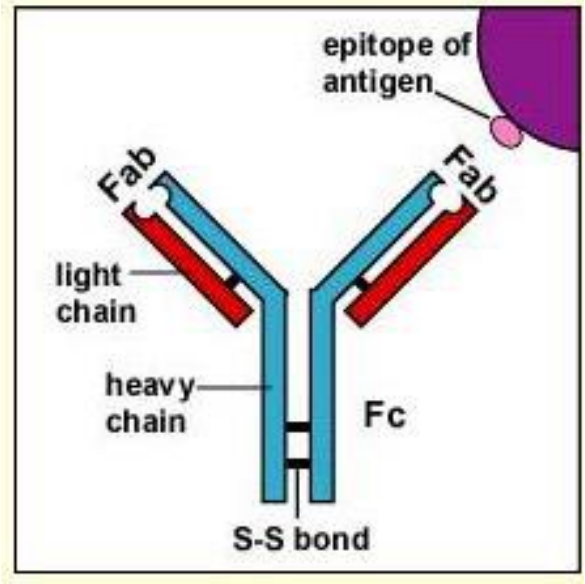


Fig1:Ab binding with Ag


Antigen
IgE
IgE Fc receptor
Signals for degranulation
Signals for cytokine gene activation
Signals for activation of phospholipase A2
Nucleus
Degranulation
SECRETED CYTOKINES


epitope of antigen
Fab
Fab
light chain
heavy chain
Fc
S-S bond

Raising Antibodies:

- Repeated injection of antigens (proteins, glycoproteins, proteoglycans, and some polysaccharides) causes the injected animal's B lymphocytes to differentiatein to plasma cells and produce antibodies.
- Members of a lymphocyte clone (descendants of a single lymphocyte) produce a single type of antibody, which binds to a specific antigenic site, or **epitope**

1. **Polyclonal antibodies :** Large complex antigens may have multiple epitopes and elicit several antibody types. Mixtures of different antibodies to a single antigen are called polyclonal antibodies.

2. **Monoclonal antibodies:** Antibodies specific for a single epitope and produced by a single clone are called monoclonal antibodies and are commonly raise d in mice.

Labeling Antibodies:

- Antibodies are not visible with standard microscopy and must be labeled in a manner that does not interfere with their binding specificity.
- Common labels include **fluorochromes** (eg, fluorescein, rhodamine), enzymes demonstrable via enzyme histochemical techniques (eg, peroxidase, alkaline phosphatase), and electron –scattering compounds for use in electron microscopy (eg, ferritin, colloidal gold.

Method

Q/ Compare between direct immunohistochemistry method(DIH) & Indirect (IHC)

- **Direct Method**, labeled Ab, Tissue Ag

Indirect Method:Secondary AB,Primary Ab, Tissue Ag

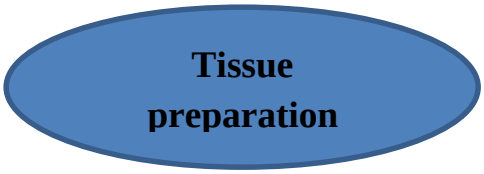
Q/ Talk about the

•**PAP Method**: (peroxidase anti-peroxidase method).

Applications of IHC

- Cancer diagnostics
- differential diagnosis
- Treatment of cancer
- Research

General Immunohistochemistry Protocol



Part 1

1. Fixation

Fresh unfixed, fixed, or formalin fixation and paraffin embedding

2. Sectioning

3.Whole Mount Preparation, Type of slides ,charge slide

Ethidium bromide is stained DNA

Part 2

1. **Antigen retrieval**
2. Two method:

Proteolyticenzyme method and Heat-induced method

2. Inhibition of endogenous tissue components

3% H2O2, 0.01% avidin

3. Blocking of nonspecific sites by use 10% normal serum

Part 3

• Make a selection based on the **type of specimen**, the **primary antibody**, the **degree of sensitivity** and the **processing time** required.

Controls

Q/ Compare between positive &Negative controls

•Positive Control

It is to test for a protocol or procedure used. It will be ideal to use the tissue of known positive as a control.

•Negative Control

It is to test for the specificity of the antibody involved.

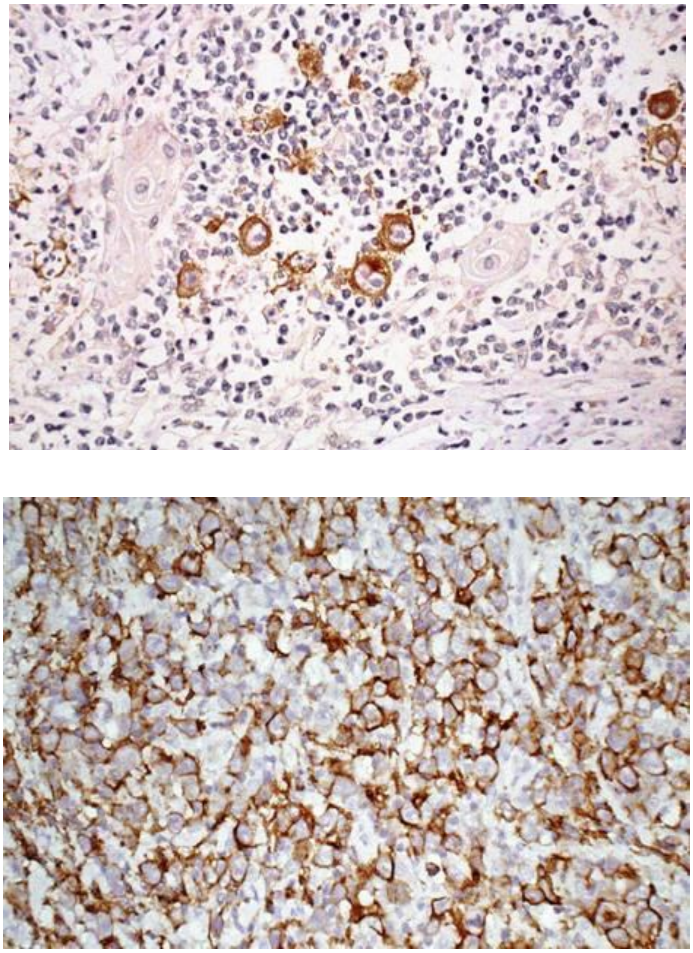


Fig2: positive &negative result.

Lec (6) **Immunotechnology**

Prof. Dr. Ekhlass Noori Ali

MONOCLONAL ANTIBODY

The knowledge that B cells are genetically preprogrammed to synthesize very specific antibody has been used in developing antibodies for diagnostic testing known as **monoclonal antibodies.** Normally, the response to an antigen is heterogeneous,because even a purified antigen has multiple epitopes that stimulate a variety of B-cell clones. In 1975,Georges Kohler and Cesar Milstein discovered a technique to produce antibody arising from a single B cell, developed a technology to fuse immortal hetero myeloma cells with B lymphocytes,

Q1/ How do PEG play role in monoclonal antibody production?

Answer: using poly ethyl glycol (PEG) to break down cell membranes and allow mixing of the genetic material from both cell types.

The resulting cell type is called a hybridoma. This hybridoma takes on the characteristics of both the lymphocyte and hetero myeloma cell, creating an immortal cell with the ability to produce antibody. which has revolutionized serological testing. For their pioneering research, they were awarded the Nobel Prize in 1984.Kohler and Milstein's technique fuses an activated B cell with a myeloma cell that can be grown indefinitely in the laboratory. Myeloma cells are cancerous plasma cells .Normally, plasma cells produce antibody,

Q/ Why myeloma cells unable to produce antibody?

So a particular cell line that is not capable of producing antibody is chosen. In addition, this cell line has a deficiency of the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) that renders it incapable of synthesizing nucleotides from hypoxanthine and thymidine, which are needed for DNA synthesis.

Hybridoma Production

A mouse is immunized with a certain antigen, and after a time, spleen cells are harvested. Spleen cells are combined with myeloma cells in the presence of polyethylene glycol(PEG), a surfactant. The PEG brings about fusion of plasma

cells with myeloma cells, producing a **hybridoma.** Only a small percentage of cells actually fuse, and some of these are like cells—that is, two myeloma cells or two spleen cells.

After fusion, cells are placed in culture using a selective medium containing

Q/ What is the purpose of the use HAT media in monoclonal antibody preparation?

Answer: hypoxanthine, amino pterin, and thymidine (HAT). Culture in this medium is used to separate the hybridoma cells by allowing them to grow selectively .Myeloma cells are normally able to grow indefinitely in tissue culture, but in this case they cannot, because both pathways for the synthesis of nucleotides are blocked. One pathway, which builds DNA from degradation of old nucleic acids, is blocked, because the myeloma cell line employed is deficient in the required enzymes HGPRT and thymidine kinase. The other pathway, which makes DNA from new nucleotides, is blocked by the presence of amino pterin.Consequently, the myeloma cells die out. Normal B cells cannot be maintained continuously in cell culture, so these die out as well. This leaves only the fused hybridoma cells,which have the ability (acquired from the myeloma cell) to reproduce indefinitely in culture and the ability (acquired from the normal B cell) to synthesize nucleotides by the HGPRT and thymidine kinase pathway **(Fig. 1).**

Selection of Specific Antibody-Producing Clones

The remaining hybridoma cells are diluted out and placed in microliter wells, where they are allowed to grow. Each well, containing one clone, is then screened for the presence of the desired antibody by removing the supernatant.

Once identified, a hybridoma is capable of being maintained in cell culture indefinitely, and it produces a permanent and uniform supply of monoclonal antibody that reacts with a single epitope.

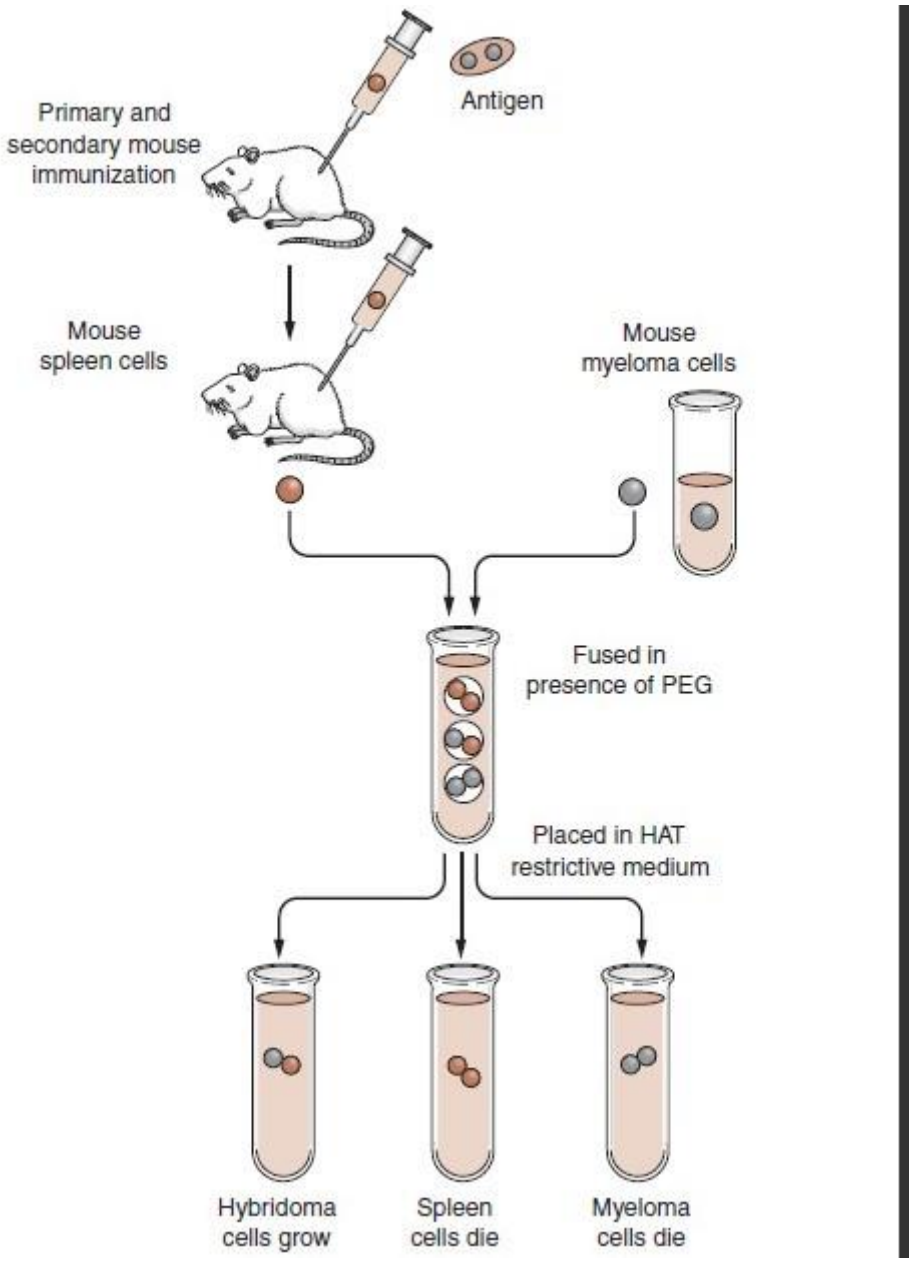
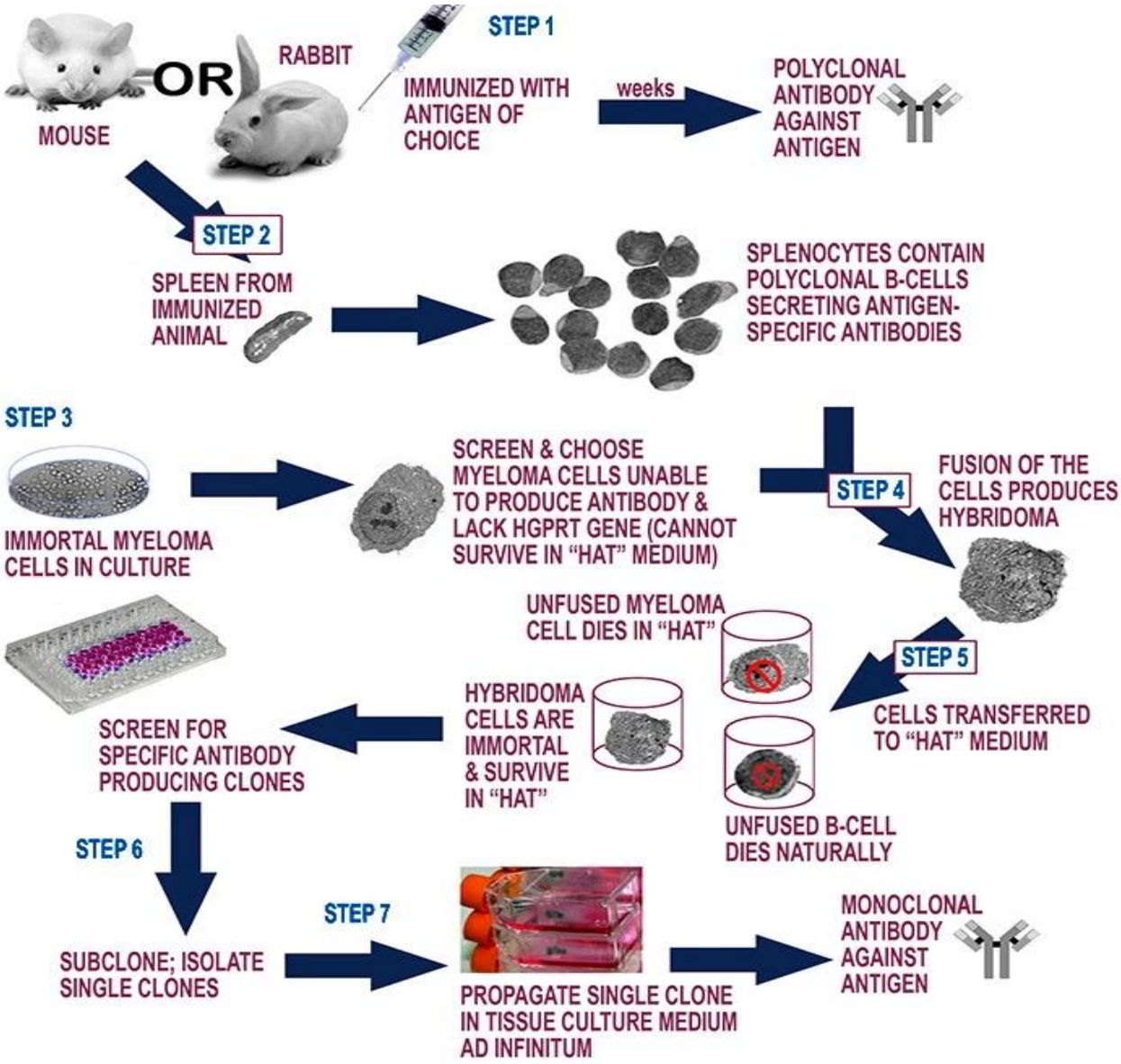


Fig1: Formation of a hybridoma in monoclonal antibody production. A mouse is immunized, and spleen cells are removed. These cells are fused with non secreting myeloma cells and then plated in a restrictive medium. Only the hybridoma cells will grow in this medium,where they synthesize and secrete a monoclonal immunoglobulin specific for a single determinant on an antigen.



Q/ Describe the method of production of a monoclonal antibody?

Clinical Applications

Monoclonal antibodies were initially used for in vitro diagnostic testing. A familiar example is pregnancy testing, which uses antibody specific for the chain of human chorionic gonadotropin, thereby eliminating many false-positive reactions.

Other examples include detection of tumor antigens and measurement of hormone levels .Recently, however, there has been an emphasis on the use of monoclonal antibodies as therapeutic agents. One of the biggest success stories is

in the treatment of two autoimmune diseases: rheumatoid arthritis and Crohn's disease (a
progressive inflammatory colitis).
Monoclonal antibodies have also been used to treat various types of cancers. In the case of metastatic breast cancer,

Uses of Monoclonal Antibodies

The greatest impact of MAbs in immunology has been on the analysis of cell membrane antigens. Because they have a single specificity rather than the range of antibody molecules present in the serum, MAbs have multiple clinical applications, including the following:

• Identifying and quantifying hormones
• Typing tissue and blood
• Identifying infectious agents
• Identifying clusters of differentiation for the classification of leukemia's and lymphomas and follow-up therapy
• Identifying tumor antigens and autoantibodies
• Delivering immunotherapy.

Polyclonal Antibodies

The immune response to an antigen generally involves the activation of multiple B-cells all of which target a specific epitope on that antigen. As a result a large number of antibodies are produced with different specificities and epitope affinities these are known as polyclonal antibodies.

For production purposes these antibodies are generally purified from the serum of immunised animals were the antigen of interest stimulates the B-lymphocytes to produce a diverse range of immunoglobulin's specific to that antigen.

The aim is to produce high titer, high affinity antibodies. Today these polyclonal antibodies are used extensively for research purposes in many areas of biology, such as immune precipitation, histochemistry, enzyme linked immunosorbent assays (ELISA), diagnosis of disease, immune turbidimetric methods, western blots and Biochip technology. Polyclonal antibodies are ideally suited for use in sandwich assays as second stage antigen detectors.

Polyclonal antibodies	Monoclonal antibodies
Inexpensive to produce	Expensive to produce
Skills required for production are low	Training is required for the technology used
Relatively quick to produce	Hybridomas take a relatively long time to produce
Generate large amounts of non-specific antibodies	Generate large amounts of specific antibodies
Recognize multiple epitopes on any one antigen	Recognize only one epitope on an antigen
Can have batch-to-batch variability	Once a hybridoma is made, it is a constant and renewable source
	No or low batch-to-batch variability

IMMUNOTHERAPY

Q/ Enumerate the applications of Immunotherapy

Immunotherapy: the another type for treatment of tumor immunology.

The possibility of stimulating the patient's own immune system to respond to tumor-associated antigens has long intrigued scientists. Immunotherapeutic methods used can be separated into two types: passive or active immunotherapy.

Passive immunotherapy involves transfer of antibody, cytokines, or cells to patients who may not be able to mount an immune response. With active immunotherapy, patients are treated in a manner that stimulates them to mount immune responses to their tumors.

Passive Immunotherapy

Passive transfer of allogeneic cellular immunity from one person to another to fight cancer has many barriers because of possible recipient rejection of foreign cells, graft-versus host disease (GVHD), and the fragility of live cells, Inducing a patient's own cellular immunity is far more likely to be successful, However, a form of GVHD called **graft versus leukemia** has been demonstrated with transfer

of allogeneic T cells and is associated with improved patient prognosis. Therefore, successful passive transfer of anticancer T cells is theoretically possible .Adaptive T-cell therapy has been attempted using several models. For example, T cells from allogeneic donors can be immunized against tumors. After recipients are immunosuppressed to prevent rejection and to eliminate T suppressor mechanisms, they receive the T cells. One strategy in this model to treat GVHD is to genetically engineer the allogeneic T cells

3-Passive transfer of antibody to treat cancer almost always employs monoclonal antibodies. "Naked" monoclonal antibodies

against cancer could induce antibody-dependent cell-mediated cytolysis (ADCC), complement-mediated lysis, or opsonization. If the antibodies are directed toward

particular receptors, they could trigger a desirable action in the cell such as inducing apoptosis or inhibiting growth signals

4. **Antibody conjugates,** or **immunotoxins,** are antibodies conjugated to toxins or radioisotopes on the premise that they can kill cancer cells while leaving adjacent cells intact.

Active Immunotherapy

The goal of active immunotherapy is to have the patient develop an immune response that will help eliminate the tumor. Nonspecific stimulation by adjuvants such as Bacillus Calmette Guerin (BCG) was first attempted, and superficial bladder cancer is still treated with BCG. Improved technology has allowed the production of novel adjuvants and selective use of stimulatory cytokines (TNF-α, IFN-γ,IL-1, IL-2, and so on) in immune competent patients to enhance the natural antitumor response and the artificial vaccine-induced response.

Other attempts at stimulating host immune systems have involved transfection of normal cells or isolated tumor cells with genes for cytokine production and injection of the modified cells into or around the tumor. This has been
done with many cytokines, including TNF-α, interferons,IL-2, and granulocyte monocyte–colony stimulating factor (GM-CSF). Of the cytokines transfected, GM-CSF has shown the most promise.

Cancer vaccines have been of great interest to researchers. When specific viruses are associated with a cancer, vaccine construction is relatively straight forward, since viral antigens are obviously foreign. The vaccine for human papillomavirus (HPV) to prevent cervical cancer is an excellent example. It is important to note that many viruses have several serotypes ,not all of which may be associated with cancer, so vaccines must be protective against the appropriate epitopes. HPV vaccines, for example, are directed epitopes that prevent initial infection with carcinogenic serotypes but do not help treat established cervical cancer, as these epitopes are down regulated in cancer cells. Therefore, a distinction exists between prophylactic vaccines and therapeutic ones.

These protocols will be important adjuncts to traditional therapies in which tumors will first be de bulked and then the immune system will eradicate residual tumor and micro metastases. The increased understanding of tumor immunology in recent years has made this a field of active study and increased optimism.

Q1/ Compare and contrast passive versus active immunotherapy, describing common techniques used in each.

Lec (7) **Immunotechnology**

Prof. Dr .Ekhlass Noori Ali

Transplantation

Transplantation is a potentially lifesaving treatment for end stage organ failure, cancers, autoimmune diseases, immune deficiencies, and a variety of other diseases. Over 28,000 solid organ(kidney, pancreas, liver, heart, lung, small intestine)transplants in particular the role of human leukocyte antigens (HLA) and the development of pharmacological agents that interfere with various components of the immune system to promote sustained graft survival .The HLA system is the

largest immunologic barrier to successful allogeneic organ transplantation. It consists of cell surface proteins that play a central role in immune recognition and initiation of immune responses. Because of the ubiquitous presence of these proteins on the surface of nucleated cells and their extensive degree of polymorphism, an allogeneic response may result in graft rejection in solid-organ and stem cell transplantation or graft-versus-host disease in stem cell transplantation.

HISTOCOMPATIBILITY SYSTEMS

The classical (transplant) HLA antigens, also known as **major histocompatibility antigens,** include the class I (HLA-A, B, and C) and class II (HLA-DR, DQ, and DP)proteins. HLA proteins are encoded by a set of closely linked genes on the short arm of chromosome 6 in the major histocompatibility complex (MHC). The HLA genes are inherited as **haplotypes** from parental chromosomes **(Fig1)**. Offspring receive one maternal and one paternal HLA haplotype. Based on this Mendelian inheritance, there is a 25 percent chance that any two siblings will inherit the same two haplotypes (i.e., are HLA identical), a 50 percent chance of being HLA haplo identical (i.e., share one of two HLA haplotypes), and a 25 percent chance of being HLA non identical (i.e., share neither HLA haplotype).

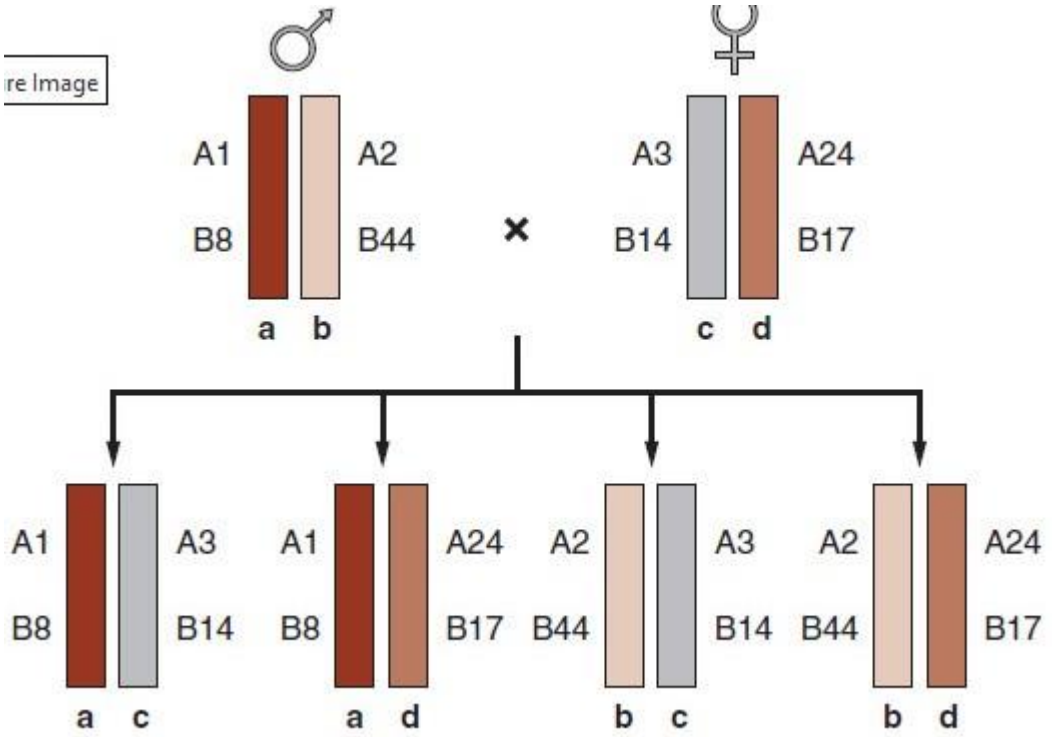


Fig1: HLA genes are linked and inherited in Mendelian fashion as haplotypes. One paternal (a or b) and one maternal (c or d) haplotype is passed to each offspring. Four different combinations of haplotypes are possible in offspring. Elucidation of haplotype sharing between siblings is an important assessment in the search for a transplant donor.

Minor Histocompatibility Antigens

A second set of transplantation antigens was identified based on studies in mice and humans demonstrating tissue rejection in MCH-identical transplants and based on outcomes of human stem cell transplants between HLA-identical siblings in whom graft-versus-host disease has developed .Early experimental studies documented a "slower" rejection pace mediated by these transplantation antigens, thus their name—minor histocompatibility antigens (mHAs).mHAs are non-HLA proteins that demonstrate polymorphism in amino acid sequence within a species. Both X-linked and autosomally encoded mHAs have been identified.Introducing a polymorphic variant of one of these proteins from one individual into another individual who possesses a different polymorphic variant (via transplantation of tissue or cells) can induce a recipient immune response to the donor variant. The immune response is mediated by CD4 and/or CD8 T cells recognizing a variant protein in the context of the recipient MHC molecule. This

response is analogous to the reaction to a foreign microbial antigen. Several different types of mHAs have been identified, including proteins encoded by the male Y chromosome, proteins for which the recipient has a homozygous gene deletion, proteins that are
autosomally encoded, and proteins that are mitochondrial DNA encoded.

MIC Antigens

The MHC class I–related chain A (MICA) encodes a cell surface protein that is involved in gamma/delta T-cell responses. MICA is polymorphic with over 50 allelic variants .MIC proteins are expressed on endothelial cells ,keratinocytes, fibroblasts, epithelial cells, dendritic cells, and monocytes, but they are not expressed on T or B lymphocytes.As such, MICA proteins could serve as targets for allograft immune responses. Antibodies to MICA antigens have been detected in as many as 11 percent of kidney transplant patients. MICA antibodies have been associated with rejection episodes and decreased graft survival.

ABO Blood Group Antigens

The ABO system is the only blood group system that impacts clinical transplantation. Anti-A or anti-B antibodies develop in individuals lacking the corresponding blood group antigens. ABO blood group incompatibility is a barrier
to solid-organ transplantation, because these antibodies can bind the corresponding antigens that are expressed on the vascular endothelium. Binding activates the complement cascade, which can lead to **hyperacute rejection** of the transplanted organ. As such, recipient–donor pairs must be ABO identical or compatible to avoid this adverse outcome For example, an individual of blood group A will possess anti-B antibodies and can thus receive an organ only from an ABO-A or O donor. Likewise, a B-expressing individual has anti-A antibodies and can receive an organ only from an ABO-B or O donor. Recently, approaches using plasma exchange and intravenous immunoglobulin administration have allowed successful transplantation of kidneys from ABO-incompatible donors by lowering ABO antibody to levels that allow transplantation to proceed without risk of hyper acute rejection.

ALLORECOGNITION

Transplantation of cells or tissues between two individuals is classified by the genetic relatedness of the donor and the recipient. An

1-**autograft:** is the transfer of tissue from one area of the body to another of the same individual.
2- **syngeneic graft :**is the transfer of cells or tissues between identical twins.
3- **allograft:** is the transfer of cells or tissue between two individuals of the same species
4-**xenograft :**is the transfer of tissue between two individuals of a different species.

Most transplantation falls into the category of allo grafting. As stated earlier, HLA disparity between donor and recipient will result in a vigorous immune response to the foreign MHC antigens and is the primary stimulus of graft rejection. The response to foreign MHC antigens is characterized by strong cellular and humoral immune responses The recipient immune system recognizes foreign HLA proteins via two distinct mechanisms—direct and indirect allo recognition **(Fig. 2).**

direct allorecognition,recipient T cells bind and respond directly to foreign (allo) HLA proteins on graft cells. Although an individual T lymphocyte can recognize self-HLA + peptide, foreignHLA proteins may mimic a self-HLA +peptide complex due to similarities in structure of the allo-HLA protein itself or to structural similarities of allo-HLA protein _ peptide.Either way, direct allorecognition is characterized by a high frequency (up to 2 percent) of responding T cells compared to the responder frequency in a typical T-cell response to a foreign antigen.The high frequency of responding T cells may be due to several factors, including 1-recognition of multiple amino acid disparities by multiple T-cell clones; 2-the presence of multiple different peptides on an allogeneic cell that are each recognized by different T-cell clones; and the presence of many foreign molecules per cells, resulting in activation of T cells with low affinity, which normally would not be stimulated. The **mixed lymphocyte response (MLR)** is an in vitro correlate of direct allo recognition.

Indirect allorecognition is the second -pathway by which the immune system recognizes foreign HLA proteins. Indirect allorecognition is analogous to the normal mechanism of recognition of foreign antigens, as it involves the uptake, processing, and presentation of foreign HLAproteins by recipient antigen-presenting cells to recipient T cells. Indirect allorecognition plays a predominant role in acute and chronic rejection.

The effector responses against transplanted allogeneic tissue include direct cytotoxicity, delayed-type hypersensitivity responses, and antibody-mediated mechanisms.Antibody may mediate antibody-dependent cellular cytotoxicity reactions and may fix complement, resulting in cell death. Rejection episodes vary in the time of occurrence and the effector mechanism that is operative.

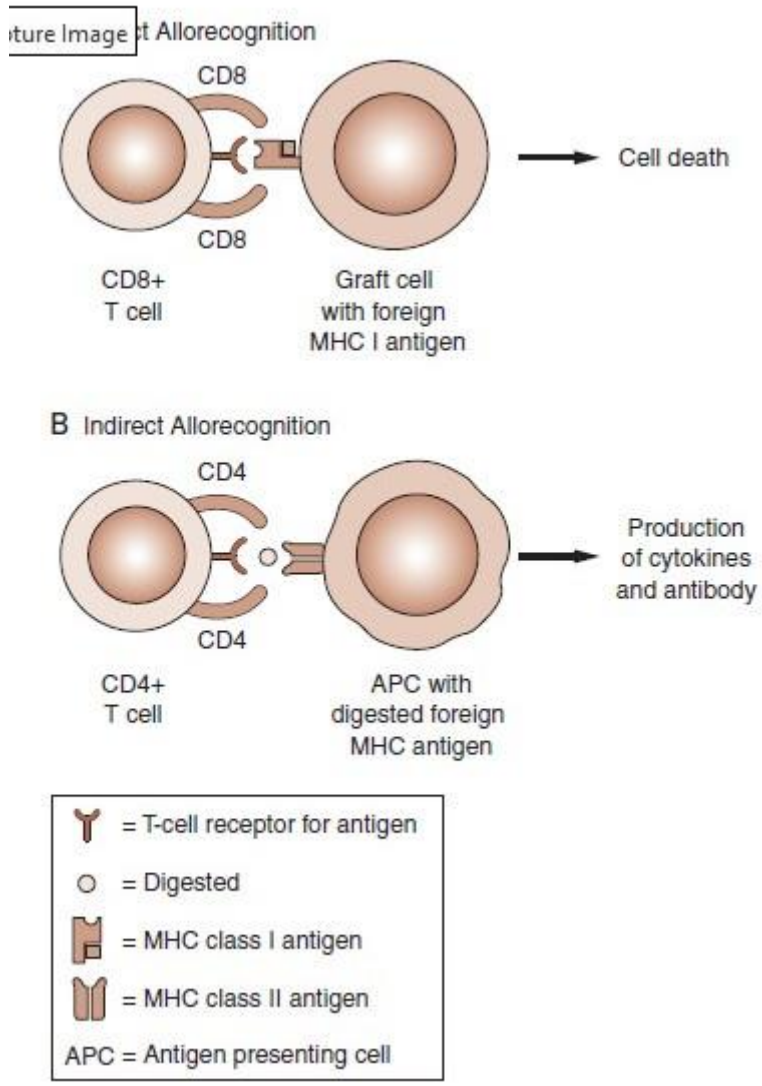


Fig2: Direct versus indirect allorecognition. *(A)* In direct allorecognition, cytotoxic T cells bind directly to foreign HLA proteins on graft cells. *(B)* In indirect allorecognition, foreign MHC
antigens are presented by phagocytic cells, and CD4_ T cells respond.

TRANSPLANT REJECTION

Hyperacute Rejection(Humoral immune response)

Hyperacute rejection occurs within minutes to hours after the vascular supply to the transplanted organ is established.This type of rejection is mediated by preformed antibody that reacts with donor vascular endothelium. ABO, HLA, and certain endothelial antigens may elicit hyperacute rejection.Binding of preformed antibodies to the alloantigens activates the complement cascade and clotting mechanisms and leads to thrombus formation. The result is ischemia and necrosis of the transplanted tissue.Hyperacute rejection is seldom encountered in clinical transplantation. Donor–recipient pairs are chosen to be ABO identical or compatible, and patients awaiting transplantation are screened for the presence of preformed HLA antibodies. In addition, the absence of donor HLA specific antibodies is confirmed prior to transplant by the performance of a cross match test.

Acute Cellular Rejection(Humoral &cellular immune response)

Days to weeks after transplant, individuals may develop**acute rejection.** This is a cellular-type rejection but may involve antibody as well. Acute rejection is characterized by parenchymal and vascular injury. Interstitial cellular infiltrates contain a predominance of CD8-positive T cells as well as CD4 T cells and macrophages. CD8 cells likely mediate cytotoxic reactions to foreign MHC-expressing cells, while CD4 cells likely produce cytokines and induce delayed-type hypersensitivity (DTH) reactions. Antibody may also be involved in acute graft rejection by binding to vessel walls, activating complement, and inducing transmural necrosis and inflammation as opposed to the thrombosis typical of hyperacute rejection. The development and application of potent immunosuppressive drugs that target multiple pathways in the immune response to

alloantigens has improved early graft survival of solid-organ transplants by reducing the incidence of acute rejection

Chronic rejection ☹Cellular immune response)

Months to years

results from a process of graft arteriosclerosis characterized by progressive fibrosis and scarring with narrowing of the vessel lumen due to proliferation of smooth muscle cells. Several predisposing factors impact the development of

chronic rejection, including prolonged cold ischemia, reperfusion, acute rejection episodes, and toxicity from immunosuppressive drugs. Chronic rejection is also thought to have an immunologic component, presumably a delayed-type hypersensitivity reaction to foreign HLA proteins. This is indicated in studies employing animal
models of graft arteriosclerosis in which mice lacking IFN gamma do not develop graft arteriosclerosis. In addition ,similar studies support an important role for CD4
T cells and B cells in this process. Cytokines and growth factors secreted by endothelial cells, smooth muscle cells,and macrophages activated by IFN gamma stimulate smooth muscle cell accumulation in the graft vasculature.

GRAFT-VERSUS-HOST DISEASE

Stem cell transplants (and less commonly lung and liver transplants) are complicated by a unique allogeneic response—**graft-versus-host disease (GVHD).** Recipients of stem cell transplants for hematologic malignancies typically have depleted bone marrow prior to transplantation as a result of the chemotherapy used to treat the malignancy.Next, donor bone marrow or, more commonly, peripheral blood stem cells are infused. The infused products often contain some mature T cells. These cells have several beneficialeffects, including promotion of engraftment, recon stitutionof immunity, and mediation of a graft-versus-leukemia effect. However, these mature T cells may also mediate
GVHD.

Acute GVHD occurs during the first 100 days post infusion and targets the skin, gastrointestinal tract, and liver.In mismatched allogeneic stem cell transplantation, the targets of GVHD are the mismatched HLA proteins, while inmatched stem cell transplantation, minor histocompatibilityantigens are targeted. The infused T cells can mediate GVHD in several ways, including a massive release of cytokines due to large-scale activation of the donor cells by MHC mismatched proteins and by infiltration and destruction of tissue.The incidence and severity of GVHD is related to the match status of the donor and recipient as well as other factors. In efforts to reduce the incidence and severity of GVHD, several approaches are taken, including immunosuppressive therapy in the early post-transplant period removal of T lymphocytes from the graft. T-

cell reduction is very effective in lowering the incidence of GVHD, but it can also reduce the graft-versus-leukemia (GVL) effect of the infused cells and increase the incidence of graft failure.Beyond 100 days post-transplant, patients may experience chronic GVHD. This condition resembles autoimmune disease, with fibrosis affecting the skin, eyes, mouth, and other mucosal surfaces.

IMMUNOSUPPRESSIVE AGENTS

There is a growing list of agents that are employed to suppress ant igraft immune responses in solid-organ and stem cell transplantation. **Immunosuppressive agents** are used in several ways, including induction and maintenance of immune suppression and treatment of rejection. Combinations of different agents are frequently used to prevent graft rejection. However, the immunosuppressed state (and graft survival) induced by these agents comes at a price of increased susceptibility to infection, malignancies, and other associated toxic side effects. There are several classes of immunosuppressive agents, which are

Corticosteroids

Corticosteroids are potent anti-inflammatory and immunosuppressive agents used for immunosuppression maintenance. At higher doses, they are used to treat acute rejection episodes.Steroids act by blocking production and secretion of cytokines, inflammatory mediators, chemoattractants, and adhesion molecules. These activities decrease macrophage function and alter leukocyte-trafficking patterns. However,long-term use is associated with several complications, including hypertension and diabetes mellitus.

Antimetabolic Agents

Anti-metabolic agents interfere with the maturation of lymphocytes and kill proliferating cells. Azathioprine was the first such agent employed. It has been replaced in large part by mycophenolate mofetil, which has a more selective effect on lymphocytes compared to azathioprine and thus fewer side effects.

Calcineurin Inhibitors

Cyclosporine and FK-506 (tacrolimus) are compounds that block signal transduction in T lymphocytes, resulting in impairment of cytokine syntheses, including IL-2, 3, 4, and interferon-gamma. Inhibition of cytokine synthesis blocks

the growth and differentiation of T cells, impairing the antigraft response. Rapamycin (sirolimus) is an agent that inhibits T-cell proliferation by binding to specific intracellular proteins, including mammalian target of rapamycin (mTOR).

Monoclonal Antibodies

Several monoclonal antibodies that bind to cell surface molecules on lymphocytes are used as induction agents and to treat severe rejection episodes. OKT3 is a mouse monoclonal antibody the binds to the CD3 receptor on human lymphocytes. Binding of OKT3 to the CD3-positive T-cell surface has several outcomes. Binding may modulateCD3 from the cell surface, rendering the affected T cells

nonfunctional. Higher doses of antibody deplete T cells from the circulation via complement-mediated lysis oropsonization for removal by phagocytic cells.

A problem with monoclonal antibody preparations administered to patients is the development of anti-mouse antibody.

Polyclonal Antibodies

Two polyclonal anti-T-cell antibody preparations are used to treat severe rejection. Thymoglobulin is an anti thymocyte antibody prepared in rabbits, and ATGAM is a polyclonal antiserum prepared from immunization of horses. Both are potent immunosuppressive agents that deplete lymphocytes from the circulation. The development of these anti-mouse antibodies can interfere with the activity of the monoclonal antibody. A drawback associated with administration of polyclonal antibody preparations is the development of serum sickness due to antibody responses to the foreign immunoglobulin

Lec (8) **Immunotechnology**

Prof. Dr. Ekhlass Noori Ali

CLINICAL HISTOCOMPATIBILITY TESTING

Appreciation of the beneficial role of **HLA matching** and the detrimental role of antibody to HLA proteins on graft survival provided the impetus for development and application of specialized testing to aid in the selection of the most appropriate donors for patients needing transplantation.Histocompatibility laboratories provide specialized testing for both solid-organ and stem cell transplantation programs. Two main activities are carried out by these laboratories in support of transplantation, HLA typing, and
HLA antibody screening/identification.

HLA Typing

HLA typing is the phenotypic or genotypic definition of the HLA antigens or genes in a transplant candidate or donor.For clinical HLA testing, phenotypes or genotypes of the classical transplant antigens or genes are determined (HLA-A, B,
Cw, DR, DQ). This information is used to find the most suitable donor–recipient combination from an immunologic standpoint. It must be stressed that other considerations go into the choice of a particular donor for any given patient, be it a solid-organ or stem cell transplant.

HLA Phenotyping

The classic procedure for determining the **HLA phenotype** is the **complement-dependent cytotoxicity (CDC)**test. Panels of antisera or monoclonal antibodies that define individual or groups of immunologically related HLA antigens
are incubated with lymphocytes from the individual tobe HLA typed. Purified T lymphocytes are used for HLAclass I typing, while purified B lymphocytes are used forHLA class II typing. After incubation with the antisera, complement is added. In the presence of bound antibody, which occurs only if the lymphocyte expresses the HLA antigen targeted by the antisera, complement is activated and cells are killed. A vital dye is then added that distinguishes live cells from dead when they are viewed microscopically. Using this assay, an extensive array of HLA antigens can be defined(Table 1).There are several limitations to the CDC method forHLA typing. Viable lymphocytes must be used, which demands timely

performance of the assay. Separation of T and B lymphocytes is required for definition of class Iversus class II antigens. The source of antisera for HLA typing is not always consistent or reliable. Thus, reagents can vary in quality or quantity over time. Finally, the level of resolution (i.e., the ability to distinguish two closely related yet distinct HLA antigens) is limited. The limits of resolution don't significantly impact the role of this technology for matching solid-organ donors and recipients. However,for unrelated stem cell transplantation, a higher level of resolution is required. DNA-based (molecular) HLA typing methods are now commonly employed in histocompatibility laboratories, because they address the limitations of CDC-based methods and are amenable to higher throughput formats.

HLA Genotyping

Molecular-based HLA genotyping methods use polymerase chain reaction (PCR)–based amplification of HLA genes followed by analysis of the amplified DNA to identify the specific HLA allele or group of alleles. The most common approaches for analysis include PCR amplification of HLA genes with panels of primer pairs, each of which amplifies specific alleles or related allele groups. Amplification is

detected by agarose gel electrophoresis **(Fig. 1).** Only those primer pairs that bind to the target gene result in detection of an amplification product. The HLA type is then identified by determining which primers resulted in amplification.

A second common approach for HLA genotyping is to perform a single PCR reaction that will amplify all HLA gene variants at a specific locus (referred to as a *generic*

Table1: Approximate number of HLA antigens and Alleles defined at the six classical transplant loci.

HLA LOCUS	# ANTIGENS	# ALLELES
A	28	506
B	61	851
C	10	276
DRB1	24	559
DQB1	9	81
DPB1	6	126

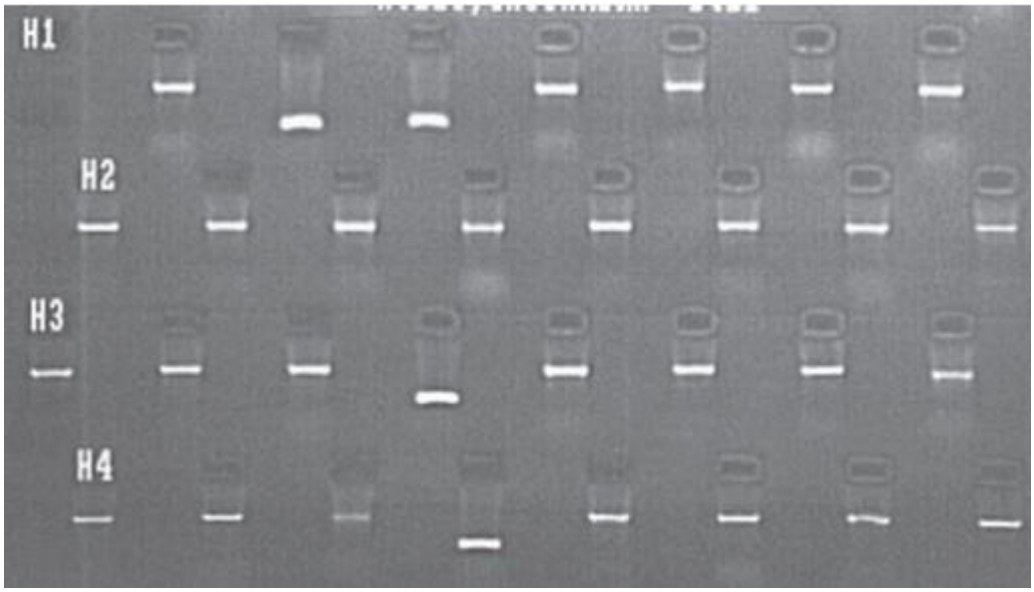


Fig1: An example of a PCR with sequence-specific primers (PCR-SSP) analysis of the HLA-DQB1 locus. Each lane of this ethidium bromide stained agarose gel contains the amplification product of an individual PCR reaction. Each reaction contains primers to aubiquitous gene and should demonstrate amplification (the larger band in each lane). This serves as an internal control to document successful amplification in each reaction. Each reaction also contains primers specific for various HLA-DQB1 alleles. An amplification product of small size is seen in several lanes, indicating the presence of the target HLA-DQB1 gene for those specific primers. The particular pattern of amplified primers is assessed to determine the HLA-DQB1type of the sample. (Color Plate 13).

amplification). The amplified gene is then subjected to hybridization with a panel of DNA probes, each specific fora unique HLA allele or allele group. Only those probes that specifically hybridize to the amplified DNA will be detected.

The **HLA genotype** is determined by assessing which probes hybridized. A third common method for HLA genotyping is sequencing of PCR-amplified HLA genes.

HLA genotyping overcomes the limitation of CDC based HLA phenotyping. Cells do not need to be viable in order to obtain DNA for HLA genotyping. Typing reagents are chemically synthesized; thus, there is no reliance on human donors of antisera. HLA genotyping can provide varying levels of resolution that can be tailored to the specific clinical need. DNA-based typing can provide results at a level of resolution comparable to CDC-based typing (antigen equivalent) or can provide allele-level results (required for matching of unrelated stem cell donors and recipients). Allele-level HLA typing has demonstrated the incredible extent of polymorphism within the HLA loci (Table 1).

HLA Antibody Screening and Identification

Antibodies to HLA antigens can be detected in candidates and recipients of solid-organ transplants. These antibodies develop in response to multiple blood transfusions; to prior HLA-mismatched transplants; and, in women, to multiple pregnancies. Because of the potential adverse impact HLA antibodies can have on graft survival, patients awaiting solid organ transplantation are screened periodically for their presence. If detected, the specificity (which HLA proteins they bind) of the antibodies is then determined so that donors possessing those HLA antigens can be eliminated from consideration for donation to that patient.

Patients are tested monthly for the presence of HLA antibodies while they are waiting for an organ offer. Antibody screening and identification is also performed post-transplantation to aid in the diagnosis of antibody-mediated rejection and to assess the effectiveness of therapy for antibody-mediated rejection.

The methods used for antibody detection and identification have changed significantly in recent years. The CDC method used for HLA typing is also used for HLA antibody detection and identification. In this case, panels of lymphocytes

with defined HLA phenotypes are incubated with the patient's serum. If the serum contains HLA antibodies, they will bind to those lymphocytes in the panel that express the cognate HLA antigen. Binding is detected by addition of complement and a vital dye to assess cell death microscopically.In some scenarios, the level of antibody in a patien tserum may be below a level detectable by the CDC assay. In these cases, antihuman globulin (AHG) may be added to the CDC assay to increase the test's sensitivity. The AHGCDC assay can detect lower levels of antibody as well as isotypes of bound antibody that don't activate complement and thus wouldn't normally be detected in the standard CDC assay. The proportion of lymphocytes in the panel (usually 30 to 60 unique lymphocyte preparations are included in the panel) that are killed by the patient's serum is referred to as the *percent panel reactive antibody* (%PRA).In addition, the specificity of the antibodies can be determinedby evaluating the phenotype of the panel cells.

Enzyme-linked immunosorbent assay (ELISA) has been developed in recent years as a substitute for CDC-based HLA antibody testing. ELISA assays utilize purified HLA antigens bound to the wells of microtiter plates.Patient serum is added to the wells of the plate, and if HLA-specific antibody is present, it will bind. Bound antibody is detected by addition of an enzyme-labeled anti immunoglobulin reagent. Addition of substrate results in a color change in the wells that have bound antibody. The wells of the ELISA plate may contain a pool of HLA antigens, thus serving as a qualitative screen for the presence of HLA antibody in a serum. Alternatively, each well may contain HLA antigens representing a single donor and thus can be used in a fashion analogous to a CDC-based analysis,allowing %PRA and specificity to be determined.Another approach for antibody detection and identification is flow cytometry. Antibody in patient serum can be incubated with latex beads that are coated with HLA antigens, either from a single donor or a single purified HLA protein. Patient serum is incubated with the beads, andbound antibody is detected by adding an FITC-labeled anti-IgG reagent **(Fig. 2)**. A more recent version of flow cytometry–based antibody detection is the multiplex bead array system that can assess binding of up to 100 different HLA antigens in a single tube using a dedicated flow-based

detection system. Flow cytometry–based methods are the most sensitive technology for detecting HLA antibodies. In addition, they can provide the most specific determination of the specificity of HLA antibodies when beads coated with a single HLA antigenic type are used .Once a donor has been identified for a particular patient ,**a donor–recipient cross match test** is performed to confirm the absence of donor-specific antibody. Donor lymphocytes are incubated with recipient serum in a CDC assay to verify a lack of binding as detected by microscopic analysis after addition of a vital dye. Alternatively, binding of antibody can be detected by flow cytometry using an FITC-labeled anti-IgG reagent. As for antibody screening and identification, the flow cytometric cross match is the most sensitive method for detecting donor-specific antibody.

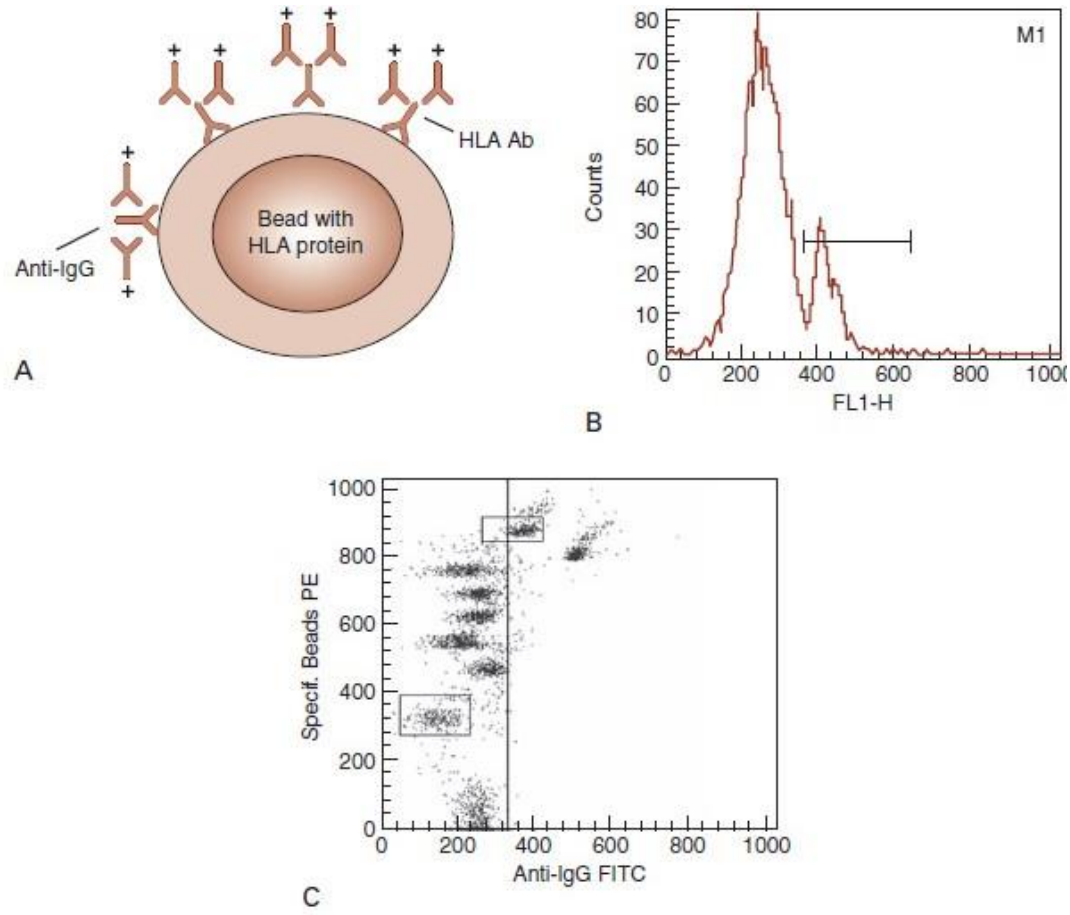


Fig2: *(A)* Flow cytometric detection and identification of HLA antibodies employs latex beads that are coated with HLA proteins from individual donors or single HLA molecules. For qualitative determination of thepresence of HLA antibodies, multiple beads, each coated with the product of an individual donor, are pooledtogether so as to represent the majority of common HLA antigens. They are then incubated with patient serum. Todetermine the

specificity of HLA antibodies in a serum sample, beads coated with a single HLA protein species can
be incubated with patient serum. Once incubated with serum, both bead types are then incubated with an FITClabeled anti-IgG reagent that will detect the presence of antibodies bound to the ead. *(B)* Single-parameter histogram display of an HLA class I antibody screen. A pool of HLA-coated beads was incubated with a patient serum, washed, and then incubated with an FITC-labeled anti-IgG reagent. Unbound FITC-labeled reagent was washed away and the beads analyzed for fluorescence on a flow cytometer. The large peak represents beads with no bound antibody, while the smaller peak to the right indicates the presence of HLA antibody bound to approximately 19 percent of the HLA class I coated beads. This represents a positive HLA class I antibody screen.
(C) Nine individual clusters of latex beads, each coated with a single HLA class I antigen species, are identified in the dual parameter dot plot of the serum from *(B).* The HLA antigen coated on each bead is indicated to the right of thebead. The bead coated with HLA-A11 has shifted to the right relative to the other beads, indicating that the HLA antibodies in this patient's serum are specific for the A11 antigen.