Cell and Molecular Biology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati
Week 11
Molecular Immunology
Lecture - 42
Antibody

Hello everyone, this is Dr. Vishal Trivedi from the Department of Bio-Sciences and Bioengineering, IIT Guwahati. And what we were discussing was the different aspects of immunology in this particular module. So far, what we have discussed is the basics of immunology and how the immune system protects the host from different types of external or internal antigens. So the purpose of the immune system is to resist any kind of changes in the host body, which are induced by internal or external factors. Now, in this context, we have discussed the basics of immunology in the previous lecture.

Now in today's lecture, we are going to discuss the antibodies and how they are going to be used for different types of applications in immunology. So an antibody is a Y-shaped structure. Right. And it is being produced by the B cell.

Right. So remember that when we were in the previous lecture, we discussed the humoral response, which is mediated by the antibody. So first, let's discuss the antibody structure and the classification. So, when an antibody is stimulated by the antigen, the B cells secrete antibodies with the same antigen specificity as the B cell antigen receptors, right? The identity between the binding site of the secreted antibody and the membrane-bound B-cell receptor was first demonstrated by creating a reagent that bound to the antibody secreted by a particular clone of the B-cell, and those reagents were also found to be receptors on the cells that secrete the antibody. So, secreted antibodies and the membrane-bound receptors belong to the immunoglobulin family of proteins.

This is a large family of proteins that includes both the B-cell and T-cell receptors, additional molecules from tyrosine kinases, and other immune receptors. And it has been characterized by the presence of one or more immunoglobulin domains. The immunoglobulin domain is generated when a polypeptide chain folds into an organized series of antiparallel beta sheets. And with each domain, the beta strands are arranged into pairs of beta sheets to form a compact tertiary domain. The number of strands per sheet varies among the individual protein.

The antibody molecule, the most common immunoglobulin domain, contains approximately 110 amino acids. Each beta sheet contains three to five strands. The pair of beta sheets within each domain is stabilized with respect to one another by interchain

disulfide bonds. Neighboring domains are connected to one another by stretches of relatively unstructured polypeptide chains. So within the beta strand, the hydrophobic and hydrophilic amino acids alternate, and their side chains are oriented perpendicular to the plane of the sheets.

The hydrophobic amino acids on the other sheets are oriented toward the positive sheets, and the two sheets with each domain are therefore stabilized by the hydrophobic interactions between the two sheets, as well as by the covalent disulfide linkages. So remember, in the antibody structures, you are going to find a lot of covalent interactions mediated by the disulfide bridges, right? So you are actually going to have disulfide bridges between the heavy chain and the light chain as well, right? At the end of each of the beta sheets, more closely loosely folded polypeptide regions connect one beta strand to the next, and these loose regions can accommodate a variety of amino acid side chain lengths and structures without causing any distortion to the overall structure of the molecules, and this is a very important feature. It is actually accommodating any kind of modifications or alterations to the sequence, right? Hence, in the antibody molecule, the immunoglobulin fold is superbly adapted to provide a single scaffold onto which multiple different binding sites can be built because the function of the antibody is to bind the antigens, and for that, it has to change its structure, charge distribution, and so on, right? These properties explain why the immunoglobulin domain has been used in many proteins with recognition or addition functions. The decennial domain characteristics provide a molecular backbone, while the loosely folded region can be adapted to bind specifically to many adhesive or antigenic structures. The immunoglobulin domain structure is used by many proteins besides the B-cell receptors.

The T-cell receptor is also made up of repeating units of the immunoglobulin domains. The other proteins that use the immunoglobulin domains are like Fc receptors, the T-cell receptors, CD2, CD4, CD8, and CD28. Each of these proteins is classified as a member of the immunoglobulin superfamily, and all antibodies share a common structure of four polypeptide chains consisting of two identical light chains and two identical heavy chains. Each light chain is bound to its partner heavy chain by a disulfide bond between the corresponding cysteine residues, as well as the non-covalent interaction between the VH and VL domains. and the CH1 and CL domains.

So these bonds enable the formation of a closely associated heterodimer, which actually contains the heavy chain and the light chain. Multiple disulfide bridges link the two heavy chains together about halfway down their length, and the C-terminal part of the two heavy chains also participates in the non-covalent interaction between the corresponding domains. The antibody molecule forms a Y-shaped structure with two identical antigen-binding sites at the tip of the Y. Each antigen-binding region is made up

of the antibody amino acids derived from both the heavy chain and the light chain, as well as the amino terminal domain. The heavy and light chains both contribute to each arm of the Y chain, with the non-antigenic binding domain of each serving to the extent of the antigen-binding arms.

The base of the Y consists of the C-terminal domain of the antibody's heavy chain. The overall structure of the antibody molecule consists of three relatively compact regions joined by a more flexible hinge region. The hinge region is particularly susceptible to proteolytic cleavage by the enzyme called papain. The papain cleaves and resolves the antibody molecule into two identical fragments that retain the antigen-binding specificity of the original antibody and the remaining region of the antibody molecule, which consists of a non-antigen-binding portion. The latter region, which is identical to all antibodies, is actually being called the FC region.

So the FC region is not variable between the different antibody molecules. And that's why it is also being called the constant region. Each FAB region and the AC region obviate their own particular functions during an antibody response to an antigen. The FAB region binds to the antigen, and the AC region of the antigen binds to the receptor on the phagocytic, cytolytic, or immune effector cells. In this way, the antibodies serve as a physiological bridge between an antigen present on the pathogen and the cells or molecules that will ultimately destroy it.

So a family of receptors exists, with each receptor expressed on a different type of cell and binding to a different class of antibodies. So this is the structure of the antibody, where you have, you know, this region is the antigen-binding region. So you have one antigen-binding region on this side and you have another antigen-binding region on this side. And this is the light chain, correct? So this is the light chain. This is the heavy chain.

And you have the two light chains and the two heavy chains, which are actually connected to each other by disulfide linkages at this junction. And this has been called a hinge region. On the other hand, this is the region that is constant between the antibody molecules. And this has been called the SC region. So, this is the constant region.

This is the variable region. And this is the region that binds the antigen. The heavy and light chains in an immunoglobulin molecule contain amino terminal variable regions that consist of 100 to 100 amino acids and differ from one antibody to another. And the constant region exhibits the limited variation that defines the two light chains and the heavy chain subtype. The heavy chain, which consists of gamma, delta, and alpha, also contains a proline-rich HIN region.

The amino terminal portion corresponds to the V region and binds to the antigen. Effective functions are mediated by the Kavakshi terminal domain. The mu and epsilon heavy chains, which lack a HIN region, contain an additional domain in the middle of the molecule, which is called a CHO. Defined as a carbohydrate linked to the heavy chain, right? So, this is a typical structure of the antibody, right? Depending on the different types of antibodies, such as IgG, IgA, IgD, and IgE, you will actually have the composition of the different types of heavy and light chains. And now discuss how the antibodies are, you know, different types of antibodies being produced.

and how they are actually maturing and giving you the final antibodies. Now, antibody diversity, right? So you basically have different types of antigens. You have the antigens that have been derived from the bacterial sources. You have the antigen that has been derived from viral sources. You have the antigen from the fungus, cancer.

You have so many antigens, don't you? And accordingly, you should have different types of antibodies. So how are we actually producing the different types of antibodies? So, multiple gene segments exist at the heavy chain, which are called V, D, and J. You also have multiple gene segments at the light region, which are called V and J loci, and these can be combined with one another to provide extensive combinatorial diversity. The p-nucleotide addition results when the higher pin of the DNA coding region is cleaved symmetrically, filling in the single-stranded DNA piece, resulting in this asymmetrical cleavage generating a short palindromic sequence. So exonuclease trimming sometimes occurs at the VDJ and the VJ junctions, causing the loss of nucleotides.

Non-template n-nucleotide addition in a heavy chain results from the terminal reactivity, and that mechanism actually contains the 2, 3, and 4 rise to give diversity at the junction between the gene segments, which contributes to the CDRs. So combinatorial diversity, the same heavy chain can combine with different light chains and vice versa. Thus, five mechanisms are responsible for the creation of the diverse receptors of the B-cell receptors and the antibodies that are available to the organism before any contact with pathogens or antigens occurs. Following antigen stimulation, the B cells are able to utilize yet another mechanism unique to the immune system to further diversify and refine the antigen-specific receptors and antibodies through somatic hypermutations. B cells are exposed to successive cycles of mutation at the BCR loci, followed by antigen-mediated selection in a specific, specialized region of the lymph node and the spleen.

And the end result of this process is that the average affinity of the antigen-specific BCRs and the antibodies formed at the end of an immune response is considered higher than its instigation. And this process is referred to as affinity maturation, which means

that what you are doing is generating a pool of antibodies; you are generating a pool of B-cell receptors, and then they are actually going to be selected by their interaction with the antigen. Based on this selection, you are actually going to select the high-affinity antibodies, and as a result, that antibody-containing clone is going to proliferate, giving you more antibodies. Then, talking about B cell development, the maturation. So in the bone marrow, the B cell develops by rearranging immunoglobulin genes like VDJ.

So, the immature B cell expresses IgM on its surface, right? So, IgM is a kind of antibody where you have the... So, any B-cell that binds to cell antigens is eliminated. So, that has been called the clonal deletions.

Remember that you don't want our immune system to work against the antigen what is present in our bodies. So, that is very important because if you don't destroy those cells, they will actually act against our own cells, and that's how they are actually going to be responsible for the different types of autoimmune diseases. So surviving the immature B cell further co-expressed IgM and IgD and circulated through blood, lymph, and secondary lymphoid organs like the lymph nodes and spleen. And when these molecules encounter the antigen, right? Then the antigen is actually going to start the selection process, isn't it? So mature naive B cells encounter the antigen in the lymph nodes or in the spleen. Binding of the antigen to the BCR provides the first activation signal.

So once the antibody actually encounters the antigen, it will give a stimulation signal because you have the SC region, and this SC region is going to bind to the cell responsible for eliciting the immune response. And as a result of this, this cell is actually going to get the stimulus signal, and it is actually going to produce a larger amount of antibodies. It is also going to produce other cytokines. So once this activation signal is sent, a second signal comes from the CD4 positive T cells through the CD40-CD40L interactions and different types of cytokines like IL-2 and IL-4. And then, in T cell-independent antigens like the polysaccharides, activation can occur directly through the strong B cell receptors cross-linking or the innate immune receptors.

Then we have clonal expansion. So, activated B cells proliferate, producing a population of identical B cells specific to the antigens. Some differentiate early into short-lived plasma cells in the primary foci secreting IgM, while others migrate into the lymphoid tissue and form the germinal centers. Then what will be the process in the germinal cell centers? In the germinal centers, the two key processes are the fine-tuning and the antibody response. So in the somatic hypermutations, the activation-induced cytidine deaminase introduces the protein mutation into the variable region of IgG, generating the PCR variants. And all these B cells are actually going to be selected against by the antigen.

And those who are actually going to engage the antigen are actually going to survive. And then they are actually going to start producing the IgG antibodies. Then you are actually going to have class switching recombination. So activation-induced cytidine deaminase also derived the switch from IgM.

Right. Remember, this is the antibody that is going to be produced in the beginning to the other antibody classes like IgG, IgA, and IgE, giving the antibody new effector functions. Right. Because IgM has its own function, and IgG, IgA, and IgE are actually going to have their own other functions. Now, at this stage, there will be memory formation and long-term immunity. So the B cell becomes long-term plasma cells sitting in the bone marrow and continuously secreting antibodies.

Some of these B cells are going to be transformed into memory cells so that they will actually keep a memory of this particular antigen. So as a result of this, when the same antigen comes the next time, it is not going to go through the extensive process of antigen processing, presentation, and so on. This antigen, as soon as it comes, will be recognized by these memory cells, and then they will start secreting antibodies so that you can have an early response, and that's how you can have better production. So once the memory cells are produced, which respond rapidly and robustly upon future exposure to the same antigen, antibody production starts with the naive B cells that, upon antigen encounter and an appropriate signal, undergo clonal expansions, chromatic mutations, and class switching. ultimately generating the high affinity plasma cells and the memory cells this refined process ensure a strong and adaptable immune defense So, this is all about the theoretical aspects: how the antibodies are selected from the antigen, how clonal selection occurs, how clonal proliferation happens, and so on; as a result of that, you are actually going to produce the plasma cells and the memory B cells.

But what happens when you are actually trying to develop a specific antibody? Suppose you want to develop a particular antibody against a particular antigen; then you are going to use different types of animals. You are actually going to use mice, rabbits, sheep, and goats; even sometimes you use the horse, right? So you might have heard that people are developing different types of antibodies and anti-serum against, you know, snake venom in horses. And then you are actually going to utilize these antibodies for different types of biotechnology applications. So to get you familiar with this whole process, how are you going to be generating the antibodies in a particular animal? we have prepared a very small demo clip where we are actually going to show you the salient features, how you can be able to prepare the antigens, how you are going to give the injections to the rabbit and how you are going to collect the serum, how you are going to determine the presence of antibodies and so on. After this demo, you will probably be able to understand the

whole process of antigen production in the animals, and what we have discussed is similar to what will happen inside the body.

So, what we are going to discuss in this demo is how to ensure that the antibodies are produced. I am Amogh Arun Sahasrabudhe. I work at CSIR, CDRI, Lucknow. And in today's demo, we will be discussing the different steps involved in the generation of antibodies in relatives.

So, for the first step, we require several things. France Complete Adjuvant, here it is from Sigma. We need a micro-immersive fine needle that has two openings connected by a fine needle. We need an antigen that is purified and filtered so there are no contaminations. It is a sterile solution of antigens. Then we take out some of the France Complete Adjuvant in Eppendorf and then mix it together.

Since this adjuvant is oil-based, it doesn't mix easily with the watery system, as the antigen does in the previous one; therefore, we mix them rigorously and forcefully. For that purpose, we take these two and mix this emulsion. We mix this previous antigen-containing solution with the oil-based adjuvant; after mixing them, we take it out with a needle using a syringe like this, and then we fix the micro-emulsifier needles into it and attach another syringe like this. Once you have filled this needle with your antigen and adjuvant, you push it here and then keep pushing from one side while pulling from the other side. Keep pushing from one side and keep pulling from the other side.

So this process forcefully pushes your material, I mean the oil and the antigen, through this palm needle. And with that, in that process, the emulsion is formed. Emulsion can be called water in oil or oil in water because both are in the same concentration and same volumes. So you can call them anyway.

By this method, an emulsion is formed. So, for ready reference, we have already prepared the immersion. This immersion looks white. Initially, it was two-phase, and then slowly it has turned into a single phase. Now you can push this immersion from one unit to another and from this series too.

Another syringe. So this process creates very good emulsion. This does not separate out later on when you are ready to inject it. So how do we check, then? So, for checking purposes, a drop of emulsion should be placed on the water surface like this. If the emulsion is not formed perfectly, it will spread out.

Otherwise, it will not spread. So, this is a check to ensure that your emulsion is formed correctly. So, once you find that this drop is not spreading, your emulsion is ready for

injection. So, this is the process by which you prepare the emulsion for injection. So this is the first step in the gradient inversion.

So now let us understand why we prepare for the inversion. We have checked that the inversion is formed. Now the purpose of making the emulsion is that you have an antigen, and through the antigen, you can raise antibodies; but after emulsifying them, you actually make the antigen release slowly, so it is a sustained-release kind of preparation. So that the antigen is exposed to the system in a systematic manner, more and more memory cells are generated, and that is the sole purpose of having immersion. Otherwise, if you inject the antigen as such in EBS or in another watery system, it will spread out in the body and will be cleared away by the immune system readily, and no memory cells will be generated.

So, this is the main purpose of preparing the immersion. So now we have prepared the immersion. We have come to the animal house. This is our rabbit, which will be immunized, and before immunization, we have to take pre-immune blood so that we can better compare the serum and the anti-serum. So, we will now get to start how we immunize it.

So, now we are preparing to immunize. The first important thing in all these chemical processes is that we have to avoid the pain towards the end. So, for that purpose, we have to strain very well because we have to inject. Therefore, we strain the removal in a way that it has less and less pain, and the movement is also less. So, we inject this into the thigh. We have to catch hold of both blades, and we have to sterilize the area using alcohol.

We have to look at the thigh muscles. They should be cleaner and more visibly clean. The skin should be clearly visible. There are two kinds of injections that we administer. One is intradermal and the other is subcutaneous.

So today we will be doing a subcutaneous injection. This is our immersion that we have prepared with macromolecules. We have seen the ideas. This is the area where we would like to inject. We have to take out all the air from the syringe and the media. We have cleaned the area again and then applied some antiseptic powder, which is vitamin powder, so that the infection cannot develop later on.

This is enough for the area of injection, and then we slowly release and leave the animal relaxed while it is immunized. To strain the animal in a towel or this kind of cloth. So the advantage of having this cloth to strain is that the animal has its claw inside and outside of this cloth, and then it cannot move. So we have to restrict the movement when it is

similar in the scalar and in the round on this block.

So we use this bit. Now we strain the animals on this block. We keep the animal relaxed on this block. Make sure that the ears are outside and the animal is trained properly so as to reduce its movement and now it is ready to bleed. We will bleed the animal from this mid-ear vein and we rub it so that it gets tender and the circulation is faster. The vein will also expand and more and more blood will flow into it.

This is the method that we normally apply. When the vein is properly visible, this is the mid-air vein from which we will lift. We will sterilize it slightly using alcohol. using a 20 gauge needle which is wide enough to give sufficient width we will pick the grain and collect the weight then we to stop it we just If less and less pain, you can collect the bleed like this. Now we have to make sure that no further bleeding occurs, and we will wipe away whatever blood is outside using sterile water.

Wipe out all the blood here and everywhere. We will wipe everything down with water so that the vane becomes cool and gets sunlight. We will just check if it is still bleeding, so keep it pushed. Until the bleeding stops. Now I think the blood has stopped coming out, and now we apply some antibiotic; here, in this case, it is Betadine powder, so that there are no further infections or inflammation in the rabbit. This also ensures that this is your pinna, and if there is any inflammation, it will cause some pain, so it will avoid that kind of pain as well.

We have isolated approximately 10 to 12 mL of blood. This will give us approximately half the volume of the blood serum. This will be coagulated, and that will be coagulated with sodium citrate for one hour, and then it will be kept in a photic citrate for one day so that the blood is shrunk properly and the serum is slowly taken out. And then we will isolate the serum, add some preservative like sodium azide, and keep it at minus 20 or minus 18 degrees as the environment. And then we also taste simultaneously the title of it and its specificity using the ELISA test and the rest of the tests as well.

Now you can see the whole element, which is relaxed. I think it really has a spin or a negligible plaything. And this is actually very important for handling an animal, ensuring that in whatever procedure you go through with the animal, it should not experience pain. Maybe if the procedure is painful to understand, it is a painful procedure; you anesthetize it. Since this procedure is not painful, it has been administered.

So this is a very important step to ensure that the animal has no pain. What should we do next? You have got to know all the steps, and we are developing it a bit. And we have prepared the medicine. We injected the medicine. We isolated the blood after

administering booster doses.

And the whole process is in the lab. I think most of this is possible. And we like it. Now, once you have produced the antibodies, these antibodies can be used for conducting different types of antibody-antigen interaction studies, and based on the antibody-antigen interactions, you can develop various techniques that can be used for different types of immunological assays so that you can use them for diagnostic purposes. Detect, you know, identifying a particular pathogen, or you can use that for identifying a particular disease, and so on. So how do the antibody and antigen interact, right? So there are many factors that are actually going to govern how the antibody and antigen interact, right? So antibodies, or immunoglobulins, are the Y-shaped molecules that are produced by B cells. Remember, not the B cell, but the plasma cell, right? Each antibody has a unique region called the paratope that specifically binds to a particular part of an antigen known as the epitope.

So it is actually the interaction between the epitope and the paratope. The binding is highly specific and occurs through non-covalent interactions including hydrogen bonding, electrostatic forces, van der Waals forces, and hydrophobic interactions. The strength of the antigen and antibody interactions is determined by the affinity, avidity, cross-reactivity, and specificity. So in the affinity, the combined strength of the known covalent interaction between a single antigen-binding site of an antibody and a single epitope is the affinity of the antibody for that particular epitope. Then within the avidity, the strength of the multiple interactions between the multivalent antigen and the multivalent antibody is called the avidity.

High avidity antibodies actually have low affinity. Then you also have cross-reactivity. So an antibody produced for an epitope of an antigen also cross-reacts with the epitope on an unrelated antigen. And this is called cross-reactivity. So cross-reactivity can occur because the two antigens share chemically related epitopes or the affinity of the antibody for the original epitope is greater than for the cross-reacting epitopes. Then you also have specificity, the ability of an antibody to distinguish between the two different types of antigens. Now, based on the antigen-antibody interactions, people have developed different types of immunological reactions, right? So the first reaction is the precipitation reaction where you can actually use the antigen to precipitate the antigens, right? So the interaction between the soluble antigen and the antibody is called the precipitation reaction.

The antibodies involved in the aggregation of soluble antigen are called precipitants. When the antibody and the soluble antigens are mixed in solution, the bi- or multivalent nature of the hemoglobin allows a single antibody molecule to bind to more than one

antigen. If the antigen is polyvalent, for example, if more than one antibody binds to the antigen molecules, then it will bind to multiple different antibodies. Eventually, the resulting cross-linked complex becomes so large that it falls out of the solution in the form of a precipitate. Now, examples of the precipitation reactions: you can have the precipitation reaction in the solutions. So, a soluble immunoprecipitation can be used to purify the antigenic molecules from a heterogeneous mixture of soluble molecules or to remove the particular antigen from the solution.

Immunoprecipitation occurs only when the antibody and antigen are essentially equivalent, and the antigen or antibody access to monovalent binding is favored and does not result in the formation of a precipitate. Then you can also have the precipitate react in the solutions. So immune precipitate can now form not only in solution but also in agar mix. So when the antigen and antibody diffuse from one another, a visible line of precipitation will form. As in a precipitation reaction in solution, a visible precipitate occurs when the concentrations of the antibody and antigen are equivalent to one another.

So immunodiffusion in the gel is rapid, easy to perform, and surprisingly accurate. So, in the outer loamy method, the most frequently employed variation of gel immunoprecipitation, both antigen and antibody diffuse readily from each other, thereby establishing the concentration gradient and the relative anterior interactions at which the maximum lattice formation turns out to be equivalent, resulting in a visible line of precipitation, or the precipitate line, forming in the gel. So, basically what you have is: suppose you have two wells, right? So if in one of the wells you put the antigen and in another one the antibody, they are both actually going to diffuse into the agar, right? And they will both interact. And as a result, they are actually going to precipitate in the center, right? So they can actually give you the line of precipitate, right? And this happens because at this point the antibody and antigen react in equivalent amounts, and as a result, they are actually going to get precipitated, come out of the solution, and give you a white precipitate. More sophisticated analyses can provide information regarding the extent of cross-reactivity and the antibody preparation with the related antigens. Then we also have the agglutination reactions, don't we? So agglutination reactions are always used for blood group typing, right? So you can actually use the agglutination reaction for blood group typing.

So the cross-linking that occurs between the dye or multivalent antibodies and the multivalent bacterial or other cellulose results in a visible clumping of the complex. Formed between the cell bearing the antigen and the antibody molecule. This clumping reaction is called agglutination, and the antibodies that produce such a reaction are called agglutinins. Agglutination reactions are identical to precipitation reactions. The only difference is that the cross-linked precipitate is visible to the naked eye because of the

large size of the antigen.

One of the simple examples is that you are actually going to detect the antigens on the surface of the RBCs in an assay that is called hemagglutination. A bacterial infection is the result of the bacterial antibodies specific to the surface antigen on the bacterial cell. Such antibodies can be detected by the bacterial agglutination reactions. The principle of bacterial education is identical to hemagglutination, but in this case, the visible pellet is made up of bacteria crosslinked by the antibacterial antibodies. So, apart from these reactions, you can also use the antibody-antigen interactions to develop a much more sophisticated technique, which is called the enzyme-linked immunoabsorbent assay or the ELISA.

So, ELISA is an array for the quantification of either the antibody or the antigen using the enzyme-linked antibody and substrate that form the colored reaction products. The conjugated enzymes are selected based on their ability to catalyze the conversion of a substrate into a colored fluorescent or chemiluminescent product. The number of variations of the basic ELISA assay has been developed. Each type of ELISA can be used quantitatively to detect the presence of antibodies or antigens. Alternatively, a standard curve based on the known concentration of antibody or antigen can be prepared and used to determine the concentration of a sample.

So you can have three different types of ELISA or four different types of ELISA. You can have the direct ELISA, the indirect ELISA, the sandwich ELISA, or the competitive ELISA. So let's talk about the indirect and direct ELISA. So antibodies can be detected or their concentrations determined with an indirect ELISA. So serum or the other sample containing the primary antibody is added to an antigen-coated microtiter plate and allowed to react with the antigen attached to the well. And then you are going to remove the free antibodies by washing, and then you are actually going to put the secondary antibody, which is going to be coupled to the enzyme, and then the free antibody is washed away again, and the substrate of the enzyme is added.

The amount of colored, fluorescent, or luminescent reaction produced is actually a direct measurement of the amount of antibody present or the amount of primary antibody present in that reaction. A direct ELISA would detect the amount of antigen on the plate using enzyme-coupled antibodies, and it is rarely used. So this is the simple, you know, the basic principle that you are actually going to have the antigen-coated wells. Then you are going to add the primary antibodies.

Then you are going to wash, and then you are going to add the secondary antibodies. The secondary antibody will have the enzyme attached to its constant region. And then, when

you add the substrate, it is actually going to give you the product. Then we're also going to have the sandwich ELISA. The purpose of the sandwich ELISA is to measure the level of the antigen.

So the antigen can be detected or measured by the sandwich ELISA. And in this technique, the antibody, rather than the antigen, is immobilized onto a microtiter plate. So basically, you have a microtiter plate on which you are going to immobilize the antibody, which is called the capture antibody. A sample unknown containing the amount of antigen is allowed to react with the immobilized antibodies. And remember, this immobilized antibody is called the capture antibody. And after the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antibody, right? So after any free second antibody is removed by washing, substrate is added, and the color reaction is produced.

The common variation on this assay is the biotin-linked secondary antibody, and then you can add the enzyme-linked avidin in an additional step. Sandwich ELISAs have proven particularly useful in the measurement of soluble cytokines such as TNF-alpha and other types of culture supernatants. And they can also be used for cytokine measurement in the serum and body fluids. So basically what you are supposed to do is first put the capture antibody onto the wells. Then you are going to add the antigen, right? And what will happen? This capture antibody is going to bind to the antigen.

And then you are going to use another antibody that is going to bind to another epitope of the same antigen, and then you are going to add the substrate, and it will give you the color, right? The amount of color is going to be the direct reflection of the amount of antigen that is present in that particular reaction. Sandwich ELISA is being used to measure cytokines such as TNF-alpha, IL-1, and IL-8, as well as other cytokines that are very important for different types of immunological conditions. So what we have done is that we are actually going to take you to my lab where the students are going to show you a demo video of how you can perform the sandwich ELISA. and how you can measure the amount of cytokines present in the biological fluid.

Remember, in this particular demo, we have not used secondary antibodies. Instead, we have used alternatives like the streptavidin-avidin system. And that's how we are actually going to show you a much more advanced system where we are not using the antibodies, the secondary antibodies. We are actually using the more advanced system where we are putting the stepped avidin and avidin system so that it becomes easier and easier. In this video, we will demonstrate how to use a pathogenic infection detection kit and what the underlying principle of that kit is.

So mostly the detection kits work on immunoassay. So, what is an immunoassay? In immunoassay, we will use specific antibodies, like monoclonal antibodies, against disease-specific or pathogen-specific antigens; then we will develop the substrate, which will produce a positive color that will be detected by spectrophotometer readings. The steps include that the first step is to coat the polyvinyl chloride plate with the capture antibody following the capturing of the actual disease-specific antigen. Suppose in most cases, in viral infections, it will detect the coded protein, and in bacterial infections, the external polysaccharide; these kinds of antigens will be detected once the antibody is coated on the plate. Then we will incubate with the sample taken from the patient, whether it is saliva or serum. After incubation, we will wash properly, then incubate again with the primary antibody specific to that particular antigen; it should mostly be a monoclonal antibody; otherwise, the detection will be nonspecific in the next step.

After washing the unbound primary antibody, we will incubate it with the secondary antibody that is septravidin conjugated to HRP. So once the secondary antibody incubation is over, we will wash and add substrate solution; mostly it is TMD or variants of TMD that are also available for enhanced chromophore detection. Then we will read the color development using a spectrophotometer.

These are the main steps, so in step by step. Now we will demonstrate how to perform the immunoassay. In order to perform an immunoassay, we need the following materials. First, we need a polyvinyl chloride plate, 96-mil plate, which should be flat-bottomed. Then the other materials we need are coating buffer, which is a bicarbonate buffer system with a pH of 9.

6. Then add 100 microliters; dispense 100 microliters each into these vials. So, once the dispense is over, we store this plate at 4 degrees Celsius preferably, but we can incubate it at room temperature for 2 hours as well. If you are incubating at 4 degrees Celsius, you have to keep it overnight; otherwise, 2 hours is enough. We have the coating buffer, so I have dispensed it into the reservoir, and then I will take the capture antibody. We have to see the dilution; we have to follow the manufacturer's instructions for dilution; otherwise, improper dilution may give false positive results or no results at all.

So, once it is over, we have to dispense 100 microliters into each plate after proper preparation. Once the material is dispensed into the plate, we have to cover the plate with parafilm or a covering plate. Then we will incubate this plate for two hours at room temperature. Post-incubation, we have to remove the unbound solution.

So we will remove that. So next we will wash the plate with TBST buffer, which should be pH 7.4. We have to wash at least 3 times properly; then we are going to block with the

blocking buffer, which contains 3% BSA in TBS. In the next step, we will dispense 100 microliters of blocking buffer into each well. So it will cover the non-specific area where there is no capture antibody, so that the reaction is specific to a particular antigen. The blocking should be done for at least 2 hours at room temperature or overnight at 4 degrees Celsius.

So here we have done it at room temperature. Once the blocking is over, we have to remove the remaining blocking buffer, wash three times, and incubate with the primary antibody. So, I am going to do that. In this step, we have to dilute the primary antibody with the assay diluent or blocking buffer, then mix properly and dispense 100 microliters each into 96 wells. And incubate at room temperature for 2 hours. Once the primary antibody incubation is over, we have to remove the unbound antibody and wash with the TBST at least 3 times so it will remove the unbound primary antibody, and in the following step, we will incubate with the.

After incubating the HRP-conjugated secondary antibody for two hours, we have to remove the secondary antibody and wash thoroughly. After washing, we will incubate with the substrate solution, which is usually TMB (tetramethylbenzidine). So, we will dispense the substrate solution into the wells. So, once the dispensing is over, we have to keep it at room temperature for some time until we can see a visible blue color. Once the reaction is over, we have to stop it with 2 normal HCl or sulfuric acid after 15 minutes. If you observe the pet, we can see that the blue color intensity in some of the plates is very high, while in some of the wells it is very low.

So, that means whatever wells are giving an intense blue color indicates that the concentration of the antigen is very high. So, at this moment, we have to stop the reaction; otherwise, all the wells may turn blue. So, we cannot identify positive samples versus false positives. So, there may be some artifacts.

So, that is why we have to stop the reaction using 2 N HCl or H_2SO_4. We will add 2 normal H_2SO_4 to stop this reaction. So, as we can see, the blue color turned into yellow. So, we have to read this in a spectrophotometer at 540 nanometers and 450 nanometers to get absolute values of these things. So this is a qualitative purpose as well as a quantitative purpose. Qualitative purpose in the sense that if you are using samples from patients, you have to do it in triplicate, and you can just identify whether that particular person is positive or negative. In another case for quantitative purposes, you need to have various varying concentration titrations of the particular antigen that the disease causes.

So, in this case, you have to dilute that antigen in different concentrations, and you have to develop the assay. In the same way developed here, you have to plot a standard graph

against the concentration versus the absorbance we have taken from that value; you will come to know what the unknown person's antigen titrations in their serum or saliva are, so that's why it is a qualitative as well as a quantitative method. With this, we can understand that this method can also be applied for the detection of various chemicals, various drugs in blood, such as drugs used for hallucination purposes, recreational drugs, and some of the drugs used in pharmaceuticals. Now, the fourth one is the competitive ELISA. So in a competitive ELISA, it is very sensitive and extremely effective for measuring the amount of antigen.

And this antibody is first incubated in a solution with a sample containing the antigen. The antigen-antibody mixture is then added to an antigen-coated microtiter plate. The more antigen present in the initial solution, the fewer free antibodies will be available to bind the antigen-coated well. After washing off the unbound antibody, the enzyme-conjugated secondary antibody specific for the isotope of AB1 can be added to determine the antibody binding to the well. In the competitive edge system, the higher the concentration of the antigen in the original sample, the lower the final thickness, right? So this is what the competitive ELISA: first, you are going to capture the antigen present in the solution using the primary antibodies, and then you allow this to immobilize onto the well, right? And then you are going to add another antibody.

So, this antibody will bind to this antibody, and it will actually give you the color. Now, once you have developed the antibodies and actually characterized them with the help of the different types of ELISA techniques, what you can do is, once you know, suppose you have developed the antibody and would like to use these antibodies for different types of applications, then you will be able to use them. Right. So let's see what the different types of applications of the antibody are. So antibodies are being used in passive immunity.

So remember, passive immunity means that you are actually going to get significant help from the reagent. Right. So passive immunity involves the transfer of preformed antibodies from an immune individual to someone who is not immune, providing immediate but temporary protection against the disease. Antibodies are key components of passive immunity. They are introduced into the person's body either naturally or artificially to help neutralize the pathogen or the toxin. So you can have natural passive immunity, which happens when you receive antibodies from your mom.

So during pregnancy, maternal IgG molecules cross the placenta and protect the fetus from infections like tetanus, rubella, and measles. Or you can actually get the natural passive immunity from the breast milk, right? So the maternal IgA antibody in breast milk protects the infant mucosal surfaces against infection, especially in the respiratory

and gastrointestinal tracts. Then you can have the artificial passive immunity, right? So artificial passive immunity is where you actually inject the antisera into the individual. There are several scenarios, such as having an immune deficiency, exposure to toxins or venom, or experiencing rapid responses to deadly infections like COVID-19 or the coronavirus. So when you are exposed to a pathogen, it is actually going to cause serious illness or death before a person's own immune system can respond. So in those cases, what you are going to do is take the antibodies from a different person, and that antibody from the different person is going to give you temporary protection until your body is ready to fight, right? So you can actually do that under the immunodeficiency, which means you might have some kind of defects because of which you cannot produce your own antibodies in a timely manner, right? So one example is acquired immunodeficiency or AIDS, right? So this is exactly a very serious problem when a person gets AIDS because HIV does not allow the body to respond to an infection and produce the different types of antibodies.

Or you can actually get exposure to the venom. So venom actually acts so fast that before the immune system can recognize it and produce the antibodies, it actually kills the person. Right. So to get the protection against these life-threatening toxins, what you do is actually inject the antibody against the venom protein directly. Then we can also use the antibody as a blocking agent, can't we? So antibodies can be used as blocking agents by binding to a specific molecule or receptor involved in a disease process, preventing their activity and thus reducing the harmful effects. So these blocking agents are, you know, antibodies; when you use the blocking agent, it can probably take care of the allergies and address any kind of receptor that has been involved in different types of, you know, pathological outcomes.

So you can actually use this for targeting the immune cell movements, or you can also use these blocking agents, like antibodies, to interfere with the key inflammatory signals or the immune cell functions, helping to calm down the overactive immune system. And that's how you can manage autoimmune diseases. Remember, I just said right when we were discussing antibody development that you are supposed to remove the self-reactive antibodies, but if you cannot remove the self-reactive antibodies or some of the self-reactive antibodies present in your body, then they are actually going to react against your own body antigens. To avoid that, what you can do is just add the blocking antibodies, and they will actually block the antigen in such a way that it should be hidden from the immune system.

And that's how it is actually going to give you temporary relief from any kind of adverse immune reactions. Then we can also use the antibodies as anti-cancer agents. So you can actually use the antibody to kill the cancer cells. So antibodies can target the tumor-

specific antigens using molecules found only on the cancer cells, making them a potentially powerful tool to identify and destroy the tumors. So you can actually use either the active or the passive immunizations, or you can be able to use the blocking factors in the serum.

Active immunization means stimulating the body's own immune system by giving a tumor antigen, hoping it protects against its own tumor response. Or the passive immunization where you can directly provide the anti-tumor antibodies that are ready to bind to the tumor cells and help eliminate them. Or, you can use the blocking factors. So scientists found that the serum from the animals or patients with the progressive tumor could block the immune responses, especially the killing activity of the cytotoxic T cells measured by the, you know, the cell-mediated lymphocytes or CML. So this blocking factor can also include not just the antibody by itself, but often the antibody-tumor antigen complexes that interfere with the immune attack. So in these cases, what you can do is use these kinds of blocking factors, which can actually block the interaction of the antibody with the antigen on the tumor cells.

Then you can also use the, you know, the, so what happens is that the antibody may bind to the tumor antigen and mark them, making it harder for the T cells to recognize and kill the tumor cells. So what you can do is prepare the antibody-antigen complexes, which will block the XC receptor on immune cells like NK cells and macrophages, inhibiting antibody-dependent cell-mediated cytotoxicity, or ADCC, which is going to be another mechanism for killing tumors. So despite these challenges, some clinical treatments today still use antibodies for cancer therapy, but they often cannot be aimed specifically at tumor-specific antigens because of the risk of these blocking effects. Then you can also use the antibody to perform different types of experiments.

So you can use the antibodies for immunohistochemistry so that you can use them for different types of experiments. So immunohistochemistry is a laboratory technique that uses antibodies to detect and visualize specific antigens. In the thin slices of the tissue, helping the researcher and the clinical study where particular molecules are located within their biological context. So you can actually use the antigen-antibody interactions, or you can use the enzyme-coupled antibodies for these particular applications. Then you can use the fluorescently conjugated antibody so that, you know, the antibody is, you know, whatever the signal is, it's actually going to give you a glow, right? You might have seen the immunolocalized antigen on the thin slices using immunohistochemistry.

Or you can use the direct and indirect detection approaches. You can use the secondary antibodies, and so on, right? And then in human chemistry, the antibodies serve as a precise targeting tool to detect the antigen in the tissue sections. When linked to the

enzyme or dye, they allow the visualization of the exact location of these molecules under the microscope, providing insight into the tissue structure, disease status, and protein localizations. Then you can also use the antibodies for targeting the antigens, right? So you can use the monoclonal antibodies, which are powerful in cancer therapy because they specifically recognize and bind to the specific antigen on the surface of the cancer cells. This makes them ideal for targeting the antigen, meaning they deliver the therapeutic agent precisely to the diseased cells while sparing the normal or healthy cells.

There are two main types of molecular antibodies. You can use either the unmodified antibodies or the conjugated antibodies. So in unmodified antibodies, you are just going to simply use the antibodies, and they are actually going to work. So, for example, in B cell lymphoma, the cancerous B cells are expressing a unique antibody on their surface. So scientists can generate a mouse monoclonal antibody that specifically recognizes this. So when injected, these monoclonal antibodies bind only to lymphoma cells and activate the complement system, which leads to cell lysis and leaves the normal cells unharmed.

Similarly, you can use the conjugated antibodies. So in this case, you can actually couple the different types of drug molecules, or you can use that for, you know, killing the cancer cells. So to enhance the killing power, monoclonal antibodies can be attached to toxins, radioactive isotopes, or chemotherapy drugs. And this approach turns the antibody into a guided missile. So it carries the toxic agent directly to the cancer cells. One of the examples is a radioisotope-conjugated monoclonal antibody, like ibuprofen, that delivers radiation directly to the CD20-positive B cells in non-Hodgkin lymphoma.

Similarly, you can use immunotoxins where a toxin is linked to an antibody that kills tumor cells without damaging normal cells. And this targeted delivery is crucial because it minimizes the collateral damage to the healthy tissue and focuses the therapeutic attack on the tumor, improving the treatment efficacy and reducing the side effects. Then you can also use the antibody as an enzyme. So you can actually use the antibody for, you know, behaving like an enzyme.

So the antibody with an enzyme-like activity is referred to as the enzyme. Enzymes are a specialized type of protein that act as catalysts. They don't just bind their targets but catalyze the chemical reaction by stabilizing the donation states just as the natural enzymes do. So, normally, monoclonal antibodies are used for their specific binding abilities, and they can precisely recognize and attach to the target molecules. But researchers realize that this precision binding can be harnessed for more than just recognition. So scientists can design antibodies that bind specifically to the transition state of a chemical reaction.

And by doing this, the antibody lowers the binding energy barrier and speeds up the reaction just like the enzyme does. So, these enzymes, like antibodies, are called ebzines, and they are a novel tool because they combine the specificity of antibodies with the catalytic activity of enzymes. So this is all we have discussed so far about the antibodies. So we have discussed what the structure of the antibody is. We have discussed the diversity of the antibody and how the diversity of the antibody is produced by clonal proliferation and selection by the antigen molecules. And then how these antibodies can be produced in an animal system, right? So we have shown you a demo video where the students are actually explaining how you can produce antibodies in the rabbit.

Subsequent to that, we also discussed the antibody and antigen interactions and how the antibody interaction can be exploited to design different types of immunoassays. So we have discussed the precipitation reactions. We discussed the equipilation reactions. And then we also discussed the enzyme-linked immunosorbent assays or ELISA assays. So within the ELISA, we discuss all the variants, right? So we have discussed the direct ELISA, indirect ELISA, sandwich ELISA, and the competitive ELISA. While we were discussing the sandwich ELISA, we also took you to my lab where the students showed you how you can do the sandwich ELISA to measure the cytokines from the, you know, the culture supernatant.

And then subsequent to that, we have also discussed the different applications of the antibodies. So, with this, I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss some more aspects of immunology. Thank you.