Immunology
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Lecture No -26
Tools and Techniques

Welcome to today's lecture. Today actually what we are going to study is different tools and techniques tools and techniques used in study immunology or the different techniques which is also known as immuno techniques that we will discuss today. This lecture actually we thought to give today before 2 main reasons 1 is the tools and techniques that we are going to discuss that will help to understand many experiment that we are going to discuss in future classes.

So you can understand better if you know what are the techniques? If I tell that just the name immuno localization you may not understand what is that mean or what is the outcome of that. So that is why we decided to discuss few techniques to understand the immune system better number 1 and number 2. I am sure by now so many days just you are listening t-cell b-cell MHC you on MHC 2, t-cell receptor b-cell receptor macrophage, dendritic cells.

So you kind of monotonous lecture one after another that continuously you are discussing definitely where that is our main goal but before that if we understand few techniques or how this antigen antibody we determine. If you remember like the primary responses negative response we showed you a graph. So at the very beginning there was no antibody and then it is increasing so how this graph is made because somebody need to measure the amount of antibody how much antibody is there in the serum or if you remember the table where we saw that what is the amount of different isotopes of antibody in serum like how much milligram is there so how we get that information.

So to understand that; so we need to know a few techniques, so, today what we are going to do we are going to discuss some tools and techniques that we normally use to study immunology. And one more important thing is that the techniques that we are going to discuss in next 4, 5 lectures different techniques. This is not restricted to immune system only because immuno

techniques are very popular I mean you believe me that very, very popular to study any almost most of the branch of biology or life science like microbiology, molecular biology, proteins, cell biology these are used everywhere.

Because using these techniques we can understand many things we that you learn with time and this is one important thing that these techniques are used almost every branches of life science and second is these techniques most of the time. Most of the time is very, very cheap you do not need to have instrument or equipments or costly equipments you do not need much expertise but definitely you have to do the experiment by yourself to know the tidbits.

We are not going to go detail about the technique mostly what we are going to do is try to understand the principle of the techniques or tools and what information we can get and how it looks like whether it is a number or it is a photograph or it is something else. So these we are going to discuss. So the first definition wise immuno techniques are you can say any techniques which use antibody for one of the reagents then it is we call immuno techniques.

So any techniques we use antibody as one of the major reagents or most important reagents we call it immuno techniques. So let us start. So today's like I mean lecture 26 it is a tool and techniques. So first what we are going to do is we are going to discuss the immunity techniques and 1 of the major reagents of these techniques should be antibody you know that antibody.

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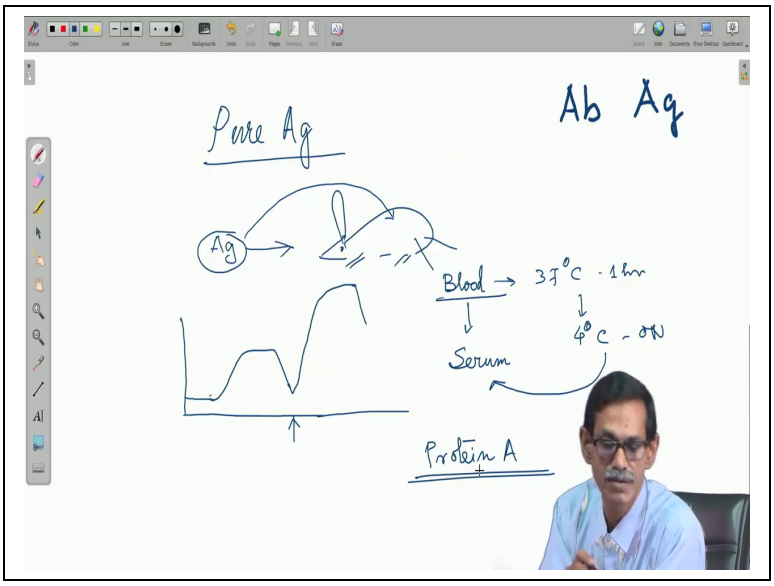

We write like Ab in sort and antigen we write Ag in salt so antibody and antigen we are going to use Ab and Ag very frequently. So now the first thing if you would like to find antibody what you need? You need antigen you need antigen and if you are studying any particular antigen specifically. First thing what we should have in our hand is pure antigen, we should have pure antigen. And most of the time what is this pure antigen?

Pure antigen is that means you have to have a pure protein in your hand. So pure protein means you have to purify the protein that source of protein may be natural source like from say if you want to purify protein from cell you can extract that total protein from cell and purify your protein of interest. I am not going to go all these details I will get the protein because it is not of class you will need to discuss just or you can pure express the protein in recombinant way and then purify your recombinant protein of your protein of interest.

Like you prune the gene express the protein, you can express the protein in bacteria you can express the protein in yeast, the baculovirus system, animal cell, plant cell whatever the original source of the protein whether it is a recombinant or native source you have to purify the protein. Protein purification there are several techniques you can for affinity purification and you can ion exchange chromatography and then you can purify by immuno precipitation that will come and discuss what is this ummino precipitation is?

So then your protein is your hand and you have to raise the antibody. So normally what we do today is the antibody because one of the reagents of ummino techniques is antigen only. If you are looking for antibody or measure antibody, detect antibody then you need pure antigen in your hand that is your bait. So antibody will bind and you can measure that the antibody part or if you would like to characterize the antigen or purify the antigen or do some localization of the antigen so then what you have to do you need antibody in your hand.

So in it is general, so if you need to characterize or know about the antigen you need antibody. If you want to know anything about the antibody then you need antigen. So antigen you have to have by purification and expression by in recombinant system. There are several methods so I am NOT going. Assume that I have pure antigen in my hand. So if I have pure antigen in my hand what I have to do to raise antibody?

Normal practices we inject that antigen into animal. So you can use a mouse you can use rabbit you can use goat, monkey, horse depending on how much antigen you antibody you want. If your requirement is very little you can use small animal. If your requirement is very high you can use big animal and if you use big animal there is 1 more factor is there if you use big animal then you need more amount of pure antigen.

So antigen purification or pure protein in your hand pure means pure to homogeneity that means no other protein contaminants should be there because if you inject 2 protein into animal antibody will be raised against 2 proteins. But if I would like to work with 1 protein that should be very, very pure that should be in our hands. So pure protein means pure to homogeneity nothing else is there. So if you want to use big animal you have to have good amount of pure protein.

And that is not always easy because having pure protein and good or large amount is one of the key factors to raise antibody. So in laboratory purpose or to do is most of the time if it is not a commercial requirement in laboratory purpose we do not need that much. So mostly we use rabbit, rabbit is fine, we use mouse. So you have pure antigen in your hand? So pure antigen you have to inject into animal.

So you have to inject into animal and so that injection what we will do that injection that antigen will you have to administered into animal different way. Many of you I am not going to discuss about detail of it why and when but there are many ways you can administer you can do intramuscular, intrapreneurs, intraperitoneal, subcutaneous, oral. So normally we do intramuscular in laboratory practice for raising antibody.

And that will discuss little more detail about this in vaccine production or during that what is vaccine how you can produce or how you can therapy. So if you inject the antigen and what will happen you remember this I am sure that you remember this graph. So initially few days you would not see anything then it will increase then grow come down then what we will do is today's more and good quality antibody we will inject again antibody in the same animal twice.

Say maybe after three weeks of first injection we will do the first booster and what will happen you know that it will reach maximum good amount good quality and IgG type. So these we will continue. So if you need war again you inject again because it is already primed. At this stage so when these first booster those; what we do is we isolate or take some blood. We isolate the blood and from that blood we purify the serum we purify serum.

How you get the serum you know difference between serum and plasma I am sure you know. So plasma is I am repeating again plasma is if you isolate the blood just sent refused immediately in presence of same anticoagulant what will happen all the blood cells like WBC, RBC all this will precipitate and then you will find a liquid part or fluid part at the supernatant. So that supernatant still contains the clotting factors.

So what will happen if you keep that fluid it will clot? So this is plasma, but if I isolate the blood and after isolation of the blood if you allow them to stay say normally what we do is we keep it at 37 degree centigrade say for 1 hour after that what will happen most of the blood will clot. And even up after clotting some fluid you still there that is actually serum. So after 37 we keep it 4 degree overnight 4 degree centigrade overnight and then next day we just collect the very centrifuge the cells and slowly without disturbing much.

Because if you disturb it then RBC lies and it will a nice red color you can avoid that by handling gently. So that 4 degree then you will centrifuge and collect the fluid that is serum. So what happened all the clotting factor you know that that will be separated by that. So these serum actually contain these serum contains the antibody. And this antibody we can purify and this antibody we can use directly the serum can be used for different techniques or in some cases where we need very good quality antibody or some techniques need pure antibody.

So because serum contains one serum any serum you take from the bovine, rabbit, human you know the major constituents of the serum is what? Major conception of the serum is the albumin, serum albumin. So it if there is too much serum albumin that may mask the antibody molecule. So some many times what we do we purify the antibody from that serum. So I am not going detail here at this moment but we can purify the antibody just by affinity chromatography.

Those who are interested go and read that affinity purification of antibody using protein A. Protein A affinity purification is normally used to purify IgG. So that way what you can do from the serum only IgG you can separate. So now after this and your thing is done, so it will take time because you need if your protein then inject you have to wait 2 to 3 months and then after that you isolate the draw the blood.

Then you isolate the serum and you test because we are not going to kill the animal ok animal will still survive we are not killing or sacrificing the animal to get all the blood. So keeping the animal life or alive we isolate some or draw some amount of blood from there we are select serum. And that serum will contain antibody, antibody against what antibody against of that pure antigen that you injected protein of interest that proved antibody you can purify using protein A.

So now this is your reagent to study. Now this is your reagent to study so what we will do with this.

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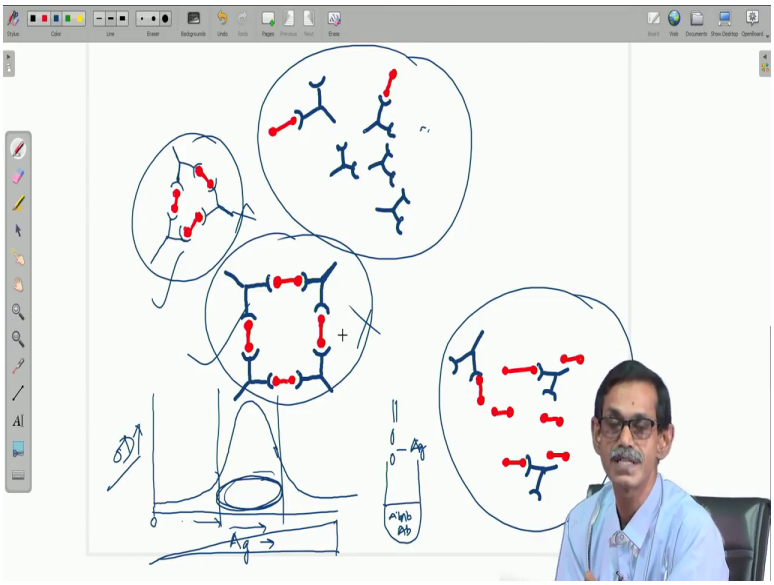

So now if I say that this antigen you purified is not good so that is not going to work better, so how do you check? What is this antibody is good or bad or is at all antibodies produced or not because many times what happened your antigen may not be good and antigen may not be good means it may not have any b-cell epitopes. If there is no b-cell epitopes, no antibody if there is no t-cell epitopes no antibody, so normally it is very rare cases that there is no b-cell epitope or no t-cell epitopes of the protein.

Generally rule of thumb is if the protein molecular weight is more than 5 kilo Dalton normally it is act as immuno reactant. You remember hope I hope that you remember what is antigen and immunogen I am repeating again. Antigen is the molecule to which antibody can bind. It can be anything antibody can bind his antigen. But if antigen you inject into animal if it can elicit immune response that is called immunity.

That means any antigen can elicit immune response is antigen sorry it is immunogenic that means what? All immunogenic are antigen but some antigen may not be winner yet that means antibody can mind but it cannot elicit immune response. So in that kind of thing non immunogenic antigen if you inject into anymore then it will not raise any antibody but you can check that normally it is not that common.

So normally most of the proteins are immunogenic so they are good enough to raise the antibody in animal. But still you have to check how than anybody is. So what we do is the one of the very simple test is immuno diffusion very simple test is immuno diffusion. What we do there? In immunodeficient there are single immunodeficient or double immunodeficient 2 things is possible. So for that what we will explain is for immuno precipitation sorry immuno diffusion that means what is happening in this here in immuno diffusion.

I also this picture I will just listen to me first. So I will explain this whatever you are seeing I will explain this why this is here. So in immuno diffusion what we see is if antigen and antibody binds are react they will precipitate and we can see a very nice precipitation line or precipitate in line. So if you see any precipitation that means antibody is there. So what is happening you can write it also agglutination or blood group test it is just the precipitation or agglutination kind of thing.

So what we can do is suppose this is I am seeing a graph assuming that we can see the precipitations we can see. Now in a tube in a tube you take a solution where lots of antibody is there this is antibody solution. So huge amount of antibody is there then your drop wise your drop wise you are adding antigen. So initially what happened initially your concentration of antigen if this is the antigen concentration you are adding so amount of antibody is fixed your drop wise adding antigen.

So initially antigen concentration at the very beginning it is zero and then gradually it is increasing. So if I draw this way it is the amount is increasing in I mean the antigen concentration is increasing. So what we will see is initially there is no precipitation at the middle there will be precipitation so this is the amount of precipitation I am trying. There you will see that you will see the precipitation.

Again it will not, so what you will see if you keep on adding the solution is clear, clear, clear and suddenly you see some cloudy precipitation is happening. And then cloudiness is increasing then if you keep on adding then again that cloudiness will be cleared. And this region that where you

are going to see the cloudiness. How you can measure that cloudiness you can measure the cloudiness by simple spectrophotometer.

So if I measured by OD, so the turbidity will increase and if the turbidity increase you can see that OD is increasing. So what do you will increase then again going down back to the minimum one. This region where do you see the precipitation or you see the cloudiness is called zone of equivalence. Why initially there was no precipitation initial what was the condition? You know that I already told that antigen-antibody interaction is not a covalent interaction, it is a non covalent interaction.

So there is depending on the affinity they bind and open bind and open it is a continuous process. If the high affinity the after binding it will remain for a long time if it is low affinity it will bind after sometime it will open and it will find another molecule of antigen it will bind there open another bind there. So it is they will continuously exchange and there will be equilibrium. So at the early stage when there are a huge amounts of antibody.

So antibody amount is very high what will happen in when the antibody amount is very high condition is assumed like this. That means too many antibody molecule only few this red is antigen and blue is antibody. So, too many antibodies but very few antigen molecules are there. So they will make like this. So individually they will find so there will be no because to precipitate something it should be a little bit heavier or make a bigger complex so that it pulled out.

So as long as this is there you will not see any I mean as long as this is there, so at that time you would not see any precipitation but if you keep on adding there we time where they will find this or this kind of structure. So it is just the ratio of antigen and antibody is such so they will make a nice kind of structure or bigger structure. So for antibody for antigen 3 antibody antigen they will make bigger structure you have you might have seen much better picture in when I was showing you the slide same slide.

I mean similar structure. So when they make this big structure then they cannot stay in the solution anymore they will precipitate, clear. Now if this region or this concentration or this ratio will reach at certain point say maybe this is the region where this kind of structure this kind of structure are formed again if keep on continuing the antigen. So you are you saw the antibody and keep on using and I will keep on adding the antigen what will happen?

In that case this is the scenario where antigen number is much more than your antibody. Again what will happen antibody will be diluted and you would not have this kind of structure. If there is not this kind of structure or this kind of structure will break and that will be diluted. So you will have this kind of distribution you again you lose the precipitation. So at a particular dilution or particular ratio of antigen and antibody will form this kind of structure only then it will precipitate.

This is whatever I am talking this is whatever I am discussing right now it is I am talking about that antigen-antibody reaction in solution. But here you cannot see the precipitation line so that is why the technique immuno precipitation that we are using.

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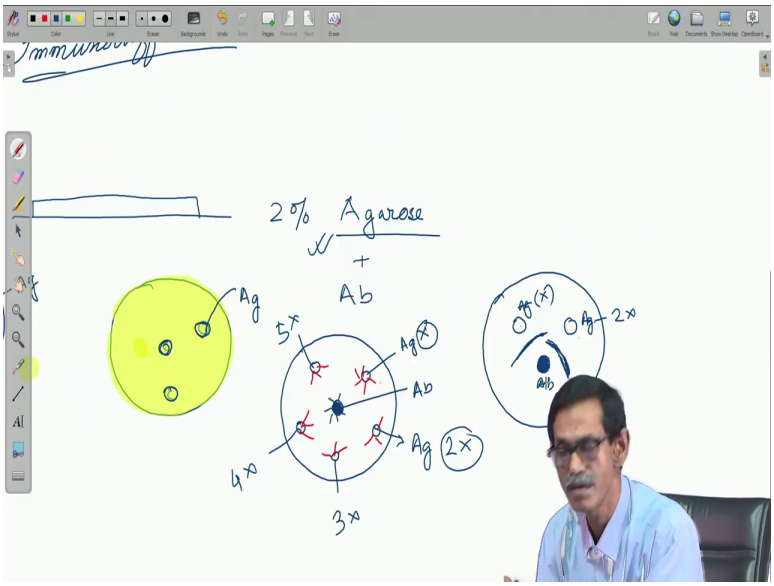

So in immuno diffusion what is happening there are single or radial immunity diffusion and another is double immuno diffusion single immuno diffusion what we do is you know if we know the other plate, so you make a 2% augur solution and may put on a slide or in a Petri plate.

So you have you have a sorry you have a glass plate so I will assume and then you put some 2% or 1.8% Agar normally we are using Agur not other that we are going using in bacteria not 2% Agur solution we had so it is the in one slide.

So it is not one dimensional or what we can do is we can use the Petri plate I am sure all of you know what is Patri plate is where you grow the bacteria and that Petri plate you pour the other how thick may be say 3 to 4 millimeter thickness and then you make hole different holes just punch a glass pipe or Pasteur pipette kind of thing and make a hole or well kind of thing. So while you are pouring this agarose what you can do is you can mix your antibody.

So that means what happens your antibodies everywhere your antibody your antibodies everywhere. So if this is a same plate suppose this is the antibodies everywhere this is your antibody it is everywhere you cannot see that definitely you cannot see that because it is not visible. But in this hole in this hole or well now you add antigen, antigen solution that pure antigen solids parentage any you have you pour antigen so what will happen?

If there is now I am drawing this again like this and there is a hole here antibody is everywhere equal concentration and you are putting antigen here and this antigen what will happen with time it will diffuse and it will diffuse sidewise I mean according to radiation it will diffuse. So while it will diffuse in different direction antigen has a particular concentration well it will diffuse.

So at the very close to the well suppose this is a world full of antigen very close to the weld and is no concentration is very high and more it go it will be diluted. So at a particular concentration while it is diluting at a particular concentration what will happen it will reach that zone of equivalence zone of equivalence that means the ratio will be perfect to make precipitate and we perfect to make precipitate.

Then what we will see we will see I mean this is the thing so what we will see this is a nice circular Petri plate this is a nice circular pretty plate let me take this little this side this is a nice circular Petri plate and you have a well which you would not see anything because all neither antigen or antibody is colored. So what we will see and the next day if you keep this plate or

overnight at 15 degree centigrade while you keep in 50 degree centigrade diffusion rate is high in a high temperature we keep it 15 degree centigrade because less chance of contamination because bacteria may grow in the Agur.

So that we would like to avoid that and diffusion will be a little slower. So chance of formation of precipitate is more. So what will happen you will see a very I am drawing the yellow it is actually it is very faint milky white so you will see very nice ring. You will see very nice ring so next morning you come you see against the light you take the light against the light you say very nice faint milky white ring. If ring is there if ring is there that means antibody is there because what you did you to isolate the serum mix with the agarose and plate it.

And you know antigen is there because you have purified antigen you use it before but antibody is there or not you did not know. If you see the precipitation line then on a very nice circle that means antibody is there. So if this is the case what is the advantage you do not need any instrument except 1 Petri plate a little bit agarose you can tell whether antibody is there or not. So and these thing you cannot I mean well you can tell that how, what is the amount of antibodies good or bad.

But normally we do not do use this we use double diffusion. What is there in the double diffusion we in double diffusion same Petri plate that means this. So in the same Petri plate what we do is we make multiple hole multiple hole means there is 1 hole in the center then radially we will put dip make different hole in better his uniform distance is better if you can punch it. In the center in the center hole we add antibody and in different hole you can do either way.

In the center suppose in the center you add antigens either way is possible. So in center you add antibody and in different hole you use antigen of different concentration. Suppose this is X this is 2X amount then 3X amount and then 4X amount and then 5X amount this amount X amount 2X amount whatever may be 2 micro gram 1 microgram that you can measure. So and you do the same thing what will happen antibody will diffuse all direction equally.

Same way if the antigen color if I make red that will also diffuse in all direction in all from all well. So what will happen for each case there will be a precipitation line. So if you have this amount of antigen it will dilute it very quickly. If you have more amount of antigen it will be diluted I mean in comparison to X, 5X what will happen the dilution will be much less in comparison to X. So if I now draw a result if I now make 1 well here where we put antibody here and 1 well here antigen another level antigen same amount if I give.

What will see at a particular distance from this well we will see a precipitation line. Same distance I will see a precipitation line so next morning I will see a nice faint milky white or good milky white precipitation line here. So if the amount of antigen if this is antigen and here is antibody are same. So say suppose 2 microgram 2 microgram serum I do not know and if I give double amount of antigen here.

What will happen; the whole amount of antigen means double amount of antigen means this will be if this is say 2X amount this is X amount where it will form? Because if this is X if this is the proper dilution that dilution will reach little far because it will dilute more. So instead of like we will get this will be closer to this antibody one.

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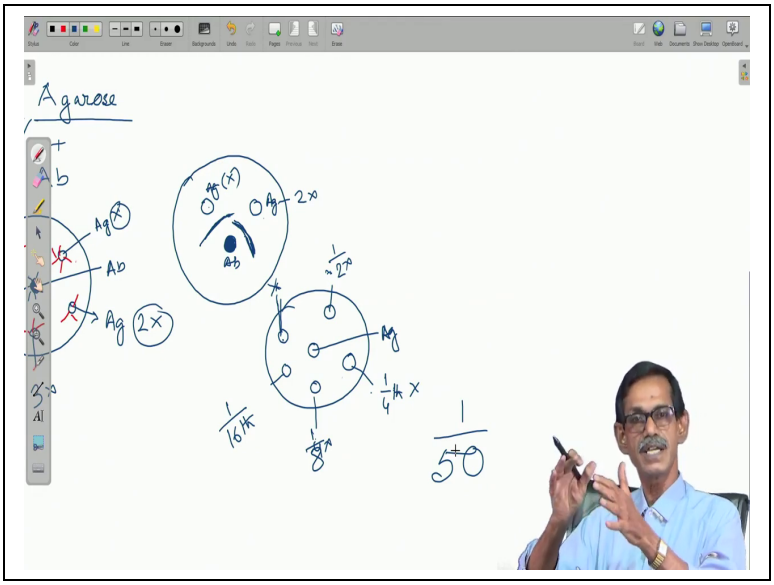

Same way we can just do in center we can keep antigen and the serum we isolate we get say X amount we give to X amount then 3X amount then 4X amount or we cannot increase what we

will do this is undiluted. Then you dilute it 0.2 times then 0.3, 0.4 you dilute so one is directly the serum then you direct half, so let me put this way half then 1/4X then 1/8X what will happen more you diluting than antibody the amount of antibody is less ok.

And here suppose you give 1 by 16th dilution of serum. So X will give you a better precipitation line and gradually the antibody is diluted you will see the line are getting faint and faint and 1/16 you may not get anything. So what this will tell? This will tell suppose you get a very faint precipitation line even in 150th 1 by 50 dilution is also giving you precipitation line that means what? That means your antibody amount in the serum is very good because even you dilute at 50 times still you are getting precipitation.

So this way a double diffusion can give you an idea how good your antibody amount and definitely whether antibody is produced or not you can figure it out. This is called ouchterlony double diffusion method. So you just type in the net immuno diffusion and search for picture you will get variety of picture variety of this kind of drawing much better because this is drawn on scale and compass and using and there are some real-life picture and you have an idea what is immuno diffusion is.

So thank you for today or this lecture in this lecture we are going to discuss another technique till then bye.