Experimental Biotechnology

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Lecture – 27
Antibody Generation

Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering, IIT, Guwahati. And today we are going to start the new topic and that topic is the immunology. So, immunology is the field of science, which basically studies the immune system. So, before getting into the details of the immunological tools, what is available with us and how to exploit them?

We have to understand how the immune system works and so, that you will be able to understand in which context we are going to be able to utilize these tools and how to exploit them for understanding the different types of biological problems.

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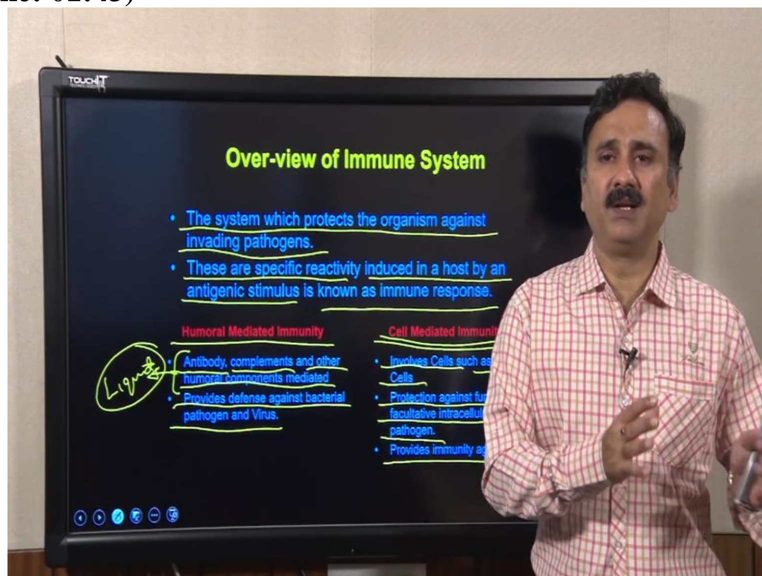

So, immune system is the system is which protects the organism against the invading pathogens and these are a specific reactions, which induced in a host by an antigenic stimulus is known as the immune response. So, once a foreign antigen such as the bacteria, virus or fungus get entered into the host, it actually recognizes this particular organism as the non self and in response to the non self, the immune system is that it is not exerting the reactions, which actually are going to destroy this foreign organisms.

So, collectively all the responses what are being developed against a foreign antigen is known as the immune response and these immune responses could be of 2 different types, it could be a humoral mediated immunity or the cell mediated immunity. In the humoral immunity a mediated immunity mainly the antibody complement and other humoral components are mediating this particular type of immunity which means, all humoral response means, all response what is being mediated by the liquid component.

For example, if the cell is secreting some cytolytic enzymes cell are secreting the antibodies cell are secreting the compliments and all these are actually circulating within the blood and they are actually continuously monitoring the foreign organisms. So, as soon as the foreign organism enter into the body, this humoral response are actually recognizing these cells and then either they are killing these cells or they are actually taking the help from the cell mediated immune response.

That is why the humoral response provides the difference against the bacterial pathogen as well as the viral pathogen. So, the humoral response is the ugly response, which is being developed against the very deadly pathogens such as the bacterial response or the viral response, for example, when we get the cold or influenza infections, we the first response, which comes is the easy humoral response that is, the virus is been recognized by the antibodies.

As well as the other components of the humoral response and they try to elicit the immune response. Similarly, if there will be a bacterial infections, the complement as well as the related system is actually been activated, and that is how they destroy the bacterial cells humoral response always take the help from the cell mediated response to create more robust and long lasting response.

So, the other response is the cell mediated immunity, where you involve the cells such as the T and B cells, it protects the organism against the fungi virus and facultative intracellular bacterial pathogens, and it provides it also provides the immunity against the cancers. So, the cell mediated immune response is more robust, it long lasting and it actually creates a memory within the organisms.

So, whenever there will be a second round of entry of the similar pathogens, it actually instantly recognizes this pathogen and creates the robust immune response and the cell mediated immune response is against the fungi, viruses and especially the intracellular pathogens. For example, the mycobacterium tuberculosis and in addition to that, when the cell is getting irregular or cell is actually getting transformed into the cancer cells, the cell mediated immune response is also acting against the cancer cells to eliminate them from the body.

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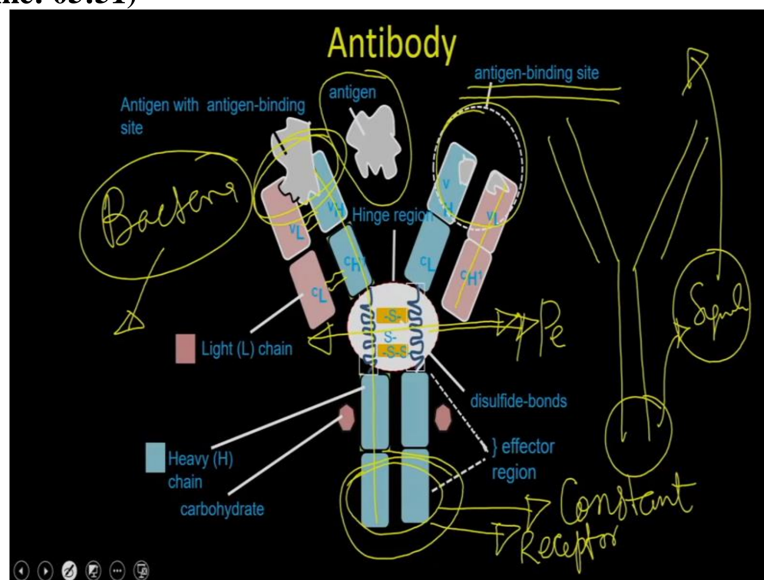

So, the within the humoral response, the antibody is the central molecule, which is taking place or which is actually governing the different types of immune responses involving the different types of cells. And that is why before getting into the detail of the further immune responses, it is important to understand the structure of immune antibody, so that you will be able to understand the interaction of the antibody with the antigen as well as the other applications of the antibody. So, antibody is a Y shaped structure or Y shaped molecule.

Which actually contains the 2 chains one is the light, where money is the heavy chain and the other one is called as the light chain this, so, you have the 2 heavy chain and the 2 light chains, which are being bound to each other by the sulphide bridges between the heavy as well as the light chain and apart from that, so, this Y shaped molecule has 2 region. One is the heavy chain region which is called or the C terminal region which is called as the constant region.

And the top region which is of formed by the light chain as well as the heavy chain is called as the antigen binding site. So, you can imagine that whenever there will be an antigen, which is coming into the vicinity of the antibodies, it is actually interacting with this particular portion and this particular portion is recognizing this antigen and if the interaction is stable enough, it actually goes and binds de-antigen binding site.

Apart from that this antibody is very, very susceptible for some of the proteases. So, if you, for example, if you treat this particular, the antibody with the protease, the most some of the proteases are cleaving this antibody into 2 part, but they are keeping the constant region away from the bearable region or the antigen binding region. So, that is how you can be able to fine tune the production of the antigen antibody in such a way that it is actually going to activate the cellular machinery but be it will not bind to the antigen.

So, because this region is a constant region it actually has a receptor for onto the cell surface. So what happened is when the antigen binds to this particular antibody, this constant region is binding to a receptor? And that is how it actually is governing our downstream signalling into the cell. And that is how it actually is eliciting the immune responses from those particular cells.

And that is how the antibodies which are actually circulating into the blood is binding to the antigen and that is how it is actually bringing the antigen bound antibodies to the receptor, and that is how it is actually activating those cells for causing the more robust immune response. Apart from that, because the antigen is binding to this particular antigen binding site, it is not alloying the antigen to move around.

For example, if you have bacteria, which is actually could be dangerous for the host, bacteria cannot replicate until it is freely moving. So, what happened is the bacteria is going to bind to this particular region of the molecule and as a result the bacteria is going to be sequestered into a very small area. So, the antibodies are mainly doing 2 main function one is actually sequestering the antigen to a very, very localized area.

So, that the other cells such as the macrophages and dendritic cells, and all other kinds of T cells and B-cells actually come to that particular vicinity and could be able to destroy this particular foreign antigen, apart from that this antigen bound antibody is actually going to

bind to the receptors present on the immune cells. And that is how it is actually going to activate the downstream signalling and that downstream signalling.

Could actually be able to activate the more robust immune response from those cells, either it would be in the form of production of more amount of antibodies or it could be indeed, in the form of production of the cytolytic enzymes, free radicals all other kinds of immune responses, so, that the this particular foreign antigen or foreign pathogenic organisms could be destroyed by this. So, this is the main function of the antibodies in the, which is participating in the immune response, let us see how the antibodies are being produced in a host such as the humans.

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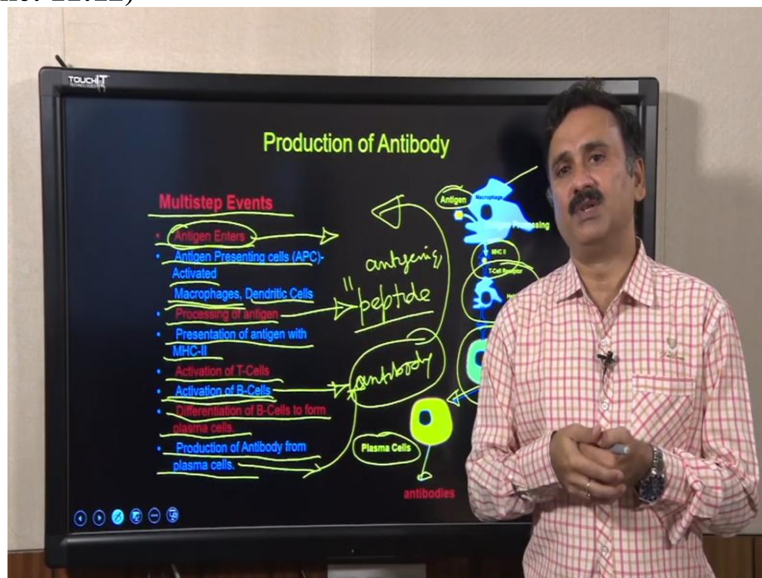

So, antibody generation is a 4 step process in an organism under the natural conditions, it has a lag phase. So, in the lag phase, what is happening is that, it is actually the in this particular phase there will be an entry of pathogen and then this pathogen is going to be contacted by the immune cells. So, this is actually a lag phase which is also could be called as the preparatory phase.

Where the foreign antigen will enter into the host body and then this foreign antigen is going to be recognized by the immune cells and then they are actually going to start the preparation for how to tackle the foreign antigen in the second phase, you have the log phase. So, then the log phase you are actually going to see, there will be an increase in the antibody production. Which means, the anti the immune cells have now acquired or understand the strategies how to tackle this particular foreign antigen.

And then they have gone through multiple processes and in which processes and after the end of these processes, they have started producing the antibodies and they as I said in the previous slide itself, that these antibodies will now start sequestering this antigen. So, that it should not be able to spread throughout the body. Then in the third is the plateau phase the plateau will be the equilibrium between the antibody synthesis as well as the catabolism.

So, at this stage actually, the foreign antigen is about to getting de eliminated from the body or because at this stage the antibody production as well as the antibody which is been bound to the antigen or which has been already been consumed is going to be the same. That is why if you see the antibody production is going to be constant throughout this region and then you are going to have the decline phase. So, there will be a decline of antibody titer which means, the antibody production is going to be down.

So, at this stage, the infection is going to be sustained to a particular localized area. But still the active intensity infection is there. So, the antibody production as well as the destruction is going to be remain constant and then you it enters into a decline phase where the antibodies are going where the antigen is going to be eliminated from the body, which means with the organism has remove the infection from the body and that is how there will be no need to produce the antibodies and that is how the antibody production is going to be decreased.

At this stage only the organism is going to start producing the memory cells or it is actually going to train the cell so that it actually going to keep a memory of the this particular foreign antigen. So, whenever this in this happens in the second response or the second time, the time what the organism has taken for the lag phase as well as the log phase to do the preoperative step is going to be shortened.

And as a result, if the same organism comes into the second stage, it is the immune response is going to be the faster and that is what the reason is that when you are vaccinating the children is or even the adults, you are actually doing nothing, but the reducing the preparative stays so that as soon as the organisms comes, the body will start producing the antibodies and then these antibodies are actually going to start sequestering the antigen to a localized area and then start acting them, so that the immune system is going to be eliminate them.

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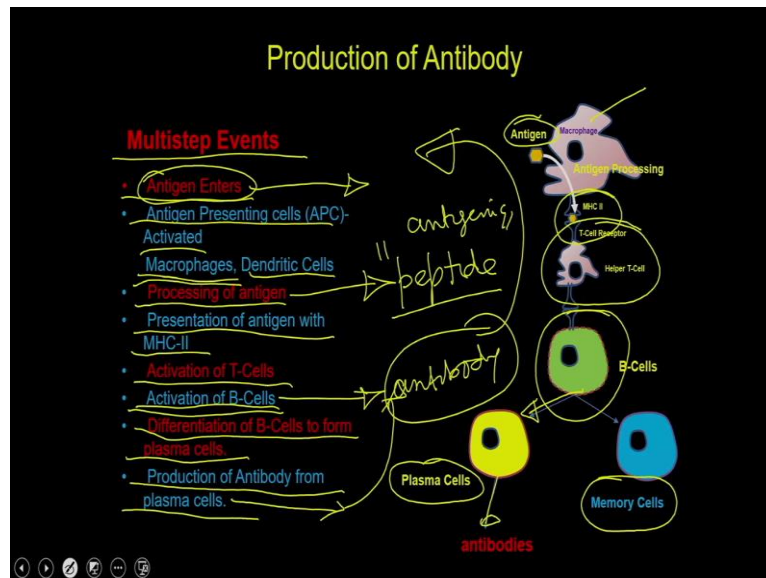

Now, let us see how what are the different events are required for getting the antibodies produced in the organisms. So, the first event is that the antigen is going to enter into the host body and then the as soon as the antigen will enter into the host body, the first cells which are going to be activated or the first cell which are going to encounter these antigens are called as antigen presenting cells, these cells are either the macrophages or the dendritic cells.

So, in every part of the body, where there is a chance that you have the entry of the foreign antigens, you have the macrophages as well as the dendritic cell for example, you have the kupffer cells, which are present in the liver, you have the alveolar macrophages, which are present in the lungs, and then you have the macrophages in every organ, so that it will be any infection which is going to that particular organ.

The first cell which is going to encounter this particular foreign antigen is these macrophages. So, what is the job of these macrophages is that they will actually going to process the antigen what is mean by process the antigen is that they are actually going to digest this antigen and they are going to generate the antigenic peptides, which means the organism is going to be destroyed and then you are actually selecting the antigens.

You are selecting the peptides which are antigenic and then these antigen presenting cells will present these antigens or the antigenic peptides with the help of the MHC class 2, once they present these antigenic peptide with the MHC class 2, these are going to activate the T-helper cells and the T-helper cells in response to the antigen presenting on to the MHC class 2 is actually going to activate the downstream B-cells and once the B-cells are activated.

They are start going to produce the antibody which actually going to again further stimulate these actions. So for the antigen is entering into the body and antigen presenting cells are actually recognizing the antigens without the help of any tool. For example, there will be a bacteria, the antigen presenting cells are recognizing the bacteria only by the proteins which are present onto the bacterial cell wall.

But, as soon as there will be activation of B-cells, the antibodies are going to be produced and then they will actually going to coat the bacteria with this particular antibody. And that actually will going to allow the antigen presenting cells to recognize these bacteria more efficiently with the help of the cell surface receptor, because now they are not dependent on the antigen which is being expressed on the bacteria.

Because the amount of antigen which is being expressed on the bacteria is very small or very little compared to the antibodies, which are going to be a bind to this particular bacteria and the receptor for that particular receptor for the antibody is going to be with going to be more efficiently bind the antigen presenting cell and that is how the whole this cascade is going to be amplified.

In the meantime, the B-cells are going to be differentiate into the plasma cells, and then the plasma cells are going to be starts producing the antibodies, these antibodies are against going to participate into the immune responses. So, this is what is going to be shown into the figure where the antigen is going to be processed by the antigen presenting cells such as macrophages, and then it is going to be expressed along with the MHC class 2 and then that actually is going to activate the T-helper cells.

And T-helper cells again going to activate the B-cells and that stage the B-cell is going to be differentiate into 2 cells, one is called as the plasma cells The other one is called as the memory cells and then the plasma cells will start producing the antibodies and these antibodies are actually going to contribute further into the more robust immune response because these antibodies are soluble in nature.

So, they will actually going to activate the more cells and more robust immune response because the purpose of the antibody is to sequester the antigen as well as to amplify the initial signal so, that you are actually going to activate the complete immune responses.

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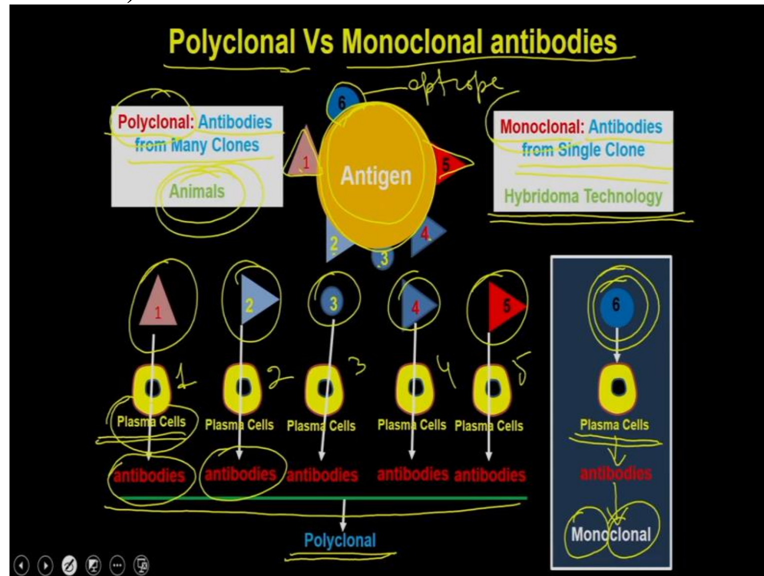

Now, let us understand what is meant by the polyclonal as well as the monoclonal antibody. So, antibodies what is being produced by an organism could be into 2 categories, one is called polyclonal the other one is called as the monoclonal antibodies. So, now, what you can imagine, what you can see is that you have an antigen, you can imagine that this is a very big protein, which actually contains the multiple types of antigenic sites.

And all these antigenic sites are called as the epitopes which means our antigen could have the multiple epitopes like 1, 2, 3, 4, 5 and 6 and all these epitopes when they are going to be processed by the antigen presenting cells are going to be expressed along with the MHC class two and subsequently. They are actually going to activate the B-cells and then the B-cells are going to activate the plasma cells.

So, in this process, what will happen is that, they are actually going to activate the multiple plasma cells. So, you are going to have for example, if you are processing the epitope 1 it is actually going to activate one type of plasma cells and that actually is going to start producing the antibodies. Similarly, you have the epitopes 2, 3, 4 and 5 all these epitopes are actually producing the different types of antibodies.

Which is antibodies, which are directed against one the antibody which are directed against 2 and so, on. So, what will happen is that, if you collect all these antibodies, which are coming from the different cells, for example, this is number 1, this is 2, this is 3, this is 4 and this is 5. So, if you are collecting the antibodies, which are coming from the 5 or more different types of cells, like different types of plasma cells, which are actually be the clones.

Then the antibody is called as the polyclonal antibody, which means the poly means, many clonal means, the clones which means, if the antibodies are coming from the multiple clones of the multiple antigens or the antigens, but a multiple epitopes of a single antigen, then the resultant antibody is called as the polyclonal antibody. Whereas, if you see the epitopes number 6, it is actually going to be processed by the single plasma cell and that actually is going to give you the antibodies.

And these antibodies are going to called as a monoclonal antibodies. Mono means single clonal means the clone. So, if a single epitopes is being processed by the single clone and you are collecting the antibodies from a single clone. Then it is called as the monoclonal antibodies which means, the monoclonal, polyclonal antibody means the antibodies from the many clones and polyclonal antibodies are going to be produced inside the animals under the natural conditions.

So, under the natural conditions when a bacteria is going to be processed in an organism, it does not produce the monoclonal antibody, it produces the antibodies against the multiple epitopes of a single antigens. Which means it could be multiple proteins and a combination of multiple proteins or it could be a multiple epitopes or multiple antigenic regions present on the single antigen irrespective of these conditions.

The purpose of producing a polyclonal antibody is that you want to increase the multiple antibodies possible for a single antigen. So, that you will be able to recognize these antigens and you will be able to create our robust immune response compared to that the monoclonal antibody means the antibodies from a single clone. Because, you cannot do that naturally because, you cannot have any way to ask the body to only select a single clone and start producing the antibodies.

It is actually been done by our in vitro technique which is called as the hybridoma technologies. So, these are the 2 ways in which you can be able to produce the antibodies one the polyclonal antibody, where you can directly use the animals or the monoclonal antibodies, where you have to use a hybridoma technology to select the clone and then ask the clone to produce antibodies. So, this is what we are going to discuss in this particular lecture.

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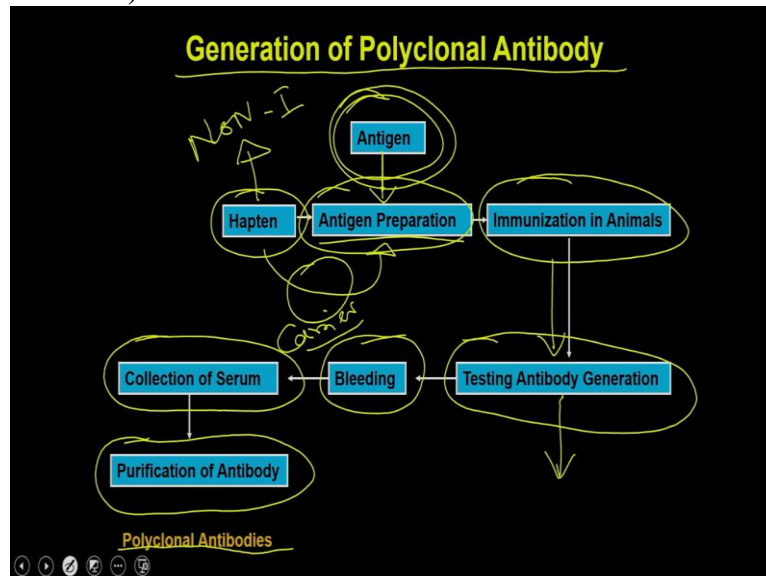

So, generation of a polyclonal antibodies in a generation of a polyclonal antibody, we have discussed when we are discussing about the affinity chromatography. So, we are not going to discuss that in detail, but except that just for your refreshing your memory, what we have discussed is that you can have the 2 different types of antigen either the hapten for hapten is also going to be present or going to be converted into the antigen with the help of carrier protein such as the kale edge or some other carrier protein.

So, that the haptens are going to be converted into the antigen haptens are the antigens, which are non-immunogenic. So, you can have the antigens, which are immunogenic which means, they can be able to create a robust immune response or you can have the antigens, which are non-immunogenic. So, these haptens are the non-immunogenic antigens, so, you have to convert them into a immunogenic antigens, and that you have to do simply by adding a carrier protein.

So, that; their size actually will go up because the haptens are non-immunogenic because they are of small size. So, you have to add tag them to a carrier protein such as the kale edge or

BSA or any other immunogenic carrier protein so, that they will going to cause an immune response then, these are the professional antigens, which are immunogen also, then you have to process them for making a preparation which means the antigen are going to mix with the you have to make the antigen ready for the immunization.

Then you have to immunize the animals by this antigen preparation, then once you immunize the animals, you have to test the animal for generation of the antibodies, once you confirm that the antibodies are being produced, then you can be able to bleed the animals and then ultimately you can collect the serum and from this serum you can be able to do the purification of the antibodies, and that actually is going to give you the polyclonal antibodies, and as I explained in the previous slide itself.

Why it is polyclonal because the antigen is going to be made up of multiple epitopes. And that is how the multiple epitopes are going to be activates the multiple B-cells and ultimately the multiple B-cells are going to be converted into the multiple plasma cells and that is how you are going to get the antibodies of from the multiple clones and that is all it is going to be polyclonal antibodies.

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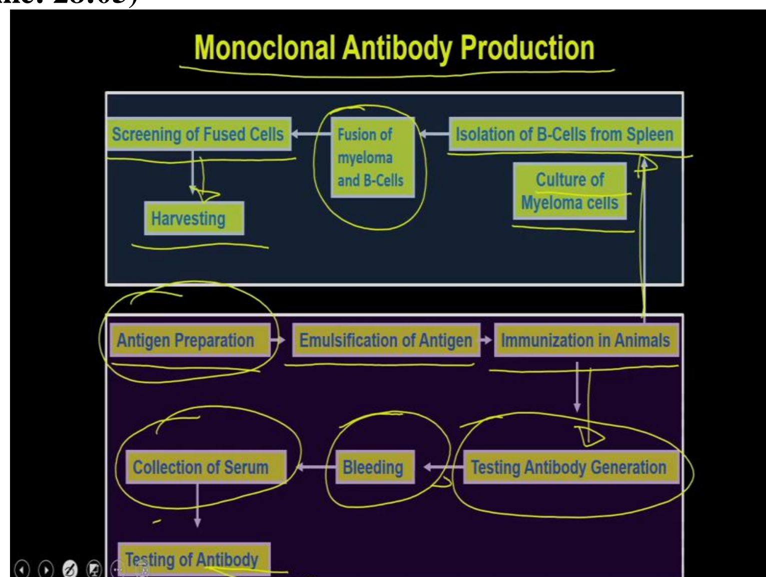

For a monoclonal antibodies, the process is little more complicated because in this you have first you are going to immunize the animals and then you are going to produce the multiple clones and then these multiple clones have to be you know screened and then you have to select the single clone. In a monoclonal antibodies, what you have to do first is take the antigens, then means you have to produce the antigen in large quantities.

Then you have to do a emulsification of this antigen, so, that you will be able to make it ready for immunization, then you have to do the immunization of these animals, then you have to test the antibodies, whether the antibodies are being produced or not, and then you are going to do the bleeding, then you collect the serum and then you test the antibodies. So, once you test that, there are sufficient quantity of antibodies are being present in this particular animal.

Then you take these animal and the prepare the B-cells from the spleen, and then you also culture the myeloma cells, and then you fuse the myeloma and the B-cells to form the hybridomas then you screen the fuse cells and then ultimately you are going to harvest the monoclonal antibodies from the single clone.

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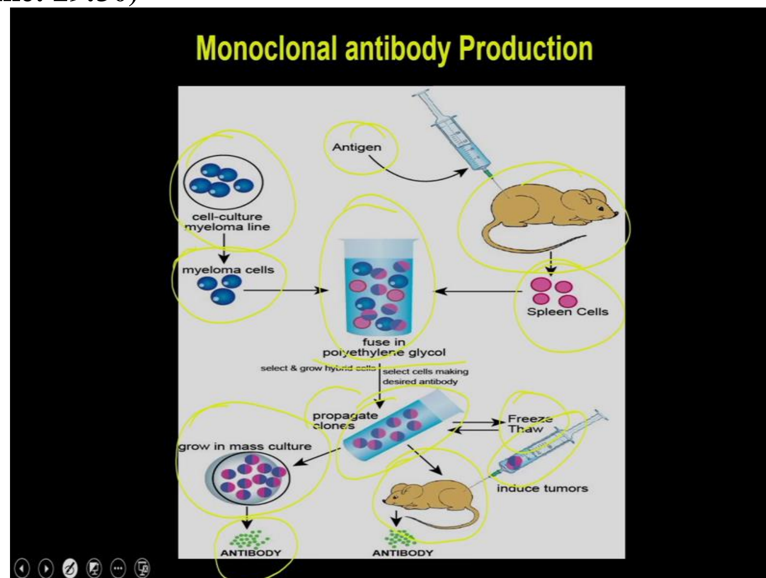

Let us see how to do the these procedures. So, in a typical monoclonal antibody production, as we discussed first you take the antigen, you immunize the animals, you collect these spleens, then, on a side you collect you culture the myeloma cell lines, you take the myeloma cells, mix them together with the fusing reactions that will contain the polyethylene glycol that actually is going to give you the hybridomas.

These hybridomas can be grown in mass culture, so that you can be able to produce the antibodies or if you can freeze these hybridomas So, that whenever is required, you can just take out these hybridoma injected into the animal, so that it will actually going to you know

recover from the from the freezing and then you can be able to produce antibodies. So, these are the multiple steps what you require to challenge the animals.

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Antigen Preparation

- Purification of antigen-The antigen used to immunize be as pure as possible. Use of pure antigen reduces the generation of cross-reactive antibodies. Two different methods to produce the large quantity of antigen for immunization purpose.

(a) Purification under Native conditions

(b) Purification under Denatured conditions

- Preparation of Immunogen- Combine 100µl of antigen (100-150µg) with an equal volume of freund's complete adjuvant to a final volume of 200µl. Mix thoroughly to obtain the emulsion using a syringe or a pipette. The perfect emulsion of the antigen can be tested by dropping a small amount into the beaker containing water. A good emulsion will not spread on water surface.

So, purification of the antigens, the antigen used to immunize to be as pure as possible use of pure antigen reduces the generation of cross reactive antibodies, we have already discussed about how to prepare the antigen you can prepare the antigen under the native condition simply by doing chromatography techniques or you can prepare the antigen under de-natural condition with the help of the electro illusions. Once your antigen is ready, then you have to prepare the immunogen.

So, what you do is you combine the 100 microliters of antigen with equal amount of fluids complete adjuvant to a final volume of 200 microliters then you mix thoroughly to obtain the emulsion using a syringe or a pipette. You have to check the emulsion whether it is a good emulsion or not simply by dropping a small drop of emulsion into water and what is the good thing is a good emulsion will not spread onto the water surface which means the emulsion is going to remain intact when even if you drop it onto the water.

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Immunization

- i. Before immunization, take out 0.1-0.5ml mice blood from the tail vein before the first injection. Incubate the sample at 4ºC at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and store it at -20ºC and labeled as pre-immune serum.
- Take out 5 mice (BALB/c strain) from the cage and sterile them by spraying 70% alcohol. Inject 200ul antigen mixture per mice. During this step either use a helper to hold the mice or use a restrain device to hold the mice. Briefly clean the injection site with 70% ethanol and inject antigen through multiple routes:
- a. intravenous- Antigen mixture can be directly injected into the tail vein.
- b. Intraperitoneal injections- While making i.p. injection avoid injecting the antigen into the stomatch.
- c. Sub-cutaneous and intramuscular injection into the tight muscle.

Then before you immunization you take out the blood and so that it is actually going to tell you whether the what is the titer of the antibodies present in the animal before you did the immunization incubate the sample at 4 degree and then you collect the serum and you can keep this serum and labeled it as a pre-immune serum which means you can collect the serum and store it at minus 20 degree and that is considered going to be a pre-immune serum which is actually going to be a control serum.

So that it will tell you what is the amount of antibodies present in its animal pay for doing the immunizations, then you are going to do the immunizations, you take out the animals or the mice in this case with the help and that a strain what you going to use is the BALB/c strain, you first sterilize them with the help of the 70% alcohol, then you are going to inject the antigen mixture what we have prepared and during this step either use as a helper.

So that we will hold mice or you can use a strain device to hold the mice briefly clean the injection site with the 70% ethanol and inject antigen through multiple routes. So you have a multiple routes through which you can be able to inject the antigens, either you can inject as an intravenous so intention mixture can be directly injected into the tail vein of this mice. So that is actually going to create very small immune response because if you inject directly into the intravenous injections, it is actually going to cause the NFL optic shocks.

First of all, and the second is it may sometime actually clear the infection clear the antigen very fast so that it will not going to create very robust immune response. So, you can only inject antigen in an intravenous mode if you know that the antigen is very, very

immunogenic. Then you can inject in an intraperitoneal injections while making IP injections avoid injecting the antigen into the stomach because you want to do an intraperitoneal injections.

But do not inject it into the stomach because otherwise, it is actually going to create a lot of trouble to the mice, then you can also do a sub-cutaneous or intramuscular injections and that you can do into the thigh muscles and these kind of injections are actually creates a robust immune response because the delivery of the antigen remains very for a very, very long time because when you inject it into the muscles or into the subcutaneously that area is in accessible for the immune system for and is not injectionable for the blood supply also.

So that is why the antigen will remain with the body for a very, very long time and that actually is going to create a robust immune response.

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Immunization

- After injection, keep the mice back to their cage.
- Combine 100µl of antigen (100-150µg) with an equal volume of freund's incomplete adjuvant to a final volume of 200µl. Mix thoroughly to obtain the emulsion using a syringe or a pipette. after 4 weeks of first injection, inject first booster dosage. Repeat booster injection 4-5 times after every 4 weeks to generate a robust immune response and development of memory B-cells.

After injection keep the mice back into the cage then you going to do another injection after some time. The only difference is that you are going to use the freund's incomplete adjuvant so in the primary injections you are going to use the freund's complete adjuvant in the secondary injections you are going to use the freund's incomplete adjuvants to a final volume of 200 microliters mix thoroughly to obtain a emulsion and this is all is going to be remain same. So when you do the secondary injections with the freund's incomplete adjuvant, it is actually going to create the memory B-cells.

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Antibody Development

- **Determination of Antibody Titre-** take out 0.1-0.5ml mice blood from the tail vein before the first injection. Incubate the sample at 4^{0}C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and determine the antibody by a indirect ELISA.

Then you are going to do a antibody titre. So before you proceed further for note taking out our spleen and generating the hybridomas you have to ensure that the antibody is being produced. So what you are going to do is you take out the blood from the mice and you know, prepare the serum from the mice and then you are going to generate determine the antibody level with the help of the ELISA. And you are going to use the indirect ELISA which are which we are going to discuss in subsequent lectures.

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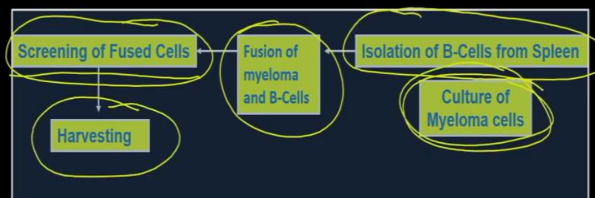
Monoclonal Antibody Production



So, up to this, we have immunized the animals and we got the antibody producing the animals. So, in subsequent to that now, since we have the immunized animals, we can further proceed to generate the hybridomas and to screen the hybridomas and then to circulate the monoclonal antibodies. So, for that first you have to do is first you have to isolate the B-cells from the spleen, you have to culture the myeloma cells.

You have to put them into the fusion reaction, so that the myeloma and B-cells are going to be fused once you got the hybridomas then you have to screen the hybridomas so that you know which clone is producing good antibodies and then you can be able to do the harvesting. So, technically, you have the more and more like 4 major steps, which you have to follow to generate the hybridomas and then to screen them to produce the to recover the antibodies from these hybridomas.

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Preparation of peritoneal excude cells

- Peritoneal excude cells (PECs) derived from the bovine hypothalamus as a feeder cells for culturing of hybridoma cells. It has following steps:
- Sacrifice the non-immunized mice either by cervical dislocation or CO2 asphyxiation.
- Soak the dead mice in a beaker containing 95% ethanol prior to start the dissection of mice in the laminar hood.
- Make small cut at the abdominal region and expose the peritoneal cavity. Inject 3-5ml of serum free DMEM into the peritoneal cavity using disposable syringe.
- Flush the peritoneum and collect the peritoneum excecude cells and plate it to the disposable petridish with 10ml serum free DMEM media.
- Count the cells and dilute the cells to $4x10^5$ cells/ml. Allow the cells to incubate for 2 days and possible contamination needs to checked before using these cells for hybridoma culture.

Before going into the hybridoma preparation, you have to prepare the peritoneal excude cells these peritoneal excude cells are actually the feeder cells which are required for culturing the hybridoma cells and preparation of the peritoneal excude cell has multiple steps. So, first thing what you have to do is you have to sacrifice the non-immunized mice either by the cervical dislocation or the CO2 asphyxiation.

Cervical dislocation is like a process which actually going to break the linkage between the ribs or between the vertebra and that is how actually it does not allow an animal to breathe properly and that is how animal is going to die. But cervical dislocation is not very popular method mostly people use a CO2 asphyxiation where you are actually simply keeping the animal in a CO2 chamber you incubate it for some time, and then because there is no oxygen present, the animal is going to die.

Then, other option is that you can actually inject the some of the anesthesia reagents like ketazine or xylazine, and that actually also is very much acceptable where you can just and anesthesia the animals and you can actually be able to go through with the process because

the ultimate aim of doing it any animal procedure is that you should give them the minimum pain and that is why the people prefer to kill them rather than keeping them under the anesthesia.

So, that they will going to feel the pain while you are dissecting them and taking out the different types of organs and cells, then you spray the animals with the 95 alcohol, so that it will start the dissection of so that it is going to be sterile because all this procedure has to be done in a in a biosafety cabinet or the laminar hood. Then you going to open the abdominal region and expose the peritoneal cavity.

Inject 3 to 5 ml of serum free media into the peritoneal cavity using our disposable syringe, flush the peritoneum and collect the peritoneum excude cells and plate it onto our disposable petridish with 10 ml serum free DMEM media then you count the cells and dilute the cells to the 4 into 10 to the power 5 cells allow the cell to incubate for 2 days and possible contamination.

Need to be checked before using these cells for hybridoma culture which means the peritoneum excude cells are going to be recovered from the peritoneum of the animals. So, this is the peritoneum of particular organisms. So, what you have to do is you just inject the needle and you know, you inject the some amount of the non- serum containing media into the belly and then you are going to recover the same cells and when you recover.

It is actually going to give you the peritoneum excude cells and these peritoneum excude cells you can keep it onto a petridish culture them for 2 days. So, that it is actually going to tell you whether the preparation is free of bacteria and other kind of contamination or not because if there will be a contamination, it is actually going to contaminate your hybridoma culture as well.

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Preparation of Spleen cells

- Sacrifice the non-immunized mice either by **cervical dislocation** or **CO2 asphyxiation.**
- Soak the dead mice in a beaker containing 95% ethanol prior to start the dissection of mice in the laminar hood.
- Make small cut at the abdominal region and dissect to remove spleen using forceps and place it in the disposable petridish.
- Inject 2-5ml serum free DMEM into the spleen and this step will swell the tissue.
- Tease the tissue with the help of forcep and released the cells into the petridish. Remove the debris and cell clump.
- Centrifuge at 50g for 5-10 mins at RT. Incubate the cells in the grey's hemolytic solution **[8ml of grey's A solution and 2ml grey's B solution]**. This step will remove the RBCs from the spleen cells leaving **myeloma cells.**
- Collect the cells and plate in the T-flask and grow up-to the mid log phase.
- Resuspend the cells in the serum free DMEM media at RT.

Then, you are going to prepare the spleen cells. So, exactly the same way you sacrifice mice as we discussed before that either by cervical dislocation or the CO2 asphyxiation, then you sterilize the animal with the help of 95% alcohol then you then you make a small cut at the abdominal region and dissect to remove the spleen using a 4 step and plate it into a disposable petridish.

Then you inject 2 to 5 ml serum free media into the spleen and this step will swell the tissue for example, if you inject it into the spleen it is going to swell then, you are going to break the spleen into with the help of the fourth step and release the cells into the petridishes, then you remove the debris and cell clump you centrifuge this at 50 gram for room temperature and then incubate the cells in in grace hemolytic solutions.

The grace hemolytic solution is a combination of 8 ml of grey solution A and 2 ml of grey solution B this solution is actually containing some of the compounds which are actually going to take up by the RBCs. And in that process, the RBCs are actually going to break open which means is by the help of the grey solutions, you are actually going to eliminate the RBC. So it actually induces the hemolysis into the system without affecting the spleen cells.

So this step is going to remove the RBC from the cells leaving the myeloma cells. Then you collect the cells and plate it into a T-flask and grow it up to the mid log phase. The resuspended the cells in the serum free DMEM media at room temperature.

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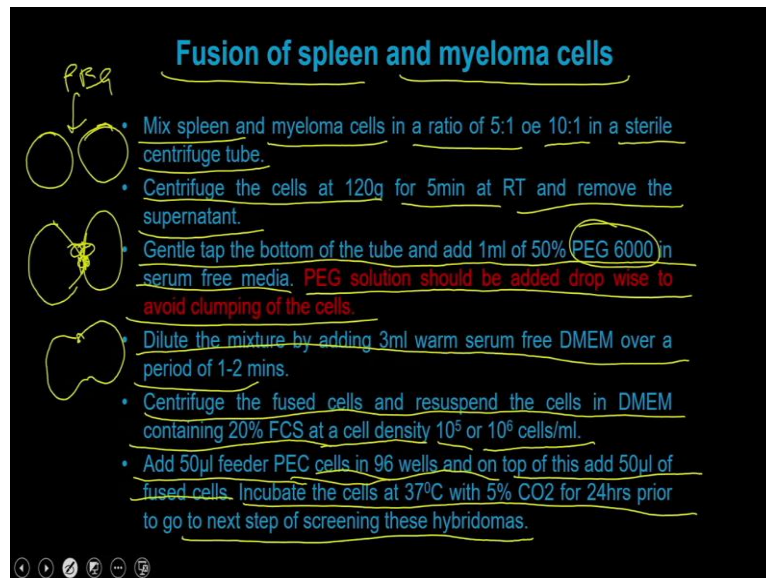

The next step, you have a myeloma cells, you have the spleen cells, and now you are going to generate the fusion reactions. Mix the spleen as well as the myeloma cells in a ratio of 5 is to 1 or 10 is to 1 in a sterile centrifuge to centrifuge the cells at 120 gram for 5 minutes at room temperature and remove the supernatant. Now gently tap the bottom of the tube and add 1 ml of 50% polyethylene glycol or PEG 6000.

In a serum free media, the PEG solution should be added drop wise to avoid the clumping of the cells to polyethylene glycol is a fusion reaction. Polyethylene glycol is actually bringing the cell membranes closer to each other. And in that process, the lipid bi-layer is going to be dissolved. And that is how the cells are getting fused. You can imagine that you have 1 cell like this, you have another cell like this and when you add the PEG the water PEG is doing is it is actually bringing these 2 cells together.

So, at this point you have these 2 cells fused to each other or they are going to be placed to each other. And now the lipids what is present here are lipid is present here is going to make dissolved each other and ultimately what you are going to get anesthesia. So if you do not do this process in a controlled fashion you are instead of getting a fusion of 2 cells, it is actually you are going to get a fusion of multiple cells and that is how you are actually going to get a clump rather than diffused cells.

Then you send diffuse so dilute the mixture by adding 3 ml of warmth over a period of 1 to 2 minutes. Then you centrifuge fused cells and resuspend a cell in a DMEM containing 20% fertile fetal bovine serum at a cell density of 10 to power 5 or 10 power 6 cells per ml. Then

you add the 50 microliter feeders PEC cells in 96 cell plate and on the top of this add the 50 micro litre of fused cells, which means you have to keep the feeder cells in the bottom and then on the top you are going to keep the hybrid cells or the fused cells.

Incubate the cells at 37 degrees Celsius with 5% CO2 prior to go to the next step of screening these hybridomas. So, then you once this is this system is ready, then you take up to the CO2 incubator and incubate for the 24 hours.

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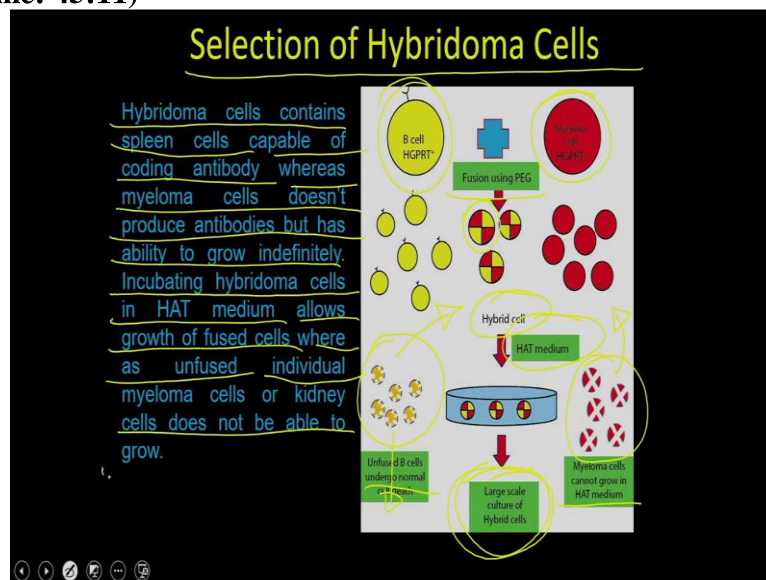

Then you have to do a selection of hybridoma cells. So, what we have done, we have just taken out the B-cells we have taken out the myeloma cells, and then you fuse them with the help of the PEG. So, now you have the hybrid cells and these hybrid cells are going to be screened with the help of the HAT medium. And what HAT medium is going to do is it is not going to allow the growth of the B-cells because the B-cells are the primary cells.

So they will going to die after some time. The myeloma cells are also not going to survive because they HAT media has some of the inhibitors which are actually going to kill the some of the crucial pathways present in the myeloma cells. What the cell is going to grow is the hybrid cell because you have the growth potential from these cells and you have the indefinite growth potential or indefinite growth phenotype from these cells.

So, the only cell is going to grow is the hybrid cells. So hybridoma cells contain the spleen cells capable of coding the antibody, whereas the myeloma cells does not produce antibodies, but has the ability to grow indefinitely, incubating hybridoma cells in HAT media allows the

growth of diffused cells whereas the unfused individual myeloma cells or the kidney cells does not be able to grow.

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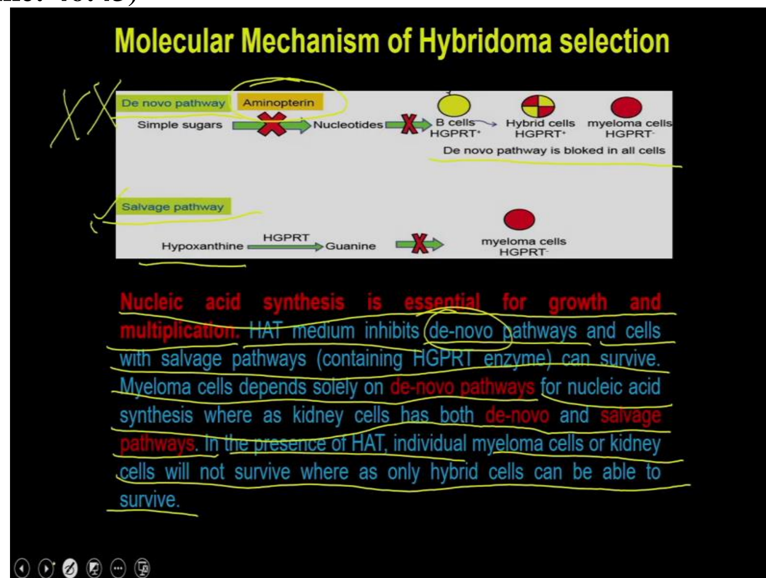

Why they are not been able to grow because the HAT has a as an inhibitor, so that actually is going to interfere with the nucleic acid synthesis. So nucleic acid synthesis is essential for the growth and multiplication of any cell. So HAT medium actually inhibits the de-novo pathway, and the cells with salvage pathway only can survive which means the de-novo what is mean by de-novo pathway.

De-novo pathway means the pathway that so you have the 2 pathway of nucleotide synthesis de-novo pathway as well as the salvage pathway. The de-novo pathway is the pathway which actually utilizes the raw material for example, the carbon dioxide, ammonia, water and all those kinds of raw material to synthesize the nucleotides either the purine nucleotides or the pyrimidine nucleotides, and then you can be able to utilize the in purine or pyrimidine nucleotides for DNA synthesis.

Whereas in the salvage pathway, you are not synthesizing the nucleotide from the raw materials, you are actually utilizing the degraded nucleotides or sometime you are also utilizing the amino acids or sometime you are utilizing the incompletely formed nucleotides as a source to synthesize the purine or the pyrimidines. And that is why the salvage pathway requires the lesser resources or it is actually a quick way of synthesizing the nucleotides compared to the de-novo pathway where you are going to start with the raw material.

So actually, the HAT media is containing the inhibitor which actually going to kill the de-novo pathway. So, what will happen the de-novo pathway is so, myeloma cells depend solely on the de-novo pathway, which means for nucleic acid synthesis, whereas the kidney cells has both de-novo as well as the salvage pathway, which means the myeloid cells are actually cannot be run the salvage pathway or if they are not going to run their metabolism until they are actually going to have the supply of nucleotides from the other sources.

Because the de-novo pathway is already been blocked, they cannot go with the salvage pathway because that is already not present in these cells. So, they are completely dependent on the de-novo pathway and since you are incubating B-cells in the HAT media, which actually contains the aminopterin and aminopterin is a is an inhibitor, which actually blocks the de-novo pathway, but it does not affect the salvage pathway, which starts from the hypoxanthine.

So, the myelomaid cells are not going to survive because there is a blockage of de-novo pathway whereas, the B-cells are can be able to synthesize because they are de-novo pathway is going to be blocked, but they have still have the salvage pathway, but the B-cells are the primary cells, they cannot go for multiple rounds of multiplication because they do not have the, they are not transformed cells.

So, they have a limited life age or limited number of multiplications through which they can undergo. So, because of that, they cannot also survive for a very very long time. So, if you grow them for 4 or 5 rounds, the B-cells are also going to die, the only cell which is going to survive is the cell which has a salvage pathway and the cell which has been transformed by the which has a transformation phenotype which means it actually can go for an indefinite period and the only cells which has this property is the hybrid cells.

So, in the presence of HAT individual myeloma cells or the kidney cells will not survive, whereas the only hybrid cell can be able to survive.

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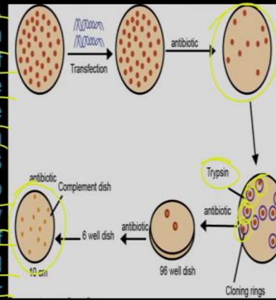
Selection of Hybridoma Cells

Once the colonies are observed, isolate these cells by serial dilution method. Delineate the boundary of each colony with a marker from the back side of the plate. Remove the media and put cloning ring to each colony. Wash the colony with PBS and add 100µl trypsin-EDTA to remove the colony. wash the colony with PBS and transfer into one well of 24 well dish. Allow it to grow and become 80% confluent. Transfer these cells to the 6 well dish in the presence of selection media and allow it to reach 80% confluency. Take a small aliquot of the cell and test the presence of antibody with ELISA.



The presence of antibody in the culture supernatant is done by ELISA.

Now, once you are done the selection of the hybridoma cells, you have to further screen the hybridomas for looking for the antibodies or you know which hybridoma is screening the more antibodies and less antibodies for that you are going to do a serial dilutions or serial dilution method. So, once the colonies are observed, you isolate the cells by serial dilution methods delineate the boundary of each colony with a marker from the backside of the plate, then you remove the media and put the cloning rings.

For example, you first got these number of hybridomas what you can do is you just put the colony you know chambers, and then you are going to add the trypsinize them with the help of the trypsin EDTA to remove the colony and then you watch the colony with PBS and transfer it into one well of 24 well dish allow it to grow and become 80% confluent the transfer these cells to 6 well dish and subsequently you can bring it to the 10 centimeter dish and that is how you are actually and then ultimately take a small aliquot of the cells and test the presence of the antibody with the help of ELISA.

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Harvesting of monoclonal antibodies

- Once the color of the medium changes from red to yellow-orange, change the medium with the fresh DMEM containing 20% FCS, 1xHAT and 20% PECs.
- The healthy cell lines that produces antibodies is transferred to the 24 well dishes containing 30-60ml of complete medium for large production of antibody.
- Harvest the supernatant by transferring culture into a tube and centrifuge it at 120g for 10min at RT. Transfer the supernatant to fresh tube and adjust the pH 7.2. Add 0.1% sodium azide and preserve the supernatant at -20°C.

Harvesting of monoclonal antibodies once the color of the media got changes from red to yellow or orange change the media with the fresh DMEM containing 20% fetal calf serum 1XHAT and 20% feeder cells. The Healthy cell line that produces antibodies is transferred to the 24 well dish containing 30 to 60 ml of complete media for the large production of antibody harvest a supernatant by transforming culture into a tube and centrifuge it at 120 gram for 10 minutes, transferred the supernatant to a fresh tube and adjust the pH to 7.2.

You can add the sodium azide as well as and you can keep are preserved this supernatant at -20 for a very very long time.

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Antibody Purification

Affinity Purification using Antigen-Coupled Column.

•Equlibration.

•Sample Preparation

•Wash the column 2 times with 10 column volume using the equilibration buffer.

•Elution

•Column Regeneration.

This we have already discussed once you got the antibodies you can be able to purify the antibodies simply by running the affinity chromatography where you have the multiple steps. First, you are going to prepare our multiple affinity column with the help of the antigen

coupled to the column. So here you can use the for example the CNBR mediated coupling to couple the antigen to the sepharose beads.

And that is how you are going to prepare the affinity column. And then you are going to do a calibration of the column with the high salt for example, you can use the 0.5 molar NaCl for that you will going to destroy the nonspecific interactions, then you are going to prepare the sample then you are going to wash the sample column 2 times with 10 column volume using the calibration buffers and that actually is going to remove the nonspecifically bound proteins.

And then ultimately, you are going to do the illusions, illusion in this case can be done either by following counter ions or you can be able to use see topic salts or you can actually play with the different types of pH, you can reduce the pH. And once you are done with elution, then you can just simply wash the column with the high salt concentrations cure topic salts and other kinds of material and then your column is ready to run for the second round.

But if you are not interested to run at that moment, then you have to preserve this column in 20% alcohol containing 0.05 percent sodium aside. So, with this, I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss about the interaction of the antigen and antibodies and how you can be able to use that property of the antibody to recognize the antigen as a tool to perform different types of experiments. So with this, I would like to conclude my lecture here. Thank you.