Eukaryotic Gene Expression:

Basics & Benefits

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Lecture No. # 34

DNA vaccines

Welcome to this lecture on Eukaryotic Gene Expression - Basics and Benefits. The last few classes, we have been discussing about delivering genes into mammalian cells as well as directly into humans, and what are the principles behind this, what kind of vectors are being used to deliver genes into both eukaryotic cells in culture as well as directly delivering genes into humans and what kind of benefits have come out of it, especially in the area of gene therapy, how people are trying to introduce genes, express genes with the aim of curing genetic disorders. We discussed at length, what are the plus points and minus points, what are the problems, and what are the prospects and so on and so forth. Today, I am going to introduce to you another very interesting and exciting area of research that again came out of research aimed that expressing genes in mammalian cells and mammalian tissues, especially in animal and human tissues.

The question we are going to ask today is that, if we can introduce genes directly into humans and with the with the with the scope of curing genetic disorders, can we also introduce genes directly into humans and express them, so that, we can induce an immune response against a foreign antigen or a pathogenic protein and thereby protect such individuals, immunized individuals, against diseases caused by those pathogens, that is, can we use genes directly as vaccines? So, this is an area that I come to be known as DNA vaccines or genetic immunization, again a very important area that developed out of a very simple gene delivery and gene expression systems. So, let us spend some time to understand what is this area of research called as DNA vaccines or genetic immunization, and how did the story began and what are the and where this area is heading to.

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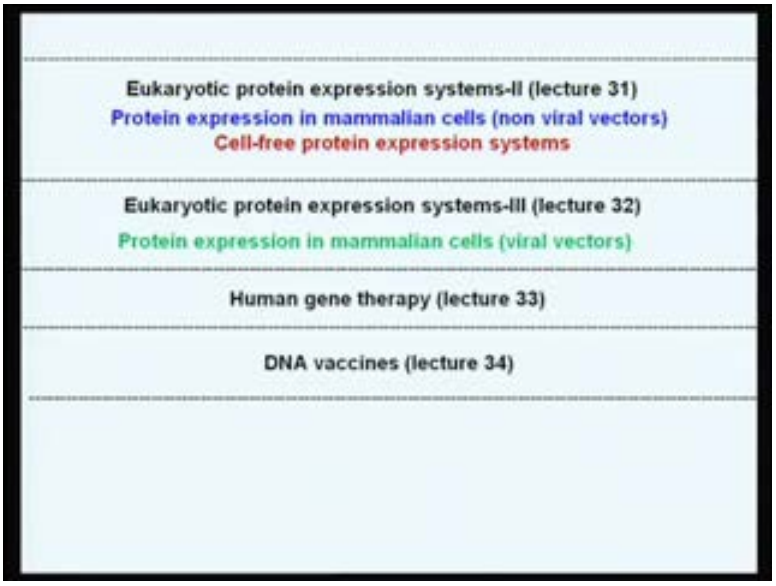

So, we have been discussing the eukaryotic expression systems. We discussed the various non viral vectors, viral vectors that are being used for delivering genes into mammalian cells and in the last lecture, we discussed at length, how people are attempting to use this non viral and viral vectors to express genes directly in humans, with the aim of curing genetic disorders, what are the limitations of this approach and so on and so forth. how far we have succeeded. So, what I am going to discuss today is about another very interesting and exciting area of research called as DNA vaccines. As I said, the purpose of this lecture is to introduce to you, the concept of inducing an immune response against a foreign antigen by directly expressing genes encoding these foreign antigens in the body. Let me first explain to see how this area of research began and how it developed and where we are hearing to.

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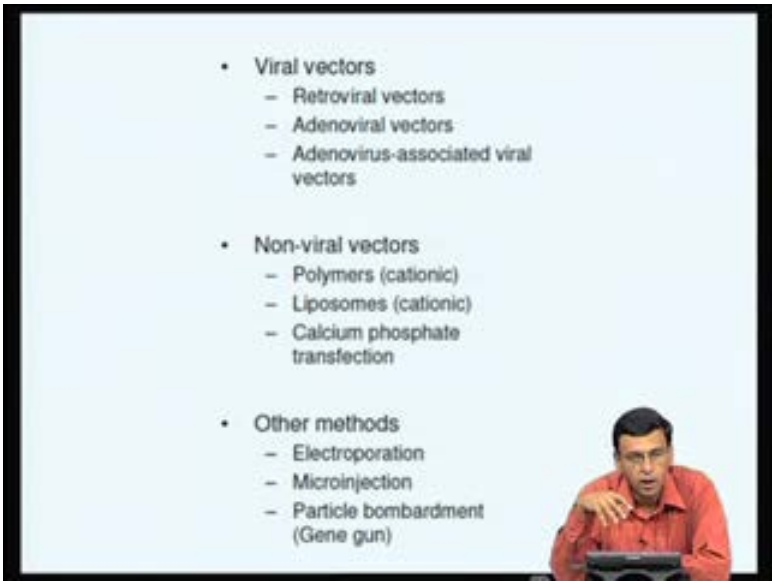

So, this just slide summarizes the varies viral and non viral vectors as well as other physical methods that people are using to introduce genes into animal tissues as well as cell lines with the aim of enhancing protein production both in cells and culture as well as in humans and animal systems. For example, we have discussed various viral vectors that are being used; retroviral vectors, adenoviral vectors, adeno associated virus vectors and so on and so forth. We also discussed how people are also trying to use a wide variety of non viral vectors, such as, mixing with cationic polymers, liposome's calcium phosphate transfixion and so on and so forth and even electroporation, micro injection and particle bombardment. The whole purpose of using all these kinds of vector systems is to enhance the delivery of genes into maximum number of cells, either in the culture, or in the animal or human tissues and enhance the expression of the genes encoded by these vectors.

Because gene therapy requires that the protein encoded by the gene has to be expressed at clinically or therapeutically significant levels, only then that you can call gene therapy as successful. So, with the aim of enhancing the efficiency of transfixion as well as enhancing the expression of the transgene, a number of these vector systems are being used to have some therapeutic potential.

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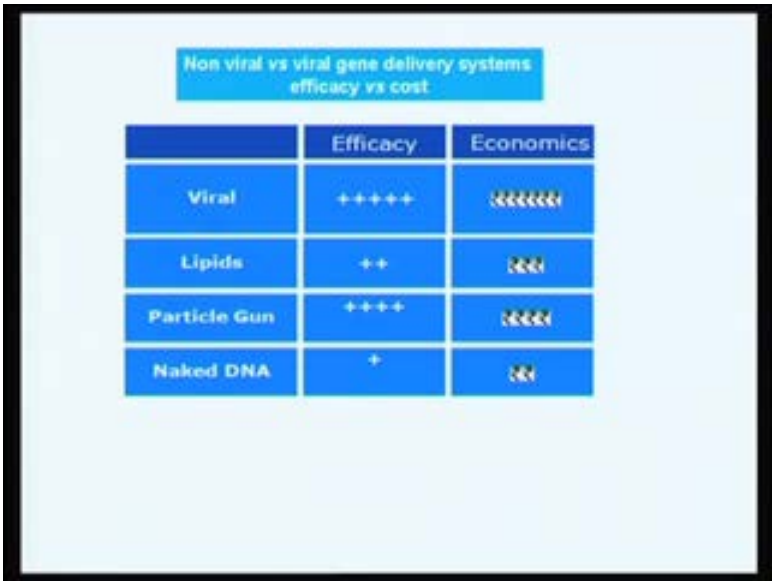

This slide just tells you what are the major advantages and disadvantages of this various vector systems. For example, if we take the viral systems, they are compared to non viral systems, the viral systems are highly efficient in terms of gene delivery, and, but, in terms of economics, they are very expensive. It cost a lot of amount lot of money to clone your genes to viral vectors and generate a recombinant virus in sufficient titters and use them either in vivo or ex vivo approaches, whereas, you contrast a you look at the various non viral vectors. Either you complex the gene of your interest, lipids or you use what is called as a gene gun or a particle gun, where you take your genes or plasmids, coat them with gold particles and directly shoot them into tissues. We will discuss this in little while from now. The purpose of all this what I am discussing here is to see, if we look at the efficacy versus economics, techniques which are very good in terms of efficiency of gene delivery as well as expression of the genes, they are also very expensive among all these things. If you try to inject your plasmid DNA that is also known as a naked DNA, directly into tissue, it is very inefficient. As I said, the cell membrane is negatively charged and poses a barrier for the entry of the negatively charged DNA inside the cells. Therefore, if you tried to just inject directly into your plasmid DNA into cells or tissues, the cells do not take up the DNA very sufficiently. So, in terms of efficiency of gene delivery and expression, the plasmid DNA, trying to introduce an expression plasmid clones is the most or the most least efficient of all the vector systems we have discussed so far and that at the same time, it is the most economical method of expressing genes.

So, it is for this reason, it is because of this major limitation, that is the DNA or the plasmid DNA Oran expression vector encoding a foreign gene cannot be taken up by mammalian cells very efficiently that we are trying to put them in viruses or we are trying to complex them with the lipids and so on and so forth; to see whether we can enhance the efficiency of gene delivery and gene expression. But today, I am going to focus on this least efficient or most inefficient method of introducing genes into the cells and explained to you how this very inefficient. But, very economical method of gene delivery holds a very new application in the in the in the form of inducing an immune response for a foreign protein or a foreign antigen and how this field has now come to be known as genetic immunization or DNA vaccination.

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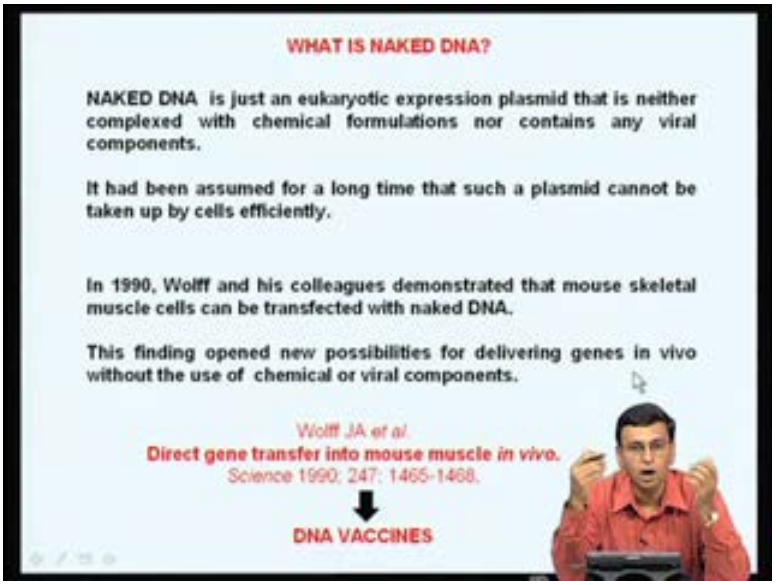

Now, what is a naked DNA? A naked DNA is nothing but it is an eukaryotic expression plasmid, that is not complexed with any any chemical formulations, or does not neither it contains any viral components. So, it is just a eukaryotic expression plasmid, it contains a mammalian promoter for driving the expression of the foreign genes and other sequences which are required for a typical mammalian expression. plasmid The problem of using such an expression plasmid for introducing or for expressing genes in mammalian cells is that, it is known for a long time that such plasmid DNA is not taken up by efficiently by mammalian cells, as I said. This is the reason why, you have to complex this DNA with either lipids or do electroporation or try to do particle bombardment or use viral vectors and so on and so forth. This is the major limitation of

using directly administering the DNA into cells directly the DNA, but very interestingly, in the year 1999, Wolff and his colleagues demonstrated that skeletal muscle cells of mouse can be transfected with naked DNA, this was the very surprising cell they got and this was actually published in the very prestigious scientific general journal 'Science' in the year 199. This entitled direct gene transfer into mouse muscle in vivo because, till then, people thought that no tissue can take up just DNA directly, you need to do all these gimmicks that I have explained so far. Only then, some efficiency can be brought into. So, this possibility, this finding that skeletal muscle of mouse can be directly transfected with a DNA opened up new possibilities for delivering genes in vivo without using any chemical or viral components. So, it created lot of excitement.

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Now, let us spend some time to examine how exactly these people came up with this novel observation. As I said, people were trying to cure genetic disorders using non viral approaches and one of the very intensely Ah investigated areas of research, in area of gene therapy, is to see if you can now take this expression plasmids of DNA and complex with a cationic lipids. Now, this cationic lipids are positively charged lipids and you complex these DNA with this positively charged lipids and then add this lipid DNA complex to the cells so that this DNA can be efficiently taken up by cells and your gene of your interest can be efficiently expressed. So, a number of research groups were actually trying a number of cationic lipids formulations to see how we can enhance the expression or efficiency of gene delivery into mammalian cells. in the I have just listed

out a various papers, how people are trying to see whether, it for example, can we cure cystic fibrosis by using this kind of a cationic lipid mediate gene delivery or can we cure certain kinds of cancers such as human melanoma or can we introduce into the nasal epithelium of patients suffering from cystic fibrosis and so on and so forth. But, these two articles which are listed here in blue color is the work being carried out by a person called named Phillfelnker in a company called y cal in United States. They were also trying to develop a number of cationic lipid formations, to see which of these lipid formulations if you mix with the DNA can be most efficiently taken up by mammalian cells or animal tissues in vivo so that they can now be taken up for gene therapy and so on and so forth.

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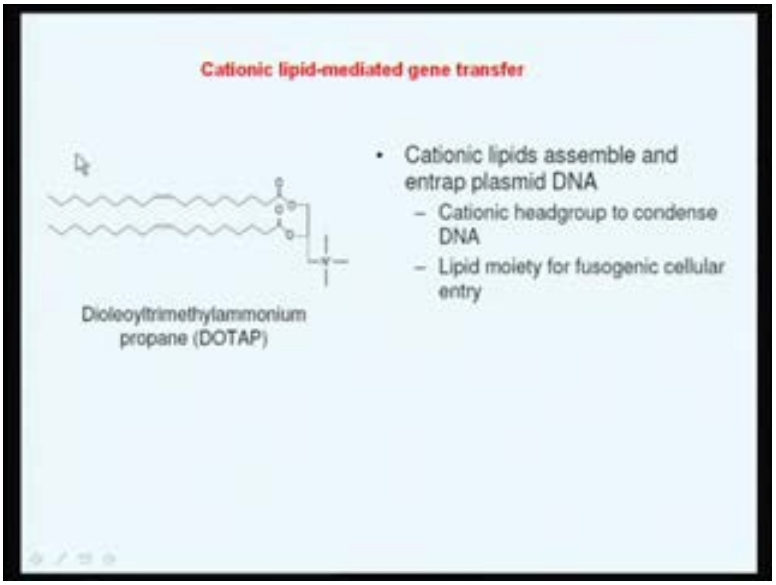

For example, this is one of such one such lipid formulations they were trying, called as DOTAP, which contains a positively charged head and a hydrophobic tail. and When we take such cationic lipids and then put the DNA, the plasmid DNA gets entrapped, interacts with this positively charged head group and the DNA gets entrapped by a lipid particle. and If you now add this cationic lipid DNA complexes into cells, the lipid fuses with the cell membrane and the DNA is delivered inside the cells. So, a variation of these cationic lipids, a number of cationic lipid formulations are being synthesized by organic chemists and they were trying to see which of these lipid formulations, if we mix with a DNA preparation can and then you inject such cationic lipid DNA complexes into

various tissues either by intravenous route or intra muscular route, which of them will most efficiently deliver your genes or a plasmid DNA into efficiently into tissues.

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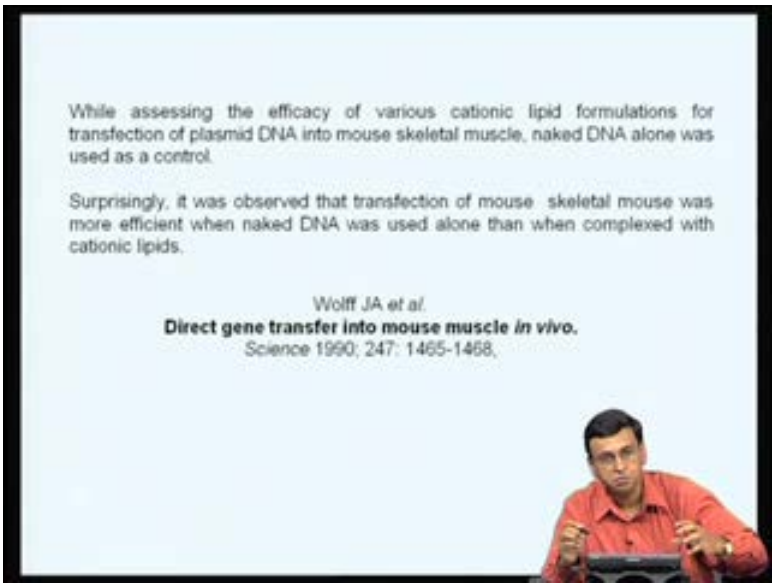

When they were doing this, that is when they were assessing the efficacy of these various cationic lipids formulations for transfecting plasmid DNA into mouse skeletal muscle. They were using actually just the plasmid DNA alone as a control; that means, plasmid DNA mixed with a number of different cationic phosphorylations, for which the plasmid DNA alone served as a negative control. So, what was expected is that, the plasmid DNA alone should not get into the muscle tissue very efficiently, whereas, when you mix this plasmid DNA with other lipid formulations, they enhance there should be an enhancement in the efficiency of gene delivery into the tissue. Very surprisingly, what they found is, that transfection of mouse skeletal muscle was more efficient when naked DNA was used alone rather than when complexed with cationic lipids. So, this is a very surprising result they got, which was got published in the general journal 'Science' in the year 199. So, the expected result was that when we take this plasmid DNA and mixed with various cationic lipid formulations, one or the other lipid formulations should efficiently promote the efficiency of gene delivery into the mouse skeletal muscle; but, surprisingly, these people found just injecting plasmid DNA dissolved in cell line was being taken up by the mouse skeletal muscle much more efficiently than mixing this DNA with various cationic lipid formulations. This was kind of, against the dogma that time, because, DNA is cannot be efficiently taken up by mammalian cells.

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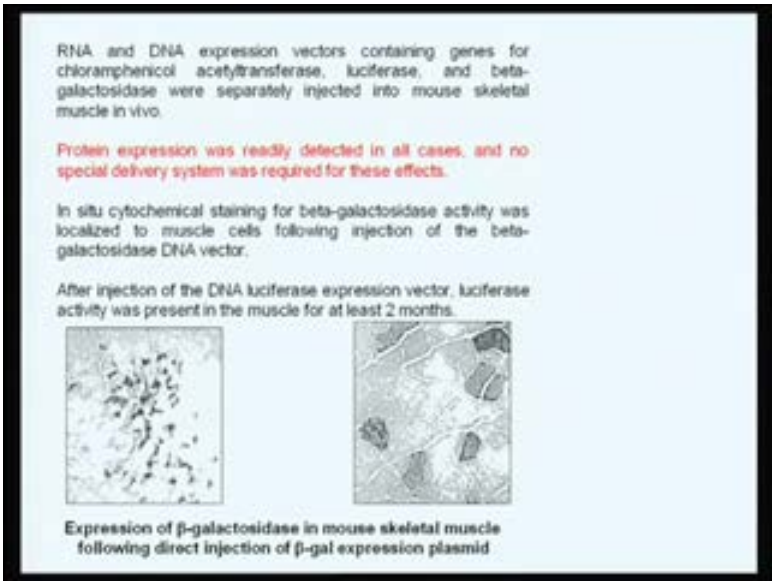

In fact, this is these are some of the highlights of this research paper, which was published by this group. They were actually using a number of genes encoding reporter reporters like chloramphenicol transferees, luciferase beta and galactosidase, because, it is very easy to detect the expression of these genes reporter genes. So, you take eukaryotic expression plasmids coding for either chloramphenicol transferase, luciferase or beta galactosidase, which are all very well known reporter genes and then you complex them with various cationic lipids and inject them into the mouse skeletal muscle and see which of the tissue will show high levels of beta galactosidase activity or chloramphenicol transferase activity or luciferase activity, so, that that lipid can now be taken up and seen whether it can be used for gene therapy. That was the whole idea when we are doing all these things, they found an instead of mixing with all these cationic lipids, if we now take this plasmid for coding either of this reporter genes and directly just dissolve them in cell line in phosphate buffered cell line and inject them into muscle tissue, you can see in and around injection site, if you now look for beta galactosidase activity, you can see blue color, cells expressing beta galactosidase, indicating that cells in and around the site of injection have actually taken up the plasmid DNA. and The gene has that has gone inside the cell and the gene is getting expressed and you are able to get beta galactosidase activity. This is just the magnification, one such area. You can actually see of the various muscle cells, here are a few muscle cells which are blue in color. Of course, this is a black and white picture, but, these are actually blue colored cells indicating that these cells are have actually taken up the plasmid DNA which has

been designed in cell line and the beta galactosidase gene expression can be detected inside this muscle fibers. So, this was a very very surprising observation, because, none of the tissues are supposed to take up just the plasmid DNA alone; it requires a lot of other gimmicks to get into the mammalian cells.

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So, this created a lot of excitement and that is the reason this paper got published in Science, a very prestigious journal. So, the people thought, on one hand people are trying a number of gimmicks using viral vectors and non viral vectors to introduce genes into mammalian tissues and human tissues to enhance the expression of transgene so that, you can have applications in the area of gene therapy and here, you hear a situation where you do not require any of these delivery systems, you simply take a plasmid DNA, dissolve in saline, simply inject into the skeletal muscle, the muscles cells are seems to able to take up the DNA and express. So, this immediately raised a huge possibility that, instead of putting beta galactosidase, or chloramphenicol transferase, or luciferase genes can you now put a gene coding for a factor 8 for example, which is a clotting factor. and In patients suffering from hemophilia, for example, they do not produce factor 8 and therefore, if you now simply take the gene coding for factor 8 and put it in an expression plasmid. and If you simply inject this expression plasmid into muscle skeletal muscle, will now the factor 8 be made inside the muscle cells and will it be secrete into the blood stream, so that, the factor 8, the hemophilia patients can be cured of this disease?

The reason why this kind of a thing is very attractive is because, for as you know hemophilia is a X-linked disorder affecting 1 in 10,000 males and this disease disease is due to defect in the gene coding for blood clotting factor called factor 8 and normal circulating levels of factor 8 is about 200 nano grams per ml. So, if we are normal, if you do not have any blood clotting disorders, our blood contains about 200 nano grams per ml of this clotting factor. That is why, whenever there is a cut and when we bleed, immediately bleeding stops. But, not in the therapeutic patients suffering from hemophilia in which whom, this gene is defective.

Even if you can have 20 to 40 nano grams per ml, that is 1 10^{th} the actual levels, one in normal individuals; it is still considered clinically or clinically significant. So, the aim of gene therapy researchers who are trying to cure hemophilia is to see if we can develop a gene delivery system to express factor 8, at least to this levels, which is even 1 10^{th} of that found in normal individuals so that, these people does not have to take factor 8 injections and so on and so forth. So, one of 1^{st} aims of this kind of a direct gene transfer using naked DNA was to see, if you now take a plasmid DNA and put a gene coding for factor 8 and just inject them into the skeletal muscle of this individuals, can you get this much level of factor 8 in the blood circulation? But, soon it became very clear that intramuscular injection of plasmid DNA encoding genes like factor 8 gene did not result in the production of clinically significant levels of factor 8. So, although the studies with the reporter genes were very exciting, when it actually came to very important therapeutic genes, they found that this technique failed to deliver; clinically significant levels of proteins could not be produced using this naked DNA injection. So, which does that it cannot be really used for gene therapy.

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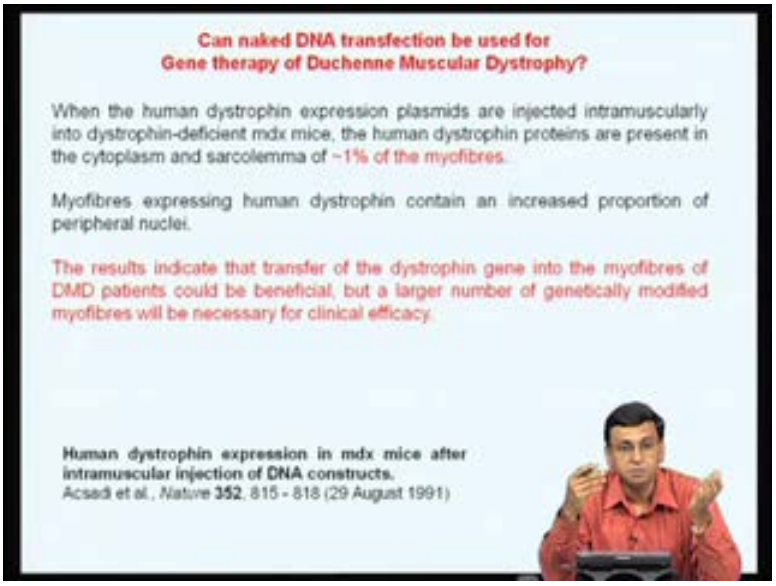

Similarly, people were trying to see if can, you by simply introducing this kind of a naked DNA, can you cure diseases like Duchene muscular dystrophy? Do you know Duchene muscular dystrophy is a skeletal muscular disorder where the protein called dystrophic is not produced in these individuals and therefore, they suffer from this disease. So, the again, the idea is to see, can we now introduce the dystrophin genes into the skeletal muscle fibers of this individuals, so that the dystrophin is now expressed in these muscle fibers and they can be cured off the disease. But again, when they did the same naked DNA injection, they found that only one percent of the myofibers could be transfected with the plasmid, So, indicating that transfer of dystrophy genes into the myofibers of Duchenne muscular dystrophy patients could be beneficial, but a larger number of genetically modified myofibers will be necessary for a clinically meaningful result. So, both using things conditions like hemophilia or Duchenne muscular dystrophy, it became very clear, this technique of directly injecting plasmid DNA into muscle cells to express genes of your interest may not be suitable for the purposes of gene therapy; this is the paper that came out sometime in 1991.

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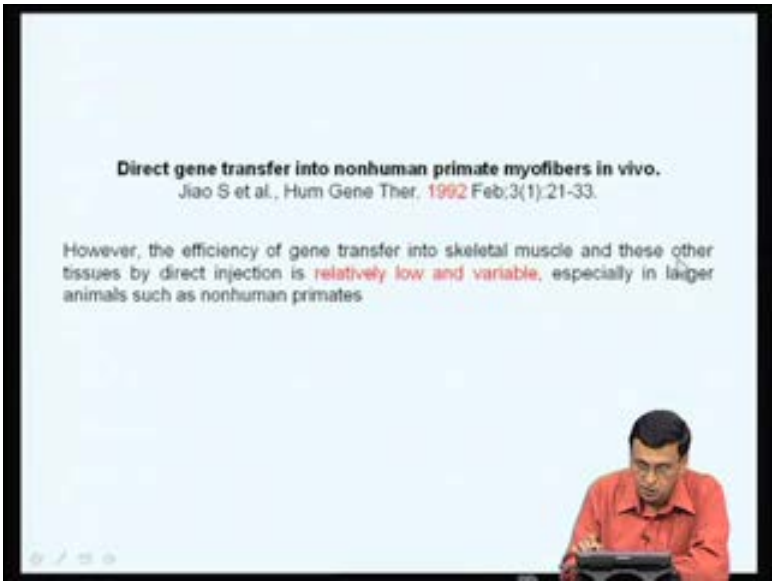

The other thing that became very evident is that, while the naked DNA injection seems to be reasonably efficient in mouse skeletal muscle, when there it is same thing for higher mammals like primates, the primate muscle fibers were not getting as sufficiently transfected as that of mouse skeletal muscle, indicating that there are species specific differences. The efficiency of naked DNA uptake is much more efficient in the mouse skeletal muscle, but is not as sufficient in primate muscle muscles. So, the efficiency of gene transfer into skeletal muscle in primate muscle fibers is relatively low and variable, indicating that this may not really work in humans.

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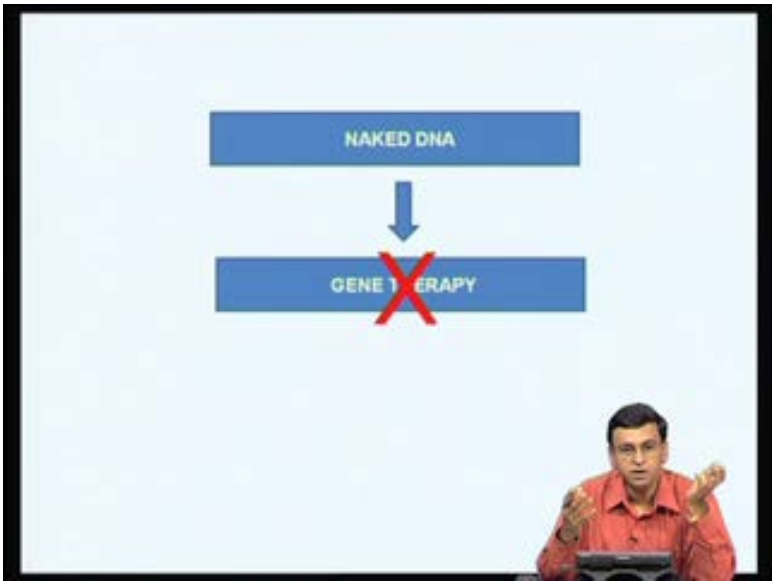

So, the initial excitement that naked DNA or just directly injecting plasmid DNA can have applications in the area of gene therapy soon turned out to be a false hope, So, because, the efficiency of gene delivery is not very efficient and therefore, it cannot be used for gene therapy. So, it failed as a gene therapy technique.

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Then this as I said, the first first report came in the year 1990 and for the next 2 to 3 years, lot of people tried to see whether they can use this technique for gene therapy. but It became very clear that the technique is very inefficient, therefore, cannot be used for gene therapy. So, the excitement died down, then this paper came in the year 1993 entitled 'heterologous protection against Influenza by the injection of a DNA encoding viral protein'. What this group headed by Ulmer et al actually demonstrated is that, instead of taking genes coding for things like factor 8 or dystrophin, you now take a gene that codes for a foreign antigen of a virus array pathogen. So, you replace the gene therapeutic genes like dystrophin or factor 8 and put a gene that codes for a foreign antigen like a viral protein and now if you take this plasmid and inject to the skeletal muscle of a mouse, the foreign antigen gets expressed in the skeletal muscle and it should get should be presented to the immune system of our body. and this The immune system in conjunction to either class 1 MHC c or class 2 MHC, image c when they expressed for an antigen, we should be able to develop either a pluripotent antibodies or a pluripotent and toxic real imposition spots and this is what this author showed here.

So, although this technique of naked DNA injection filled failed as a gene therapy technique, when they look a gene that goes to a influence of a Influenza protein, influence as antigen, Influenza as is a very nasty virus, causes a lot of problems, even now. And it is very difficult to develop influence of Influenza vaccines. So, if you now take a gene that codes for what is called as a nucleoprotein of an influence of Influenza virus, put it in a expression plasmid driven by a cytomegalovirus promoter, simply if you inject into the mouse skeletal muscle, they found the influence of Influenza protein that is getting expressed inside this animal tissue is able to invoke a protectable immune response. This is what was showed immunization of mice by intra muscular injection of plasmid DNA encoding an influence of Influenza viral protein generated specific cytolytic T cells and protected mice against subsequent challenge of a live Influenza virus.

This is a very novel concept because, if you want to induce an immune response against the foreign antigen, you have to take this foreign antigen, you have to produce, like we discussed in the earlier classes, like hepatitis B antigen for example. If you want to make hepatitis B vaccine, you have to take the gene coding para proteins beware as expressing ease cells, purify this protein, mix with an adjuvant such as alum and then only if you inject then only, you will get an immune response. But, here, we are saying that, you do not have to do any of these things. Simply take the gene coding for a foreign antigen, clone it into a mammalian expression plasmid, make this plasmid in large amounts in bacterial cells and simply inject the plasmid DNA into the mammal, in this case, mouse. And This antigen is now made inside the muscle cells and is now able to induce an immune response, which means, you do not have to express the foreign protein or you do not have to purify a prior protein; direct injection of gene coding of foreign antigen can induce an immune response in a mammalian system, which is a very very novel observation in the earlier early 1990s.

This is generated a lot of interest. So, number of b reviews, news and views came out in the journal saying that naked DNA points to vaccine. So,. So for far, the dogma has in that, only when you immunize animals with proteins or by carbohydrates or conjugates of proteins, you can induce a immune response, but, for the first time, these people have demonstrated that you do not need a protein or a carbohydrate base immunization, genes can be directly injected into the body and these genes when injected into the body, the

proteins will be made in C 2 in situ, inside the cells of mammal and it can induce an immune response. So, genes can be used as vaccines which created a laugh lot of excitement.

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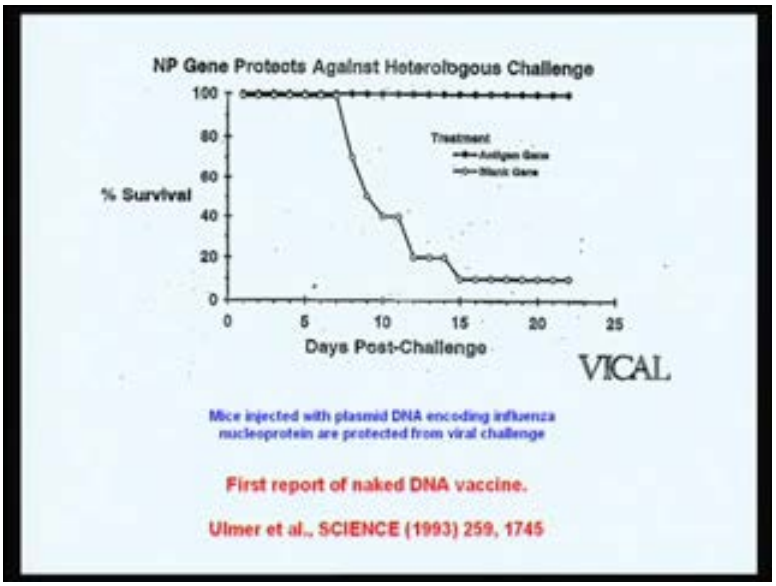

This is the actual experiment that was done by this group, which was published in this paper. They took these mice and injected them with a plasmid DNA that codes for what is called as a nucleoprotein of the Influenza virus and then, after giving 2 or 3 immunization challenges, this mice will be actual Influenza virus. And as you can see here, if you look at the person survival verses days after viral challenge, as after 5, 10, 15, 20, 25 days, by 15 days all the control mice which were not expressing the antigen they all died because of a virus challenge. But, all those mice which are actually expressing the nucleoprotein are or immunized with the nuclear protein expressing plasmid, they were all protected.

The other important point in the experiment is that the challenge virus that was used is called as the heterologous challenge, because, the nucleoprotein gene came from a different stain of a virus and the challenge virus was a another strain, which was unheard of the in the case of Influenza, because Influenza virus keeps changing its surface epitopes very often. and Therefore, a vaccine which was made, for example, in the previous year we will not work against the strains which are in the field the following year. So, every year, vaccinologists are vaccine manufactures, how have to go to the

field, see what kind of Influenza virus are circulating in this that particular year and then use this virus strains for making new vaccine. So, every year, you have to go to the filed, take up the circulating viruses and then make them as vaccine, develop them as vaccine strains. So, this major problem is the Influenza, but here, we have a situation, this is actually shown in this clear.

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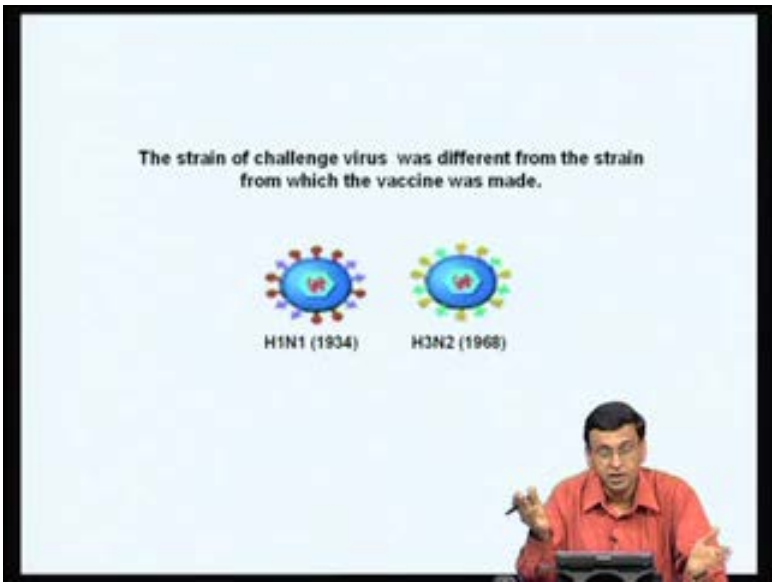

The antigen gene coding for the nucleoprotein of Influenza virus came from a virus strain called H 1 N 1, which was isolated in the year 1934 from patients, but the virus which was challenged challenge virus was a virus strain that created an epidemic in the year 1968. So, the vaccine was made from a different strain of Influenza virus, the challenge virus is a different strain. But still, it had it conferred protection, indicating that there was a cross strain protection using the DNA vaccination.

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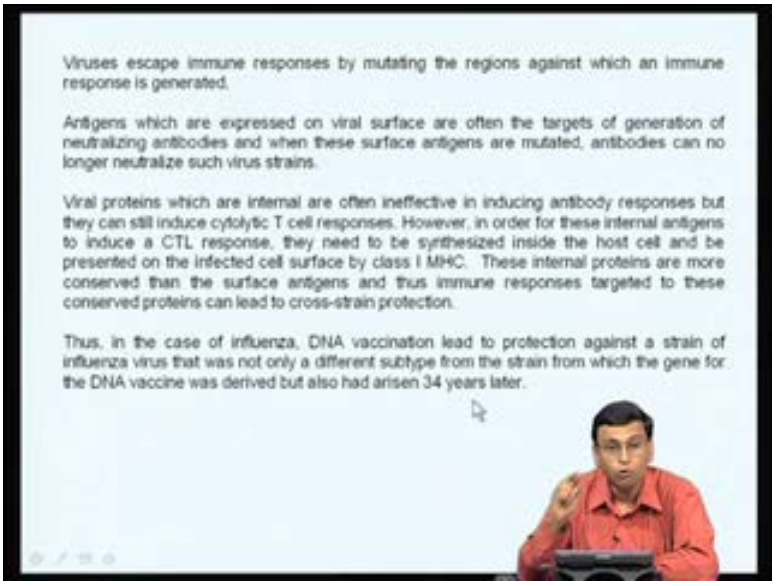

Now, let us I have just described in detail some some little bit of immunology behind this, viruses escape immune responses by mutating the regions against which an immune response is generated. This is what I told, usually when you take influence of virus in an inactivated Influenza virus and give it as a vaccine, all the antibodies will be generated against what is called as the hemagglutinin, which is present on the surface of the virus, but, since these viruses keep changing this hemagglutinin protein now and then, if you make a vaccine against one particular strain and strains which are change the hemagglutinin a few seasons later, is no longer responds to the vaccine. will no longer to the vaccine The vaccine will not be able to protect such kind of a strains, that is why you need to keep changing their vaccines strain as often as possible. So, antigens which are expressed on the viral surface are often the targets of generation of neutralizing antibodies and when these surface antigens are mutated, antibodies can no longer neutralize the virus.

This is the problem with viruses like Influenza or your malaria parasites or even H I and so on and so forth because, they keep changing the surface antigens. On the other hand, viral proteins which are internal are often ineffective in inducing anti body responses, but they can induce very potent cytolytic T cell responses. In order for an antigen to induce an antiviral response, they should be present on the surface of the virus. Only then, the antigen presenting cells can see them and then induce an antibiotic response. But, for inducing a cell mediated immune response, the antigens have to be synthesized inside the

host cell and this these antigens as have to be presented in complex with class 1 MHC on the surface of the infected cell; in this case on the surface of the transfected cell. And then, this will be recognized by this CTL and you get certain the (( )) cell responsibility can be induced. So, in order for the internal antigens to induce a cytotoxic T cell info site response, they knew need to be synthesized inside the host cell and be present on the infected surface by class 1 MHC, this these internal proteins. So, unlike the surface proteins which are present on the viral surface, these internal proteins are highly conserved. They do not change that often as a surface as the surface proteins do. So, the internal proteins are often conserved between various clinics or various strains of this virus. So, this is the region, these people actually use the nucleoprotein of a different strain and when the immune the cytolytic cytolytic T cell response that are generated by this nucleoprotein is able to control confer protection is a totally different strain, which was which came out almost 13 years later because, the nucleoprotein is pretty conserved in this planes.

So, DNA vaccination lead to protection against a strain of an Influenza virus that not not only was a different subtype from the strain from which a gene was of the DNA vaccine was derived, but it has aroused in 34 years later. So, the nucleoprotein gene came from a strain which was in the year 1934 and the challenge virus came out in the year 1968. So, even after 30 years, this nucleoprotein gene is able to protein confer cross-strain protections. So, it was a very major breakthrough.

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So, this created a lot of excitement among vaccinologists indicating that almost all major news papers reported this finding as a very anxiety exciting really very and. In fact, this was being called as a third vaccine revolution in the area of vaccinology and this year 1993 actually marked the beginning of this DNA vaccine research. The reason why I am emphasizing is that, you can see here, a very simple gene delivery technique. Here, you simply take a gene coding for a viral protein, put it in mammalian expression vector, just inject them in to skeletal muscle and the antigen is made inside the cells. and The antigen is expressed and you are able to induce an immune response. So, a simple gene delivery technique which failed as a gene therapy technique became a very very promising technique for inducing immune response and created a whole field of research called DNA vaccination or genetic immunization.

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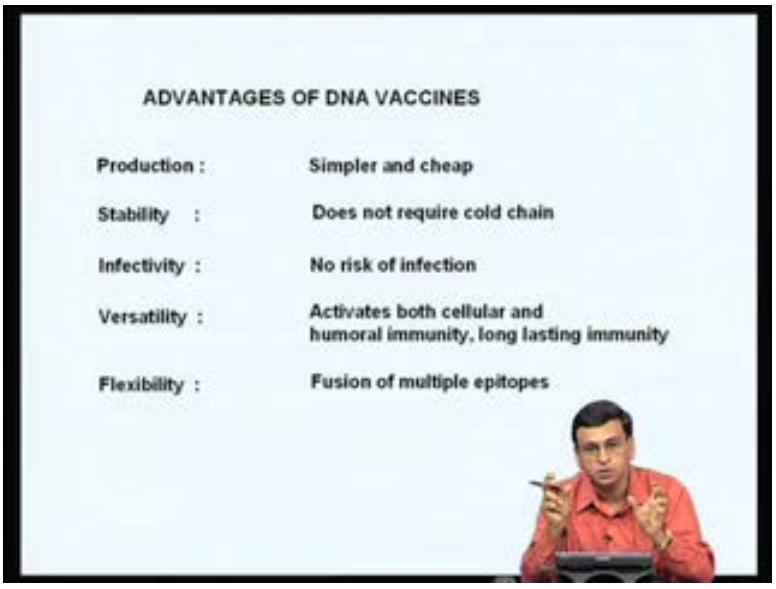

What are the advantages; why people were excited about this DNA vaccines? They have lot of advantages compared to the conventional vaccines, which is either live attenuated vaccines or inactivated vaccines or recombinant protein based vaccines or carbohydrate vaccines, because, they can be they are very simple and easy to produce. Cloning a gene into a eukaryotic expression plasmid and making this plasmid in large amounts in E. coli is much more less expensive and much more simpler than infecting cells with viruses, killing the viruses or developing value a live attenuated strains of viruses are or making recombinant proteins, purifying this proteins and giving it as vaccines. So, producing a plasmid DNA is much cheaper and much simpler than producing other kinds of vaccines.

The other important advantage of DNA vaccine is that, it does not require a cold chain. All the other vaccines that I talked so far has have to be refrigerated whereas, DNA is much more stable than proteins or inactivated or live attenuated viruses.

So, plasmid DNA need not be stored at the clod chain of refrigerators, it can be stored at room temperature. In fact, almost 20-25 percent of the vaccine cost often goes or for maintaining what is called as a cold chain, that is, from the at the place of manufacture to the ultimate place of fuse, you have to keep this vaccine in refrigerated condition. So, if you can develop a vaccine which does not require this cold chain, that the cost of vaccine can be dramatically brought out down. So, there was lot of excitement when people showed that DNA can be directly used as a vaccine, that suggested that, you do not you do not store them in refrigerators. So, the cost of vaccines can be dramatically brought down. It is non-infectious and there is no risk of infection because you are not dealing with pathogens. Remember, if you have to make a live attenuated vaccine or if you have to make a killed vaccine, you have to infect cells with the virus, grow this virus in large amounts; either you use this attenuated viruses or killed viruses as vaccines that means, you have to deal with the pathogens. Here, we are only dealing with 1 or 2 antigens of the pathogens. So, it is non-infectious and it is very versatile because, it can activate both cellular as well as humeral humoral harm of the immune response. You can get a cell mediated immune response cytotoxic T cells, you can also get very efficient anti-bodies. and The other advantage is that, there is lot of flexibility. You stretch search whatever genes you want, you can put different epitopes, you can make synthetic genes containing different epitopes from different antigens and express them so that, you can develop polyvalent vaccines. So, because of this these numerous advantages, DNA vaccine generated lot of excitement.

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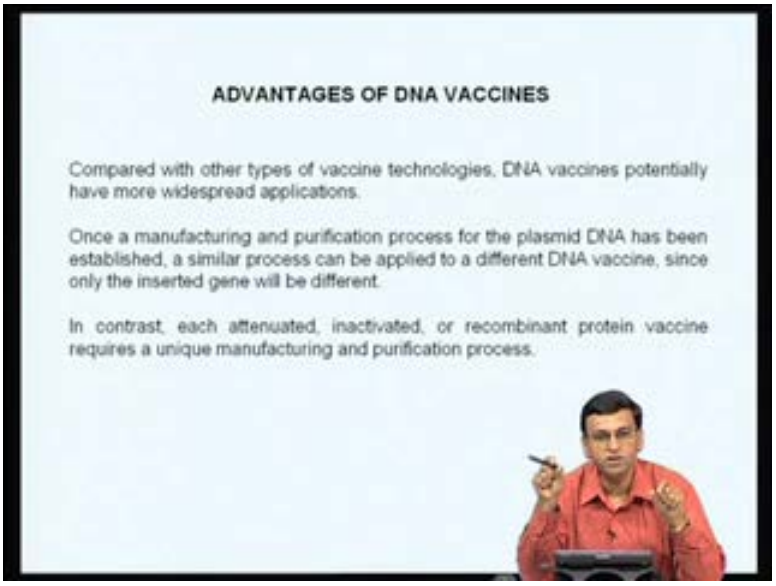

Similarly, the other advantages are as compared to the other types of vaccine technologies, DNA vaccines have much more wide spread applications. For example, once a manufacturing and purification process for a plasmid DNA is established, a similar process can be applied for other different vaccines. Only difference will be there for a one case For a example, you say if you want to make a influence of Influenza vaccine, you would be putting in influence of Influenza gene, a gene coding for Influenza protein whereas, if you want to develop a vaccine for a malaria for example, instead of Influenza gene, you will be putting a gene coding for malarial antigen. But otherwise, rest of the vector is the same. So, in terms of manufacturing plasmid DNA, whether it contains Influenza gene or a malarial antigen does not make any difference. So, the manufacturing process will be more or less the same, only the inserts will be different. The genes will be different so, that means, you can, using the same manufacturing facility, you can produce different different DNA vaccines, which is a tremendous advantage whereas, if you have to make attenuated or inactivated or recombinant protein vaccines, each process is different for a different vaccine. A process which is used for making recombinant hepatitis B vaccine cannot be used for making a polio vaccine, because, these manufacturing process are totally different. So, all these advantages make the entire area of DNA vaccine research very very exciting. So, lot of interest for generated to see this simple gene delivery gene expression technology, if it can it be developed as a very novel form of immune inducing, immune responding mammalian systems. a Once people demonstrated that just by injecting DNA plasmid,

DNA encoding foreign antigens can induce immune response in skeletal muscle, people ask the question why skeletal muscle, can you give to other tissues?

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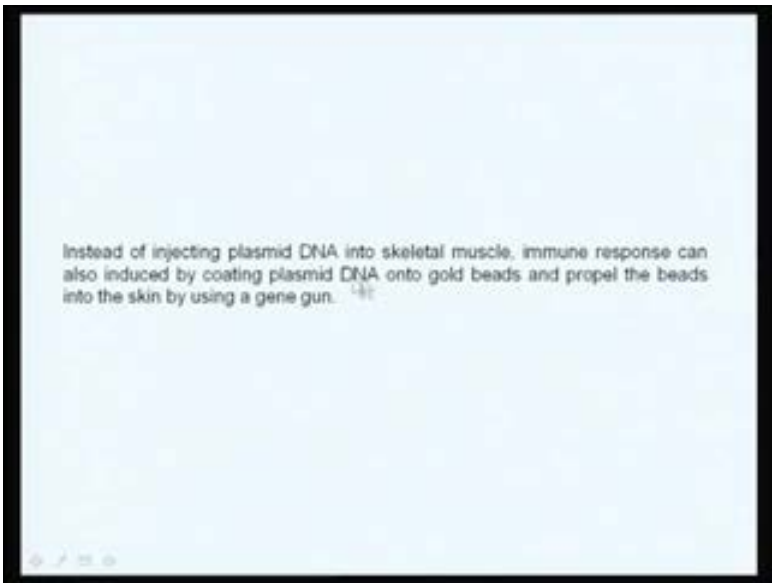

In fact, instead of injecting plasmid DNA into skeletal muscles, if you now take this plasmid DNA encoding foreign antigen and mix it with gold particles and then shoot them, propel them into the skin by what are called as gene guns, then they found the efficiency of human responses much more. You can get a more efficient human response if you introduce this foreign genes or foreign expression plasmid encoding foreign genes into skin using biolistic methods than simply injecting using syringes into skeletal muscles.

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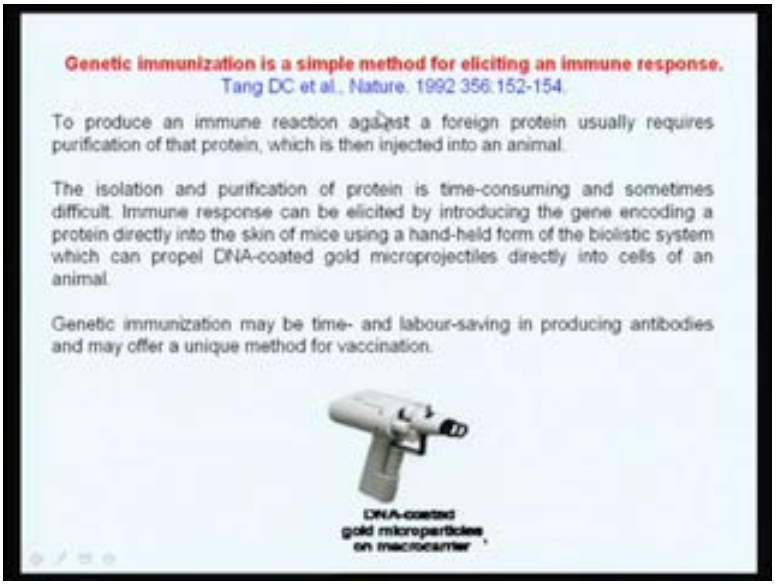

So, new devices called as gene guns were actually developed. For example, a paper that came in nature in the year 1992 entitled to genetic immunization a very simple method of eliciting immune response suggested that, for inducing the dogma till then for example, to produce an immune reaction against a foreign protein, you actually actually had you need to have a purified protein of that particular organism. Then only you can, if you inject that influence along with an adjuvant, you get an immune response. But, the isolation and purification of proteins is time consuming and sometimes very difficult if these antigens are produced in very small amounts. If you cannot express them properly, then you cannot make a vaccine for that particular antigen. Immune response can be elicited by introducing the gene encoding protein directly into the skin using a hand held form of a biolistic biolistic system, which can propel DNA coated gold particles directly into the cell. This is the picture of what is called as a gene gun, marketed by company called Bio-Rad. So, instead of instead of making the protein, purifying the protein and then mix them with adjuvant and injecting to induce immune response, here is a very simple method. You simply take the gene pulled a mammalian expression plasmid and then mix it with certain gold particles and using this kind of a gene guns, you can just shoot them into skin and you get a fantastic immune response against this foreign antigen.

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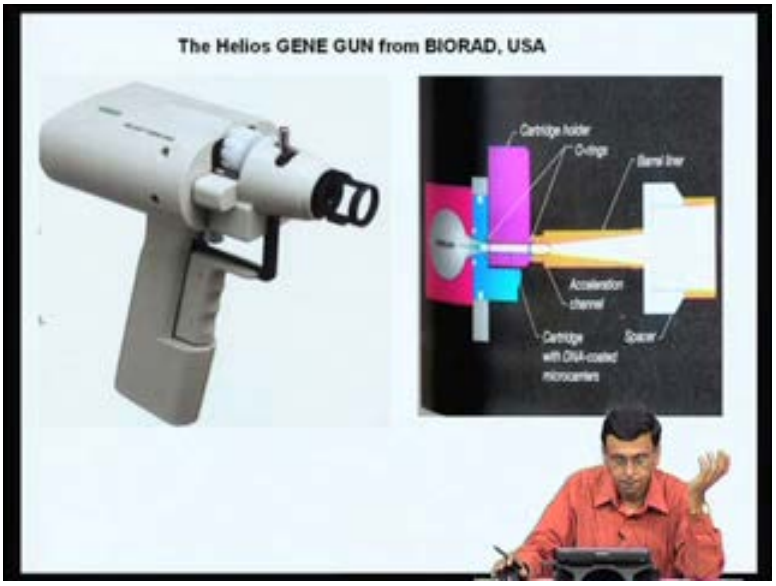

So, this is the enlarged picture of this gene gun called has the Helios gene gun which was marketed by Bio-Rad. So, you can simply take your DNA containing your foreign antigen, foreign gene, coat them with gold particles and simply put it into this device and just shoot them into your skin. and The DNA will go into the skin and the antigen will be made inside the skin cells and you will get a immune response.

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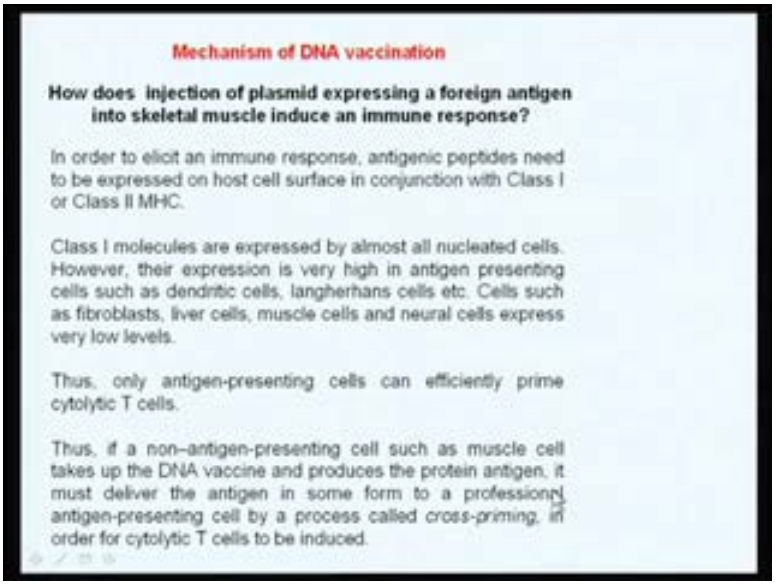

So, we will spend couple of minutes to understand how exactly this DNA vaccine is able to induce an immune response; what is the mechanism by which injecting this plasmids

either into skeletal muscle or shooting them into skin is able to induce an immune response. So, how does injection of plasmid expressing a foreign antigen into skeletal muscle induced an immune response? Now, in order to elicit an immune response, antigenic peptides need to be expressed on the host cell surface in conjunction with class 1 or class 2 MHC. I am sure all students were who studied basic immunology know that this if you is what a cytotoxic T lymphocyte response.

Response The antigens has to be synthesized inside these cells through the intracellular pathway and should be expressed along with class 1 MHC, and only then, you will get a cytotoxic T lymphocyte response. If you want an efficiency antibody response, it has be expressed in conjunction class 2 MHC and then you get a very efficient antibody response. So, class one M class 1 MHC molecules are expressed by almost all nuclear T cells; however, the expression of these class MHC molecules is very high in antigen presenting cells such as dendritic cells, langerhans cells etcetera. whereas, Other somatic cells such as fibroblasts, lever liver cells and muscles cells do not express very high levels class 1 MHC antigens. What it tells you is that, if you introduce your DNA into somatic cells such as skeletal muscle cells, because the muscle cells do not express class 1 MHC at very high levels, the antigen presentation will not be very efficient, whereas, if the plasmid DNA is taken up by professional antigen presenting cells such as langerhans cells, are entitled cells since they express very high levels of MHC class 1, the antigen presentation will be much more efficient. So, only antigen presenting cells can efficiently prime cytolytic T cells, only they can induce cytotoxic T lymphocyte response. So, if a non-antigen presenting cells such as muscle cells take up DNA vaccine and produces the foreign protein or protein antigen, it must deliver this antigen in some form to a professional antigen presenting cells by a process known as cross priming in order for cytotoxic T cells to be induced. So, what is happening? When you inject your plasmid DNA, initially people thought is the muscle cell which is expressing the foreign antigen and is presenting the antigen in conjunction with MHC class 1. MHC,, But, people realized that muscle is not a very good antigen presenting tissue. People then realized that what is happening when you inject a foreign gene, when you make this injection containing foreign DNA, you are actually attracting some of the antigen presenting cells size to the of site of injection and it is these antigen presenting cells which are taking the plasmid DNA. and The foreign antigen is getting expressed inside these presenting cells and these antigen presenting cells now express this foreign antigen in conjunction is with

class 1 MHC. and that is why we are getting very potent cytotoxic T lymphocyte response.

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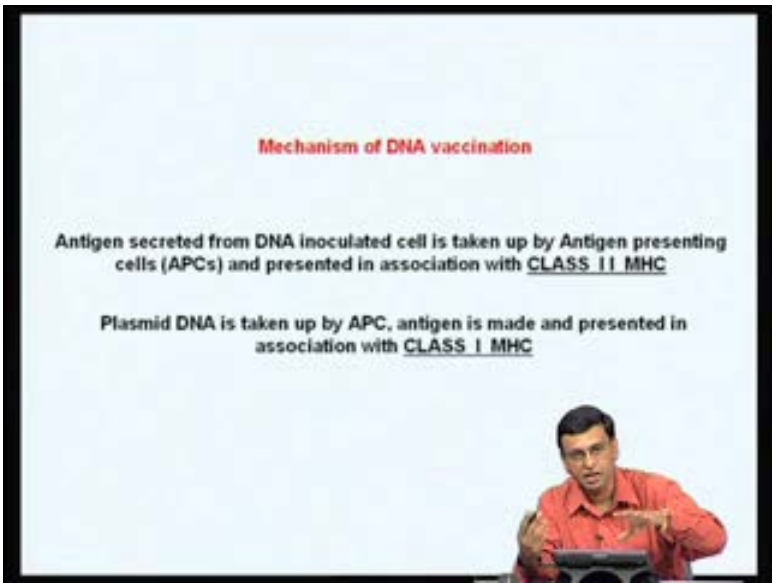

So, what is the mechanism of DNA vaccination? The antigen that is secreted from the DNA D N A inoculated cells is being taken up the antigen presenting cells or the antigen presenting cells are directly transfected with the plasmid DNA, because they can directly take up the plasmid DNA by they can phagocytosis or any other mechanism. and When they present in associated association with class 2 MHC, then you get a very potent immune response. So, it is not directly the muscles cells that is actually responsible for this potent immune response, but it is the cross priming, it is the expression of this foreign antigen by the antigen presenting cells that is a responsible for this potent immune response generated by DNA vaccination.

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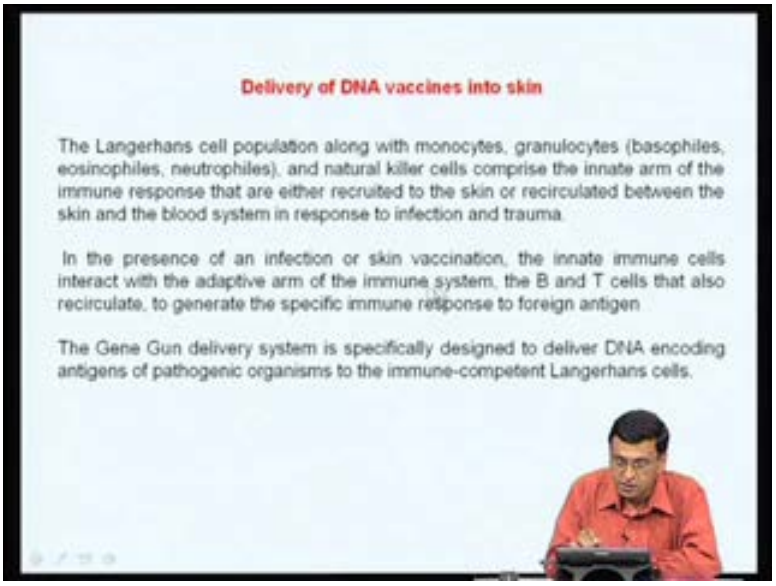

That is why, when you inject this DNA into skin, you're getting much better immune response than injecting into muscle cell. This is because, skin is a much better immune servalent organ compared to skeletal muscle because, number of antigen presenting cells like langerhans cells dendritic cells are present beneath the skin. Because of that, the skin is the first line of defense. Therefore, when you shoot this DNA into skin or when you do an intra dermal immunization rather than the muscular immunization, the chances of your plasmid DNA being taken up by the antigen presenting cells or the chances of the foreign antigen that is secreted from the skin cell and being taken up by this antigen presenting is much higher. So, you get much better antigen presenting cell presentation when you inject your foreign naked DNA into skin rather into skeletal muscle. So, the langerhans cell population along with monocytes, granulocytes and natural killer cells comprise the innate arm of the immune response that are either recruited to the skin or re-circulated between the skin and the blood system in response to infection or trauma; even by injecting, you are actually creating a trauma.

So, in the presence of an infection or a skin vaccination, the innate immune cells interact with the adaptive arm of the immune system, the B and T cells that are also in recirculation, to generate a specific immune response to foreign antigen. So, the gene gun delivery system is specially designed to deliver DNA encoding antigens of pathogenic pathogen organisms to the immune competent langerhans cells. So, that is why, the intradermal immunization or intradermal delivery of genes was found to be

much more better than intra muscular immunization, as far as genetic immunization of DNA vaccine was concerned.

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So, the first paper on DNA vaccine appeared in the year 1993. Since then, as you can see in this slide, the number of papers were published. Actually, to demonstrate whether it is a viral disease or a bacterial disease, parasitic disease or even cancer, if you take the corresponding gene or the relevant gene, put it in expression plasmid and either inject into skin or into muscle, you can evoke a protective immune response in animal models. This was demonstrated in the next 5 years, following the 1993 paper. In fact, more than 42 phase 1 and phase 2 clinical trials in humans were initiated. Because of this euphoria, because all this plasmid DNA vaccination seems to elicit a product protective immune response in animal models such as mice and monkeys and so on and so forth. So, people ask the question, will this magic work in humans? So, human trials were initiated to see will if DNA vaccines work in human beings. A new website called DNA vaccine dot com was initiated and even today, if you go to the website, you can get updated with all the excitement that is going on in the area of DNA vaccination.

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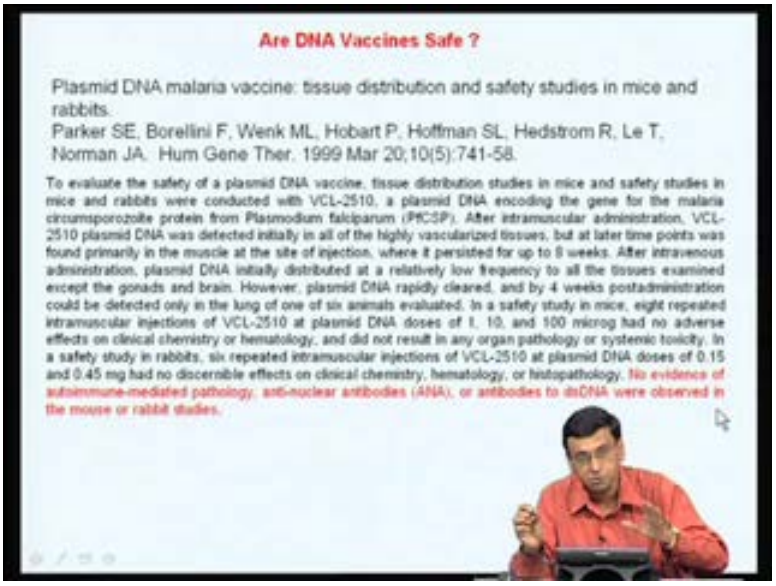

People started asking the question, are DNA vaccine safe? A very systematic study was conducted by this group and was published in the general journal ‘human gene therapy’ in the year 1999 to see how safe is this plasmid DNA immunization is and people were actually shown, I will not go in to the details of the paper, that following the plasmid DNA immunization in mice, there is no evidence for autoimmune mediated pathology, anti nuclear antibodies or antibodies to double stranded DNA were observed in the immunized animal decading deciding that the plasmid DNA immunization is pretty safe.

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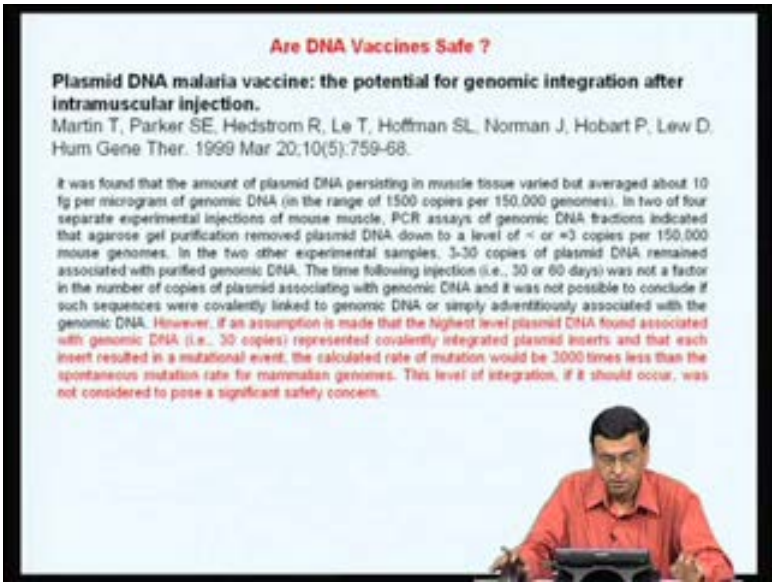

The other major concern that the DNA vaccination had was, when you inject this plasmid DNA into muscle cell or skin cell, will this plasmid DNA go on and integrate into the genome? Remember, in the last class when we were discussing about retroviral mediated gene transfer, one of the major problems of retroviral gene transfer is the random integration of the virus into the chromosome, resulting in the activation of certain oncogenes like l m o two and so on and so forth, leading to leukemia. Now, that was the major drawback of retroviral mediated gene transfer. In the case of gene therapy, in the same way, when you now take plasmid and inject in a muscle cells, people asked the question if this plasmid DNA now goes and injects randomly into the chromosome of this muscle cells or skin cells, can it activate an oncogene or can it inactivate a tumor suppresser gene? As a result, it can have very important harmful effects.

And a detaied study was again carried out and reported in human gene therapy in 1999. The crux of this study, we can read this abstract of this paper, but, what it was shown is that, the chances of this plasmid DNA going and integrating into the chromosomal DNA is 3000 times less than a spontaneous rate of integration, chances of spontaneous integration, indicating that this the chance that you inject a plasmid goes and integrates and as a result causes the disorders or disease is 3000 times less likely to happen than that happens in a very spontaneously. The chances of genomic integration are very very minimum.

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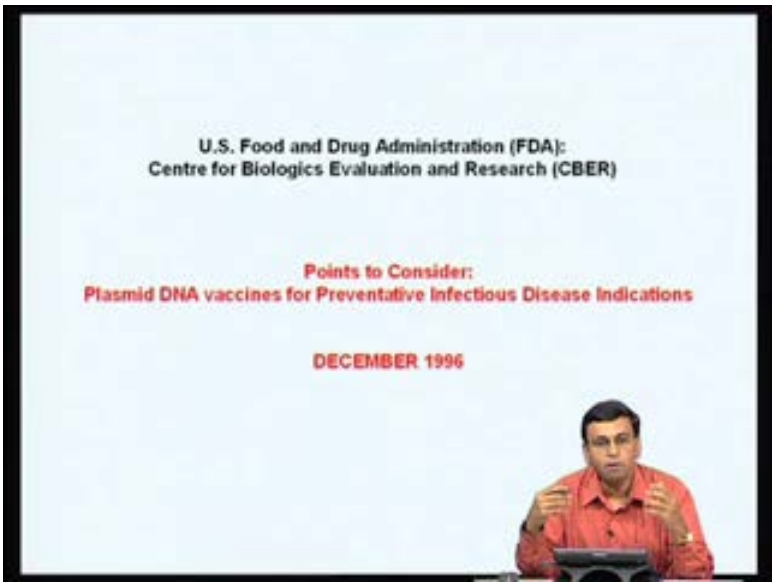

The United States food and drug administration which is the watch dog for giving regulatory approvals for any new drug or a vaccine that comes to the market actually passed the centre for biologics evaluation and research to actually bring out guidelines. So, if somebody wants to develop a DNA vaccine for a particular disease, how should they proceed, what kind of qualities of plasmid in DNA they have to make, what is the manufacturing practice, what kind what is the contaminants that should be the avoided in the plasmid in preparation and so on and so forth. So, this agency came out with a document called points to consider for plasmid DNA vaccines for preventing preventive infective indications in the year 1996. All this these things tell you the seriousness with which researchers as well as companies came in to see whether you can take this new and exciting development of DNA vaccination and make it into a reality. In fact, just 3 years back, in November 2007 the centre for biological evaluation and research of the food and drug administration of nitro United States came out of the with another new sets of the guidance for the industry to entitle consideration for plasmid DNA vaccines for infectious disease indications; They revised the guidelines and came up with this is the way you have to make plasmid DNA if you want to inject in to human beings and bring this DNA vaccine as a product into the market.

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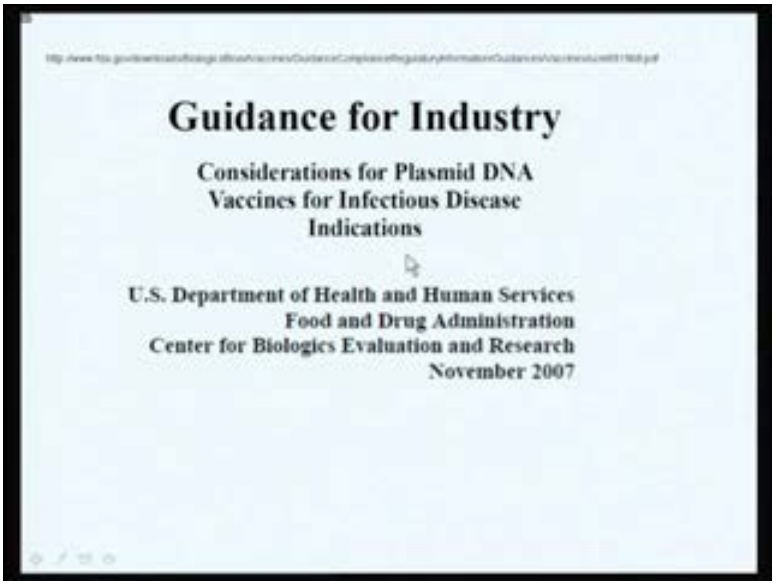

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Many exciting things happened, companies actually started using all these guidelines and see where can they actually make a clinical great plasmid DNA. So, if for example, if I am interested in making a DNA vaccine for malaria or DNA vaccine for hepatitis B, I clone this antigen into plasmid, go to this companies and say can you please make using the guide lines given by u s f d a USFDA or the centre for biological u s US machine, can you use what is called as a good manufacturing practices and make plasmid DNA in a pure form so that they can be used for human clinical trail? So, a new industry came up of down to see how you can manufacture plasmid DNA in large amounts, because you require grams and milligrams amount of DNA if you have to inject into humans. So, the entire plasmid DNA in a manufacture has to be scaled up. So, huge normal methods of plasmid DNA in a purification that was developed to see how you can purify plasmid in a large scale using fermenters and using novel downstream process. So, a new industry came up following this very simple in a gene delivery technique. I will not go into the details; a number of guide lines came up to see what kind of manufacturing practices are needed to be make if you have to make a DNA vaccine for human use, what kind of safety precautions one has to take if you have to examine this DNA vaccine in humans, what all the parameters that you have to be checked if you have to convince the regulatory authorities that is the DNA vaccine is actually safe. I will not again go into the details, all guide lines were actually formulated and these are all taken from this documents, which were actually prepared from by the centre for biological evaluation and research, CBER c b e r.

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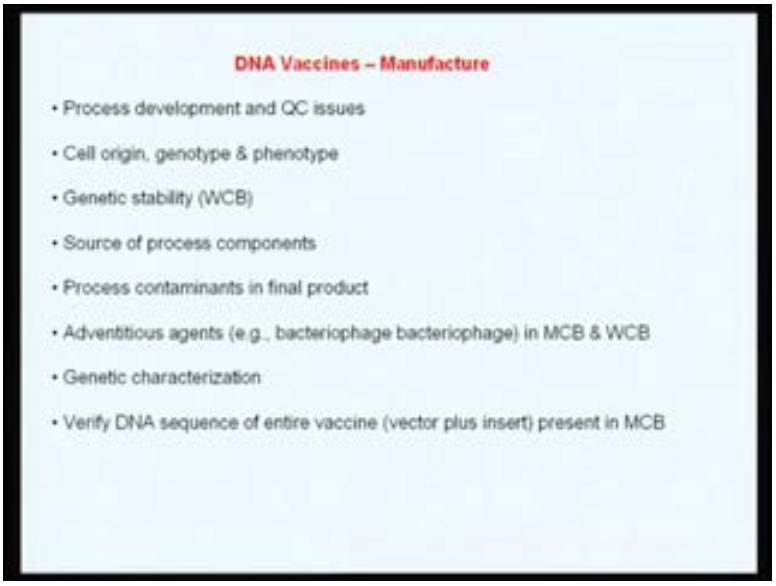

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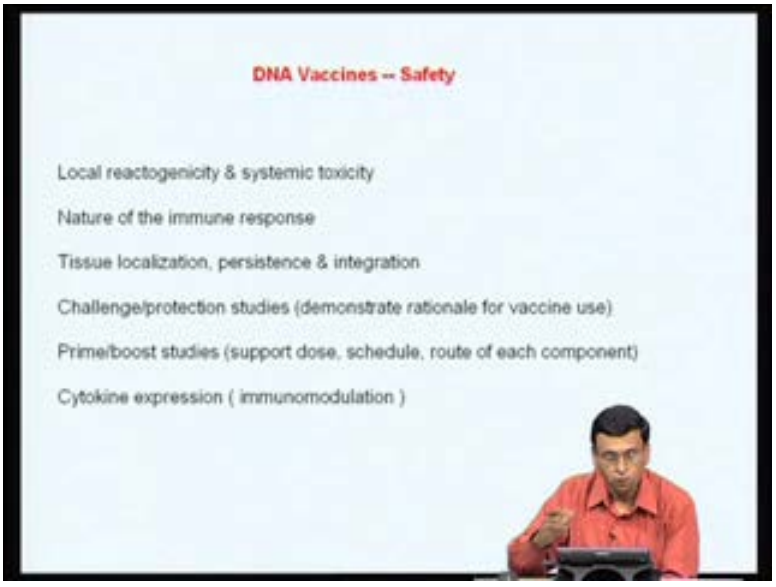

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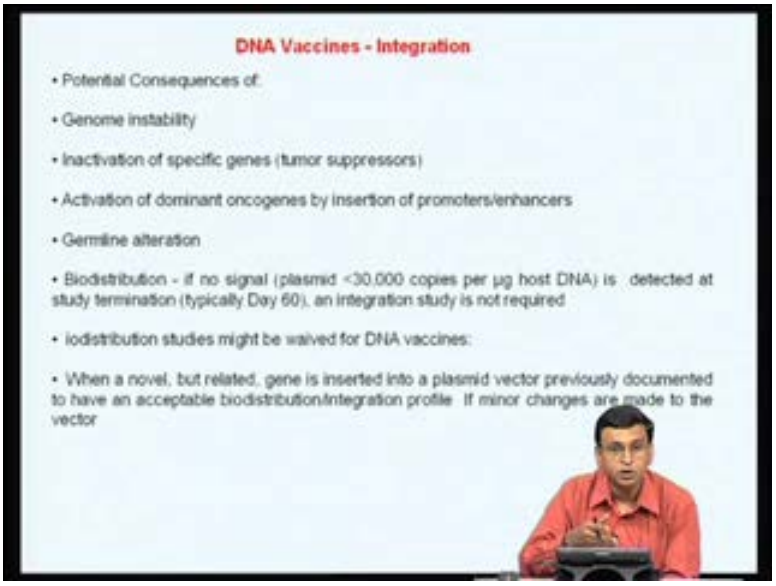

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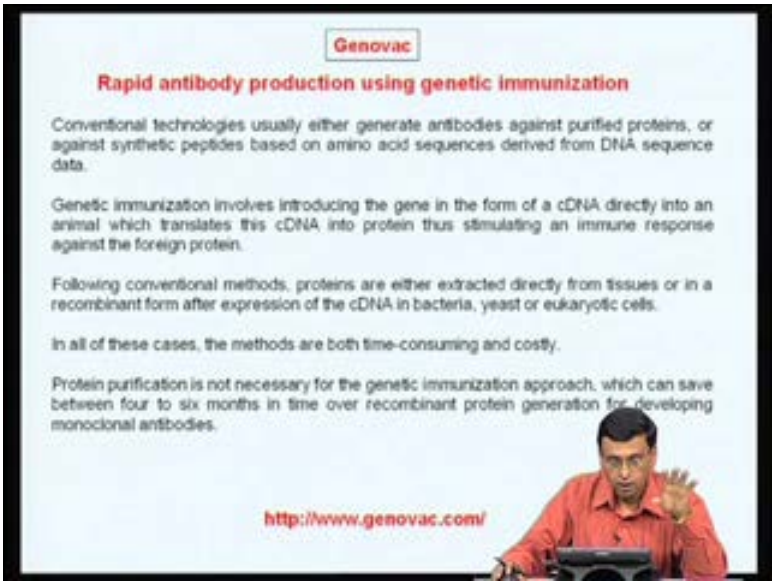

So, other interesting things that are happened following this discovery of DNA vaccination or genetic immunization is that, people thought companies like genovac came up with this idea of that, we will make, suppose if you want to make an antibody for a particular gene, particular protein, you do not have to go and then express this protein, clone this gene into bacterial vector or purify the protein mix it with an adjuvant adjunct and then immunize animals for its antibodies. They said, we will use the genetic immunization procedure and make antibodies and give it to you. This created lot of excitement. Rapid antibody production using genetic immunization, that means, you do

not have to express this proteins and in bacteria, you do not have to mix them in its adjuvant and then you do not have to raise antibodies. You can directly inject immunize animals with these genes and you can make antibodies, which has tremendous scope for basic research, especially in this era of human genome being sequenced that the number of genes, new genes being discovered. We still do not know the function of many of these genes. So, one of the first thing that we want to understand the function of genes is that, you need to make antibodies against in this proteins and see where are this these proteins getting expressed, what kind of proteins this proteins is interacting with. For all these things, antibodies are very very important tools to understand the function of normal genes. So, using this approach, what it told is that, if you have a new gene and if you want to make an antibody for a protein, called gene you do not have to take this gene, you express this gene into organisms like bacteria, purify the protein and then use it for immune response. You can simply take the gene and directly put in a eukaryotic expression plasmid, immunized mice or rabbit and you can generate a antibodies. So, a new company called genovac came up with a number of advantages to see how genetic immunization can be directly be used for rapid antibody production. I gave it just a generalize scheme, one can go the website of the company to get more details as how you can directly introduce antibody production by genetic immunization using mice or rabbits.

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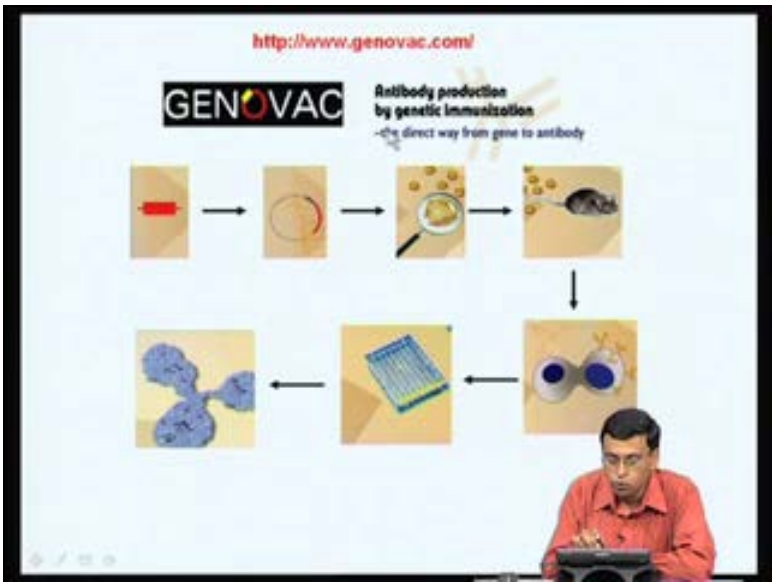

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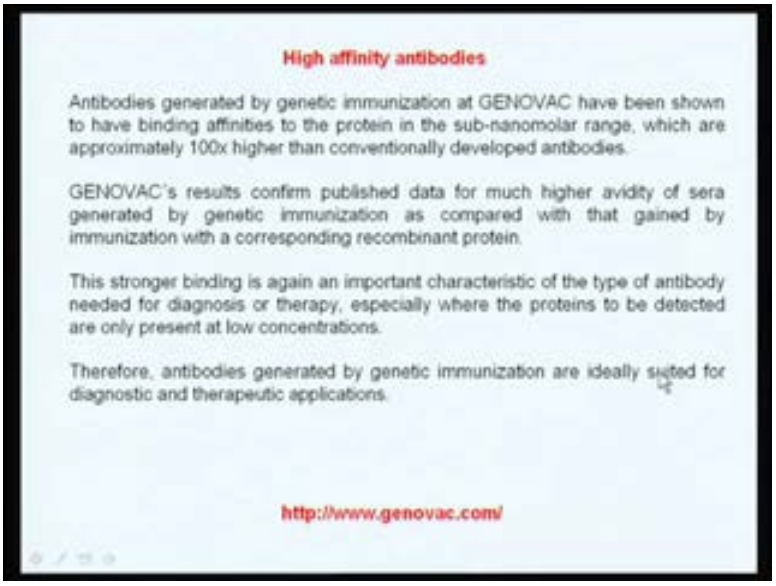

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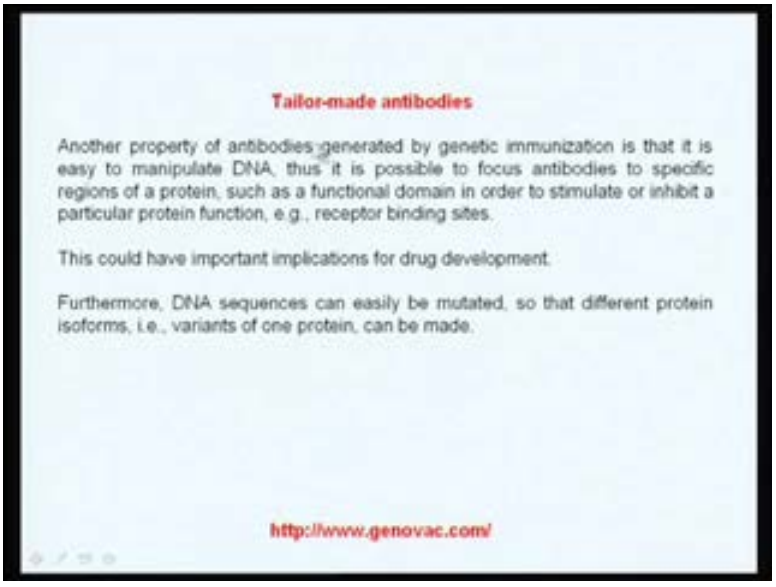

The company actually demonstrated that, you can actually demonstrate and develop very high affinity antibodies using the DNA immunization procedure and these antibodies generated by the genetic immunization are ideally suited for diagnostic and therapeutic applications. The other important advantage of this kind of a genetic immunization approach is that, you can make tailor-made antibodies. For example, if you want only a small antibodies made against the very small region of a protein, you can just delete that, you can clone only that particular region of the gene and express it by a put it putting in an expression vector and you will get only antibodies only agreeing in that particular

epitopes. or You can stitch different epitopes of a protein, put them together and see whether you can develop antibodies only agreeing those epitopes. all By using genetic engineering cloning techniques, you can do all kinds of manipulations, genetic manipulations and you can get tailor-made antibodies for any numbers of epitopes that you want. You can stitch various epitopes together and put those gene epitope genes encoding epitopes together and you can generate antibodies for this multi epitope based vaccines. So, tremendous excitement was there in the by this discovery of this DNA vaccination. In fact, there is a very nice review that is actually says genetic immunization for antibody generation in research animals by intravenous delivery of plasmid DNA that came up in the general journal called 'bio techniques' in February 2006. and One can read this review to understand the scope of using this kind of a genetic immunization approach for inducing an immune response and generating an antibodies.

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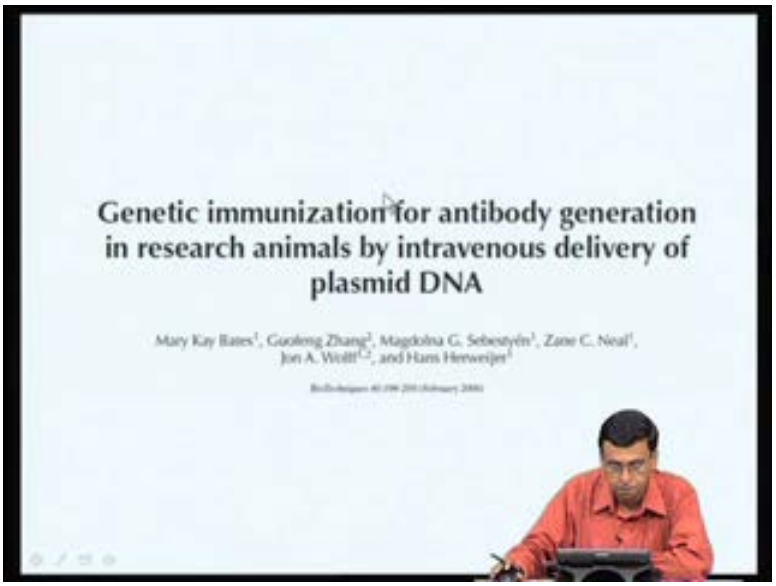

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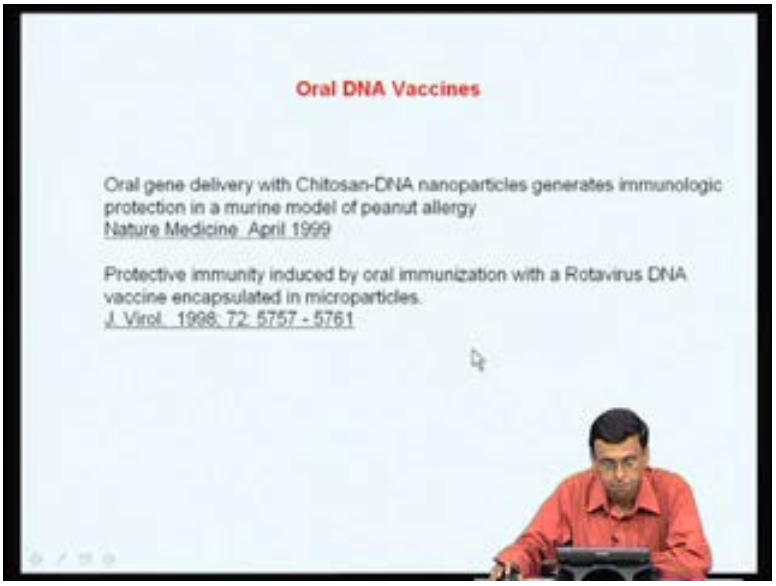

Again people came up with variation of this basic technique. Instead of injecting DNA into skin or injecting DNA into muscle, why cannot you take the DNA by oral route? over all root. So, two papers I am just coding quoting it as example, that were published in 1980 1980-99 overall gene delivery of chitosan DNA nano particles generates immunological protection in a marine or this peanut allergy. In another case, for a rotavirus productive immunity induced by overall immunization of rotavirus DNA vaccine encapsulated in micro particles. So, you do not need biolistics, you do not need gene gun immunization, you do not have to inject plasmid DNA. If you simply swallow DNA complex with certain cytosomes or micro particles, it will be taken through the overall root oral route and you can get an immune response. So, overall oral immunization using plasmid DNA encoding foreign antigens is possible.

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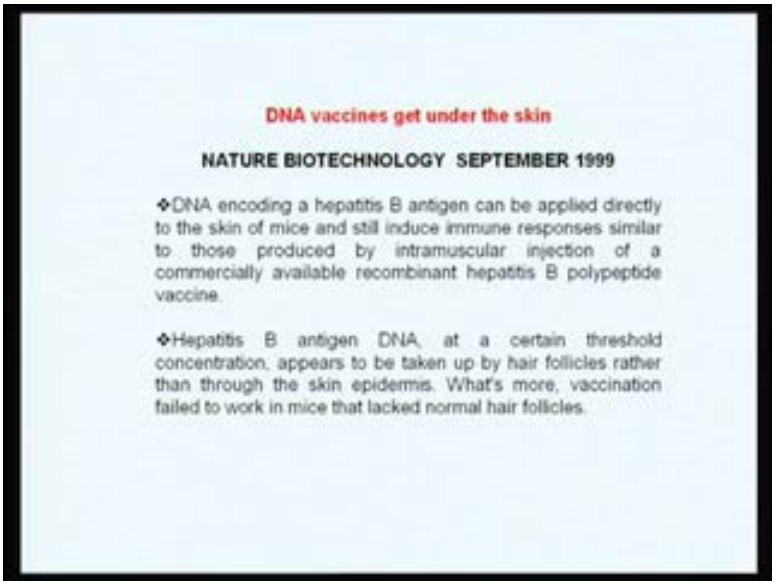

Another very interesting paper came in 1999 saying that, actually DNA is actually taken through the force in the skin. If you have mice which do not have the hair follicles, they said you do not get an immune response, but if we have mice which are have hair follicles, you get a much a better immune response, indicating that when you immunized through the intra-dermal root, the DNA is actually taken up through the hair follicles and you can get a robust immunization immune response indicating that this route of the route of DNA delivery is through the hair follicles of the skin rather than the other routes roots.

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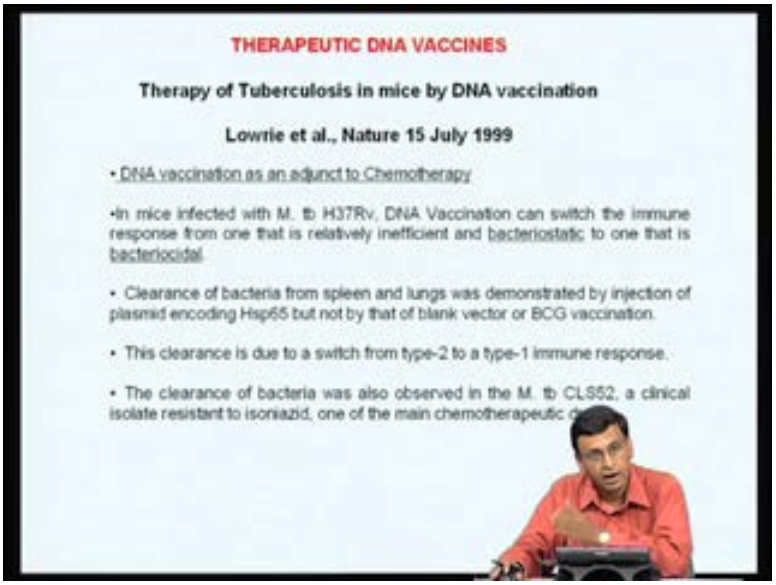

I think I will not go to the details of therapeutic DNA vaccines. People thought DNA vaccination in combination with chemotherapy can be used for treating diseases like tuberculosis, this is like tuber closes again, a proof of principle paper was published in 'Nature' in 1999, DNA vaccination as an adjust adjuvant through chemotherapy. Again, I will not go into the details, but, what this tells you is that the excitement that was generated by this very simple and versatile technique. Simply take a gene, put in a equate expression plasmid, dissolve in if you now simply inject, you can get the antigen can be expressed even though at low levels. But, it is good enough to induce a protective immune response. It have has generated a tremendous excitement between 1993 and till date, tremendous amount of this is going on, in this area of research.

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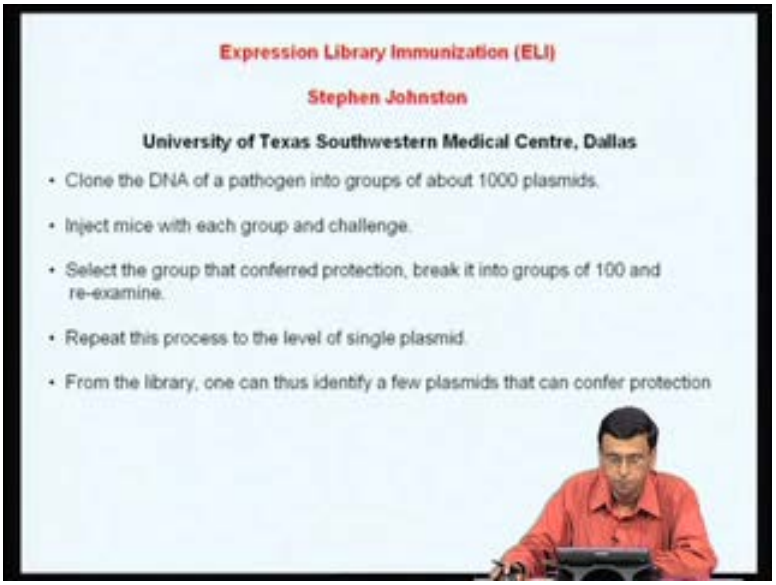

I will not go into the details, again Stephen Johnston came with this concept what is called as a expression library immunization, where, for example, if I want to make a DNA vaccine from malaria, I do not know what are the protective antigens. What he says is that simply cDNA library in a mammalian expression vector and simply inject this serial in library into the mice or make batches. Take 10 10 different C D in a cDNA clones and immunize and then see which one of this C D and then, if you challenge this mice and then see which one of this C cDNA when expressed, can induce a productive immune response. So, by this, you can identify naval novel pr, otective antigens for complex dieses diseases like malaria, HIV, into Huber closes and tuberculosis, called as an expression library immunization.

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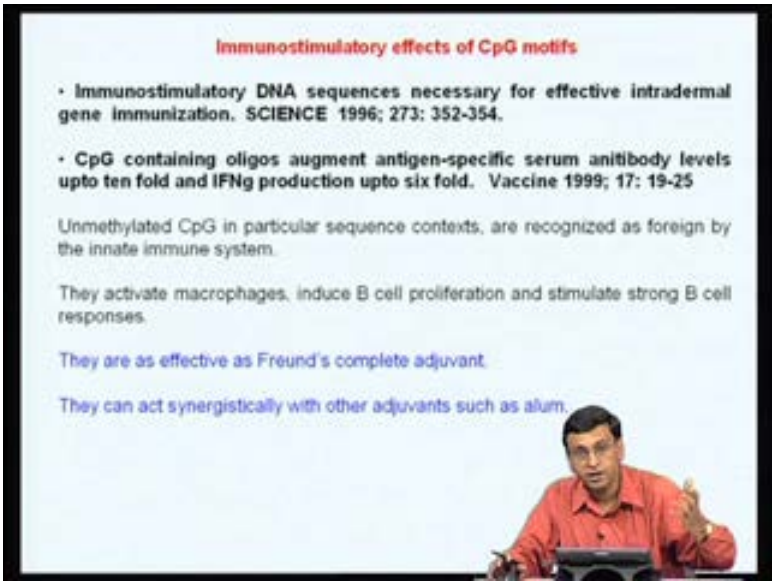

People again came up, it is a very very interesting finding, that these CpG sequences which are present in the bacterial vector can actually act as immune stimulator motives, because, these CpG sequences in the bacterial DNA or not methylated whereas, the mammalian CpG sequences are maculated methylated. So, when you inject a plasmid DNA containing non-maculated non-methylated CpG, these non-maculated non-methylated CpG sequences can actually act as an immune stimulant remotives; from again, this initiated a new area of research in the area of adjuvants. People thought, these immune stimulatory motifs of the CpG sequences can actually be as effective as friend's Freund's complete adjuvant or alum. So, a new area of adjuvant research was initiated indicating that these immune stimulatory sequences or DNA sequences can be used as adjuvants.

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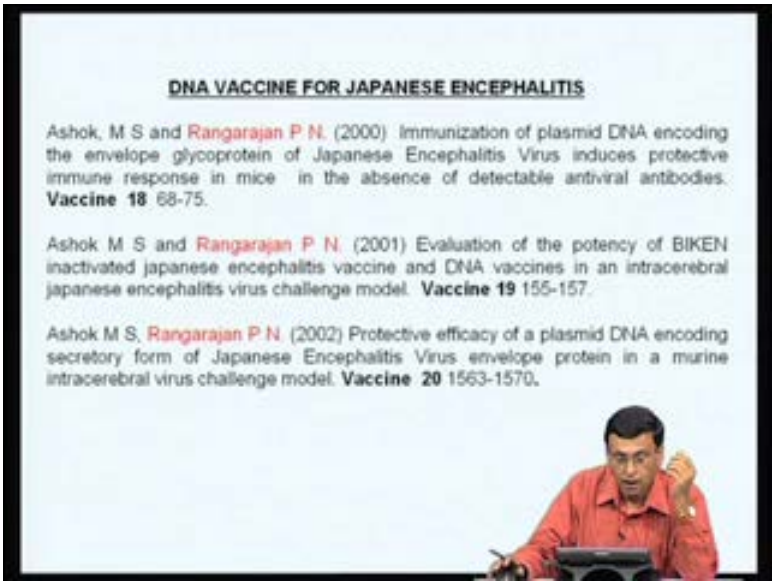

Our own lab with at Indian institute of science demonstrated in principle, that you can actually develop DNA vaccines for Japanese encephalitis. I will not go into the details, the results are published in 'vaccine'. We also tried to develop a DNA vaccine for rabies in collaboration with a company called Indian neurological limited in Hyderabad in India. And In fact, regulatory procedures are currently on and hopefully a product will come out sometime in the near feature, for a new form of a DNA based rabies vaccine for veterinary use entailed entitled as dinarab.

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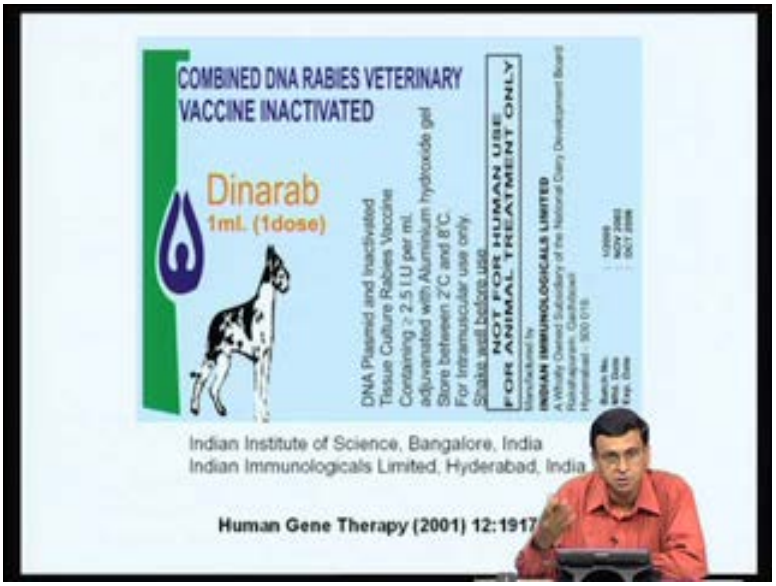

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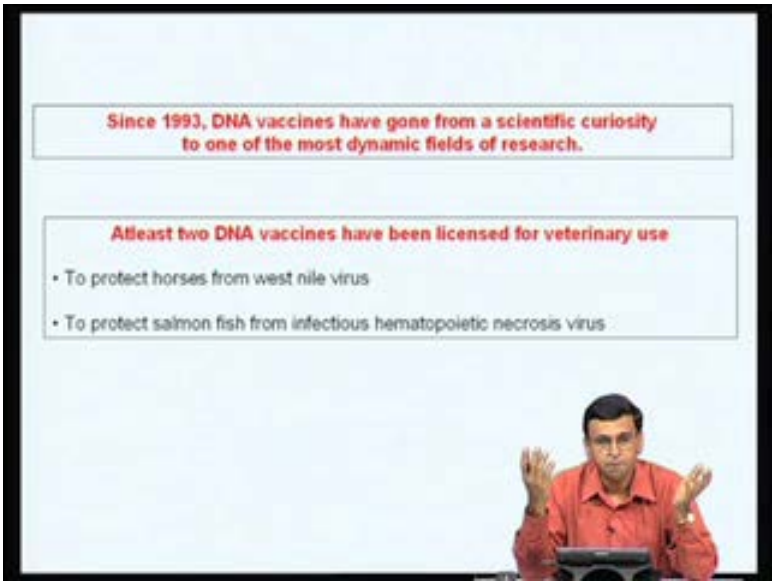

So, an area of research which began in the year 1993, the first paper on DNA vaccine was published in 1993 created an entire. It created a lot of eforea euphoria and lot of optimism. But, a question comes in addition to all this publications, and all this eforea euphoria, did any DNA vaccines really came out into the market? As I speak, today at least 2 DNA vaccines have been licensed, but both are for veterinary use. 1 DNA vaccine has been licensed for use in horses for protection against West Nile virus and another DNA vaccine has been licensed for protection against salmon fish.

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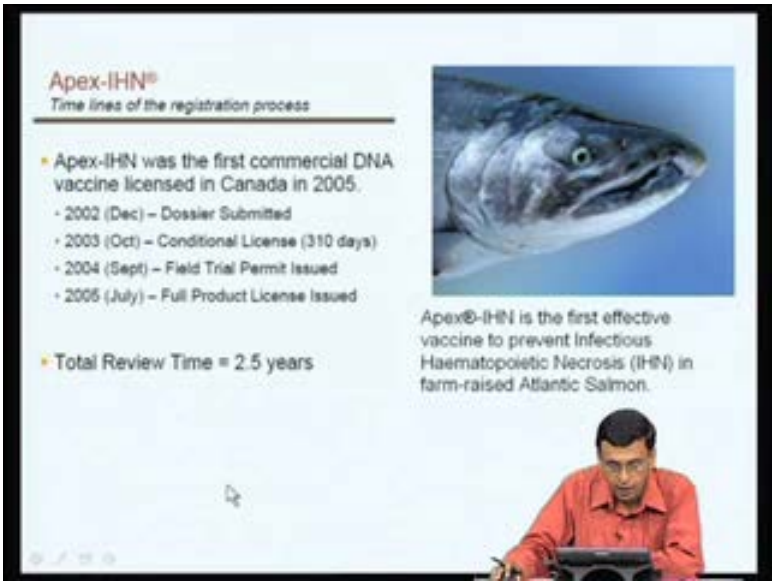

So, this is the DNA vaccine, West Nile DNA vaccine called as West Nile innovator DNA, was brought out by company called 4 bridge, a division of voith and another DNA vaccine called as apex IHN. A Canadian company brought out this vaccine against a viral disease against the fish. So, at least 2 DNA vaccines are currently in the market and hopefully our vaccine will be third, if we get the regulatory approvals for bringing into the market.

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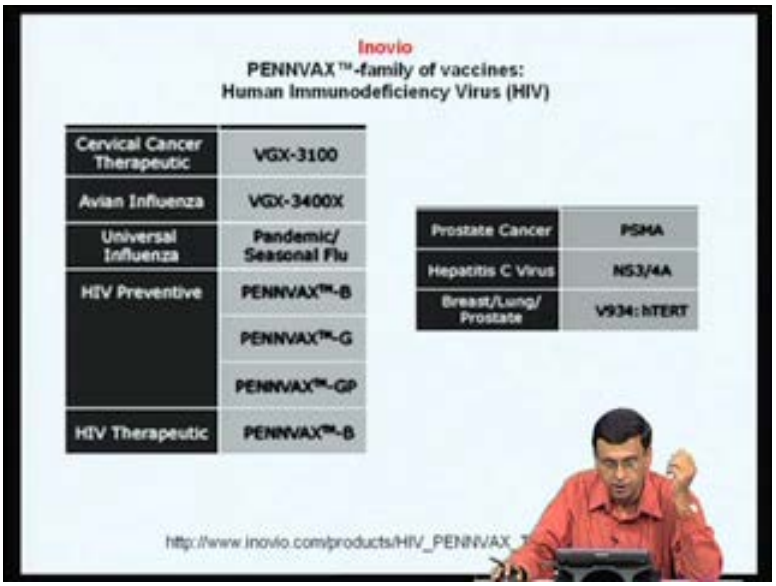

A number of companies are now trying to use this principle of DNA vaccinations to see if we can we develop vaccines for disease like cervical cancer, Influenza, HIV, hepatitis C, cancer and so on and so forth. I will not go into details, a company called innovio for example, once used this DNA basic technology to see where is you could develop vaccines for all these diseases, one can go to the website and look in, to get more details. A company called powderject came up with very small devices to see if can you inject yourself, can we have DNA vaccine cartridges you can immunize yourself; tremendous excitement was initiated.

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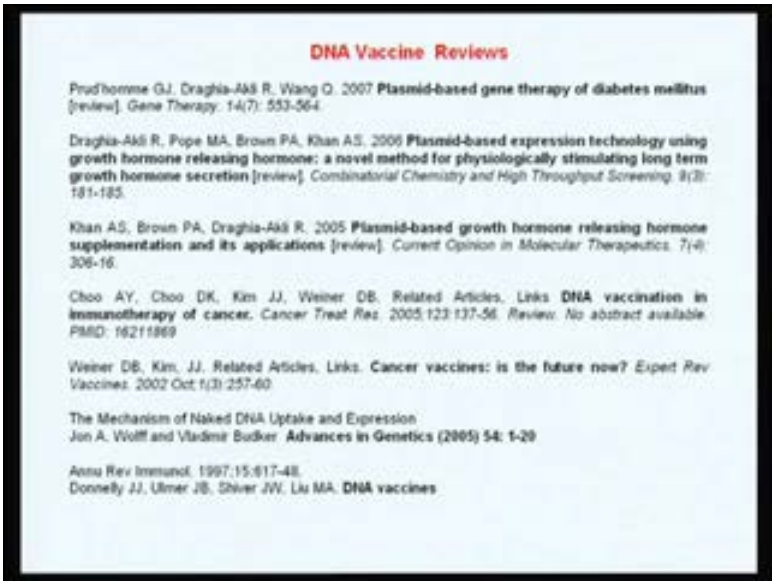

So, the purpose of this lecture, what I tried to tell you in the last one hour is to see how a very simple DNA delivery technique which failed as a gene therapy technique, but found a new innovation and found a new use as a novel form of inducing immune response in animals and humans. So, a technique which failed as a gene therapy technique found very new applications in the form of DNA vaccination or genetic immunization and how in the last 17 years, from 1993 to till today, how a whole new area of a research was developed based on this very simple gene delivery and gene expression technique. and At least two products are currently in the market, both for veterinary diseases and hopefully some DNA vaccines for human use will soon be approved in the years to come. So, I have listed a number of review articles one can actually go through and then enlighten yourself more about the DNA vaccines; very nice review articles are there.

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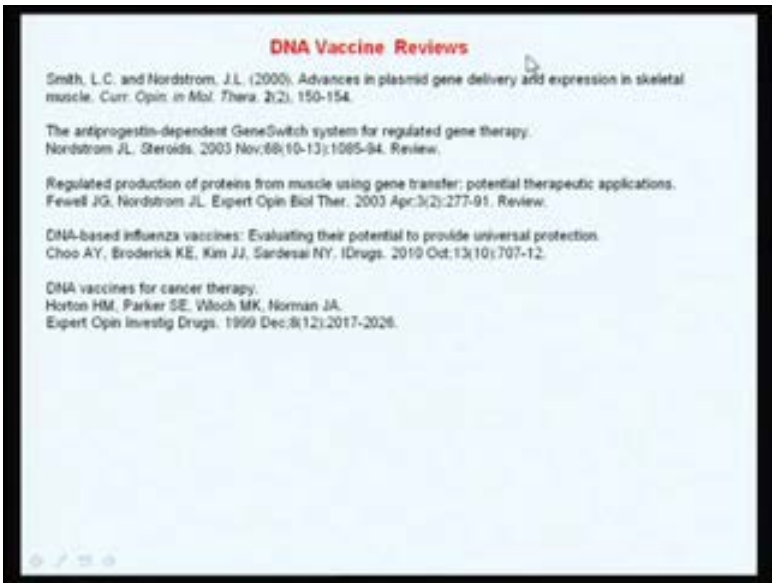

And one can read up many of these review articles and see how people are trying to develop DNA vaccines. In additional, if you go and visit websites like the DNA vaccine dot com, you can get more and more information of how this very simple, versatile technology of gene delivery and gene expression has tremendous promise of a developing into a novel form of vaccination for animals and humans, thank you.