

# **Development of a DNA Vaccination for Ebola Hemorrhagic Fever Using the VP40 Epitope**

## **Literature Review :**

### **Introduction**

In human medicine, there are a large number of disease with various serious and deadly consequences upon infection. One of the diseases with the most notorious public image is Ebola virus, a viral hemorrhagic fever with African and Asian origins. However, despite its well-known status in popular culture and high rate of mortality, there is still no effective vaccine against this pathogen. The main population at risk for this disease is the low income populations in Africa, though a serious danger is also posed to researchers who work with primates. In the interests of protecting both these already unfortunate populations and ensuring the safety of research workers, we have chosen to pursue a vaccine to create immunity to Ebola virus.

### **Disease Mechanism**

Ebola hemorrhagic fever multiplies in the body and systemically involves the liver, lymphoid organs, kidneys, blood vessels, heart, stomach, and intestine. The cell types initially attacked by the infection include the phagocytes, endothelial cells and hepatocytes. This virus has 17 potential *N*-linked glycosylation sites on its surface containing seven genes that code for proteins (Alazar-Dany et al, 2006). The production of these proteins leads to an increase in permeability of the blood vessels in the tissues. In addition, virus infection leads to an increase in inflammatory cytokine production, which leads to massive systemic inflammation that destroys the body's tissues. Internal bleeding throughout the entire body follows as fluids leave the blood vessels. Both large and small forms of the glycoprotein are secreted from cells infected by Ebola and are encoded by the same gene, but have different cellular targets (Alazar-Dany et al, 2006). The smaller protein binds to the neutrophil receptors, leading to a failure of their function and limiting of the immune response. The large proteins bind to the receptors on the endothelial cells. This binding permits the protein spikes to enter and infect the cells, where replication takes place in the cytoplasm (Harper et al, 2007).

### **History & Treatment**

Ebola hemorrhagic fever was first located around the Ebola river valley in Zaire around 1976. Since that time there have been sporadic outbreaks. These outbreaks have occurred in the Democratic Republic of Congo, Gabon, Sudan, the Ivory Coast, Uganda and the Republic of Congo. Ebola hemorrhagic fever is believed to infect no certain population demographics other than the central region of Africa. (Sullivan et al, 2003)

The Ebola virus currently has no effective vaccine for humans. However there has been proof of a successful antiviral intervention against Ebola in non-human primates. Research is still being done to determine why the intervention works in non-human primates and not in humans is still being analyzed. (Sullivan et al, 2003) Currently the treatments for Ebola are considered the standard. The treatment includes balancing patient fluids and electrolytes, maintaining the patient's oxygen status and blood pressure and treating any opportunistic infections that might arise while the immune system is functioning poorly. These procedures may seem ineffective because the mortality rate in humans is around 80% and as of now there is no evidence on why some people infected with the virus recover with no problems and others are not able to ward off the disease.

## **Ebola Virus Structure**

Ebola virus is one of the two members of the family *Filoviridae*, along with Marburg virus, which cause hemorrhagic fevers in humans and non-human primates. The virus particles are encapsulated in a lipid membrane derived when budding from the host cell, which have a consistent diameter of 80 nm but may vary in length.

Ebola virus is a negative stranded RNA virus, containing one single-stranded 18.9kb molecule of RNA per virion. The virus genome encodes eight proteins, which are found in two separate areas of the virus structure. The core ribonucleoprotein complex is made up of the proteins NP, VP35, VP30, and RNA-dependent RNA polymerase, as well as the RNA genome. The lipid envelope contains the rest of the proteins, namely GP, sGP, VP40, and VP24. These eight proteins contain a total of ten genes: 3' non-coding leader, nucleoprotein, virion protein (VP) 35, VP40, glycoprotein (GP1), GP2, VP 30, VP 24, RNA-dependent RNA polymerase, and 5' non-coding trailer. (Hoenen et al, 2006)

As of February 2007, six of the Ebola virus proteins have had their structures evaluated and submitted to the the Protein Data Bank (PDB). They are VP40 (PDB accession number 1ES6), GP2 (PDB: 1EBO, 2EBO), VP40 (N-terminal domain) + RNA (PDB: 1H2C, 1H2D) and VP30 (PDB: 2I8B). (Hoenen et al, 2006) Since the crystal structures of VP30, VP40, and GP2 are the only ones currently available, it is most logical to revolve a structural analysis of Ebola virus around these proteins.

The protein VP40 has a total of three structures available for reference, as it appears in a variety of forms. While VP40 is a monomer in solution it has been shown to also have hexameric and octameric forms, both of which are important in the viral life cycle. (Nguyen et al, 2005). It is a matrix protein, primarily responsible for the characteristic filamentous shape of filoviruses. It is also strongly associated with budding and virion assembly. (Hartlieb et al, 2006) Most important to this vaccine proposal, a 2005 study revealed that treatment with VP40 and glycoprotein alone were enough to prevent a lethal infection of Ebola virus in rodents. Studies of this model in non-human primates are underway, although there are no published results of these to date. (Warfield et al, 2005)

GP2 has two crystal structures available for study. It is located in the virion envelope, and is a glycoprotein. Studies have shown that GP2 is important in increasing host cell membrane permeability, and since there is such a large amount of cell death in an Ebola infection this implies a great role of GP2 in pathogenicity. (Han et al, 2006)

The final protein available for study, VP30, is located in the core of the virion. VP30 is a vital Ebola virus transcription factor. For this reason, its inhibition has been a target for anti-viral drugs. One promising study successfully prevented VP30 oligomerization, thus inhibiting Ebola virus replication. (Hartlieb et al, 2003)

Further research investigating individual proteins and their implications in virulence has identified several key components of Ebola virus pathogenesis and some additional potential vaccine epitopes. For example, mutations in NP and VP24 have been associated with increased virulence in mouse and guinea pig models, making them potential anti-viral targets. (Hartlieb et al, 2003) VP24, specifically, has been shown to block interferon signaling, partially disabling innate immunity.

It should be noted that the virulence of Ebola makes a live-attenuated virus dangerous to any person, so a less risky alternative must be sought. Based on these concerns, a VP40-GP vaccine is likely a very valuable lead to follow. Inactivated vaccines have not to date had any significant success, nor have any recombinant virus or DNA vaccines. (Hoenen et al, 2006) The present research on the structure of Ebola viral proteins implicates VP40 very strongly as a vaccine target.

## **Epidemiology**

The epidemiology of infection by the Ebola virus has been well researched and documented due to the seriousness of the disease's effects. Through investigation, four distinct strains of the virus have been isolated, and the cause of the outbreaks associated with them defined. The strains include two which have proven lethal to humans, the Sudan and Zaire Ebola viruses, along with two strains that may infect humans but have not been known to cause fatalities, known as the Ivory Coast and Reston Ebola viruses. (Cohen, 2004)

The Ebola virus incubation is generally found to be five to ten days, though cases have been observed for as short as two and as long as 21 days. No natural reservoir of the virus has yet been identified, though humans are often exposed through live and deceased monkeys. (Sullivan et al, 2003) Testing by purposeful infection of a variety of animals with Ebola found that certain bats were capable of carrying and replicating the virus without showing any clinical signs or symptoms. In addition, it is hypothesized that dogs may act as a reservoir for the virus, though none have been found naturally infected in the wild.

Transmission of the virus occurs mainly in the later stages, when the patient is suffering severe hemorrhaging, diarrhea and vomiting. Contact with these bodily fluids is an easy way to be exposed to and infected by the virus. In some cases, contact with mucous membranes and skin has also been known to transmit the virus. (Sande, 2007) Airborne transmission has occurred in monkeys, but is viewed as unlikely in human cases.

Prevention of secondary transmission, human to human, has been proven to be a fairly simple affair. Sterilization of instruments and facilities, along with basic hygiene practices has proven effective in avoiding infection. Many of the outbreaks observed for this virus occurred in sub-optimal hospital conditions, many through the re-use of needles. (Aitken et al, 2001) Nurses often become infected through neglect of barrier nursing techniques, such as wearing protective clothing and patient isolation, and then may pass the disease on to their other patients. In contrast, prevention of primary infection is extremely difficult due to the large amount of uncertainty surrounding Ebola in nature. Without positive identification of the natural reservoir of the disease, it is impossible to reliably avoid contact with infected animals. (Weir et al, 2001) This also makes it difficult to track or control the virus spread.

Each strain of the Ebola virus previously mentioned has had at least one notable outbreak. In the case of the Zaire strain, the most deadly of the four, the first recorded outbreak occurred in Yambuku, Zaire in 1976. A person who had been traveling returned to Yambuku, and was incorrectly diagnosed with malaria. In the course of his treatment, he was given an antibiotic shot, and the needle was then used to inoculate other patients. (Aitken et al, 2001) This lack of sterile procedure led to the infection of many other people. Further spread came through the nurses' lack of protection and hygiene, along with burial procedures of the deceased that included flushing of the digestive tract and washing of the corpse.

The second strain that has caused human fatalities, the Sudan Ebola virus, was first detected in 1976 as well, in the city of Nzara, Sudan. Cotton factory workers were the first infected, but the main host for the outbreak was a nightclub owner. (Galbraith et al, 1980) During his treatment at a local hospital, staff and patients were infected through, once again, lack of hygiene and use of infected instruments on multiple patients. This strain of the virus has most recently been found in Yambio County, Sudan, in 2004.

The first of the non-lethal strains is the Reston Ebola virus. This is very deadly in the crab-eating monkey, native to the Philippines. This is the only Ebola strain with a non-African origin, and is distinct from the other three serotypes. It was imported to the United States through infected animals, and researchers in contact with the monkeys later tested positive for the virus, though no deaths occurred. This particular serotype has had multiple outbreaks starting from 1989 in Reston, Virginia, USA. (Peters, 2007) Later cases were recorded in 1990 in Virginia and Texas, then in 1992 in Sienna, Italy, followed by a final outbreak in 1996 in Texas once again. All these outbreaks occurred in

populations of monkeys being used for research.

The final type of Ebola virus is known as Ivory Coast Ebola virus. Only one human case has occurred, in a researcher who performed necropsies on the bodies of chimps found deceased in the Tai Forest of Cote d'Ivoire in Africa. Dead chimps were found through 1994, and the virus was isolated from their tissues. (Aitken et al, 2001) The human case was not a fatality, with symptoms similar to Dengue fever and complete recovery occurring within six weeks.

## **Vaccine Description**

### **Vaccine Type:**

A vaccine for Ebola Hemorrhagic Fever is needed because of its continued emergence. In this case, the researchers chose to create a DNA vaccine because contains modified and inactivated genes from the Ebola virus but no infectious material. This is essential because Ebola is such a dangerous disease that any sort of infectious materials would be far too dangerous to use as a vaccine. Because Ebola virus replicates in the body at such a high rate, host immune defenses and protein synthesis of the infected cells overwhelm the inflammatory systems and adaptive immune response to the infection. The vaccine contains three plasmids, which are closed loops of the DNA, one each encoding the internal nucleoprotein and the surface glycoprotein of the virus.

At the same time, some cell types are targets of the infection. (Sullivan, 2007). With a DNA vaccine, after identifying a single gene from a pathogen and artificially copying and multiplying it, the gene is then injected into the muscle. Muscle cells take up the injected genes and integrate it into their genome, leading to eventual transcription of the product the gene depicts. The [immune system](#) will recognize the product produced by the host cell as foreign respond to it just like it would in any infection or vaccination. In this vaccine, it was decided to segregate the VP40 gene of the Ebola virus. This gene, unique to Ebola, will stimulate an immune memory to the virus and increased response to actual infection in the future. (Oxford, 2006).

### **Administration & Adjuvants:**

In the administration of a DNA vaccine, an initial injection of cardiotoxin diluted with 0.9% NaCl sterile saline must be given. The toxin, by causing some minor muscular damage, leads to a healing process that allows the DNA of the vaccine to be more readily taken in by the host's cells. The saline is required not only as a diluent, but also to maintain the osmotic pressure of the area, so that cells are not unnecessarily damaged or destroyed. By administering this mild toxin approximately five days before giving the DNA vaccine, optimal results can be expected.

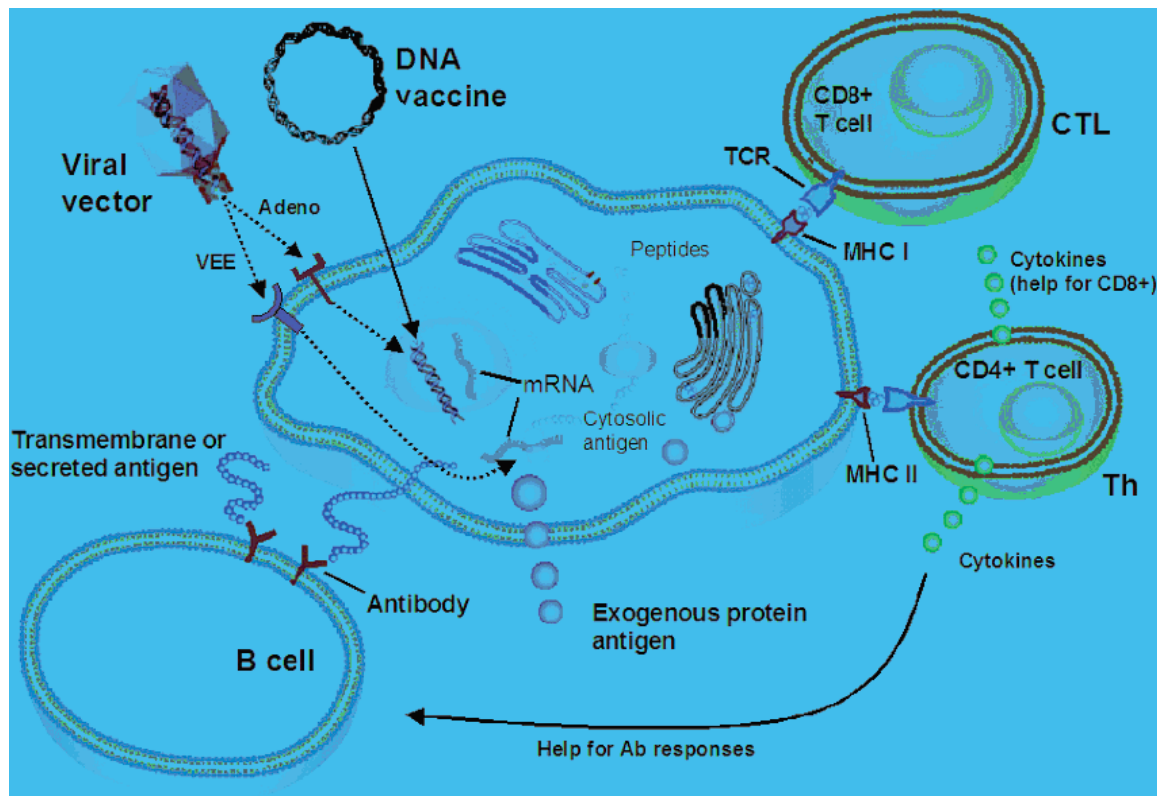
### **Creation of Immunity:**

The DNA vaccine itself consists of DNA plasmids, circular loops of genetic material, containing the VP40 gene from Ebola virus. These plasmids, while carrying the desired gene, also act as the adjuvant to stimulate B cells. These are injected into the subject in a solution of phosphate buffered saline (PBS). The plasmids consist of DNA that is massive enough to be detected and phagocytized by macrophages, which can continue on to present parts of the plasmid, including the VP40 gene potentially, on class II MHC to B cells specific for that antigen. The plasmids that are taken in by the host muscle cells will adopt the DNA within, integrating it into their own genome. In those cells, the VP40 gene will be expressed, transcribed and the protein itself will then appear in the cell's membrane on MHC Class I. This protein will still appear as foreign to the immune system, stimulating

a response by T cells to what appears to be a virus infected cell.

Intramuscular injection of the vaccine has been shown to lead to those cells taking up the DNA and, as the plasmid has been altered to contain a eukaryotic promoter, will induce transcription in the nucleus and translation to VP40 in the host cell cytoplasm. This should lead to both T and B cell immunity. Class I MHC will present the VP40 on the muscle cells, just as with all other intercellular proteins. Professional antigen presenting cells also take up the DNA, particularly dendritic cells or Langerhan's cells in the skin. These cells, once presenting VP40, can activate cytotoxic T cells in the thymus. Once activated, these cells can then recognize any infected cells presenting VP40 on their MHC CI. The strong cytotoxic T cell response to DNA vaccines is a notable staple of their function and is particularly attractive, therefore, in a vaccine against viral pathogens such as Ebola virus. It is additionally favorable to other types of vaccines since the intracellular production of antigen, followed by MHC CI presentation of it, mimics a real viral infection, thus providing a better immunity. (Hyugen, 2005)

Antibody production is a result of cross-priming in the dendritic cells (DCs). Once DCs present the VP40 antigen on MHC Class II, they can activate helper T cells in the thymus, which then induces B cells to proliferate and produce antibodies. Since VP40 is a glycoprotein, the activation of B cells is vital as this is an antigen they are very likely to encounter in the event of infection. DNA vaccines have not shown the striking B cell response that they have for CD8+ cells, however there has been evidence that both these pathways are being activated by DNA vaccines. A DNA vaccine to HBV has been shown to elicit both CD8+ T cell immunity as well as antibody production. (Hanke, 2006).



**Figure 1.** (Bråve , 2007) Viral vectors access the cell by specific receptors and replicate or directly express their proteins intracellularly. As examples, the VEE vector, an RNA virus, enters through its cellular receptors and expresses its encoded protein in the cytoplasm. The adenovector, a DNA virus, enters through other receptors; its nucleic acids go to the nucleus, but the genome does not integrate. Plasmid DNA enters the cell nucleus inefficiently when simply injected or more effectively via

electroporation. The encoded proteins are then expressed, secreted for activation of B-cells and antibody production, or presented as peptides that bind to MHC class I or class II for cellular immune activation.

### **Problems & Risks:**

The DNA vaccine is a very promising vaccination, but like all other vaccinations there may be some problems that can occur. One of the biggest problems is that the vaccine may cause an autoimmune disease to develop. The definition of autoimmune disease is a disease caused by the reaction of a host antibody to substances that occur naturally in the body. During the stages of developing a vaccine avoiding this problem is a huge concern. The reason that this could be a problem in the fight against the Ebola virus is because of the speed at which Ebola is contracted. If a patient becomes autoimmune the vaccine may help against the Ebola virus, but the immune system may then be unable to recognize common pathogens that affect our immune system. This would be due to the body's responses being completely overwhelmed by the constant reaction to its own cells. If this were to occur then the subjects who showed symptoms of autoimmune disease wouldn't be able to ward off simple viruses like the common cold, for example.

Another problem that occurs in DNA vaccines is the possibility of insertional mutagenesis. Insertional mutagenesis could occur if the introduced DNA integrates improperly into the host genome. If this process were to occur it could cause what is known as carcinogenesis, which causes the cell's oncogenes to be turned on. When oncogenes are turned on they cause the cells to become malignant, producing a tumor from uncontrolled division. In order for this vaccine to work all of these problems have to be addressed from all directions.

In order to minimize these problems that may occur with DNA vaccines several tests should be done. These tests should include tests on the subject's immune system, for example measuring antibodies and antigens. The subjects should be injected with a test antigen to elicit an immune response and then the subject should be injected with the same antigen days later and the antibody titers should be measured. The healthy immune system should elicit a quicker and larger immune response after second exposure. Doing these tests will show whether the subjects immune system will be able to handle the DNA vaccine.

In order to maximize the effectiveness of this DNA vaccine post immunization tests have to be completed. Repeatedly measuring the subject's progress as far as production of antibodies and measuring of the overall symptoms that the subject is experiencing in order to improve the vaccine over time. The reason that this is important is because viruses change over time in order to be able to continue to infect cells, and if the progress of the subjects that have been given the vaccine isn't monitored it makes it very difficult to improve the vaccine to prevent certain symptoms or long-term damage. This will also provide valuable information about the duration of immunity created by the vaccine, allowing the development of a vaccination schedule to maintain immunity.

### **Immunity Assessment & Vaccine Trial:**

#### **Administration:**

The vaccine will be administered as an intramuscular injection of plasmids containing the Ebola VP40 gene. This is essential as the muscle cells are required to adopt the injected viral DNA into their own genome. The DNA will then be expressed by those cells, leading to an immune response. Additional plasmids in the intracellular environment will be identified and phagocytized by immune cells.

## **Antibody Production:**

A series of ELISA tests will be performed to verify antibody production, and to determine which isotopes will be made. The 96-well plate will first be incubated with purified VP40, also having a set of wells with a negative control of no antigen. The primary antibodies will be from serum samples from the study subjects, as well as a positive control of purified VP40 and a negative control of serum of a subject never exposed to the vaccine or Ebola virus. The secondary antibody used, which will bind to the primary as a marker, will be one of the following: anti- $\mu$  chain, anti- $\alpha$  chain, anti- $\delta$  chain, anti- $\epsilon$  chain, and anti- $\gamma$  chain. The secondary antibodies will be covalently linked to a color-changing reagent, to then detect which wells (and thus, subjects) test positively for each type of antibody. It is hypothesized that there will be IgM and IgG especially.

Antibodies will be most effective in preventing an infection by opsonizing free viral particles and preventing binding and entry into host cells. VP40 is a surface protein, so the antibodies should be effective in binding VP40 in vivo. This job would be primarily performed by IgA and IgG, and in the circulation this job is performed by IgM. The activity of IgM is perhaps the most important, since the viral titers of Ebola virus in the blood are exhibited to be very high. It is not ethical to test the effectiveness of the vaccine by infecting human subjects with the virus as it would be with an animal model. It is then proposed that to test the effectiveness, an analogy to the TB skin test could be utilized. Injection of purified VP40 is harmless, but should elicit an immune response visible as inflammation in the area of injection. If infection is shown to be blocked in an animal model, particularly if it is proven in a non-human primate, and a skin test in the human subject is positive after vaccination, it can be correlated that an effective humoral response is being mounted in the subject.

## **B Cell Immunity Evaluation:**

The initial test of antibody titers would take place two weeks after vaccination, with an additional test taking place at four to eight weeks. Based on previous research, it has been found that two weeks are required for the beginning of an immune response to a DNA vaccine, with titers increasing until up to eight weeks later. These titers would be acquired using a simple ELISA test, with plates incubated with the VP40 epitope exposed to test patient serum. Secondary antibodies would be specific to the variety of antibodies produced, most likely IgG and IgA in the initial test at 2 weeks and IgM in the later test. This is very similar to the test for antibody type, except in that these tests will be identifying the actual titer of the VP40 antibodies present. Concentrations of antibodies to the Ebola VP40 would indicate the level of the immune response being initiated by the vaccine. These antibodies are produced by B cells activated by exposure to the pathogen, so antibody presence and titer is a strong indication of B cell immunity.

## **T Cell Immunity Evaluation:**

There are several tests that measure different levels of cells and their effectiveness. For Ebola virus a test that would be very useful is one that shows the levels of cytokines. A good test is the ELISA test. It measures the frequencies that are present, the type of cytokine that is present and the amount of the cytokine, through creating a secondary antibody that is specific for the cytokine. This test can be very useful in the Ebola virus in order to determine if these cytokines have the capability of affected the behavior of the infected cells in the body. If the ELISA test shows low cytokine activity there may be a possibility that the subjects immune response isn't noticing the foreign antigens and stimulating an immune response where these cytokines can be made. If the levels of cytokines are high then the body is producing these proteins to ward off the infection to the best of its capabilities. The

ELISA test is a very easy test to conduct and the results are extremely helpful in the treatment of the subject and evaluation of vaccine efficacy.

Another test that is important with a DNA vaccine is one that measures the level of memory cells and one that measures the effectiveness of those cells. A good test for that is simply called CD4 count, which can sufficiently show the strength of the immune system. The test is conducted using a blood sample from the subject, looking at the high or low levels of the memory cells. If the levels are high then the viral load is low, which allows the body to elicit an effective immune response. If the memory cell level is low then the viral load is greater than the defense cells ability to rid the body of infection. The CD4 count test is very useful in almost all viruses that are associated with the DNA.

Both of these tests are capable of providing prompt results, due to short experiment time and high accuracy. These results are in depth and show exactly what the body's immune response is doing. For the best results for both of these tests they have to be done continuously in order to determine the status of the patient. It is best if these tests are done soon after the vaccination in order to show the improvement of the subject over time. If these levels get considerably more toward the expected results over time then the body is able elicit an immune response that is accurate to rid this disease. Testing for these cytokines and the effectiveness of memory cells is important after any vaccination. The results of these tests may also make it possible for researchers to improve current vaccines as the body becomes immune to the current DNA vaccination for Ebola or the Ebola virus changes, by allowing such changes to be identified and monitored.

### **Problems & Trial Assessment:**

The Ebola virus infection usually occurs within 14 to 21 days. With induced cytopathic effects, new information has been presented about the immune responses and pathogenesis because this virus does not display a high degree of variability like other viruses utilize to evade host immunity. However, the glycoprotein of the Ebola virus modifies the target cell function, which then illustrates a perfect strategy for immune evasion. A disruption occurs in inflammatory cell function due to the cytotoxic effects of the glycoprotein on both the endothelial cell and macrophage function. By disrupting the expression of the cell surface of immune recognition molecules and adhesion proteins, Ebola virus can interrupt the processes needed for cytolytic T cell function and immune activation. After two months of lethally challenged injection, strong immunity with both nucleoprotein and glycoprotein immunization was shown. After four months, immunity from nucleoprotein was not retained and good immunity from the glycoprotein vaccines was noted. After examination, immunization fully neutralized the infection, which means no traces of the Ebola virus were found as well as any toxicity and evident side effects.

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**Figures:**

Figure 1: Vaccine Delivery Methods Using Viral Vectors, Andreas Bråve, Karl Ljungberg, Britta Wahren, and Margaret A. Liu *Mol. Pharmaceutics*; 2007; 4(1) pp 18 – 32