

Ebola DNA Recombinant Vaccine for the Zaire Strain Targeting African Populations Exposed to the Virus

Literature Review:

Ebola hemorrhagic fever is a deadly disease resulting from infection by Ebolavirus (EBOV). This negative stranded RNA virus belongs to the *Filoviridae* family and has four subspecies, three of which infect humans. The *Reston ebolavirus* strain originates in Asia and has never been reported to cause disease in humans. Among the remaining strains, *Zaire ebolavirus*, *Sudan ebolavirus*, and *Ivory Coast ebolavirus*, *Z. ebolavirus* (ZEBOV) is the most virulent and can cause mortality rates as high as 90% (Hoenen 2006). Symptoms of Ebola hemorrhagic fever appear in infected individuals after about a week of viral incubation and in severe cases death can occur within a few days (Pourrut 2005). Zoonotic transmission of the virus is most often a consequence of contact with a various species of monkey carcasses while transmission between humans is facilitated through direct person-person contact (blood, semen, secretions, or organs), contaminated needles/syringes, and aerosol droplets (Pourrut 2005,). Oddly enough, aerosol transmission does not appear to be the main route of transmission in humans unlike many other infectious diseases (OLS, 2002).

Ebola is one of the most severe viral infections that human can catch and the mortality rate have been known to reach 80-90% in outbreaks (Bray 2005). Ebola specifically affects Africa where it infects human and nonhuman primates. The virus outbreaks not only cause a sense of panic and fear but they also have had an effect on the socioeconomic status of the country itself. EBOV was first observed in 1976 in Sudan and the Congo; there were over 500 cases, including at least 350 deaths. Since then, the following outbreaks have occurred, all on the African continent: 1979, in Sudan; 1995, in Democratic Rep. of the Congo; 2000, in Uganda; 2001-2002, in Democratic Rep. of the Congo. The natural reservoir of the disease has yet to be identified and humans are at this point are expected to be the only accidental host (OLS, 2002). Currently there is no antiviral treatment or vaccine and the outbreaks are unpredictable, have increased in frequency, and in some areas it appears to be an endemic. The infection presents itself with flu-like symptoms that soon progress to fever, severe vomiting and diarrhea, bleeding from severe blood coagulation defects, decreased blood pressure, hypotensive shock, and eventually the collapse of the circulatory system. Death typically occurs within one to two weeks after symptoms occur unless the person falls in the 10-20% that show clinical improvement around the second week and eventually recover from the infection (Bray 2005).

The structure of EBOV plays an important role in the virus' pathogenicity. Each virion is rod shaped, measures about 80nm in diameter, and varies in length but on average are about 1250nm long (Hoenen 2006). The central core of the virus is known as the ribonucleoprotein (RNP) complex and is comprised of a helically wound single-stranded, negative sense RNA that codes for seven genes. Along with the RNA, the RNP complex contains nucleoprotein (NP), virion protein 35 (VP35), virion protein 30 (VP30) and RNA-dependant RNA-polymerase (L). Surrounding the RNP complex is a lipid envelope which contains the remaining coded proteins: glycoprotein (GP_{1,2}), virion protein 40 (VP40), and virion protein 24 (VP24) (Hoenen 2006). The glycoprotein units

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are responsible for receptor binding, virus-cell membrane fusion, and viral entry, making this protein a key component in the virus' pathogenicity (Manicassamy 2005).

EBOV enters the body through the mucosa and lesions in the skin and begins infection by targeting macrophages and dendritic cells (DCs). Several different proteins are utilized to act as cellular attachment molecules for binding to target cells. Various different types of C-type lectins and integrin receptors which target DCs, macrophages, liver, and lymph node cells have been speculated as cellular attachment molecules making EBOV effective for binding to several cell types (Hoenen 2006).

Innate immune response to EBOV infection involves several responding cell types that are either stimulated or inhibited when the pathogen is introduced. Initial infection causes an inflammatory response alongside a cytokine production. In addition, interferon (IFN) response is crucial to defending against this virus and mice models have shown that a lack in IFN signaling leads to death. EBOV suppresses responses to IFN- α and IFN- γ by using VP24 to block IFN signaling and VP35 to block phosphorylation of IFN-regulatory factor 3, which is a transcription factor for IFN production. DC infection also plays a significant role in immune response (Feng 2007). After infection of these cells, DCs can no longer produce pro-inflammatory cytokines or express molecules, such as CD80 or CD86, which support T-cell proliferation. In addition to IFN and DC infection, natural killer (NK) cell number drops during the first few days. While these cells are not directly infected with the virus, the decline in numbers hinders the usual perforin and granzyme release from NK cells (Geisbert TW 2000).

Adaptive immune response to EBOV infection also has an important role and has been shown to differ in the nonfatal and fatal cases. In nonfatal cases of EBOV, patients present with specific IgM antibodies early on in infection followed by IgG antibodies a few days later. Fatal cases have a low percentage of IgM and no IgG antibodies are detected for EBOV. T-cell activation in fatal cases has been found to decrease significantly prior to death and CD4⁺ and CD8⁺ lymphocytes undergo "bystander apoptosis" even though they are not directly infected with the virus. Due to comparisons between fatal and nonfatal cases, it has been suggested that the response to the virus early in infection determines the outcome of the disease; early inflammatory response usually results in survival (Hoenen 2006).

Currently the treatment for Ebola is primarily supportive including intravenous-fluid replacement, administration of pain relievers, and typical nursing measures. At this time there are no specific antiviral drugs to treat Ebola, however, there are several experimental approaches that have demonstrated some success (Hoenen 2006).

The main target for current vaccine development thus far has been the glycoprotein because it is the only protein that has been encoded by the Ebola genome and proven that it has cytotoxic effects. The first successful DNA vaccine for Ebola was tested in guinea pigs. The animals were immunized with plasmid DNA that had the Ebola virus glycoprotein encoded. None of the control animals survived, and the survival rate of those that were immunized showed a correlation with the antibody titer. DNA vaccines have definitely been useful and effective in rodents, however, they have been found to be less effective in various primates and demonstrate an even lower affectivity in humans (Nabel 2003).

Recombinant vaccines have also been considered, although there are numerous concerns about the safety of a recombinant Ebola vaccine for humans. As of now there

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are single vaccines that have been shown to protect for twenty-eight days after vaccination, and even more recently there was a recombinant vaccine developed that will protect guinea pigs from the Ebola infection after a single intranasal infection (Nabel 2003).

There have been numerous methods used to improve the immune response in primates which involve the use of DNA immunization that are followed by a boost of adenovirus. In one study macaques were immunized against Ebola with a plasmid DNA containing the Ebola glycoprotein gene and then they were boosted a few months later the recombinant adenovirus that expressed the identical protein. The study resulted in all four vaccinated macaques surviving and it was the first time a vaccine was produced that protects against Ebola infection in primates. A large downfall to vaccines based on adenoviruses, however, is that pre-existing immunity can compromise the efficacy (Nabel 2003).

Due to over expression of transcription factor (TF) causes the development of disseminated intravascular coagulation (DIC) there have been efforts put into discovering a method of inhibiting this process. There are a lot of concerns in using anticoagulants when treating hemorrhagic fever disease, yet a recombinant nematode anticoagulant protein c2 can be administered up to twenty-four hours post infection and has demonstrated a 33% survival rate in what would normally be a lethal infection (Hoenen 2006).

Finally, Passive immunization is another possible consideration for the treatment of hemorrhagic fevers although the usefulness in treating Ebola specifically is unclear. During a specific outbreak in Kikwit in 1995 sera from recovering Ebola patients was used to transfuse into severely ill patients that had hemorrhagic fever symptoms due to an infection and all but one of the patients survived. It is not known what stage they were in or what types of additional care they may have received (Hoenen 2006).

Ebola has been suggested to be a bioterrorist threat but because infected individuals die within 6-10 days, spread of the disease is less likely to occur. Since the symptoms are of excess bleeding and secretions, it is easily transferable to individuals who come into contact with a sick patient. With the advent of vaccine therapy against EBOV it would be suggested that those who live in the tropical rainforest ecosystem of Africa and who are health care workers or interact with patients infected with Ebola should also be required to become vaccinated.

Description of Vaccine

An effective vaccine against EBOV will induce both humoral and cell-mediated immune responses without serious side effects. An intramuscular vaccination consisting of a multigene plasmid DNA serum will be given to target populations. The actual vaccine will consist of EBOV-specific epitopes derived from the glycoproteins (GPs) of the virus. These GPs have been modified by deleting genes in the transmembrane region in order to eliminate the cytotoxic effects produced by normal length GPs. In addition to the deletions in the coding regions for the GPs, insertions into the gene sequence were added to increase the expression of such proteins on human cells. The GP region is

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inserted into a bacterial plasmid, each consisting of several genes coding for these viral protein parts (Martin 2006). When the serum is injected into the cells, they are transported to the nucleus of the cell and undergo transcription of the DNA, followed by translation of the sequence into proteins. Therefore, it is the host cell that is manufacturing large quantities of the viral proteins which initiate an immune response (Nabel 2003).

In order to help stimulate adaptive immunity, an adjuvant known as an immune stimulatory complex will be added to the vaccine. It will consist of a matrix of Quil A with subunits GP1 and GP2 of Ebola's enveloped glycoprotein. Quil A is a detergent whose small micelles will contain the glycoprotein subunits. Once in these micelles, the subunits can fuse to an antigen-presenting cell, allowing them to enter the cell's cytosol. Fusing to the antigen-presenting cell is less complicated for the subunits, since the detergent will convert the soluble protein antigens into particulate material, which is easier for an antigen-presenting cell to digest. The antigen-presenting cell then travels to the nearest lymph node and stimulates a response to the viral protein. This response will be very similar to the response created by a virus infected cell. Once in the lymph node, cytotoxic T cells specific for Ebola glycoprotein will be created. Similarly, helper T cells will be activated in this manner as well, leading to the stimulation of B cells that will produce antibodies against the virus. Additionally, macrophages will also be stimulated by the glycoprotein subunits and antigen-specific helper T cells. Aluminum hydroxide gel will also be present in the adjuvant composition, in order to delay the release of antigen and enhance macrophage uptake. This is important because if the entire antigen is released too quickly, macrophages and neutrophils will bind to it, and the glycoprotein won't have a chance to travel to the lymph system to induce antibody production. As for the enhanced macrophage uptake, this will provide a stronger innate response and increase the chances of an antigen-presenting cell fusing to the glycoprotein.

Ebola's glycoprotein is produced by transcriptional editing, which involves adding an Adenine residue into a combination of seven other Adenine residues (Dowling, 2007). This group encodes for a secreted protein, and with additional editing, GP is produced. The glycoprotein is then cleaved by a furin-like enzyme in the Golgi network into two subunits, GP1 and GP2. Through disulfide bonding, the two subunits bind, and the complex binds to the viral membrane (Dowling, 2007).

Glycoprotein is highly glycosylated, and it has been suggested that this might mask epitopes that could otherwise be neutralized. This is because the glycosylated region creates steric hindrance, which prevents neutralizing antibodies from binding (Dowling, 2007). One study found that by altering or removing an N-linked glycosylation site in the GP2 subunit (amino acid 565), the glycoprotein's antigenicity was decreased. This was because the mutation prevented disulfide bonding between GP1 and GP2. When GP1 and GP2 cannot bind, the antigenic conformation of glycoprotein cannot be formed (Dowling, 2007). After observing the success of mutating glycoprotein, the proposed vaccine will contain a glycoprotein possessing the described N-linked glycosylation mutation.

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Today, no current Ebola vaccine has been approved due to the fear of the virus mutating back to an infectious strain. Usually this fear arises when a live-attenuated virus vaccine is injected because since the virus is still alive, it can continue to replicate. While the strain may contain several mutations in genes encoding important proteins, the chance that the virus could continue to mutate and turn back into the pathogenic state is possible (Janeway, 2005). However, this risk has been limited because the proposed vaccine is a DNA recombinant vaccine, which will only use mutated glycoproteins inserted in bacterial plasmids. The mutated glycoproteins will contain deletions in the transmembrane region and mutations in the subunits. The transmembrane region deletions, in theory, will limit the cellular toxicity that is seen when wild type glycoprotein is used (Martin, 2006). Similarly, the *E. coli* DNA plasmids used are incapable of replicating in animal cells. Hence, if the improbable occurred and the glycoproteins mutated, the cell would not allow the generation of the virus within it (Martin, 2006). Furthermore, a similar DNA vaccine for HIV was based on the same type of design and principles, and it proved to be safe and immunogenic in healthy individuals (Martin, 2006).

Additionally, since Ebola's glycoprotein possesses high antigenicity, there is a valid fear that the glycoprotein subunits used in the adjuvant and plasmids could bind and become virulent. However, by removing the N-linked glycosylation site in the GP2 subunit, no disulfide bonding can occur between the GP1 and GP2 subunits; therefore, the antigenic form of glycoprotein cannot be produced (Dowling, 2007).

Previous studies in animals have confirmed the use of plasmid-based vaccines to increase immunogenicity against EBOV. A study involving cynomolgus macaques that were injected intramuscularly with a GP plasmid based vaccine and vectored by an adenovirus, were shown to have induced protection against a lethal challenge of Ebola virus. This study deleted portions coding for the GP transmembrane in order to eliminate the cytopathic effects that the wild type have been speculated to express. Even with this deletion, the humoral and cellular immune responses were strong and induced a successful response when challenged. GP-specific IgG titers were observed along with high CD8⁺ T cell numbers in vaccinated animals as compared to controls (Sullivan 2006). Another study conducted with a chimpanzee adenovirus-based vaccine vector was tested in guinea pig models. When vaccinated guinea pigs were challenged with a lethal dose of EBOV, complete protection against the Ebola strain was observed. The animals showed no clinical signs of sickness or weight loss (Kobinger 2005). Although each of these cases used adenovirus as a vector in the vaccination process, each vaccine was comprised of a recombinant DNA plasmid consisting of the EBOV-GP subunits. While the effectiveness of the transmission of the vaccine into the host is significant, the GP subunits are the most important factor to whether or not an immune response is built against the viral proteins. Therefore, the studies which find success in animal models offer valid support for this recombinant vaccine.

Description of Immunity Assessment:

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The vaccine is administered in 4.0 mg dose. Two immunizations will be given twenty-eight days apart to complete the series. The vaccine is designed to be intramuscular, thus it is injected into the deltoid muscle of the arm. Enzyme-Linked ImmunoSorbent Assay, or the ELISA test, is the biochemical technique used to detect the presence of the specific antibodies against the Ebola virus. Two antibodies are used by the ELISA test, one is specific to the antigen, and the other reacts with the antigen-antibody complex and couples to an enzyme. This technique is extremely useful in determining serum antibody concentrations fighting the Ebola infection. Furthermore, the western blot test is used to detect the level of glycoprotein (GP Z) and nucleoprotein (NP) in the sample. The western blot test uses gel electrophoreses to separate the native proteins by size and shape. The proteins can then be transferred out of the gel and onto a membrane to be probed with antibodies specific to GP Z and NP. This process is helpful in confirming the level of antibody response against the Ebola virus (Martin, 2006).

The western blot and ELISA test results ensure that antibody isotypes IgG and IgM are present after the vaccination. IgG is the most abundant immunoglobulin and it is equally distributed within the blood and tissue liquids. IgG is capable of binding to many viruses, thus Ebola virus, and protects the body against the pathogen by complement activation, neutralization, and opsonization for phagocytosis. In general, if IgG levels are increased it signifies an active immune stimulation response against the Ebola virus. IgM is present on B cells and it is the primary antibody against A and B antigens on red blood cells. IgM is the largest antibody in the circulation and it appears early on in an infection and typically does not reappear upon second exposure to the pathogen. Therefore, the level of IgM is useful when diagnosing infection by the Ebola virus because if they are present in a patient's serum it implies a recent infection (Martin, 2006).

The Ebola virus-specific neutralizing antibody can be measured by a pseudotyped virus neutralization assay. Neutralization occurs when antibodies recognize the Ebola virus and immediately bind to a viral receptor. This act is an attempt to block the preferred receptor on the virus which will inhibit the virus from binding and infecting a host cell. This is typically a preventative method although it can become pathologic if the antibody neutralization becomes inadequate. For instance, if the antibody does not cover the viral receptor properly it can promote and enhance viral infectivity rather than inhibiting the process. Testing for neutralization provides useful information concerning whether or not IgG is present and attempting to inhibit the Ebola virus (Martin, 2006).

It is possible that the T cell responses also contribute to protection against the Ebola virus along with the GP-specific IgG. The effectiveness of a vaccine is highly dependent on the memory T cells produced. The ELISPOT is used to test vaccine induced T-cell responses by measuring the number of cytokine secreting cells in each well. Because IgG is used to bind to many viruses including Ebola, it is used as the pre-coat for the wells as the anti-cytokine antibody. After incubation, washing, and addition of the chromogenic substrate and washing again, the spots are counted to determine the release of cytokine by T cells. Positive response is perceived to be found for CD4+ T cells by binding to APC class II MHC and CD8+ T cells by binding to class I MHC. CD4

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is the glycoprotein found on T helper cells, a co-receptor, used to signal the cascade of an activated T cell. CD8 is a transmembrane glycoprotein found on cytotoxic T cells that also serves as a co-receptor by binding to MHC.

Cytotoxic T lymphocytes bind to the Ebola infected cell using class I MHC to signal apoptosis. The Chromium Release assay is used to measure the percent of cytotoxicity for various ratios of CTL infected cells. The infected cells are labeled with ⁵¹Chromium before the CTL are added; the amount of ⁵¹Cr released into the supernatant is proportional to the number of infected cells killed. When infected cells are incubated with the ⁵¹Cr they take it into their cytosol. The CTLs are added and as the Ebola infected cells die, they release the ⁵¹Cr into the supernatant. The specific release is the amount of ⁵¹Cr after the cells are centrifuged. Negative controls measure spontaneous release from infected cells by eliminating CTLs. Positive controls measure maximum release by lysing infected cells with detergent. The percent cytotoxicity or amount of Ebola infected cells is calculated using the formula: $(\text{specific release} - \text{spontaneous release}) \div (\text{maximum release} - \text{spontaneous release})$, the higher this percentage, the more Ebola infected cells have been killed by cytotoxic T lymphocytes.

There are a number of adverse events that may occur throughout the year long study for each participant. It is important to make note of these unexpected problems especially if the subject decides to continue participating. Procedural mistakes may occur when performing the assays so it is important to have enough sample so tests can be done repeatedly for accuracy and correct timing. The chromium release assays is being done in vitro which is also more effective. The assays only works best if there are at least an equal number of CTLs to infected cells. If proper ratios are not available the assay will not be performed accurately.

Each subject will provide blood samples through out the course of 52 weeks. Beginning with the first immunization soon after diagnosis of virus followed by the second immunization 28 days later, subjects will visit again at weeks 8, 12, 24, and 52. Blood samples will undergo various tests including ELISA, western blot, ELISPOT, and chromium release to determine immunogenicity progression. Monitoring the subject throughout the course of a year will insure subject's safety to vaccine trial as well as an in depth understanding of the immune response to the virus. Accuracy of the study will also depend on consistency of study population demographics. This may include initial health status of infected individual as well as, equal gender, BMI, educational level, and geographic location.

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