A Live Attenuated Recombinant Vaccine to Prevent West Nile Virus Infection in Elderly Individuals

LITERATURE REVIEW

EPIDEMIOLOGY AND POPULATION AT RISK

West Nile Virus (WNV), an arbovirus in the family *Flaviviridae*, does not cause illness in every person it infects. In the majority of cases, individuals with healthy immune systems are never diagnosed with WNV because their cases are either asymptomatic or subclinical (Weiss et. al, 2001). In approximately 20% of WNV cases, however, patients develop West Nile Fever, which includes a variety of influenza-like symptoms (Hubalek, 2001; World, 2007). In below 1% of WNV patients, the central nervous system (CNS) becomes infected, increasing the mortality rate drastically (World, 2007; Chowers et. al, 2000). In a 2001 study on the Israel outbreak of WNV, results showed a 14.1% mortality with CNS involvement and a correlation of increased mortality in WNV cases involving immunocompromised or elderly individuals (Chowers et. al, 2000). Between 41% and 55% of WNV neurological infection survivors reported that they had not made a complete physical, functional, or neurological recovery. Only 37% of subjects with WNV neurological infection experienced recovery in all three areas (Campbell et. al, 2002).

WNV is transferred from birds to humans via a mosquito vector. Recently, it was discovered that WNV can transmit through breast milk from mother to baby, from acquiring a intrauterine infection during pregnancy, from exposure to infected laboratory specimens, from receiving an organ transplantation that carries the infection, from infected blood transfusions and from frozen plasma (Batalis et. al, 2005). Additionally, a public health study performed by Tackett et. al suggests that poverty rate, temperature, and mosquito surveillance methods influence the rate of WNV-related mortalities (2007).

WNV is of epidemiological importance because it has caused significant outbreaks in many countries. In the United States, the Center for Disease Control (CDC) reported in 2006 that there were a total of 4,219 cases of WNV, 34% of which were West Nile meningitis or encephalitis, 62% of which were West Nile fever, 4% of which were reports of clinically unspecified cases, and 161 reports of fatalities (Center, 2006). Because the virus can propagate in over 100 avian species, it has the potential to transmit globally at an increasing level (Turell et. al, 2002). Since the 1999 movement of the virus into New York City, the incidence of WNV has increased every year. Although United States WNV-related fatalities have fallen since their peak in 2002 at 284 deaths, the grave potential for WNV to spread through the global avian population and continue infecting humans, along with the severity and consequences of WNV CNS infection, necessitate an effective vaccine (Center, 2006). Because WNV infections are most life threatening in elderly and immunocompromised populations, this proposed vaccine targets these two focal groups and has been constructed with consideration of a weakened immune system.

STRUCTURE

WNV is an enveloped virus and its outer membrane is characterized by icosahedral symmetry. The outer surface of the virus, which has a diameter of 50nm, does not contain any spikes or other surface projections (Kramer et. al, 2007).

The interior of the virus is made up of a nucleocapsid core, which contains several copies of the single-stranded, positive-sense RNA genome. The genome of the virus contains approximately 11,000 base pairs and consists of both a 5’ and 3’ untranslated region, as well as a single loop open reading frame (Kramer et. al, 2007). Upon translation of the viral genome, a

single polypeptide encoding 10 different proteins is formed. Cleavage of the polypeptide into individual proteins is performed using both viral and host proteases. Three of these cleaved viral proteins are structural: the capsid protein (C), a group of envelope proteins (E), and the premembrane protein (prM). The seven remaining viral proteins are nonstructural (Nybakken et. al, 2006). The structural proteins are vital components in the formation of new viruses, while the nonstructural proteins are essential in viral replication and assembly. The nonstructural proteins also serve as a method to evade the responses made by the host's innate immune system (Kramer et. al, 2007).

DISEASE MECHANISM

WNV follows a mosquito-bird-mosquito transmission cycle, and only involves humans when the virus is transmitted from bird to mosquito, and then from mosquito to a human host (Samuel et. al, 2006). Once the mosquito has inoculated the host, the virus then replicates in the dendritic cells. Dendritic cells then move the virus to the lymph nodes, where further replication occurs. After primary inoculation, WNV can spread to other organs in the body of the host, such as the brain, liver, spleen, pancreas, and lungs (Campbell, 2002).

INNATE IMMUNE RESPONSES

Nucleic acid sensors, such as the Toll-like receptor 3 (TLR3), are important in host recognition and response to RNA virus infections. These responses can begin to work after viral infection or ligand binding to the receptor. The double-stranded viral RNA is also involved in host recognition, as it is sensed by cytoplasmic double-stranded RNA sensors (Samuel et. al, 2006). IFN-α, and IFN-β are necessary to prevent WNV from replicating and spreading to nearby cells (Fredericksen et. al, 2004 and 2006). IFN-γ prevents viral replication, which limits the spread of WNV to the CNS. Recent studies show RIG-I and IRF regulatory factor 3 (IRF3) respond to WNV infection and appear to interfere with poly(I-C)-induced IFN responses.

Complement is a necessary feature of the immune system to help protect against WNV infection by all three complement pathways: classical, lectin, and alternative (Samuel et. al, 2006). Macrophages engulf WNV and control infection through direct clearance of the virus, producing nitric oxide intermediates, enhancing antigen presentation, and secreting cytokines and chemokines. Dendritic cells function as antigen-presenting cells which also produce IFN-α/β after infection. The γδ T cells are part of the early immune response, limiting WNV infection by reacting with viral antigen in the absence of innate antigen processing. The γδ T cells help to control the replication of WNV and to aid in the generation of the adaptive response. Natural killer (NK) cells control infection by recognizing and eliminating infected cells.

ADAPTIVE IMMUNE RESPONSES

Neutralizing antibodies are directed against a region of the E protein on WNV, while other antibodies recognize the prM protein (Colombage et. al, 1998; Falconar, 1999; Pincus et. al, 1992; Vazquez et. al, 2002). There are three fields on the WNV E protein that aid in viral latching, entrance, and assembly. Domain III (DIII) has been suggested to be the receptor-binding domain (Samuel et. al, 2006). Domain II (DII) contains a fusion loop, which is involved in viral attachment. Finally, domain I (DI) assists in structurally rearranging the E protein, which is required for viral fusion. Antibodies specific for DIII are able to inhibit viral fusion after viral attachment (Gollins and Porterfield, 1986; Nybakken et. al, 2005). When a WNV-infected cell is recognized, WNV-specific cytoxic T lymphocytes proliferate and strengthen the adaptive

response through the release of inflammatory cytokines (Douglas et. al, 1994; Kesson et. al, 1987; Kukarni et. al, 1991). The T lymphocyte can then release perforin and granzymes to lyse infected cells. CD4+ T cells help control the WNV infection by furthered activation and stimulation of CD8+ T cells, cytokine release, and B cell activation (Samuel et. al, 2006). B-cells and IgM antibodies confine the viral infection to the serum in order to prevent the virus from reaching the CNS. Specific neutralizing antibodies are produced after primary WNV infection. It is still unknown how the specificity of epitopes contributes to memory responses. According to Samuel et. al, it is probable that T cells play a crucial role in prevention of a second WNV infection. Multiple research studies support this notion and show that T cell memory is key to the prevention of WNV infection. These studies have shown that many different WNV vaccine preparations do induce the production of T memory cells (Anraku et. al, 2002; Monath et. al, 2006; Yang et. al, 2001).

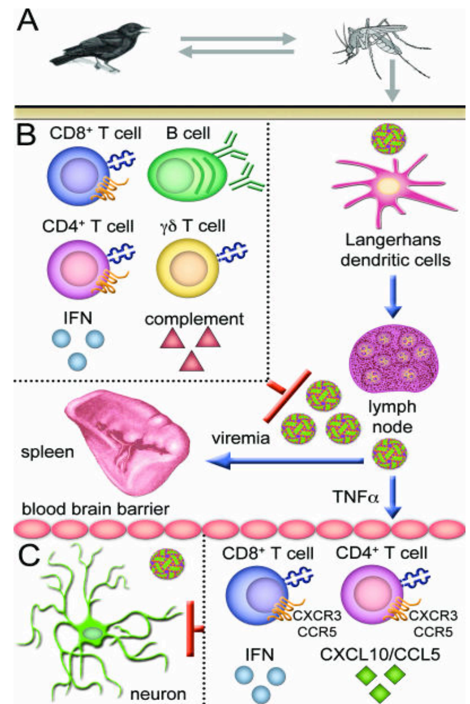

Figure 1: Diagram depicts the transmission cycle and disease mechanism of WNV (Samuel et. al, 2006).

Central Nervous System Responses

WNV is acquired through peripheral inoculation and then spreads to the CNS. To effectively clear infection from the CNS, there must be only limited damage to nonrenewing cells, such as neurons (Griffin, 2003). IFN-α/β, CD8 and CD4 T cells help control replication of WNV in neurons. Chemokines CXCL10 and CCL5, and ligands CXCR3 and CCR5 recruit CD8* and CD4* T cells, along with monocytes, to the CNS. The roles of CD8 and CD4 T cells are very important in host immunity (Samuel et. al, 2006).

A simple diagram derived from Samuel *et. al* can be seen to the right. This figures summarizes the immune response after an initial WNV infection.

Current Treatment/Vaccine

There are currently no curative treatments or approved vaccines for WNV, although there are experimental vaccines undergoing human testing. One such vaccine trial is the ChimeriVax-WN02, including the placebo YF 17D. Both the vaccine and the placebo contain similar elements, but the ChimeriVax-WN02 contains a genetically engineered WNV sequence (Monath et. al, 2006). In a 2006 ChimeriVax-WN02 trial, the ChimeriVax-WN02 was observed to be less neurovirulent than the YF 17D. The higher dosage ChimeriVax-WN02 had less viremia than the lower dose – possibly due to the lower dose inducing a less powerful innate response and a less rapid adaptive response. ChimeriVax-WN02 caused all subjects to develop a higher titer of WN02-specific neutralizing antibodies by day 21, and two subjects developed neutralizing antibodies by day 10. Another observation showed WN-specific T cells were higher in ChimeriVax-WN02 than IFN-gamma-producing T cells in YF 17D vaccine, showing a positive adaptive immune responses.

Another possible vaccine undergoing studies utilizes a live attenuated vaccine derived from Dengue viruses. Dengue viruses, also in the Flavivirus family, are transmitted by mosquitoes (Huang et. al; 2005). There is a promising WNV vaccine which utilizes Dengue 2

(D2) strain primary dog kidney (PDK)-53. This vaccine is prepared by passing the wild-type D2 16681 strain 53 times through the PDK cells. This vaccine is tetravalent and therefore targets all four Dengue serotypes. Additionally, the D2 PDK-53 vector was engineered to include the prM-E genes that are seen in all other flaviviruses.

DESCRIPTION OF VACCINE

VACCINE TYPE

A live attenuated recombinant vaccine has shown to produce a strong neutralizing antibody response (Monath et. al, 2006). Additionally, this vaccine scheme proved to prevent viremia after a single dose (Dauphin and Zientara, 2006). Under several observations, ChimeriVax-WN02 has shown promising results in its stimulation of cell-mediated immune responses (Monath et. al, 2006). We followed the successful trials performed by Monath et. al, and this vaccines utilizes a live attenuated recombinant vaccine. Because the ChimeriVax-WN02 vaccine has proven to elicit a T-cell memory response and decrease viremia, we chose to use this vaccine and change the adjuvant and concentrations that were used by Monath et. al.

ChimeriVax-WN02 uses a live yellow fever 17D strain as the vector and incorporates the E coding genes from all Flaviviruses. The structural protein genes, YF-prM-E gene, were replaced by WNV-prM-E gene. Only structural genes were utilized, as non-structural genes have shown to be less effective at eliciting an immune response in other WNV vaccine trials.

ADJUVANT

An encapsulated antigen adjuvant composed of degradable polymer (DL-lactide-co-glycolide)-entrapped antigen is included in the vaccine concoction (Hanly et. al, 2007). The degradable poly-entrapped antigen adjuvant slowly releases the antigen into the system. Adjuvants create a postponed antigen liberation into the body's systems in order to travel to the spleen and/or lymph nodes. This way, the adjuvant will increase the secondary response without encountering innate immune recognition. The use of an adjuvant and antigen is predicted to produce more neutralizing antibodies with less antigen concentration in comparison to just live-attenuated antigen alone. The goal is to produce a moderate to high titer with high affinity antibodies. In order to achieve this, low amounts of live-attenuated antigen are used (Hanly et. al, 2007).

CONTROL AND PLACEBO

The control for the vaccine is YF-VAX 17D, a common yellow fever vaccine (Monath et. al, 2006). The placebo used consists of buffer (Tris), NaCl, a sugar alcohol component (sorbitol), and water. The yellow fever control is appropriate because the recombinant ChimeriVax-WN02 vaccine is constructed from an infectious cloned strain of yellow fever virus 17D. The difference between the two vaccines is in the premembrane and envelope proteins, as the ChimeriVax-WN02 has altered premembrane and envelope proteins. YF 17D is an excellent control because YF 17D and ChimeriVax-WN02 share similar properties, such as the location of infection and replication in hosts. The two only differ in their rates of replication.

TYPE OF IMMUNITY PLAN IT ELICITS

Upon injection of vaccine dose, the live attenuated serum bypasses the physical barriers of the skin and enters into the blood stream. The poly-entrapped antigen adjuvant ensures there is time for the live-attenuated antigen to be released in a more ideal location, such as the spleen

and/or lymph nodes (Hanly et. al, 2007). Because of the delayed time release, many more altered antigens are able to use phagocytes to aid in the activation of the secondary immune response. Macrophages detect the invading antigen by engulfing them, and this response is increased via complement activation (classical, lectin and alternative) and/or TLR-3 recognition (Parham, 2005). Soon after, chemokines and cytokines are released, and subsequently neutrophils, macrophages and NK cells are recruited to the infection site. Antigen phagocytosis is performed by macrophages, NK cells and neutrophils. The dendritic cells activate the adaptive immune response and present the live-attenuated antigen on MHC I and MHC II. Circulating small lymphocytes then identify invaders, and this pushes for selection, growth and differentiation of B, T memory, CD4+ and CD8+ T cells (Parham, 2005). When the dendritic cells bind to the antigen receptors, replication of helper T (T_H), cytotoxic T (T_C) and memory (T_M) cells is stimulated. The T_H cells travel to the infection site to secrete cytokines. These cytokines result in the increase of inflammation and the stimulation of B cells to make WNV prM/E-specific Ig receptors. In contrast, Tc cells kill infected cells. The T lymphocytes discharge granzymes and perforins to eradicate infected cells. Afterwards, the plasma cells (activated B cells that have proliferated and differentiated) secrete WNV prM/E-specific antibodies. Eventually, the WNV adaptive immune response becomes specialized and prepared for a WNV invasion (Parham, 2005; Hanly et. al, 2007; Samuel et. al, 2006; Monath et. al, 2006).

Although, the concentration of antigens in ChimeriVax-WN02 is enough to encourage an adaptive immune response, it would be wise in future experiments to inoculate boosters afterwards to ensure immunity. This experiment will help identify a specific period in which the antibody titers are declining, thereby indicating when a booster shot should be administered, if necessary (Hanly et. al, 2007).

Problems that Can Happen and Ways to Minimize Them

The live attenuated recombinant vaccine can have adverse effects in immunosuppressed individuals. For example, elderly individuals and young children are hopeful recipients of this vaccine, yet they also tend to have sub par immune responses. It is possible that the vaccine could cause an unexpected infection in these individuals, and thus precautions were taking regarding dose concentrations. The initial human trials will begin with the lowest possible dose concentration. The subjects' immune responses will be measured from this baseline. The concentration will be increased only until a sufficient immune response can be recorded. This gradual increase in concentration will minimize the opportunity for true infections to develop in persons with weakened immune systems.

It is also possible that study results will be skewed due to unexpected high viremia in some subjects. This can occur due to an increase in replication at the skin and lymphoid tissue before an immune clearance has occurred (Monath et. al, 2006). In order to avoid this data anomaly, it will be necessary to conduct blood tests prior to measuring the immune response. The blood test analysis will identify if an immune clearance occurred or not.

When any live vaccine is used, it is always possible that the live attenuated construction could revert to a pathogenic state. This has been minimized in our trial through the use of structural proteins. Because non-structural proteins were not included in the vaccine, there is a lower probability that the live vaccine will revert to a pathogenic state. The use of low concentrations will also decrease the chances of a reversion occurring, for a lower concentration creates a smaller pool for mutation events.

MAXIMIZING EFFICIENCY AND SAFETY OF THE VACCINE

Replacing the YF-prM-E gene of the yellow fever virus with the inserted WN-prM-E genes increases the WNV structural gene production. The removal of genes necessary for pathogenicity eliminates the yellow fever virus' pathogenic characteristics and ensures the vaccine's safety. The use of a live attenuated vaccine reinforces the production of prM-E gene. The increased production of prM-E gene in the yellow fever virus shell improves its translation efficacy. In addition, the E protein gene contains specific functions for attachment, entering, and assembling in the mutated yellow fever virus, thus ensuring a host infection (Monath et. al, 2006). Therefore, safety is maximized through the removal of pathogenicity, and the vaccine efficiency is maximized through genetic engineering and the use of a live specimen.

DESCRIPTION OF IMMUNITY ASSESSMENT

IMMUNOLOGICAL TESTS TO MEASURE VACCINE EFFICACY

A test that quantitatively measures antibody production is necessary to determine if the immune system is making an effective response against antigen. In order to determine if the subjects are making the proper responses after vaccination, the amount of IgM will be measured. The Ig**M** **A**ntibody **C**apture **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay (MAC-ELISA) will be used to measure the level of IgM secretion by B lymphocytes. The MAC-ELISA is preferred over the traditional ELISA because of the assay's ability to more specifically bind only IgM, resulting in fewer occurrences of false positives (Prince and Hogrefe, 2005). The test will be administered 7 to 10 days following immunization, allowing sufficient time for IgM secretion by B lymphocytes.

The first step of the MAC-ELISA is to coat microtiter wells with anti-human anti-IgM antibodies. Nonspecific protein, such as powdered milk, is added to coat any areas of the well where the anti-human anti-IgM failed to bind. The wells are then washed with a buffer solution to remove any unbound molecules. Serum that has been obtained from the subject is diluted and then added to the wells. The wells are then incubated for several hours and then washed. The presence of the anti-human anti-IgM antibodies allows all of the IgM molecules in the serum to be captured, not just those specific to WNV. WNV antigen is then added to the wells and binds to the IgM molecules that are specific for the virus. The wells are incubated and then washed. An enzyme-linked, monoclonal antibody specific to the flavivirus group is added as a secondary antibody. The wells are again incubated and then washed. A chromogenic substrate is then added. A color change indicates the presence of IgM specific for WNV within the subject's serum (Prince and Hogrefe, 2005). These results show that the vaccination is successful in stimulating an immune response against WNV.

The measurement of T cell memory is an essential indicator of the ability of the vaccine to elicit immunological memory. The **E**nzyme-**L**inked **I**mmuno**spot** Assay (ELISPOT) is a test that can measure cytokine secretion by T lymphocytes. Specifically, the ELISPOT assay will be used to measure IFN-γ secretion by CD4+ T helper-1 cells (T_H1). The test will be administered 7 to 10 days after initial administration of the vaccine to allow sufficient time for memory helper T cells to develop.

There are several steps in order to effectively perform the ELISPOT assay. Microtiter wells are first coated with a primary antibody specific to IFN-γ (anti-IFN-γ antibody) (Decker, 2006). Powered milk is then added to cover any areas of the wells where the primary antibody did not bind. The wells are then washed with a buffer solution to remove any unbound molecules. The T_H1 cells that have been isolated from the subject's serum are added to the

wells. These cells have been isolated using flow cytometry. In this technique, fluorochrome-labeled antibodies are directed toward a specific marker on the cells of interest. In this case, the fluorochrome-labeled antibody is directed toward the CD 272 marker that is present only on T_H1 cells (Abd Serotec, 2006). The antibody is added to the serum, incubated, and then run through the flow cytometer (Decker, 2006). The stream of serum run through the flow cytometer is a single cell wide. Each cell goes past a detector that measures the level of fluorescence. The T_H1 cells will have a high fluorescence due to antibody binding and can therefore by separated out from the remainder of the serum. The wells are incubated for several hours or overnight to allow for secretion of IFN-γ by the T_H1 cells. Once cytokine secretion has taken place, the IFN-γ will bind to the anti-IFN-γ antibodies and the wells are again washed. A secondary antibody that is coupled to an enzyme and is specific for a different epitope on IFN-γ is added, and the wells are incubated to allow sufficient time for binding. The wells are washed to remove any unbound secondary antibody, and then a chromogenic substrate is added. A color change in the substrate due to the enzymatic activity indicates that the secondary antibody was able to bind IFN-γ and is indicative of a positive result for T_H1 cell memory as well as production of the cytokine IFN-γ.

VACCINE TRIAL

A Double blind study will be conducted on 300 human subjects. Patients who could have potentially already been exposed to WNV or other flaviviruses will not be included in this study (Monath et. al, 2006). This includes military personell and travelers to tropical locations. Additionally, patients who have immunocompromising conditions, such as HIV or hepatitis, cannot participate in this study. Therefore, blood will be screen for such conditions before the subject can begin participating in the trial. If the blood tests show that there is any large variance when compared to typical laboratory blood test results, the patient should be excluded from the study as well. This is crucial because the results cannot be standardized if they do not share a common baseline starting point. Also, pregnant or lactating individuals cannot participate, for the fetus or infant could become infected.

The subjects will be inoculated with either 0.5mL of ChimeriVAX-WN02, 0.5mL YF-VAX or 0.5mL of placebo. Patients will return every day for 30 days to monitor their viremia levels. Their antibodies will be tested 5 times during this 30 day period, on days 0, 7, 14, 21, and 30. T cells will be measured four times during the 30 days, on days 0, 10, 20, and 30. Subjects will note their symptoms, daily temperatures, and reactions in a journal for the duration of the study. The subjects' serum will also be sampled and analyzed during the year after the study and beginnings 4 months after the study. This will help to assess the level of sustained immunity after vaccination.

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