

# West Nile Virus DNA Vaccine for clinical trial in the Western States

## Team 4

### Literature Review:

The West Nile Virus (WNV) is a member of the Japanese Flaviviridae family, specifically the Flavivirus genus. The mosquito-borne WNV was historically found in Africa, West Asia, and the Middle East. While isolated incursions into Western Europe occurred during the twentieth century, the WNV gained little medical attention until it made its first major appearance in North America during a series of outbreaks in New York City in 1999, where viral encephalitis began appearing in a cluster of elderly patients (Hayes *et al.*, 2005). This outbreak was significant because it heralded a new serotype of WNV, termed NY99, that deviated from the WNV's historically low virulence, and unleashed a rapidly spreading epidemic in the United States, one that is not only spreading across the country but also into Canada and South American (Gubler, 2007). Historically, the disease, West Nile fever, caused by the WNV, produces flu-like symptoms that generally disappear within several days without causing long-term harm. However, the NY99 serotype that appeared in New York City is the most virulent strain to date and has increased the potential to lead to a neuroinvasive form of WNV, causing infections of the central nervous system, termed West Nile Encephalitis and/or meningitis. While West Nile fever is of minor worry, the high prevalence of neuroinvasive disease in America garners great concern, due to the lack of specific treatment targeted at the illness. Currently, only symptoms can be treated. Between 1999 and 2004, more than 7,000 neuroinvasive WNV cases were reported in the US alone (Hayes *et al.*, 2005). According to the October 2007 CDC MMWR West Nile Update, 42 states reported to the CDC 3,022 WNV cases in 2007, 76 of which were fatal (CDCa, 2007).

WNV is an enveloped virus with a single-stranded positive-sense RNA genome containing a single open reading frame for 10 proteins. Seven proteins are nonstructural and mainly drive replication of the viral genome: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Dauphin and Zientara, 2006). They also attenuate host antiviral response (Chung *et al.*, 2006). The three structural proteins are the capsid (C) protein, premembrane/membrane (prM/M) protein, and envelope (E) protein. The RNA genome is packaged within capsid protein C. The prM/M and E proteins are surface proteins that form heterodimers until the prM/M protein is cleaved to the M protein, which then allows the E protein to form homodimers. E protein function includes: virion assembly, cell receptor recognition and binding, fusion with host membranes, and stimulation of B and T cell responses (McDonald *et al.*, 2007). The M protein also participates in virion assembly, host range, tissue tropism, replication, viral assembly, and stimulation of B and T cell immune responses (Chung *et al.*, 2006). However, it is the E protein that is the most immunogenic of the membrane proteins, invoking neutralizing antibody responses, especially at its EIII epitope. The West Nile Virus binds, using the E protein dimers, to an unknown protein on host cells and enters the cell through a vesicle in a manner similar to that of endocytosis. The prominent binding theory suggests that the E protein interacts with highly sulfated heparan sulfate (HSHS) residues, which are often present on the surface of many cells. Once the virus enters the cell, a drop in pH theoretically exposes a hydrophobic domain of

the E protein, facilitating fusion of the viral and host membranes, allowing the rRNA genome to release into the cytoplasm and begin immediate translation because of its positive-sense RNA (Deubel *et al.*, 2001). The resulting proteins assemble and then copy complementary minus-strand RNA from the genomic RNA, which are copied again to form positive-strand RNA. Genome replication occurs in the perinuclear cytoplasm. Translation occurs in the rough ER. Once enough C, E, and M proteins are formed, viral assembly can occur in the lumen of the endoplasmic reticulum, grouping the proteins with new RNA genomes. Mature viruses are either transported out of the cell via the secretory pathway or through budding from infected cells (Deubel *et al.*, 2001). Viral reproduction usually occurs in the tissue and lymph nodes near the viral entry site. Progeny virions travel from the lymphatic system to the blood and then spread through the body (Hunt *et al.*, 2007).

WNV has several defense mechanisms that make it difficult for the immune system to mount an effective response. One mechanism causes a reduction in complement-mediated lysis. The nonstructural protein NS1 is a secreted glycoprotein that induces decreased complement activation. The secretion of NS1 in high amounts into the blood is associated with the development of neuroinvasive disease, causing inflammation in surrounding tissues. Secreted NS1 also binds to the infected cell, recruiting, binding, and activating plasma glycoprotein factor H (fH), which is a key regulator of complement activation. When activated, fH cleaves C3b, causing the destruction of C3b and C5b-9 membrane attack complexes. NS1 inhibition of the alternative complement pathway causes a decrease in complement-mediated lysis of infected cells, efficacy of B and T cell priming, opsonization and neutralization of infected cells, and the recruitment of other immune cells through the release of chemokines, such as MIP-1 $\beta$ , MIP-3 $\beta$ , or RANTES (Chung *et al.*, 2006). Another means through which WNV evades the immune system is specific to serotypes found in the United States. These serotypes block the interferon  $\alpha/\beta$  receptor, preventing interferon-mediated immune response and allowing infected cells to replicate and release copies of the virus (UT Southwestern Medical Center, 2006). These two immune evasion techniques make it difficult for the immune system to construct an efficient defense when first infected, allowing the virus infection to spread. The uniqueness of the American WNV serotypes in their ability to interrupt interferon signaling helps to explain the increased virulence of these strains.

WNV is a vector-transmitted, blood-borne pathogen similar to the yellow fever virus, dengue virus, and Japanese encephalitis virus (Chung *et al.*, 2006). The disease vector responsible for most human infections is mosquitoes. The disease is most commonly transmitted when a human is bitten by an infected mosquito. The mosquito usually acquires the virus as a result of feeding on the blood of infected birds. Because humans are infected through mosquito bites, WNV is seasonally epidemic, with peak activity typically recorded from July through October. However, as one travels further south, peak season lengthens. While mosquito-borne infection is the most common, Americans have contracted WNV through receiving blood transfusions and organ-transplants from infected donors. In addition, fetuses can contract the disease while in utero should the mother become exposed, and there is a possibility that infants can contract the disease as a result of being breastfed by an infected mother. Lastly, laboratory personnel working with live WNV have contracted the disease, as well as avian workers handling infected turkeys, raising the disturbing possibility of infection through aerosol exposure (Hayes and Gubler, 2006). Some of the symptoms associated with the infection include fever, headache, stiff neck, unconsciousness, disorientation, coma, tremors, convulsions, muscle weakness, vision loss, numbness, and paralysis. These symptoms can last several weeks.

Neurological effects could persist indefinitely. About 20% of the people infected with WNV have flu-like symptoms, swollen lymph nodes and skin rashes. These can persist between a few days and several weeks. About 80% of people infected with WNV will not exhibit any symptoms (CDCb, 2006).

The introduction of WNV to America first began with a NYC outbreak in 1999, where 62 elderly New Yorkers developed West Nile fever, leading to 59 cases of neuroinvasive disease and 7 deaths. Epidemiological data and theory suggest that the WNV arrived in America from infected patients traveling from Israel, where a WNV outbreak had occurred (Gerhardt, 2005). The disease began to spread rapidly among the avian population, and human spread followed thereafter. For instance, in 2000 the only human WNV cases occurred in New York, 19 of which progressed to the neuroinvasive form, subsequently leading to 2 deaths. However, that same year, WNV-infected dead birds were officially identified in 12 Atlantic coast states. In 2001, WNV avian infection spread toward the western bounds of Florida, Iowa, and Louisiana, as well as north into Canada. Also in 2001, WNV spread among humans, moving both westward and southward into the Caribbean, Central, and South America. 66 WNV infections occurred among Americans, 64 of which involved neuroinvasive disease, resulting in 9 deaths. However, it was not until 2002 and 2003, once the WNV had transversed the United States to finally reach California, that the WNV reached epidemic proportions. In 2002, there was an increase in human infection rising to 4156 reported cases, of which 2946 cases developed the neuroinvasive form, causing the deaths of 284 people. Infections increased again in 2003, with the epicenter identified in the Midwestern states. Human infection rose to 9862 reported cases, 2866 neuroinvasive cases, and 100 deaths. Fortunately WNV infection decreased among humans during 2004: 2593 cases, 1142 instances of neuroinvasive disease, and 119 deaths. Unfortunately, cases began to rise again afterwards. Case numbers rose to 3000 reported cases in 2005, leading to 1294 neuroinvasive cases and 119 deaths. In 2006, infection numbers further increased to 4261 cases, 1455 neuroinvasive cases, and 194 deaths (Gubler, 2007). From January 2007 to October 2007, 3022 human cases have been reported, 76 of which were fatal (CDCa, 2007). While infection rates are again increasing, they occur with varying annual frequency across the United States and are too low to provide protective immunity (Hayes *et al*, 2005). The unlikelihood of developing any protective immunity is problematic, given the increase in infections, and is one of the instigators for the nation-wide call for a vaccine.

WNV is theorized to have spread so easily across the United States because the serotype introduced was new and possessed increased virulence from previous forms of WNV. In addition, its abundance of vertebrate hosts and vectors make transmission across distance easier. So far in 2007 alone there have been 1,489 dead corvids and 435 other dead birds reported, as well as horse infections in 31 states. The western United States has harbored 3 infected canines, 26 infected squirrels, and 3 unidentified infected animal species. Moreover, 7208 infected mosquito pools have been identified in 32 states (CDCa, 2007). Efforts to stem the spread of early infection include preventing large mosquito populations from occurring and tracking WNV in wild animals, birds, and horses. Education efforts about how to prevent mosquito population growth and what to do when one encounters dead birds help to educate the public on how to lower the likelihood of contracting the disease. People of all ages are susceptible to West Nile fever. However, the elderly and the immunocompromised are at higher risk for developing the neuroinvasive form of the disease, due to their weakened immune systems, which make them more susceptible to infection. People with the highest likelihood of developing any form of WNV live in areas with greater mosquito exposure (Hayes and Gubler, 2006), such as people

living in the states of California, North Dakota, South Dakota, Colorado, Nebraska, Montana, and Wyoming (CDCa, 2006).

Given these statistics, and the absence of a specific WNV treatment, it is apparent that there is a growing need for a WNV vaccine. Much research has been conducted to develop a vaccine for the human population and a vaccine for the equine population, which has faced devastating results from equine infection, resulting in death and event cancellation (Dauphin and Zientara, 2006). Two vaccines have been licensed for equine use, whereas no human vaccines have been licensed so far. Equine vaccines are of significance to the development to human vaccines, as the WNV disease mechanism seen in horses is comparable to that seen in humans. One licensed equine vaccine employs a classical vaccine using whole formalin-inactivated WNV, which uses two doses to confer a 94% protection rate present after 12 months. Since it is an inactivated vaccine, there is no risk of the animal contracting WNV from the vaccine. However, despite an initial 94% protection rate, immune system response is weak and requires frequent boosters, preferably every 6 months, to maintain immunity. The other licensed equine vaccine is a live canarypoxvirus recombinant vaccine, which expresses the WNV preM/M and E proteins in the absence of viral replication. Since the virus does not replicate, there is little danger of infection as a result of vaccination. Vaccine efficacy is increased through the use of a Carbopol adjuvant. Two doses, plus annual boosters produces a quick onset of protective immunity. In addition to inducing immunity in horses, the vaccine has been shown to produce similar results in both cats and dogs (Dauphin and Zientara, 2006).

WNV vaccines currently in development that are targeted at humans include two live-attenuated virus vaccines, the yellow-fever WNV (Chimerivax-WN02) and dengue 4-WNV vaccines. It should be noted that both yellow-fever and dengue are similar to WNV. In both vaccines, the respective E and preM/M proteins are replaced with those from WNV, allowing for strong immune responses in primates, especially in the form of neutralizing antibodies (McDonald *et al*, 2007). Human clinical trails are currently being used to determine the safety and efficacy of Chimerivax-WN02 vaccine, but it has not yet been approved for use (Monath *et al.*, 2006). Other WNV vaccines in development are protein-based subunit vaccines using WNV-NS1 and the E-protein as target antigens, such as one that fuses the EIII domain of the E-protein to a modified bacterial flagellum (McDonald *et al*, 2007). Such a vaccine shows potential for eliciting both innate and adaptive immunity but has not yet been approved for human trial. DNA vaccines targeting the E protein in mice, horses, and birds have also elicited promising immune responses that warrant further investigation of DNA vaccines for treatment of WNV in humans (Chang *et al*, 2001).

While there are many potential human WNV vaccines, none have been approved for use. This proposal presents a human WNV DNA vaccine that, unlike live-attenuated vaccine designs, poses no risk of viral infection, and is superior in simplicity, cost effectiveness, and stability.

## West Nile Virus DNA Vaccine for clinical trial in the Western States

### Vaccine Design:

Since the NY99 WNV strain has a very high virulence, live WNV vaccines for humans have definite drawbacks that may prove insurmountable (Seregin *et al.*, 2006). Consequently, in order to elicit a strong immune response without the risk of active infection, we propose the development of a DNA vaccine, injected intramuscularly, that is capable of stimulating both adaptive humoral and cellular immunity. DNA vaccines employ plasmids containing the genomic DNA of the antigen to which one wants to elicit an immune response. In this vaccine, the target antigens will be the E, M, and NS1 proteins. The E and M proteins are the only envelope/membrane proteins present for the immune system to identify on the surface of the WNV, so they serve as the antigens most likely to elicit an effective immune response. Targeting the E protein is especially important since it has the highest immunogenicity. Also, the E protein is the main antigen target for neutralization, and since effective antibody neutralization of WNV in the body would prevent or greatly limit the severity of an infection, developing strong neutralizing antibody humoral immunity, in addition to cellular immunity, is essential. The NS1 protein, secreted by infected cells, interferes with complement activation by binding to the surface of infected cells and is one of the main instigators in the increased virulence of the NY99 strain. Immunizing against the NS1 protein would help the immune system recognize it as foreign and induce a stronger response.

In designing the vaccine, the DNA encoding the genes for the E, M, and NS1 protein antigens will be incorporated into a plasmid. In addition to the genomic DNA, DNA encoding a human promoter region will be incorporated preceding each coding region and transcription termination and polyadenylation sequences will be incorporated after each coding region. The intramuscular injection of DNA alone induces both humoral and cellular immunity to the E, M, and NS1 proteins, but the use of adjuvants increases the strength of the immune response. In this vaccine, the immune response will be enhanced through the inclusion of a plasmid encoding the cytokine GM-CSF and a plasmid encoding the cytokine IL-2. Multiple experiments have shown that the presence of IL-2 will serve to induce the proliferation and activation of T cells in response to antigen introduced by the DNA vaccine. GM-CSF is also an effective adjuvant for DNA vaccines, because it serves as a ligand for TLR-9, which activates dendritic cells (DCs), which will phagocytose and present, alongside macrophages, the injected DNA that does not enter a muscle cell. The presence of GM-CSF has been shown to increase the vaccine-elicited CD4<sup>+</sup> T cell and antibody responses (Greenland and Letvin, 2006). A mixture of these three plasmids, one plasmid containing the E protein, M protein, and NS1 protein DNA, one plasmid containing IL-2 DNA, and one plasmid containing GM-CSF DNA, will be injected intramuscularly using a gene gun.

Once injected into the body, the WNV DNA vaccine can begin to induce an immune response along both the cellular and humoral pathways. The cellular pathway will be initiated when the virus is either injected into muscle cells or into antigen presenting cells (APC) such as DCs. Once the plasmid enters the cytoplasm, expression of the antigen genes occurs. Once proteins have been translated, the host cell identifies them as foreign and processes them as intracytoplasmic antigens, cutting the proteins into small peptide fragments. The resulting peptides are presented on MHC class I molecules (MCHI). It should be noted here that the INF  $\alpha/\beta$  receptor of infected cells will not respond normally or reliably to interferon stimulation, essentially eliminating that form of immunologic action (UT Southwestern Medical Center,

2006). APCs presenting antigen peptides on their MHC I travel to secondary lymphoid organs. DCs that have bound WNV antigen will be induced to travel to the secondary lymphoid organs once activated by the binding of GM-CSF (injected as DNA and expressed by host cells, to increase the amount of GM-CSF, creating a more efficient activation of DC cells) to TLR-9. In the secondary lymphoid organs, circulating T cells will “sample” the APCs, especially the DCs, for MHC-WNV peptide complexes, searching out foreign antigen to which they are specific. Some T cells will identify and bind to the MHCs presenting WNV peptides. CAMS LFA-1 on T cells and DC-SIGN on DCs draw the two cells together so that should the T cell receptor (TCR) be specific for the WNV peptides the two can bind. Once the specific TCR interacts with the MHC-WNV peptide complex, and the CD28 receptor of the T cell receives co-stimulation by the DC membrane molecule B7, the WNV peptide specific (either to the E protein, the M protein, or the NS1 protein) T cell is activated. Once T cells are active, they start synthesizing the IL-2 receptor's  $\alpha$ -chain (IL-2R, also called Tac), increasing the IL-2R affinity, allowing for lower IL-2 concentrations to signal clonal proliferation of WNV peptide-specific CD8<sup>+</sup> T cells. It is at this point where the IL-2 expressed as a result of its DNA being injected as a plasmid as part of the vaccine comes into play. The presence of IL-2 signals clonal proliferation of WNV peptide-specific CD8<sup>+</sup> (cytotoxic) T cells, increasing the rate of cellular immune response. The activated Tc cells (cytotoxic T cells) undergo clonal proliferation for 4-5 days and then differentiate into armed effector cells, which are stimulated by the presentation of MHC-WNV peptide. The presence of VLA-4 instead directs them toward virus-infected tissue or infection sites, where the Tc cells will destroy infected cells. In order to do so, the Tc cells will bind the infected cell (in this case, the cell presenting a WNV peptide on its MHC I), inducing the cell to undergo apoptosis via perforins and granzymes. In addition to stimulating cell death of infected cells, Tc cells release IFN $\gamma$ , TNF $\alpha$ , and TNF $\beta$  to activate macrophages to destroy any free virus. Tc cell release of IFN $\gamma$  helps to directly inhibit viral replication. It also increases the production of MHC I by activating IL-1 and TAP expression in WNV-infected cells. Lastly, IFN $\gamma$  released by Tc cells will recruit and activate more macrophages to be effector cells as well as APCs. When the infected cells apoptose via Tc cells, viral E, M, and NS1 proteins not yet presented will be expelled into the body and available for phagocytosis by APCs: macrophages, neutrophils, DCs, and B cells. These APCs will breakdown the protein in into small peptides in the endosome and then present these peptides on MHC Class II molecules (MHC II).

The mature naïve T cells stimulated by WNV peptides displayed on MHC II will receive signal #1 (weak signal that antigen is present) and be activated as helper CD4<sup>+</sup> T cells (Th cells). Th1 will activate Tc cells and macrophages, through secretion of IL-2, IFN $\gamma$ , and TNF $\beta$ , in order to stimulate further cellular immunity and induce inflammation in order to cause an efflux of more immune cells (i.e. monocytes) to the site of infection. Th1 will secrete chemokines like CCL2 to direct migration of phagocytic cells to the infection site. Th1 will also activate macrophages to more efficiently fuse their lysosomes and phagosomes. Lastly, Th1 cells will activate B cells, secreting IL-3 and GM-CSF, which will induce B cells to produce opsonizing antibodies IgG and induce the bone marrow to produce more leukocytes (Greenland and Letvin, 2006). Th1 cells will also aid in the destruction of infected cells through binding their FasL with the Fas on infected cells, resulting in apoptosis. The activation of Th2 cells allows them to activate and induce naïve B cells to divide and secrete IgM. Th2 cells will also secrete cytokines IL-4, IL-5, and IL-6 to induce B cells to produce neutralizing antibody, which is essential for fighting virus infections. Also, Th2 cells will activate B cells that have phagocytosed any WNV

antigen floating freely. This is important since the B cell receptor (BCR) must be able to specifically bind and crosslink the WNV-antigen for phagocytosis to occur.

Once humoral immunity has been activated, B cells specific to the WNV antigens will bind to and present WNV peptides on MHCII. Two pathways of humoral immunity will be activated in order to produce a WNV-antigen specific response. The first pathway occurs when the B cell recognizes the WNV-MHCII complex bound to a Th2 cell. Next, the CD40L on the Th2 cell will bind CD40 on the B cell, costimulating B cell activation and increasing expression of B7 on B cells. In addition, Th2 cells will release cytokines IL-4, IL-5, and IL-6 to signal the B cell to begin expansion and differentiation into antibody secreting plasma cells. Initially, the secreted antibodies are IgG and IgM; however, after B cells undergo isotype switching and somatic recombination, IgA will also be secreted. Isotype switching is important as it allows for increased Ig molecule diversity without sacrificing antigen-binding specificity. Several rounds of proliferation allow B cells to differentiate into antibody-secreting cells. The second way naïve B cells are activated is by migration to the primary follicle, which contain follicular dendritic cells and naïve resting B cells. B cell division occurs, producing germinal centers. Rapid proliferation and somatic mutation occur at the germinal centers, facilitated by the presence of Th2 cells. After dividing, B cells will be selected to survive, through the process of affinity maturation and clonal selection, based on the strength of their affinity/avidity for WNV peptides presented on DCs. The WNV-peptide specific (either for E protein, M protein, or NS1 protein) surviving B cells will either differentiate into memory B cells and travel to both the bone marrow and lymph or differentiate into plasma cells that will travel to the site of infection via circulation and secrete antibody. Memory B cells inherit the genetic mutations that occur during the germinal center reactions. Such somatic mutations and gene rearrangements resulting in isotype switches will allow memory B cells to stimulate a rapid humoral response to fight any future WNV infections.

The plasma cells will secrete IgM and IgG antibody. IgG will neutralize antigen, especially the EIII epitope of the E protein. IgG and IgM will induce opsonization of antigen, allowing for free antigen uptake and destruction. IgM and IgG will also induce the complement pathway. However, the presence of NS1 will greatly counteract complement pathway efficacy, but the presence of neutralizing antibodies should help neutralize NS1, therefore reducing or eliminating future threat of NS1 as an inhibitor of the complement pathway. Lastly, antibodies will also activate NK cells and ADCC. Any cell that was injected with the antigen DNA may express viral proteins signaling “infection.” Antibodies bound to this cell would then recruit NK cells through interaction with the NK cell’s Fc receptors, inducing NK cells to destroy the infected cells through apoptosis using perforins and granzymes.

These immune responses will have the end result of producing memory T cells and memory B cells specific to the E, M, and NS1 protein antigens. The memory B cells will mainly produce neutralizing IgG antibodies specific for the proteins, especially the EIII epitope of the E protein. By having a quick humoral memory response, neutralizing antibodies will be induced faster in the future to neutralize free WNV and any secreted NS1 proteins from infected cells. Also, a quick cellular memory response will begin destruction of infected cells promptly. A quick immune reaction is essential to counter the WNV’s ability to weaken complement response and block the interferon  $\alpha/\beta$  receptor. The immune system’s ability to effectively contain and eliminate the WNV is essential for preventing the neuroinvasive form of the disease.

Two potential concerns regarding DNA vaccines include possible incorporation into human cellular DNA and vaccine-induced development of autoimmune disease. Concern over

integration into cellular DNA postulates that integrated viral DNA could cause insertional mutagenesis resulting in the development of cancer through activation of oncogenes or suppression of tumor suppressor genes. However, research suggests that no evidence of DNA integration near the spontaneous mutation frequency exists. DNA integration has been documented at a level three times below the spontaneous mutation frequency (Ledwith *et al.*, 2000). Nevertheless, the FDA requires that potential DNA vaccines provide data on the plasmid's potential for cellular DNA integration (Smith and Klinman, 2001). Another concern is that DNA vaccines could induce auto-antibodies against cDNA, producing systemic autoimmune disease, and that DNA vaccines could result in localized inflammatory responses against the cells presenting the antigen, producing an organ-specific autoimmune response. However, studies on the ability of DNA vaccines to induce autoimmunity in mice show that the low level of autoantibodies produced as a result of DNA vaccines are insufficient to cause autoimmune disease or increase the severity of any existing autoimmune disease (Smith and Klinman, 2001). Moreover, early human Malaria and HIV DNA vaccines show no detectable anti-double-stranded-DNA or antinuclear antibodies (antibodies indicating autoimmune disease) after vaccination (MacGregor *et al.*, 1998; Le *et al.*, 2000). Despite evidence that DNA vaccine-induced autoimmunity is statistically unlikely, the FDA requires that autoantibody levels in DNA vaccinated patients still be tested to ensure the continual safety of vaccine recipients (Smith and Klinman, 2001).

There are no real statistically relevant disadvantages to a DNA vaccine. There are, however, a great many number of advantages. DNA vaccines are relatively simple compared to more traditional vaccines. DNA propagation is much more affordable than the development of live-attenuated vaccines or recombinant vaccines. Quality control of DNA vaccines is much simpler as well, given that genomic DNA isolation of the target antigen, incorporation into the plasmid, and replication of the plasmid to prepare an adequate dosage amount can be easily monitored. DNA vaccines are also significantly more stable than their traditional counterparts (Seregin *et al.*, 2006). This is a very promising advantage, because it allows for easier storage and transportation, especially in more inclement locations. Should this vaccine be successful, a DNA vaccine would be easily transported not only in America, but since the vaccine targets proteins found in all serotypes, it should prove effective with other serotypes in more inhospitable locations such as the Middle East and Africa. Lastly, because it does not employ a live virus, the vaccine is safe for use among immunocompromised patients. In order to maximize the effectiveness and safety of the vaccine, GM-CSF and IL-2 cytokine adjuvants will be used to ensure a sufficient and timely immune response. Recipients will also be monitored at regular intervals, including time of injection, for adverse response such as the development of autoantibodies or anaphylaxis. Should an adverse response occur, the recipient will either not continue to receive injections or will do so with appropriate preventative measures such as a preliminary dose of Benedryl to prevent any hypersensitivities.

## West Nile Virus DNA Vaccine for clinical trial in the Western States

### Vaccine Efficacy:

The proposed WNV DNA vaccine seeks to induce immunity to the WNV strains prevalent in the United States, especially NY99. Vaccine efficacy will be demonstrated through a clinical trial involving healthy volunteers of both sexes, ages 18-50, who ideally engage in outdoor activities. Volunteers will be drawn from a target population consisting of states with the highest infection frequencies: CA, ND, SD, CO, NE, MT, and WY (CDCa, 2006). Vaccination will begin in April, three months prior to the onset of the peak infection season, which typically lasts from July to October. During the clinical trial, three doses of the DNA vaccine will be administered through intramuscular injection at intervals of 21 days, in order to induce a mounting, strong immune response. Criteria for a successful trial includes: minimal evidence of side effects after each vaccine injection, laboratory tests indicating both cellular and humoral immunity were strongly stimulated as a result of the vaccine, and minimal toxicity among vaccine recipients. Vaccine participants will be observed through the peak infection season to determine if any patients become infected via mosquito bites.

Two weeks after each vaccine dose has been administered, allowing time to build adaptive immunity, laboratory tests will be run to determine if the desired immune responses have been stimulated. Because West Nile is a virus, the desired immune response will include neutralizing antibodies (specifically IgG and IgM) and cytotoxic T cell responses. The following tests can be run on blood collected from the vaccine recipients to measure cellular and humoral immunity.

Laboratory tests to measure cellular immunity to WNV include  $^{51}\text{Cr}$  Chromium (Cr) Release assay for WNV specific  $\text{CD8}^+$  Tc cells,  $\text{INF-}\gamma$  ELISpot Assay, and Intracellular Cytokine Staining (Horton and McElrath, 2003). For the proposed vaccine to be effective, it must be proven that each of the antigens included in the vaccine are capable of inducing a cellular response among Tc cells, to ensure the destruction of infected cells, and among Tc cell production of cytokines such as  $\text{INF-}\gamma$  and IL-12, which work to activate macrophages and DCs and increase their expression of MHC I, as well as enhance Tc cell and NKC cytotoxicity, respectively. Without such a response, successful immunity against WNV would not be possible.

In the  $^{51}\text{Cr}$  Release assay, target cells (cells infected with WNV) are labeled with  $^{51}\text{Cr}$ . This is done by exposing infected cells to a wash with  $^{51}\text{Cr}$ , since cells will readily intake  $^{51}\text{Cr}$  into their cytosol but will not release it spontaneously. Once the target cells are labeled with  $^{51}\text{Cr}$ , they will be exposed to the blood of those who were vaccinated. If the vaccine is effective, the serum will contain  $\text{CD8}^+$  Tc cells that will kill the labeled target cell. If the cells are lysed, the  $^{51}\text{Cr}$  will be released into the supernatant, confirming that there are  $\text{CD8}^+$  Tc cells specific for WNV in the serum. After centrifugation of samples to separate out the cells, the amount of  $^{51}\text{Cr}$  released can be measured. These samples will be compared to positive and negative controls to determine if the vaccine was successful in inducing an immune response for WNV-specific  $\text{CD8}^+$  Tc cells. Positive and negative controls can be done with samples of blood known to have been exposed to WNV and samples known to not have been exposed to the virus. Four separate  $^{51}\text{Cr}$  Release assays will be performed: (1) using E protein peptides presented on MHC I as the target cells, (2) using M protein peptides presented on MHC I as the target cells, (3) using NS1 protein peptides presented on MHC I as the target cells, and (4) using WNV-infected target cells. It is essential to the success of the vaccine that  $\text{CD8}^+$  Tc cells specific for each of the three

vaccine antigens are present in measurable amounts in recipients' blood, as well as for the Tc cells to identify and bind to said antigens when they are in their natural state as part of the WNV.

The next test, the INF- $\gamma$  ELISpot assay, will measure the ability of T cells to secrete INF- $\gamma$  in response to a specific antigen. PBMCs, or peripheral blood mononuclear cells (i.e. lymphocytes & monocytes), from a person that is vaccinated are added along with a specific WNV peptide (either from the E protein, M protein, or NS1 protein) to a 96-well nitrocellulose plate that has been coated with antibodies to INF- $\gamma$  (anti-INF- $\gamma$ ). A control is also run at same time with wells containing PBMCs and complete RPMI (10%FBS and 1% penicillin-streptomycin) medium only (without WNV peptides). The plates are then incubated overnight at 37°C. If WNV-specific T cells are present in the PBMCs, they will be stimulated by their related WNV peptide to secrete INF- $\gamma$ . The anti-INF- $\gamma$  antibody bound to plate binds the secreted INF- $\gamma$ . The next day, the PBMCs are removed and the wells washed. Anti-INF- $\gamma$  antibody labeled with biotin is then added. This second antibody binds to a different epitope on INF- $\gamma$  than the first antibody. A sandwich of INF- $\gamma$  between two anti-INF- $\gamma$  antibodies is produced. Unbound Antibody is washed away with buffer. Then, streptavidin-enzyme conjugate is added. Streptavidin binds very tightly to the biotin linked to the second anti-INF- $\gamma$  antibody, creating a biotin/streptavidin complex bound to the enzyme (usually HRP). The enzyme substrate is added, resulting in the formation of a colored product in the wells. Each spot corresponds to an antigen-specific cell that has secreted INF- $\gamma$  in response to its related WNV peptide. The number of spots corresponds to the number of antigen-specific T cells present among the PBMCs. This assay will be repeated three times, each time using either E protein, M protein, or NS1 protein peptides, in order to ensure that T cell secretion of INF- $\gamma$  occurs in response to the three antigens included in the vaccine. WNV-specific CD8<sup>+</sup> T-cell responses can be distinguished from WNV-specific CD4<sup>+</sup> T-cell responses in these assays by removing CD4<sup>+</sup> T cells prior to the assay using magnetic beads labeled with anti-CD4 antibody. These CD4-depleted cells are assayed along with the normal PBMC. If responses are seen in the PBMC wells but not the corresponding CD4-depleted wells, the responses are due to WNV-specific CD8<sup>+</sup> Tc cells. If responses persist in the CD4-depleted wells, these are due to WNV-specific CD4<sup>+</sup> T cells. The same test can be done to measure IL-12 presence as well.

Another test, intracellular cytokine staining (ICS) is a flow cytometry method for counting the amount of antigen-specific, cytokine-secreting T cells. PBMCs from the vaccinated patient are stimulated with WNV peptides (from the E, M, or NS1 proteins) in the presence of co-stimulatory antibody against CD28 and CD49. Negative control test are run with PBMCs and co-stimulatory antibodies but no WNV peptides. Positive control test are run with super-antigen like bacterial toxins. These tests are incubated for 6 hours at 37°C in the presence of brefeldin A or monesin, which are inhibitors of protein transport through the Golgi body. This prevents cytokines secreted by WNV-specific T cells, due to WNV peptides, from permeating the cell membrane, allowing the cytokines to accumulate inside the cell. Responding T cells can be seen using fluorescently labeled antibodies specific for IL-12. Responding cells can also be distinguished by additional, lineage-specific antibody markers like, CD3 and CD8 antibodies for CD8<sup>+</sup> Tc cells. They can be fluorescently labeled and then analyzed using flow cytometry.

The INF- $\gamma$  ELISpot assay and the intracellular cytokine staining assay measure the same effector function, that of cytokines, but have different advantages. The ELISpot assay requires fewer cells and is less technically demanding than ICS, but the ICS allows for precise identification of T cells using lineage-specific markers to recognize the responding cell population. They can be used together for a more precise measure of immunity.

Laboratory tests to measure humoral immunity include complement fixation test for neutralizing antibody and Enzyme-Linked ImmunoSorbent Assay for WNV IgG antibody. Complement fixation test for neutralizing antibody allows for the determination that anti-WNV antibodies are present in recipients' blood. For this test, sheep RBC's (sRBC) and anti-sRBC (antibody made against sheep RBC), complement, and WNV are needed. The protocol is as follows:

1. Place collected vaccine recipients' serum in a sterile test tube.
2. Add WNV virus. If WNV antibody is present in recipients' serum, then antibody-antigen complexes will form. If no antibody is present, no complexes will form and antigen will float around free or unbound
3. Add complement that will bind antibody-antigen complexes if they are present
4. Add sRBC and anti-sRBC which will form a complex
5. Incubate at room temperature for 15 minutes and then centrifuge.
6. Remove tubes from the centrifuge and observe the amount of hemolysis by comparing the intensity of the red color to control groups or using colorimetry

If antibody is present in recipients' serum, then complement will be completely bound, resulting in little sRBC lysis. If antibody is not present, then complement will bind anti-sRBC + sRBC complex, resulting in lysis of the sRBCs, which causes the red color. This test will indicate whether antibody for WNV has been produced in response to vaccination.

Since neutralization of viruses is essential to stop viral spread from cell to cell, and in the case of WNV, also important to stop secreted NS1 protein from impeding the complement pathway, it is essential that the proposed vaccine stimulate the presence of IgG antibody, which is essential for effective viral neutralization. To test specifically for the presence of IgG against E protein, M protein, and NS1 protein, as well as its ability to bind to such epitopes on the whole WNV itself, Enzyme-Linked ImmunoSorbent Assay will be used. The protocol is as follows:

1. Place single antigen type (either E, M, NS1 protein, or WNV) in a microtiter plate and allow the antigen solution to incubate 30-40 minutes to allow for optimal binding saturation to the plastic.
2. Remove unbound antigen with buffer and cover with unrelated protein from powdered milk, blocking any sites that might nonspecifically bind antibody and again remove unbound protein by washing with buffer.
3. Add serum sample from vaccinated patient and allow specific antibody to bind to the antigen (E, M, or NS1 protein). Remove any unbound antibody by washing.
4. Add anti-human gamma chain that will bind human IgG's Fc region. Remove unbound antibody-enzyme complex. Note: anti human-IgG can be made by injecting mice with human IgG. The mouse's immune system will produce antibody against human IgG
5. Add chromogenic substrate such as pNPP or ABTS, which are colorless substrates that enzymes (i.e. alkaline phosphatase [AP] or horseradish peroxidase [HRP]) will convert to a colored product. Incubate the plate until color develops.
6. Measure color in a spectrophotometer. The more intense the color development, the more IgG to specific antigen type is present in the patient's serum.
7. Compare to positive and negative controls. To run a negative control test, omit the antigen. To run a positive control test, substitute patient serum with serum from a patient that is known to have WNV infection.

The test will be repeated four times to identify the presence of IgG specific for E protein, M protein, and NS1 protein, as well as for IgG capable of binding to the antigen proteins when they exist in their normal state as part of the virus.

Lastly, laboratory tests for antinuclear antibodies and antibodies against double-stranded-DNA will be conducted to assay possible autoimmune antibodies/antigens. The test will be performed using an ELISA assay. All specimens determined to be positive are confirmed using HEp-2 cells, and if positive, the titer and pattern will be reported. The test uses anti-human IgG conjugate, since many normal individuals have low levels of anti-nuclear antibody-IgM. Isotype switching of anti-nuclear antibodies from IgM to IgG is usually followed by the onset of autoimmune disease states (ARUP 2007).

The Phase I WNV Vaccine Trial will be considered successful if the following occur: induction of strong stimulation of cellular and humoral immunities, no significant development of autoimmune antibodies, and low levels of hypersensitivity reactions. Successful vaccines are instrumental in reducing infection. With a successful WNV DNA vaccine, we expect the number of WNV infections and deaths in high infection rate areas to measurably decline. Moreover, since the antigens included in the proposed DNA vaccines are common to all WNV serotypes, this vaccine will have potential application for inducing immunity worldwide.

## References:

- ARUP. 0050080: Anti-Nuclear Antibodies (ANA), IgG Screen with Reflex to IFA Titer. Salt Lake City (UT): ARUP Laboratories; 2007 [cited 2007 Nov 14]. Available from <http://www.aruplab.com/guides/ug/tests/0050080.jsp>
- CDCa. 2007. West Nile Virus Update --- United States, January 1 -- October 16, 2007 [Internet]. MMWR 56(41): 1084-1085. [Cited 2007 Nov 1]. Available from <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5641a4.htm>
- CDCb. West Nile Virus: What You Need to Know. CDC Fact Sheet [Internet]. Atlanta (GA): Centers for Disease Control and Prevention; 2006 Sept [cited 2007 Nov 11]. Available from [http://www.cdc.gov/ncidod/dvbid/westnile/wnv\\_factsheet.htm](http://www.cdc.gov/ncidod/dvbid/westnile/wnv_factsheet.htm)
- Chang GJ, Davis BS, Hunt AR, Holmes DA, and Kuno G. 2001. Flavivirus DNA vaccines: current status and potential. *Annals of the New York Academy of Sciences* 951: 272-85.
- Chung KM, Liszewski MK, Nybakken G, Davis AE, Townsend RR, Fremont DH, Atkinson JP, and Diamond MS. 2006. West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proceedings of the National Academy of Sciences of the United States of America* 103(50): 19111–19116.
- Dauphin G and Zientara S. 2007. West Nile Virus: Recent trends in diagnosis and vaccine development. *Vaccine* 25(30):5563-5576.
- Deubel V, Fiette L, Gounon P, Drouet MT, Khun H, Huerre M, Banet C, Malkinson M, and Desprès P. 2001. Variations in Biological Features of West Nile Viruses. *Annals of the New York Academy of Sciences*: 951 (1): 195–206.
- Gerhardt R. 2006. West Nile Virus in the United States (1999–2005). *Journal of the American Animal Hospital Association* 42:170-177.
- Greenland JR and Letvin NL. 2007. Chemical adjuvants for plasmid DNA vaccines. *Vaccine* 25(19):3731-3741.
- Gubler DJ. 2007. The Continuing Spread of West Nile Virus in the Western Hemisphere. *Clinical Infectious Diseases* 45:1039-1046.
- Hayes EB and Gubler DJ. 2006. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. *Annual Reviews of Medicine* 57: 181-94.
- Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, and Campbell GL. Epidemiology and Transmission Dynamics of West Nile Virus Disease. 2005. *Emerging Infectious Diseases* 11(8).

Horton H and McElrath MJ. Assays to Detect Host Immune Responses to HIV: HIV InSite Knowledge Base Chapter. San Francisco (CA): University of California San Francisco Center for HIV Information; 2007 [cited 2007 Nov 9]. Available from <http://hivinsite.ucsf.edu/InSite?page=kb-02-02-04>

Hunt TA, Urbanowski MD, Kakani K, Law LJ, Brinton MA, and Hobman TC. 2007. Interactions between the West Nile virus capsid protein and the host cell-encoded phosphatase inhibitor, I<sub>2</sub>. *Cellular Microbiology* 9(11): 2756–2766.

Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, Kumar S, Wang R, Doolan DL, Maguire JD, Parker SE, Hobart P, Norman J, and Hoffman SL. 2000. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* 18(18):1893-901.

Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, Harper LB, Schock HB, Zhang H, Faris JE, Way PA, Beare CM, Bagdon WJ, and Nichols WW. 2000. Plasmid DNA vaccines: assay for integration into host genomic DNA. *Developments in Biologicals* 104:33-43

McDonald WF, Huleatt JW, Foellmer HG, Hewitt D, Tang J, Desai P, Price A, Jacobs A, Takahashi VN, Huang Y, Nakaar V, Alexopoulou L, Fikrig E, and Powell TJ. 2007. A West Nile Virus Recombinant Protein Vaccine That Coactivates Innate and Adaptive Immunity. *The Journal of Infectious Diseases* 195:1607-1617

MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, Chattergoon MA, Baine Y, Higgins TJ, Ciccarelli RB, Coney LR, Ginsberg RS, and Weiner DB. 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *Journal of Infectious Diseases* 178(1):92-100

Monath TP, Liu J, Kanesa-Thanan N, Myers GA, Nichols R, Deary A, McCarthy K, Johnson C, Ermak T, Shin S, Arroyo J, Guirakhoo F, Kennedy JS, Ennis FA, Green S, and Bedford P. 2006. A live, attenuated recombinant West Nile virus vaccine. *Proceedings of the National Academy of Sciences of the United States of America* 103(28):10823.

Seregin A, Nistler R, Borisevich V, Yamshchikov G, Chaporgina E, Kwok CW, and Yamshchikov V. 2006. Immunogenicity of West Nile virus infectious DNA and its noninfectious derivatives. *Virology* 356(1-2): 115-125

Smith HA and Klinman DM. 2001. The regulation of DNA vaccines. *Current Opinion in Biotechnology* 12(3):299-303.

UT Southwestern Medical Center. Study unveils how West Nile virus evades immune defenses, helps set the stage for vaccine development [Internet]. Dallas (TX): University of Texas Southwestern Medical Center; 2006 Oct [cited 2007 Nov 1]. Available from <http://www.utsouthwestern.edu/utsw/cda/dept37389/files/321284.html>