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Phylogenetics and New Approaches to Vaccine Development for West Nile

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PHYLOGENETICS AND NEW APPROACHES TO VACCINE DEVELOPMENT FOR WEST NILE

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ABSTRACT

West Nile virus (WNV) was first isolated in 1937 from a febrile Ugandan woman. WNV now has a worldwide distribution from Australia and India in the east to Europe and the United States in the west. The first human cases of WNV in the United States were detected in New York in 1999. The North American isolates were also found to be highly neuroinvasive and neurovirulent and in many cases leading to mortality or permanent CNS sequelae among humans. WNV was first isolated in Louisiana in 2001. The genome of this virus named LSU-AR01was sequenced and a detailed genetic analysis revealed 26 amino acid changes in comparison to the prototypic New York-99 strain. Phylogenetic analysis using Neighbor-joining and the Bayesian approach showed that LSU-AR01 was closely related to a strain isolated from a mosquito in 1999 in Connecticut. This relationship was bolstered by a 58% bootstrap value and a 66% posterior probability by these algorithms respectively. Comparative pathology revealed that the LSU-AR01 was more neurovirulent and neuroinvasive especially at low doses indicating a virus with a competitive edge. Recombinant vesicular stomatitis virus (rVSV) based vaccines cleverly incorporate reverse genetics to recover a recombinant virus expressing a foreign antigen of interest. rVSV vectored vaccines expressing the LSU-AR01 envelope (E) glycoprotein were engineered and administered to mice in a prime-boost approach. The vaccines were able to confer high degree of protection in mice against lethal challenge with highly virulent WNV LSU-AR01. Detailed immunological analyses of immunized mice revealed the production of neutralizing antibody responses. In addition, vaccinated mice generated WNV E glycoprotein specific $CD8^{+}CD62L^{low}$ $IFN\gamma^{+}$ T cells response against WNV. Recombinant VSV expressing the Simian Retrovirus-2 (SRV-2) gag and Env gene constructs and the Herpes B virus glycoprotein D gene were constructed and characterized in cell culture experiments. The VSV-

SRV-2 gag and env recombinants generated protective immune responses in non-human primates. The VSV Herpes B recombinant vaccine will be tested in non-human primates in the near future. Collectively, these experiments revealed that VSV-vectored vaccines are highly effective in generating humoral and cellular immune responses against viral infections.

CHAPTER 1

INTRODUCTION

STATEMENT OF PROBLEM AND HYPOTHESIS

West Nile virus (WNV) was first isolated in 1937 from a febrile Ugandan woman. The virus gradually spread westward towards the United States. The first human cases of WNV in the United States were detected in Flushing, NY in 1999. Until then, WNV was one of the least concerns for humans. The new viral strains caused significant mortality among birds particularly corvids, a phenomenon not observed in the old world outbreaks. Moreover, the viral isolates from North America were found to be highly neuroinvasive and neurovirulent and in many cases leading to mortality or permanent central nervous system (CNS) sequelae among humans.

Starting in 2001, a number of WNV isolates were isolated in Louisiana from infected people and animals. Based on the initial outbreak and detection of WNV in NY in 1999, it was largely assumed that these viruses originated from the prototypic WNV NY99 strains, as it evolved and moved towards the south and west of the country. The main goal of these investigations was to generate a new vaccine against WNV based on the vesicular stomatitis (VSV) viral-vectored vaccine approach originally pioneered by Dr. John Rose at Yale University. This approach involves the construction of recombinant VSVs that express the desired immunogen. Optimal boosting immunization is achieved in this vaccination protocol by using recombinant VSVs that express different G glycoproteins, which is required for viral infectivity and spread. Most of the antiviral immune response is directed against the G glycoprotein. Therefore the use of different G variants enables boosting immunization without compromising viral infectivity and replication due to pre-existing immunity against VSV.

Previous work has shown that the WNV E glycoprotein is highly immunogenic and can elicit robust humoral and cellular immune responses against the virus. Therefore, we decided to construct recombinant VSVs expressing the WNV E glycoprotein.

In the process of identifying the most virulent strain of WNV that could be used for challenge purposes in mouse experiments, I found that a Louisiana strain isolated by Dr. Alma Roy at the Louisiana Animal Disease Diagnostic Laboratory (LADDL), LSU School of Veterinary Medicine, exhibited higher neurovirulence than the prototypic NY-99 strain in mice. This observation led us to sequence the entire genome of the LSU-AR01 virus and study its phylogenetic relationship to other known WNV strains. This work revealed that the LSU-AR01 is closely related to a 1999 mosquito isolate from Connecticut.

The main hypothesis of the proposed work was that recombinant VSV vaccines could effectively protect mice against lethal challenge with the highly virulent WNV virus strain LSU-AR01, because they could elicit robust humoral and cellular immunity against WNV.

A series of experiments detailed in this thesis established that the recombinant VSVs that I constructed were able to elicit strong humoral and cellular immune responses against the virus and protected mice against lethal challenge with the highly virulent WNV LSU-AR01 strain.

The VSV-vectored vaccine is a versatile vaccine approach that can be used to combat a variety of important pathogens. In addition to the work on WNV, I constructed recombinant VSVs that expressed the simian retrovirus-2 (SRV-2) gag and Env genes and against Herpes B virus using the B virus glycoprotein D (gD). These SRV-2 vaccines were recently tested in non-human primate vaccine studies in collaboration with Dr. Preston Marx at the Tulane National Primate Research Center (TNPRC) and were shown to protect animals against lethal challenge.

The rVSV-Herpes B gD vaccine will be tested in the near future. The construction and characterization of these recombinant viruses is included as a separate chapter in the body of the thesis.

The VSV-vectored vaccine approach could be made safer by constructing non-replicating VSVs that could be safely administered to humans. Providing essential VSV genes in *trans* through their expression in complementing cell lines and the introduction of specific mutations within the viral genome that further attenuate the virus could be easily engineered to make VSV safe vehicles for vaccine purposes.

STATEMENT OF RESEARCH OBJECTIVES

The overall objective of this thesis was to construct VSV-vectored vaccines that can protect mice against WNV infections. In the process of these investigations, additional VSV-vectored vaccines were constructed to combat SRV-2 and herpes B infections in collaboration with the Tulane National Primate Research Center (TNPRC).

I. Characterization of the WNV-LSU-AR01 strain.
   a. Comparative evaluation of pathogenicity of the LSU-AR01 WNV and the NY99 strain of WNV.
   b. Analysis of genetic differences between the LSU-AR01 and the New York 99 (NY99) strain of WNV.
   c. Phylogenetic analysis of WNV LSU-AR01.

II. Development of rVSV-based vaccine for West Nile virus.
   a. Construction and characterization of rVSV glycoprotein exchange vector vaccines expressing West Nile virus (WNV) Envelope (E) glycoprotein.

b. Development and standardization of flow cytometric assay for evaluation of cell-mediated immune response.

c. Evaluation of vaccine efficacy in mice by evaluation of humoral and cell-mediated immune response elicited by the vaccines.

d. Evaluation of the vaccine potency in mice by lethal challenge experiments in mice using the WNV LSU-AR01 strain.

III. Development of rVSV vaccines for Simian Retrovirus-2 (SRV-2) and Herpes B virus.

a. Construction and recovery of rVSV glycoprotein exchange vector vaccines expressing the SRV-2 gag protein.

b. Construction and recovery of rVSV glycoprotein exchange vector vaccines expressing the SRV-2 chimeric C-terminus truncated SRV2 Envelope (Env) glycoprotein fused to VSV G glycoprotein C terminus for enhanced recovery of the recombinant virus.

c. Construction and recovery of rVSV glycoprotein exchange vector vaccines expressing the SRV-2 C-terminus truncated SRV-2 Envelope (Env).

d. Construction and recovery of rVSV glycoprotein exchange vector vaccines expressing the C-terminus truncated Herpes B virus glycoprotein D.

This research work is presented in manuscript format. Chapter 2 describes the isolation, sequencing, sequence and phylogenetic analysis of a WNV (WNV-LSU-AR01) isolated in Louisiana and comparison with the prototypic WNV New York 99 (NY99) strain. In addition, the chapter includes a comparative pathogenesis study of LSU-AR01 and NY99 in mice.

Chapter 3 describes the engineering of a recombinant VSV based vaccine for WNV. The recombinant VSV expressing WNV E glycoprotein was tested in mice and was shown to elicit

strong humoral and cell mediated immune responses. Additionally, it completely protected vaccinated mice against lethal challenge with the highly virulent WNV LSU-AR01 strain.

Chapter 4 describes the construction of VSV vectored vaccines for Simian Retrovirus-2 and Herpes B virus. The SRV-2 vaccines have been recently been tested in non-human primates and preliminary data suggests that these vaccines are efficacious. The herpes B VSV vaccines will be tested in the future. The final chapter (Concluding Remarks and Future Challenges) provides a summary of the work described in this dissertation as well as information about potential future directions of this type of research. Appendix A contains additional phylogenetic analysis of WNV whole genomes using a Bayesian approach for phylogeny.

LITERATURE REVIEW

West Nile Virus (WNV) Virology

West Nile virus (WNV) was first isolated in 1937 from a febrile patient in the West Nile province of Uganda (Smithburn et al., 1940). The virus belongs to the flavivirus genus in the family Flaviviridae. It has an approximately 11kb single-stranded positive (+) sense RNA genome. The icosahederal virion particles are about 500Å in diameter and have 180 molecules of pre-membrane/membrane (preM/M) and 180 molecules of envelope (E) glycoprotein on its surface (Lindenbach, Thiel, and Rice, 2007). A cross sectional electron microscopic view showed highest density at the outermost layer. This lipid bilayer is 34-40 Å thick and has the transmembrane domains of preM/M and E glycoprotein weave through it (Mukhopadhyay et al., 2003).

The genome is translated as a single polyprotein (Figure 1.1), which is subsequently cleaved by host as well as virally encoded proteases to produce three structural and seven

nonstructural proteins (Brinton, 2002; Chambers et al., 1990). The structural proteins include a capsid (C) protein, a premembrane (prM) protein, and an envelope (E) glycoprotein that mediates attachment, virus-induced membrane fusion and virion assembly (Mukhopadhyay et al., 2003; Mukhopadhyay, Kuhn, and Rossmann, 2005). Host signal peptidase cleaves at the C/preM, preM/E, E/NS1, 2k/4B junctions while a virus encoded serine protease cleaves the junction between NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K and NS4B/NS5 junctions (Lindenbach, Thiel, and Rice, 2007; Roosendaal et al., 2006). The enzyme responsible for cleaving the NS1/NS2A junction is not known (Lindenbach, Thiel, and Rice, 2007). The viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) regulate viral transcription and replication, as well as attenuating host antiviral responses (Best et al., 2005; Guo, Hayashi, and Seeger, 2005; Khromykh, Sedlak, and Westaway, 2000; Lin et al., 2006; Liu et al., 2005; Munoz-Jordan et al., 2005).

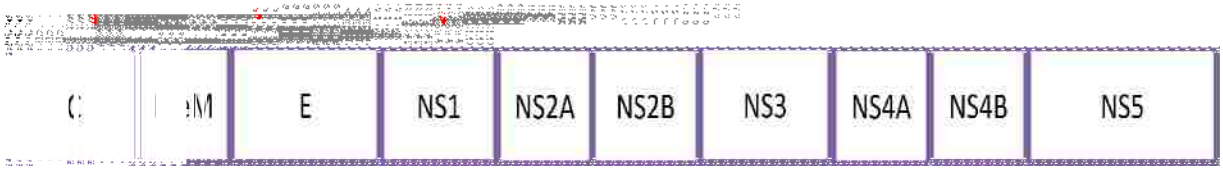


Figure 1.1: Organization of West Nile virus genome. WNV has an approximately 11kb RNA genome which is transcribed as a single polyprotein. The polyprotein is cleaved by various viral and host proteases. The Capsid (C), pre-membrane (PreM) and envelope glycoprotein (E) constitute the structural genes. WNV also encodes seven non-structural (NS) that are vital in transcription, translation and pathogenesis of the virus.

In flaviviruses, the capsid protein is an approximately 11kD highly basic protein. It plays a significant role in that it contains a C-terminus hydrophobic signal that allows for endoplasmic

reticulum (ER) translocation of the preM (Mukhopadhyay, Kuhn, and Rossmann, 2005). It is believed to play an important role in RNA binding and membrane interactions. The preM (26kD) is one of the two glycoproteins expressed on the viral surface. Its role in E gylcoprotein folding and chaperoning is critical (Konishi and Mason, 1993; Lorenz et al., 2002). It also functions in protecting the E glycoprotein from the acidic environment and consequent structural rearrangement to fusogenic form as the complex passes through the secretory pathway. (Guirakhoo, Bolin, and Roehrig, 1992; Heinz et al., 1994). The flavivirus life cycle is illustrated in Figure 1.2. The maturation of the virion particle occurs as a result of a Golgi resident furin mediated cleavage of preM to Pre peptide and the membrane glycoprotein (M) (Stadler et al., 1997; Stiasny et al., 1996; Wengler, 1989). Following this, the M and E glycoproteins associate with each other and heterodimerize.

The envelope glycoprotein is probably the best studied protein in flaviruses, especially WNV primarily for its role in receptor binding, membrane fusion and major immunogenic determinant. The WNV E glycoprotein is an approximately 53 kD type I membrane glycoprotein and has 12 conserved cysteine residues (Nowak and Wengler, 1987). The E glycoprotein has three domains (DI-DIII). DI is the central structural domain while DII is the dimerization domain. The DII domain contains a 12 amino acid (aa) long fusion loop that is necessary for virus-cell membrane fusion and for receptor binding (Pokidysheva et al., 2006). The DIII domain spans amino acids 296-415 (Beasley and Barrett, 2002) and functions in receptor recognition and binding (Anderson, 2003; Chu et al., 2005; Lee, Chu, and Ng, 2006; Volk et al., 2004).

More importantly, from a vaccinologists' perspective, a majority of the neutralizing epitopes have been mapped to DIII (Beasley and Barrett, 2002; Chu, Chiang, and Ng, 2007; Li,

Barrett, and Beasley, 2005; Nybakken et al., 2005; Oliphant and Diamond, 2007; Oliphant et al., 2005). The residues most consistently identified as neutralization epitopes on DIII are positions 302-309, 330-333, 333-365 and 389-391 (Oliphant et al., 2005). Co-crystallization studies of DIII with monoclonal antibody mAbE16 revealed that these regions mapped to adjacent Ig-like loops forming a discontinuous epitope (Nybakken et al., 2005). Pierson et al. showed that the most potent mAbs blocked infection at very low occupancy with as few as 30 of the 180 envelope proteins bound (Pierson et al., 2007).

West Nile Virus Natural Cycle

West Nile Virus (WNV) has a worldwide distribution ranging from Australia, India and China in the east to the Middle East, Africa, Europe and the United States in the west. Phylogenetic data of WNV reveals at least two distinct lineages (Bondre et al., 2007; Briese et al., 2002; Lanciotti et al., 2002; Lanciotti et al., 1999). Strains from North America, Europe, Middle East, Australia and India belong to lineage I. The Indian, Australian (Kunjin), Czech (Rabensburg) and LEIV-Krnd88-190 virus (Russia) isolates form separate sub-lineages within lineage I (Bakonyi et al., 2005; Bondre et al., 2007; Coia et al., 1988; Lanciotti et al., 2002; Scherret et al., 2001). Lineage II is mainly composed of isolates from sub-Saharan Africa and the island of Madagascar.

Following its initial isolation in 1937 in Uganda (Smithburn et al., 1940), WNV outbreaks were reported in Egypt and Israel (1951 and 1957), France (1962-1965), South Africa

Figure 1.2: WNV infects cells by receptor mediated endocytosis. The low endosomal pH triggers an irreversible trimerization of the E glycoprotein followed by the fusion of viral and cell membranes. Following uncoating of the nucleocapsid, the viral genomic RNA is released into the cytoplasm and is translated into a single polyprotein which is cleaved by viral and host proteases. Additionally, genome replication occurs on intracellular membranes. Virion particles are assembled in the endoplasmic reticulum (ER) and the immature virion particles are transported via the trans-Golgi network (TGN). Following a furin mediated cleavage of preM, mature virion particles bud out by exocytosis.

virus m
fusion and
disassembly
virion assembly
Nucleus

(1974), India (1980-1981), Ukraine (1985). More recent history includes outbreaks in Algeria (1994), Romania (1996), Morocco (1996 and 2003), Tunisia (1997 and 2003), Italy (1998),Czech Republic (1997), Israel (1997, 2000 and 2003), Russia (1999-2001), and France (2000, 2003-2004) (Bernkopf, Levine, and Nerson, 1953; Chowers et al., 2001; Dauphin et al., 2004; George et al., 1984; Gerhardt, 2006; Hayes, 2001; Hayes et al., 2005a; Hubalek and Halouzka, 1999; Joubert et al., 1970; Murgue et al., 2001; Murgue, Zeller, and Deubel, 2002; Platonov et al., 2001; Schuffenecker et al., 2005; Tsai et al., 1998; Zeller and Schuffenecker, 2004). WNV was not considered a major risk for humans until the 1996 outbreak in Romania. WNV subsequently became a major veterinary and public health concern in the Europe and the United States. The first cases of the 1999 WN outbreak in the United States were reported in Flushing , NY (Asnis et al., 2000).

WNV is primarily transmitted in nature by Culex mosquitoes. However, the virus has been isolated from more than 60 species of mosquitoes (Dauphin and Zientara, 2007; Gubler, 2007) as well as from ticks (Formosinho and Santos-Silva, 2006; Lawrie et al., 2004). Mosquito saliva is known to greatly enhance the success of the virus by acting on the host immune system in multiple ways (Titus, Bishop, and Mejia, 2006). A recent study comparing four species of mosquitoes showed that each mosquitoes was able to inject a mean dose of $10^{3.6\text{-}5.9}$ PFUs of WNV per bite (Styer et al., 2007). Approximately 10^2 PFU is additionally injected extravascularly. Mosquitoes become infected with WNV as they imbibe a blood meal from a viremic host. They can also infect each other as they co-feed on the same host (Higgs et al., 2005). The virus infects the gut epithelium cells of the mosquitoes and as viral titers increase, they escape into the hemocoel, spread to surrounding tissues, and finally invade the salivary glands and brain of the mosquito (Davis et al., 2006; Fields, Knipe, and Howley, 2007).

When the infected mosquito bites a vertebrate, WNV is transmitted intradermally. It infects and replicates in the Langerhans cells which in turn migrate to the lymph nodes. A second round of replication occurs in the lymph nodes before viremia occurs (Drebot and Artsob, 2005).

Birds are amplifiers of the disease. The North American strain of WNV is characterized by its ability to cause fatal neurological disease among many different species of birds. WNV was isolated from at least 300 species of birds and was shown to cause specific pathological changes in many tissues of at least 14 different species (Fields, Knipe, and Howley, 2007; Hayes et al., 2005a; Steele et al., 2000). Thus, birds are the most important link in WNV maintenance and transmission. American alligators (*Alligator mississippiensis*) can also become infected with WNV and transmit WNV to mosquitoes (Klenk et al., 2004; Miller et al., 2003). In addition to this, WNV has been isolated from a number of non-avian vertebrate hosts including but not limited to equines, felines, canines and bats (Austgen et al., 2004; Fields, Knipe, and Howley, 2007). Like birds, equines play an import indicator role in WNV outbreaks but are dead-end hosts. They become viremic but the viremia is not high enough for horses to serve as an amplifying host (Bunning et al., 2001). In the 1999 outbreak, 36% of the infected horses either died or were euthanized (Ostlund et al., 2001). Other studies reported a 22-28% mortality rate in horses (Salazar et al., 2004; Schuler et al., 2004). The common clinical symptoms in horses include weakness, incoordination and ataxia (Trock et al., 2001).

Human West Nile Disease

Humans, like equines, are a dead-end host as WNV does not achieve sufficiently high enough titers to be transmitted to mosquitoes. The virus has also been reported to be transmitted

in humans through modes that are not vector mediated. Reports show that WNV could be transmitted from mother to child by the intrauterine route (CDC, 2002a). Transmission through breast milk (CDC, 2002d; Hayes and O'Leary, 2004) and blood transfusion (CDC, 2002b; CDC, 2003a; CDC, 2004b) have also been documented. Additionally, the virus can also be transmitted via bone marrow transplant (Hiatt et al., 2003), organ transplantation (CDC, 2002e; Iwamoto et al., 2003) and through dialysis (Cairoli, 2005; CDC, 2004a). Finally, laboratory acquired infection has also been reported (CDC, 2002c; CDC, 2003b).

The human incubation period for WNV is 2-14 days (Gea-Banacloche et al., 2004). Approximately 80% of patients infected with WNV were found to be asymptomatic and 20% of the patients had West Nile Fever (WNF) (Gea-Banacloche et al., 2004; Hayes and Gubler, 2006; Mostashari et al., 2001). These WNF patients generally have fever and headache. Some patients may also exhibit a variety of signs and symptoms including fever, headache, muscle weakness, fatigue, nausea, vomiting, gastrointestinal manifestations, lymphadenopathy and non-pruritic maculopapular skin rash (Davis et al., 2006; Del Giudice et al., 2005; Ferguson et al., 2005). Additional non-neurological clinical manifestations include rhabdomyolysis (Jeha et al., 2003; Kulstad and Wichter, 2003), pancreatitis (Perelman and Stern, 1974), hepatitis (Sampson et al., 2000), myosistis, orchitis (Smith et al., 2004), chorioretinitis (Khairallah et al., 2004) and cardiac dysrythmias (Hayes et al., 2005b).

Less than 1% of the WNV patients suffer from a West Nile Neuroinvasive Disease (WND) including West Nile meningitis (WNM), encephalitis (WNE) and acute flaccid paralysis (poliomyelitis-like syndrome) (WNP) (Hayes et al., 2005b; Mostashari et al., 2001; Sejvar, 2007). The differences in symptoms of WNF and WND are often not black-and-white and consequently are difficult to distinguish.

West Nile Meningitis: WNM is believed to occur in ~40% of cases of WND. Symptoms of meningeal irritation, including fever, headache, nuchal rigidity, photophobia, and phonophobia are observed in these patients. Some of the patients also exhibit Kernig's and Brudzinski's signs (Klein et al., 2002). Pleocytosis of <500/mm^3 is often observed (Sejvar et al., 2005). As with WNF, the outcome of WNM is generally favorable although some patients complain about weakness and problems with memory and concentration on follow-up examination (Klee et al., 2004).

West Nile Encephalitis: The risk of developing WNE increases with increasing age. WNE can range in severity form mild encephalitic disease to a more severe from characterized by coma and death (Sejvar and Marfin, 2006). Patients with WNE exhibit depression, altered level of consciousness or confusion and personality change (Debiasi and Tyler, 2006; Sejvar, 2007). Additional symptoms include ataxia, lethargy, movement disorders, Parkinsonism**,** conjunctivitis, confusion, photophobia, slurred speech, seizures, tremors and involuntary body movements (Davis et al., 2006; Drebot and Artsob, 2005; Hayes et al., 2005b; Kramer, Li, and Shi, 2007; Sejvar, 2007; Sejvar et al., 2003a). Outcomes of WNE are not as good as those for WNM. Patients often complain of functional and cognitive problems (Klee et al., 2004). Interestingly, WNE was first reported in the United States in 1952 in New York when the Egyptian strain of WNV was used in treatment of cancer. Encephalitis was observed in 9.47% (9 out of 95) patients (Southam and Moore, 1954).

West Nile Poliomyelitis: Acute flaccid paralysis (WNP) is the result of WNV infecting the motor neurons. This results in asymmetric acute flaccid paralysis of one or more limbs (Jeha et al., 2003; Li et al., 2003; Sejvar et al., 2005; Sejvar et al., 2003b). In some patients, there may be diaphragmatic and intercostal muscle paralysis (Hayes et al., 2005b) resulting in respiratory

failure. These patients will therefore need to be provided with intubation and mechanical ventilation (Leis et al., 2003; Sejvar et al., 2003b). Areflexia or hyporeflexia, loss of bowel and bladder control are also common (Hayes et al., 2005b). A Guillain-Barré-like syndrome affecting peripheral nerves, radiculopathy and demylenating peripheral neuropthay have also been reported although true Guillain-Barré syndrome is rare (Ahmed et al., 2000; Leis et al., 2003; Park, Hui, and Bartt, 2003; Sejvar et al., 2005). Recovery is variable and in general, initial severity of the disease does not forebode poor prognosis (Cao et al., 2005; Sejvar et al., 2005).

Among WND cases an estimated 55-60% suffer from WNE with an estimated 20% case fatality. Additionally, WNP may contribute to 10-50% case mortality in humans (Sejvar, 2007). The most common victims of WNV are the very young, the elderly and those with suppressed or compromised immune systems. The first human victims in Israel in 1999 were an elderly couple 75 and 76 years old (Bin et al., 2001). Both patients had developed WNE and slipped into a coma. Of the first human cases in the 1999 New York WNV outbreak, four out of five cases developed flaccid paralysis. Three of these patients died of WNE. These patients were between 57-87 years of age (Asnis et al., 2000; Asnis et al., 2001). It is consequently evident that the risk of WN and more so WND increases with age. In the 2005 outbreak in California, 34.65% of the cases (305 out of 880 cases) were classified WND cases (Jean et al., 2007). The authors argued that male sex, diabetes and older age were significant risks in acquiring WND. Similar predispositions were seen in the 2003 outbreak in Colorado (Patnaik, Harmon, and Vogt, 2006).

Immunity to WNV and Dynamics of Virus Entry into the Central Nervous System (CNS)

Innate Immune Response: Type I interferons (IFN α and β) are among the first cytokines to be secreted during viral infections. They have been shown to counteract WNV by

restricting its tropism, reducing viral burden and preventing the death of neurons (Lobigs et al., 2003; Samuel and Diamond, 2005). IFN binding of the type I IFN receptor triggers the Janus Kinase/ signal transducer and activation of transcription (JAK/STAT) pathway (reviewed in Murray, PJ (Murray, 2007)). The STAT1 and STAT2 heterodimerize and translocate to the nucleus where they bind to IFN regulatory factor 9 (IRF9) forming a heterotrimeric complex called IFN stimulated gene factor 3 (ISGF 3). ISGF3 in turn binds to cis-acting IFN stimulated response element (ISRE) located upstream of type I IFN regulated genes transcribing ISG, dsRNA activated protein kinase (PKR), 2', 5'-oligiadenylte synthetase (2', 5'-OAS) and other genes (King et al., 2007). The PKR-mediated signaling pathway controls translation in virus infected cells while the 2′, 5'-OAS and the latent endonuclease RNAse degrade viral RNA (Gilfoy and Mason, 2007; Mashimo et al., 2002; Wang and Fikrig, 2004). WNV and Kunjin virus have evolved to stop this cascade by using NS2A which interacts with STAT1 and STAT2 thereby preventing its translocation to the nucleus (Liu et al., 2006; Liu et al., 2005). Other evasion mechanisms include the RNA helicase retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen-5 (MDA5) (Fredericksen and Gale, 2006). The virus consequently delays the activation of innate immune response buying it time to replicate unimpeded early in infection. The accumulated viral protein acts on IFN α and β (Keller et al., 2007).

IFN γ, a Type II IFN plays an important role both in innate immune response and adaptive immune response. It is produced primarily by γδ T cells, $CD8^+$ T cells and NK cells and limits viral replication by inducing an anti-viral state. IFN $\gamma^{-/-}$ mice showed increased viral burden and mortality when infected with WNV NY99 (Shrestha et al., 2006; Wang et al., 2003a). The authors argued that IFN γ played an early role against WNV. However, Wang et al. (Wang

et al., 2006b) showed that $CD8^+$ cells do not require IFN γ to mediate an immune response to the lineage II Sarafend strain of WNV.

WNV replicates through a dsRNA intermediate. This dsRNA can binds Toll-like receptor TLR-3. TLR-3 mediated secretion of TNF-α changes the permeability of the blood-brain barrier (BBB) allowing WNV to invade the CNS (Diamond and Klein, 2004; Wang et al., 2004a). WNV is known to upregulate MHC I and II, adhesion molecules like ICAM-1, VCAM-1 and E-selectin CD62E (Shen et al., 1997). Furthermore, the study showed that IFN-γ, TNF-α and IL-1 acted in synergy with WNV to increase expression of MHC class I molecules. A study of chemokine profiles in mice infected with the NY99 and the Eg101 WNV strain showed upregulation of RANTES (CCL-5), MIP1-α (CCL-3), MIP1-β (CCL-4) and IP-10 (CXCL-10) mRNAs in the NY99 infected mice. The Eg101 WNV strain showed a higher expression of B cell and monocyte activating chemokine (BMAC) peripheral sites (Shirato et al., 2004). In an interesting study, macrophage migration factor (MIF) was shown to be important in WNV neuroinvasion. It was shown that $mif^{-/-}$ mice had lower viral loads in the CNS and this rendered mice resistant to WNV lethality (Arjona et al., 2007). Therefore, MIF may also compromise the integrity of the BBB.

All three complement pathways (classical, alternative and mannose binding lectin pathway) were found to play an important role in controlling WNV infection (Mehlhop and Diamond, 2006; Mehlhop et al., 2005). Defects in Fas or perforin/granzyme exocytosis increase the susceptibility of mice to lethal WNV infection (Wang et al., 2004b).

Adaptive Immune Response: Humoral immune response plays a crucial role in protection against WNV infection (Diamond et al., 2003b). B cell deficient mice are highly

susceptible to infection and harbor increased viral load in the CNS. Passively administered antibodies can prevent disease in animals (Diamond et al., 2003a; Engle and Diamond, 2003). Similarly mice deficient in secreted IgM also suffered from higher WNV titers in the CNS (Diamond et al., 2003c).

A majority of the antibodies in flavivirus infections are directed against the E glycoprotein while a smaller population may be directed to preM/M (Colombage et al., 1998; Falconar, 1999; Pincus et al., 1992; Vazquez et al., 2002) and NS1 (Chung et al., 2006). The DII, containing the fusion loop and DIII, that contains the putative receptor binding domain of WNV E are major targets for virus neutralization. Antibodies against the DII domain were found to be protective against WNV in mice (Gould et al., 2005). However, the most potent neutralizing antibodies are those that bind to the lateral surface of DIII domain and this forms the basis of many subunit vaccines (see below). These antibodies could therefore prevent WNV from attaching to receptors or block the fusion step after the virus has attached. On the other end of the spectrum is pathology associated with flaviviral antibodies. This phenomenon is called antibody-dependant enhancement of pathogenicity (ADE) wherein antibody-antigen complexes are internalized by cells expressing FCγ receptors. This phenomenon has been well documented in Dengue (Morens, 1994; Rothman and Ennis, 1999). WNV antibodies have been shown to enhance infectivity *in vitro* (Diamond et al., 2003b). Another related phenomenon called "early-death" has also been documented in WN wherein animals with pre-existing antibodies many not respond well to WNV challenge and may succumb earlier than animals without pre-existing immunity (Morens, 1994; Rothman and Ennis, 1999). An interesting study by Pierson et al. (Klasse and Burton, 2007; Pierson et al., 2007) demonstrated the number of antibodies required

to achieve WNV neutralization. They also showed that WNV coated with sub-neurtralizing antibody conventrations could still infect cells expressing FCγRs.

Both $CD4^+$ (Burke, Wen, and King, 2004; Kulkarni, Mullbacher, and Blanden, 1991) and CD8 T^+ (Liu, Blanden, and Mullbacher, 1989) cells have been shown to respond in WNV infection. Wang et al. (Wang et al., 2003b) showed that mice infected with the Sarafend strain of WNV showed an influx of $CD8^+$ but not $CD4^+$ T cells in the brain. Additionally, $CD8^+$ T cells in the brain and spleen also expressed the early activation marker CD69 along with CD25. In another set of experiments γδ-T cells were shown to limit WNV load and thereby control viral infection (Wang et al., 2003a). A follow-up study showed that γδ-T cells were required for effective $CD8^+$ memory T cell response against WNV in mice (Wang et al., 2006a). Mice that had perforin deficient $CD8^+$ T cells showed increased viral burden in the CNS and increased mortality when infected with WNV NY99. Perforin had a more limited role in containing lineage II (Sarafend) WNV infection (Shrestha, Samuel, and Diamond, 2006; Wang et al., 2004b).

$CD8^+$ T cell play a role in recovery from WNV as well as in the associated immunopathology. In T cell deficient mice, $CD8^+$ T cells mediate recovery from a low viral challenge dose but mediate immunopathology at a higher challenge dose of WNV (Wang et al., 2003b). This suggests a separation between mortality caused by viral overload and mortality due to peripheral and encephalitic disease.

Innate Immune Response and Viral Entry into the CNS: It has been proposed that flaviviruses like WNV may enter the CNS by three possible routes. They could a) enter via

infected leukocytes, b) directly travel across the BBB or c) enter by retrograde axonal transport through the peripheral nervous system (PNS) (King et al., 2007).

The endothelial cells in the BBB form a tight junction and express very low levels of leukocyte adhesion molecules (Bart et al., 2000). The IFN-γ produced during a peripheral WNV infection upregulate adhesion molecules like ICAM-1 on the luminal surface of endothelial cells. ICAM-$1^{-/-}$ mice were shown to have a lower viral load in the CNS and were more resistant than control mice to a lethal WNV challenge (Dai et al., 2008). Secondly, the expression of MHC molecules is upregulated during flavivirus infections (Shen et al., 1997). Thirdly, TLR3 increases permeability of BBB through tumor necrosis factor (TNF) (Wang et al., 2004a). TNF in turn triggers the secretion of neutrophil attracting chemokine N51/KC (CXCL-1) (Garcia-Tapia et al., 2007; King et al., 2007) and increases expression of adhesion molecules on endothelial cells (Garcia-Tapia et al., 2007). The neutrophils generate copious amounts of nitric oxide (NO) that causes bystander cell death. These multiple events allow damage and breaching of the BBB. WNV can now enter the CNS via infected infiltrating leukocytes in a "Trojan-horse" fashion making use of the upregulated ICAMs and the breached BBB. Gracia-Tapia et al. (Garcia-Tapia et al., 2007) reported increased expression of monocyte chemoattractant protein MCP-5 (CCL-12), IFN-γ inducible protein IP-10 (CXCL-10) and monokine induced gamma interferon MIG (CXCL-9) preceding the expression of IFN-γ and TNF-α in the brain. The authors suggest that IP-10 and MCP-5 initiate recruitment of leukocytes into the perivascular spaces and activates circulating leukocytes that produce IFN-γ, IL-1α and TNF-α. The resulting increase in permeability may on one hand eliminate viral entry into the CNS but may also serve as a conduit for WNV infected leukocytes to enter the CNS. A recent study in mice and

hamsters, however, showed that increased BBB permeability did not correlate with WNV associated lethality (Morrey et al., 2008).

Flaviviruses do not seem to replicate well in endothelial cells (Khaiboullina et al., 2005) but may cause direct or indirect damage to the vascular endothelial cells (German et al., 2006). However, it has been suggested that they may be transcytosed to the paranchymal side of the CNS and cause indirect damage to the BBB (Desai et al., 1995; King et al., 2007). Flaviviruses may enter the CNS through olfactory neurons or a breached BBB (Monath, Cropp, and Harrison, 1983). WNV is capable of entering the CNS by anterior-grade axonal transport and spreads from caudal to rostral direction in peripherally inoculated mice (King et al., 2007). WNV has been detected in distinct regions of the human brain including the medulla and the thalamus both of which are the first sites that come in contact with the peripheral nervous system (PNS) (Hunsperger and Roehrig, 2006). The presence of viral antigen in the PNS dorsal root ganglion (DRG) suggests that the DRG are responsible for rapid spread of WNV from PNS to CNS. The above study also pointed out that the CNS is infected early and the virus is cleared. However, these tissues become reinfected at a later time point. The difference between a mouse that died and one that lived depended on the efficiency with which the immune system was able to clear the initial infection (two days post infection) and the amount of virus introduced via the PNS. Persistent flaviviviral infections may be another reason for mice that survived. The host immune response and neurological environment may allow for virus to be recovered in survivors (Garcia-Tapia et al., 2007).

Current Vaccines against West Nile

West Nile was first isolated more than seventy years ago (Smithburn et al., 1940). It was

considered to be one of the least worrisome flaviviruses until it invaded North America causing disease with neurological implications. Consequently, despite the long time we have known of this virus, there had been no impetus to develop a human vaccine for this disease. Although the New York epidemic in 1999 caused the government, scientific community and the industry to rethink their strategy, currently, there are no commercially available vaccines for human use. The main reason cited for this is the very low incidence of disease relative to the overall population. Another reason for not developing a vaccine is commercial interests. There is a very small to almost insignificant market for the vaccine. The prohibitively high cost-to-benefit ratio for development and marketing the vaccine severely impact its commercialization (Monath, 2001a). The vaccine company Crucell had launched an initiative to develop a WN vaccine for human use. It recently decided to abandon the pursuit and issued the following statement "Crucell has come to the conclusion that the commercial and market opportunities for its West Nile products are not as attractive as other products in Crucell's pipeline" (http://www.reuters.com/article/rbssHealthcareNews/idUSWEB473420080212). Three vaccines have been licensed to be used as veterinary vaccines. These include the inactivated vaccine by Fort Dodge licensed in 2003, a recombinant live canarypox vectored vaccine by Merial Ltd. licensed in 2004 and a DNA vaccine licensed in 2005 (Dauphin and Zientara, 2007).

Despite the gloomy outlook, a number of experimental vaccines have been successfully developed and tested. Every known technology has been used and vaccines have been tested in mice, hamsters, birds, equines and non-human primates and have been described in details below. Some vaccines are currently undergoing phase III clinical trails in humans.

Inactivated Vaccines: A formalin-inactivated whole-virus veterinary vaccine developed by Fort Dodge Animal Health, IA is sold under the brand name Innovator®. This vaccine was

shown to be safe and efficacious (Ng et al., 2003) in horses and was granted full license by the USDA. This vaccine however failed to elicit neutralizing antibodies in flamingos and red-tailed hawks (Nusbaum et al., 2003). Another experiment in golden hamsters vaccinated with the Fort Dodge vaccines showed that all vaccinated animals survived challenge with West Nile virus. Eight out of nine animals (88.8%) showed hemagglutination Inhibition (HI) and complement fixing (CF) antibodies while 55.5% animals showed low levels of WN neutralizing antibodies (Tesh et al., 2002a). The Innovator® vaccine was tested in the baboon model for WN. Vaccinated animals showed increased IgG and IgM response and high PRNT titers (Wolf et al., 2006). The animals exhibited very low viremia on challenge with WNV-OK2 strain. These results encouraged the development of the human WNV vaccine by the vaccine company Crucell as described earlier.

In 2003, Ng et al. (Ng et al., 2003) tested an inactivated equine WNV isolate on horses. On challenge, 81.8% of the control horses had viremia as compared to 19% of the vaccinated horses. Researchers tested yet another inactivated vaccine on young geese. This vaccine was prepared by formalin inactivation of WNV passaged in sucking mouse brains. The inactivated brain homogenates were blended with mineral oil and used to vaccinate geese. Eighty-five percent of the birds were protected upon intra-cranial challenge with WNV. Extensive field studies showed the vaccine to be safe and efficacious (Samina et al., 2005). The same group also developed an inactivated vaccine using the PER.C6 cell platform. They showed that 91.4% of the vaccinated geese were protected following severe intracranial challenge (Samina et al., 2007).

Recombinant/Subunit Vaccines: The WNV envelope glycoprotein from a Connecticut mosquito isolate was used to express the E gene. The protein was purified and used to vaccinate

C3H/HeN mice. The vaccine was shown to elicit protective immune response on challenge (Wang et al., 2001). In another experiment, soluble WNV E protein expressed in S2 cells was used to vaccinate mice and horses. All vaccinated mice survived challenge and both mice and horses developed high titer WN antibodies (Ledizet et al., 2005). Watts et al. (Watts et al., 2007) immunized hamsters with a carboxy terminus-truncated WNV E protein either with or without non-structural protein 1 (NS1). Some of these vaccines were administered with ISCOMATRIX adjuvant. Animals in the NS1 only group showed an 87% survival whereas animals vaccinated with NS1 and E or just E alone showed a 100% survival on challenge. In a follow-up study (Lieberman et al., 2007) by the same group, the authors characterized and reported a robust cellular immune response in vaccinated hamsters.

Domain III (D III) of flavivirus envelope gene is highly immunogenic. Researchers have successfully made subunit vaccines for Dengue (Mota et al., 2005) and Japanese encephalitis (Wu et al., 2003) using the D III region. In 2007 Chu et al. (Chu, Chiang, and Ng, 2007) constructed a similar vaccine for WNV. The vaccine was shown to elicit a strong Th1 response with production of IL-2 and IFN-γ. McDonald et al. generated a WNV EIII domain-bacterial flagellin (STF2Δ) fusion protein (McDonald et al., 2007). This vaccine was able to stimulate both innate and adaptive immune responses and protect mice against challenge. Martina et al. (Martina et al., 2008) constructed a subunit vaccine expressing the D III of WNV E gene. All vaccinated mice survived challenge as compared to 80% survival observed in a β-propiolactone inactivated whole virus vaccine.

Nucleic Acid/DNA Vaccines: A DNA vaccine expressing the WNV NY99 capsid gene was constructed and tested in mice (Yang et al., 2001). This vaccine was shown to elicit a strong Th1 immune response with a robust peak in IL-2 and IFN-γ levels. Davis et al. (Davis et al.,

2001) engineered a DNA vaccine expressing WNV preM and E proteins. The vaccine protected 100% of the mice and generated robust neutralizing antibody response in horses on challenge. In another study, a plasmid DNA encoding the infectious full length RNA genome of Kunjin virus was used to vaccinate mice. A single mutation in the NS1 gene of the Kunjin virus attenuated it in sucking mice. The vaccine was shown to protect against intracerebral and intraperitoneal challenge with both WNV NY99 and the Kunjin virus (Hall et al., 2003). In 2007 Martin *et al.* (Martin et al., 2007) carried out phase I clinical trials for a DNA vaccine expressing WNV NY99 preM and E genes. The vaccine was found to be safe and well tolerated and elicited strong humoral and T cell response. More recently, a capsid deleted Kunjin virus DNA vaccine was developed with the capsid being provided in *trans*. These single cycle viruses replicate one time to generate VLP progeny as an additional antigen and was found to be highly immunogenic in mice and horses (Chang et al., 2008).

Recombinant Virus Vaccines: The recombinant live canarypox vectored vaccine by Merial is one of the three licensed veterinary vaccines. This vaccine is known as the RecombiTEK vaccines and has the canapox/ALVAC vector expressing WNV preM and E genes. Studies by Grosenbaugh et al. (Grosenbaugh et al., 2004) showed a strong anamnestic immune response in horses that had been previously vaccinated with the Fort Dodge Innovator ® vaccine. A single dose of the vaccine afforded early protection against viremia in horses challenged with WNV infected mosquitoes (Siger et al., 2004). When vaccinated with two doses and subjected to a mosquito challenge, all vaccinated horses developed high titer neutralizing antibody response and did not show any clinical signs of illness (Minke et al., 2004). The RecombiTEK vaccine was also tested in cats and proven to be effective (Karaca et al., 2005). In a separate set of experiments ten control and ten RecombiTEK vaccinated horses were challenges by the

intrathecal route. Eight controls developed encephalomyelitis and one vaccinated horse developed mild muscle fasciculation. Overall the vaccine proved to be effective considering the challenge route (Siger et al., 2006).

A lentivirus vector based vaccine (TRIP/sE_{WNV}) was tested in mice. A single dose of this vaccine protected against lethal challenge with WNV IS-98-ST1 strain. This protection was seen as early as seven days post vaccination and also provided long lasting immunity (Iglesias et al., 2006).

A live measles virus vaccine expressing secreted envelope glycoprotein from IS-98-ST1 strain of WNV was constructed using the attenuated Schwarz strain of measles virus. This vaccine (MVSchw-sE_{WNV}) protected mice against lethal challenge with WNV (Despres et al., 2005).

Virus-like Particles and Heterologous Vaccines: A number of other approaches have been used to produce effective WN vaccines. Qiao et al. (Qiao et al., 2004) generated WN virus-like particles (WNVLPs) using recombinant baculovirus expressing preM and E genes or capsid, preM and E genes. WNVLPs expressing preM and E only generated a strong, protective and sterilizing immunity in mice on challenge. WNVLPs with the capsid, preM and E genes generated a much weaker immune reponse.

Heterologous vaccine approaches have been used based on immunity and cross protection afforded by closely related flaviviruses. An excellent example of this is the use of Israel turkey meninoencephalitis virus (ITMV). ITMV is an arbovirus belonging to Ntaya serogroup of flaviviruses and was first described in 1960 (Komarov and Kalmar, 1960). Turkeys less than ten weeks seldom showed any incidence of WNV probably due to presence of cross

reacting antibodies. Geese vaccinated with formalin inactivated ITMV vaccine showed anywhere between 39-72% survival on intracranial challenge with WNV (Malkinson et al., 2001). In 1971, Price and Thind (Price and Thind, 1971) demonstrated that hamsters vaccinated with any of four Dengue virus serotypes were protected against a WNV challenge. Hamsters vaccinated with Japanese encephalitis virus vaccine (JEV SA14-2-8), wild-type St. Louis encephalitis virus (SLEV) and with Yellow fever virus vaccine (YFV 17D) showed some level of protection against WNV. Animals vaccinated with the JEV SA14-2-8 and the SLEV vaccine were protected against WNV encephalitis and death (Tesh et al., 2002b). American crows (*Corvus brachyrhynchus*) vaccinated with wild type Kunijin virus were completely protected against WNV challenge (Hall and Khromykh, 2004).

Despite the encouraging results with cross-protection afforded by heterologous flavivirus vaccines, it was shown that humans vaccinated with either the JE vaccine (JE-VAX, BIKEN, Japan) or a Dengue vaccine (Aventis Pasteur, Lyon, France) did not show protective neutralizing antibodies against WNV (Kanesa-Thasan et al., 2002). Similar observations were made by Takasaki et al. (Takasaki et al., 2003) who showed that mice vaccinated with mouse brain derived JE vaccine were not protected against intracranial challenge with WNV but were afforded partial protection at higher vaccine doses when challenged intraperitoneally. Interestingly, mice vaccinated against WNV also elicited partial protective response against JEV (Martina et al., 2008).

Passive Antibody Prophylaxis: Hyperimmune sera have been used for passive antibody prophylaxis for many diseases and had been used in a number of experiments for WNV therapy. Pooled sera from mice that were actively immunized with WNV E protein was diluted 1:5 and administered to naïve mice. These mice were challenged with 10^1-10^6 PFU WNV after 24 hours

of passive immunization. Four out of five control mice and one out of five vaccinated mice died over a 15 day observation period (Wang et al., 2001). In a different study, immunocompetent and immunocompromised mice were administered polyclonal WN antibodies prior to infection with the virus. The antibody prevented morbidity in wild-type mice but the immunocompromised mice eventually succumbed at later time points (Engle and Diamond, 2003). Passively administered sera from immunized horses has been shown to protect naïve mice on challenge with WNV (Ledizet et al., 2005). The same group used affinity purified horse antibodies against three WNV envelope peptides to naïve mice. They found 48-59% survival rate in these mice when challenged with WNV (Ledizet et al., 2007).

Passive antibody prophylaxis has been used with a good amount of success in humans. Shimoni et al. (Shimoni et al., 2001) described a case of a 70 year old Israeli woman who deteriorated from impaired consciousness to deep coma in 72 hours after admission. She was intravenously administered Omr-IgG-am antibodies from Omrix Biopharmaceutical Ltd, Israel at 0.4g/Kg. The patients' level of consciousness dramatically improved over the next week. In a similar case in 2002, a 42 year old Israeli male lung transplant recipient with confirmed WNE exhibited deteriorating level of consciousness. He showed dramatic improvement within 48 hours of being intravenously administered Omr-IgG-am antibodies (Hamdan et al., 2002). In a third case a 55 year old man with chronic lymphocytic leukemia and hypogammaglobulinemia was diagnosed with WN. The patient was administered the Omr-IgG-am antibodies at 0.5g/kg. Unfortunately, probably because of the timing of administration and the underlying conditions, the patient succumbed thirty-two days into his illness (Haley et al., 2003).

Live Attenuated Vaccines: Live attenuated vaccines can be divided into two subcategories viz. attenuation by selection, deletion or cloning and those based on ChimeriVax™

technology. The ChimeriVax™ based vaccines will be discussed in a separate section below. A live attenuated vaccine was developed by Lustig et al. (Lustig et al., 2000) by passaging the Israeli strain of WNV in a mosquito cell line and selecting an escape mutant using monoclonal antibodies. The resulting virus WN-25A was found to be genetically stable and had lost all neuroinvasiveness. This vaccine protected 100% of vaccinated mice and geese upon lethal challenge. Pletnev et al generated two chimeric viruses (Pletnev et al., 2003). One of these had the WNV membrane precursor and envelope on a Dengue 4 (WN/DEN4) background and the other had a 30 nucleotide deletion in the 3' non-coding region of DEN4 (WN/DEN4-3'Δ30). Both these vaccines were attenuated in Rhesus macaques, elicited high neutralizing antibody titers and prevented viremia in the monkeys up on challenge. A follow-up study with the WN/DEN4-3'Δ30 virus showed that it was unable to infect geese. The resulting virus also showed reduced neurovirulence in intracranially challenged suckling mice but had lost all neurovirulence in immunocompromised mice. Furthermore, it was significantly attenuated in monkeys (Pletnev et al., 2006). In a similar study, Huang et al constructed two chimeric Dengue 2 viruses expressing WNV NY99 preM and E glycoprotein. These vaccines were shown to be attenuated and protect mice on challenge with the NY99 strain of WNV (Huang et al., 2005). In another study, a molecularly cloned descendant of the lineage II prototype B956 was generated. This virus, the WN1415, was shown to elicit a potent immune response and protect 100% of the mice on challenge. At a lower vaccine dose (55 PFU), 67% of the mice were protected (Yamshchikov et al., 2004).

ChimeriVax™ Technology Based Vaccines: To obtain license for a commercial West Nile vaccine for human use, the vaccine must amply demonstrate safety and efficacy in clinical trials. Additionally, the vaccine must be able to elicit a potent protective immune response. The

ChimeriVax™ vaccines in many ways exploits the clinical data that exists for its parent vaccine the Yellow fever 17D vaccine. The vaccine virus, known as the Asibi strain, was isolated from a patient named Asibi in Ghana in 1927 (Stokes, Bauer, and Hudson, 1928; Theiler and Smith, 1937). In 1930, Max Theiler developed the first attenuated strain of this virus which he called the 17D virus (Theiler and Smith, 1937). The vaccine has demonstrated a very good safety record of millions of doses over the years (Monath, 2001a; Monath, 2001b). The ChimeriVax™ vaccines rely of their comparative safety against this vaccine. The ChimeriVax™ vaccines therefore have a vector backbone consisting of the 17D non-structural genes. The preM and E genes of the candidate flavivirus is incorporated into this backbone generating a recombinant virus expressing the antigens of interest in a 17D background.

The first chimeras contained the Japanese encephalitis (JE) virus preM and E (Chambers et al., 1999) and were shown to be genetically stable and afford a solid protection against virulent JE virus challenge (Guirakhoo et al., 1999). The ChimeriVax™-JE virus did not infect *Aedes* or *Culex* mosquitoes (Bhatt et al., 2000). The vaccine has been extensively tested and characterized in mice (Guirakhoo et al., 1999) and Rhesus macaques (Monath et al., 2000; Monath et al., 1999). ChimeriVax™-JE has been studied in humans and a Phase II clinical trial showed its promise as an effective human vaccine (Monath et al., 2003; Monath et al., 2002). Pugachev et al. (Pugachev et al., 2003) published a detailed review on these vaccines.

Similar vaccines have also been generated and tested for all four Dengue virus serotypes (Caufour et al., 2001; Guirakhoo et al., 2001; Guirakhoo et al., 2002; Guirakhoo et al., 2004; Guirakhoo et al., 2000; van Der Most et al., 2000). The ChimeriVax™-Dengue vaccine is currently undergoing Phase II clinical trials (Edelman, 2007).

Studies in the hamster model showed that the ChimeriVax^{TM}-WNV protected hamsters and induced a strong immune response as measured by HI, CF and the plaque reduction neutralization test (PRNT) (Tesh et al., 2002a). Preclinical studies were also carried out in mice and Rhesus macaques. ICR mice that were vaccinated and challenged intraperitoneally were protected in a vaccine dose-dependant manner. Similarly, ChimeriVax^{TM}-WNV vaccinated macaques were uniformly protected against intracerebral challenge (Arroyo et al., 2004). About 50% of these animals suffered from subclinical disease post challenge and this is attributed to the aggressive route of challenge. In a second set of pre-clinical studies, the ChimeriVax-WNV$_{02}$ vaccine which has multiple point mutations was tested in rhesus macaques. The skin and lymphoid tissues were prominent sites for viral replication. Additionally, studies on human subjects revealed that the vaccine produced high titer neutralizing antibody response and antigen specific CD8^{+} T cells producing IFN-γ (Monath et al., 2006). Additionally, WNV specific CD4^{+} T cells were detected in >80% of the subjects. A Phase II clinical trial with ChimeriVax^{TM}-WNV is currently underway. It is expected to be completed by January 2009 and enroll 208 participants (Hall and Khromykh, 2007).

Vesicular Stomatitis Virus

Basic Virology: Vesicular stomatitis virus (VSV) belongs to vesiculovirus genus of Rhabdoviridae family, order mononegavirales (Lamb, 2007). It's a single stranded negative (-ve) sense RNA virus with an approximately 11 kb genome. The genomic RNA is completely covered by the viral nucleoprotein and codes for five genes (Figure 1.3). These genes from 3' to 5' direction are (leader sequence)-N (nucleocapsid protein)-P (phosphoprotein cofactor, also known as NS)-M (matrix protein)-G (glycoprotein)-L (large polymerase catalytic subunit)-(trailer) (Lyles and Rupprecht, 2007). As with all negative sense RNA viruses, the RNA genome

of VSV is never found as a naked RNA during replication. The N protein tightly associates with the genomic RNA to form a ribonucleoprotein complex forming a helical functional structure that protects the genomic RNA from nucleases and to avoid antisense associated problems (Roberts and Rose, 1998). This complex associates with the viral RNA dependant RNA polymerase (vRdRP) complex (Barr, Whelan, and Wertz, 2002; Whelan and Wertz, 1999). The L and P proteins associate to form the vRdRP complex and performs replicase and transcriptase functions (Barr, Whelan, and Wertz, 2002; Emerson and Yu, 1975; Patton, Davis, and Wertz, 1983) and makes up 60% of the genome (Schubert et al., 1985). The minimal infectious unit is 1250 subunits of the N protein along with smaller amounts of the polymerase (P and L) proteins (Thomas et al., 1985).

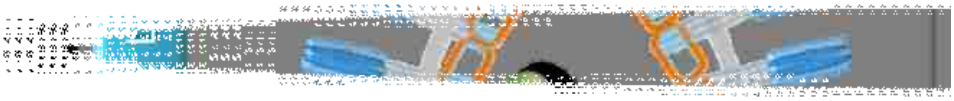
The matrix protein is the smallest VSV gene product and is believed to have multiple roles in cytopathogenesis, viral transcription and its regulation, viral assembly and budding (Blondel, Harmison, and Schubert, 1990; Carroll and Wagner, 1979; Clinton et al., 1978; Harty et al., 2001; Jayakar, Murti, and Whitt, 2000). The G glycoprotein is the only glycoprotein expressed on the surface of the virus. It is a type I membrane glycoprotein forming trimeric spikes facilitating virus attachment to host cell surface receptors and subsequent endocytosis (Kreis and Lodish, 1986; Zagouras and Rose, 1993). The G glycoprotein undergoes a conformational change at low pH (pH 6) resulting in fusion of viral envelope and endosome vesicle membrane thereby releasing the viral ribonucleocapisd complex into the cytoplasm (Roche et al., 2008; Roche et al., 2006; Roche et al., 2007).

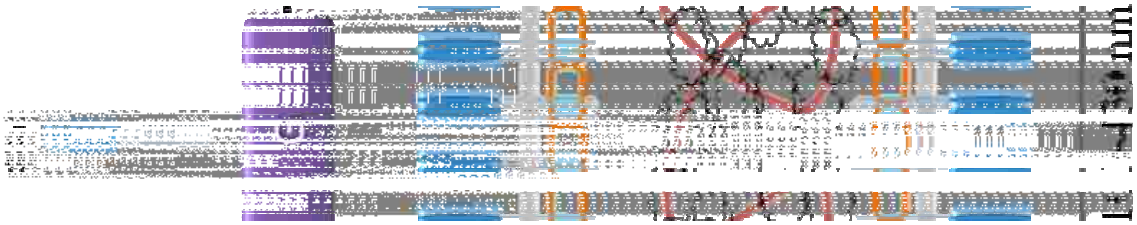
VSV infects cells by receptor-mediated endocytosis followed by a pH dependent fusing of the viral envelope with the endosomal membrane. The resulting uncoating releases the

ribonucleoprotein-polymerase complex into the cell cytoplasm. A schematic illustrating the life cycle of VSV is shown in Figure 1.4.

Transcription of the viral genome is obligatorily sequential, polar and occurs by a stuttering/stop-start mechanism (Abraham and Banerjee, 1976; Ball and White, 1976; Barr, Whelan, and Wertz, 2002). Transcription starts at the 3' end with the generation of a small leader RNA followed by the five mRNAs. Analysis of VSV genome revealed a conserved 23 nucleotide intergenic region at each gene junction that was shown to contain a putative transcript polyadenylation motif: 3'-AUACUUUUUUU-5'. This polyadenylation motif is followed by an untranscribed dinucleotide CA/GA and a transcription start signal 3'-UUGUCNNUAG-5' (Abraham and Banerjee, 1976; Ball and White, 1976; Schnell et al., 1996b). The polymerase consequently terminates and reinitiates at each intergenic junction producing five discrete mRNAs. During this process of termination and reinitiation, the polymerase may occasionally detach at these junctions but will have to restart all over again at the 3' end promoter of the genomic template. As a result, there is a pronounced gradient of mRNA transcripts and consequently proteins with the N transcript/protein being most abundant and the L transcript/protein being the least abundant (Schnell et al., 1996b) (Figure 1.5). At some point of time after mRNA synthesis begins, the virus switches from transcription mode to replication mode. Encapsidation of the nascent RNA by a complex of N and P proteins (referred to as N_0-P)

Figure 1.3: VSV genome organization and virus architecture. VSV is a single stranded negative sense RNA virus. VSV virions are bullet-shaped viruses belonging to rhabdoviridae family. They are approximately 100-430 nm long and 45-100nm in diameter. The genome is very simple and has five genes. The nucleocapsid protein (N) is tightly associated with the viral genomic RNA. The matrix protein (M) is a multifunctional protein. The polymerase subunits viz. the phosphoprotein (P) and the large subunit (L) associate to form a functional RNA dependant RNA polymerase. Glycoprotein G is the only glycoprotein expressed on the VSV virions and plays a vital role in virus attachment, entry and pathogenesis





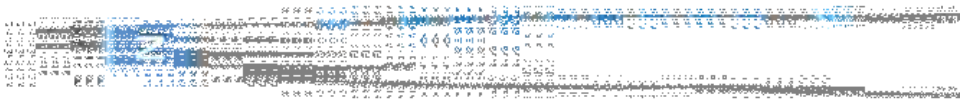


Figure 1.4: Life cycle of VSV. VSV enters the cell by receptor mediated endocytosis. This is followed by uncoating and releasing of the helical ribonucleoprotein complex. The N, P, L and M mRNAs are translated by free cytoplasmic ribosomes while the G glycoprotein is translated by endoplasmic reticulum (ER) bound ribosomes. Once threshold levels of N, P and L proteins have been translated, they participate in viral RNA replication producing the positive sense antigenomic RNA template. Negative sense genomic RNA is packaged into progeny nucleocapsids as G and M proteins are assembled into the progeny virions and they bud out. Adapted from Flint et al. (Flint, 2000).

Nucleus
Cytoplasm
- sense
L
P
N
M
+ sense
- sense
ER
G
Golgi

signals the polymerase to read through the genome by ignoring the intergenic start/stop signals (Lyles and Rupprecht, 2007). The encapsidated antigenomic RNA thus produced is used as a template for progeny genomes (Patton, Davis, and Wertz, 1984; Wertz, 1983).

VSV Natural Cycle: VSV causes a vesicular disease clinically similar to foot-and-mouth disease and afflicts cattle, pigs, and horses. The signs include vesicles and ulcers in mouth and oral mucosa, feet and teats of affected animals (OIE. Office international des épizooties (Paris), 2004). The disease is economically significant with losses resulting from drop in milk and meat production and trade barriers including livestock trading (McCluskey, Hurd, and Mumford, 1999). VSV infection is believed to be via the transmucosal and transcutaneous routes (OIE. Office international des épizooties (Paris), 2004). The virus may also be transmitted through sandflies, blackflies,

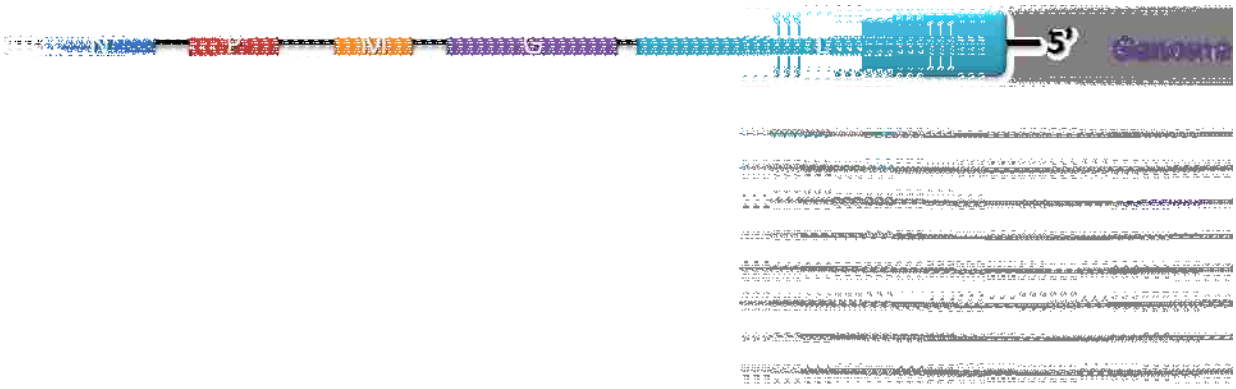


Figure 1.5: Polarity of transcription in VSV. Transcription of the viral genome is polar and is characterized by a stuttering mechanism. The transcription starts at the 3' end resulting in the generation of a small leader RNA followed by the five mRNAs. The polymerase consequently terminates and reinitiates at each intergenic junction producing five discreet mRNAs. During this process of termination and reinitiation, the polymerase may occasionally detach at these junctions but has to restart all over again at the 3' end promoter of the genomic template. Consequently, there is a pronounced gradient of mRNA transcripts and viral proteins with the N transcript/protein being most abundant and the L transcript/protein being the least abundant.

mosquitoes and certain other dipteral species (Clarke et al., 2006; Lichty et al., 2004; Lyles and Rupprecht, 2007). VSV G glycoprotein is a potent immunogen and also serves important functions in virus-entry and virus-induced cell fusion (Roche et al., 2007). Natural VSV infections of humans is rare, causing at most mild flu-like illness (Lichty et al., 2004).

VSV System: The recovery of a non-segmented negative sense RNA virus was first described for rabies virus in 1994 (Schnell, Mebatsion, and Conzelmann, 1994). The same principle was later used to recover rVSVs (Lawson et al., 1995; Whelan et al., 1995). Briefly, the anigenomic sequences were encoded on DNA plasmids under a T7 promotor. The 3' viral termini were created using Hepatitis D virus ribozyme. The nucleocapsid protein N, the the polymerase subunits P and L were provided on separate plasmids under a T7 promotor in *trans*. The four plasmids were transfected onto cell lines that either stably expressed T7 RNA polymerase or were previously infected with recombinant vaccinia virus expressing the T7 polymerase. Infectious virus was assembled after transcription and translation of the genomic RNPs. This process is explained in details in the subsequent chapters of this dissertation.

Advantages of VSV Vectored Vaccines: VSVs can be easily grown in a number of cell lines to very high titers (Roberts and Rose, 1999). They have a small, well understood genome. As compared to vaccinia virus vectors that express around 200 different proteins, the VSV G glycoprotein is the only glycoprotein expressed on the virus surface (Roberts et al., 1999). Consequently, there are fewer proteins that compete for the immune response thereby directing more resources towards fighting the antigen of interest. An additional advantage of the VSG G glycoprotein is a low degree of similarity between G glycoproteins among various serotypes of VSV and other vesiculoviruses. The G glycoproteins of VSV Indiana (VSV IN) and VSV New Jersey (VSV NJ) serotypes are around 50% identical at the amino acid level (Martinez et al.,

2003). Whereas the Chandipura (CH) vesiculovirus G glycoprotein shares about 40% sequence identity with the VSV IN or VSVNJ (Masters et al., 1989). This difference is cleverly exploited to circumvent the immune response against the vector itself as a result of primary vaccination. This abilty to generate recombinant VSVs (rVSVs) expressing the antigen of interest and serologically distinct G glycoproteins for subsequent booster vaccinations overcomes preexisting vector immunity and is known as glycoprotein exchange vectors (Figure 1.6). rVSVs can accommodate multiple foreign inserts up to 4.5 kb and incorporate about 30% foreign glycoproteins on the virion when compared to the native G glycoprotein (Haglund et al., 2000; Schnell et al., 1996a). As is evident from the VSV life cycle, VSV replicates solely in the cytoplasm through RNA intermediates therefore, it cannot go latent or insert itself into genomic DNA. rVSV based have also been shown to confer immunity in presence of maternal antibodies (Schlereth et al., 2000). rVSV vaccines can be administered non-invasively through the intranasal route and have been shown to elicit very strong humoral and cellular immune response against a variety of infectious viral and bacterial diseases.

VSV infectious viruses can be efficiently recovered by a reverse genetic approach that utilizes multiple plasmids expressing VSV genes (Schnell et al., 1996a). This method has enabled the rapid construction of recombinant VSV expressing a variety of viral and bacterial antigens.

rVSVs have been engineered to generate a large number of experimental vaccines against extremely infectious viral and bacterial diseases. rVSV vaccines influenza virus hemagglutinin (HA) protected mice against challenge (Roberts et al., 1998). VSV expressing various truncation of the HA gene was shown to be equally promising and were highly attenuated for pathogenesis (Roberts et al., 1999). Single cycle VSV vaccines expressing H5 HA

of the avian influenza virus afforded long-term protection against challenge in mice (Schwartz et al., 2007).

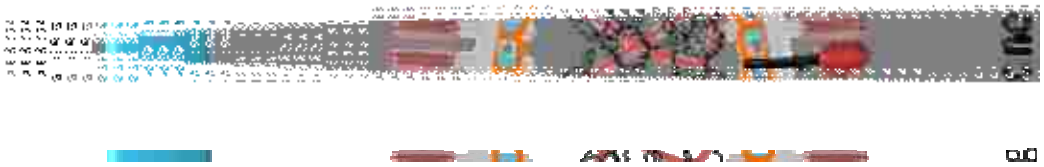
VSV expressing bovine viral diarrhea virus (BVDV) E2 glycoprotein was shown to induce strong neutralizing antibody responses in mice (Grigera et al., 2000). Similarly, rVSV expressing Respiratory Syncytial virus (RSV) F and G glycoproteins were able to protect mice against challenge (Kahn et al., 2001). The major capsid protein L1 of cotton-tail rabbit papilloma virus (CRPV) expressed on rVSVs offered complete protection of rabbits on challenge (Reuter et al., 2002; Roberts et al., 2004). CRPV E1, E2, E6, E7 antigens expressed by VSV reduced pappiloma volumes in rabbits. The greatest reduction (96.9%) in papilloma volume was observed in rVSV expressing the E7 protein (Brandsma et al., 2007a; Brandsma et al., 2007b).

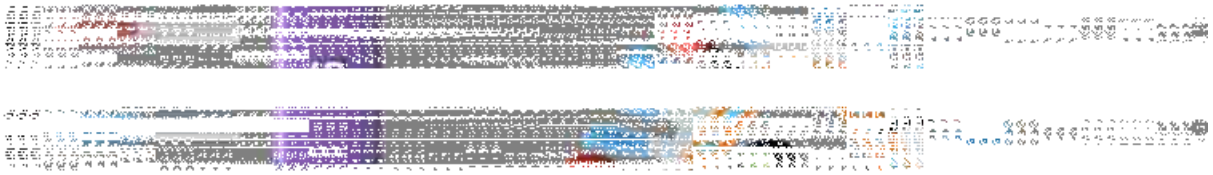
rVSV vaccines expressing Hepatitis C virus core, E1 and E2 antigens generated vigorous antibody response in mice (Ezelle, Markovic, and Barber, 2002).

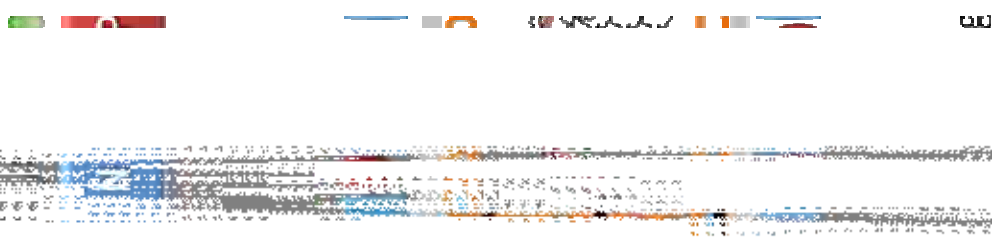
Schlereth et al. (Schlereth et al., 2003) showed that VSV expressing measles virus (MV) hemagglutinin was able to induce neutralizing antibodies and protect cotton rats against challenge.

Single cycle rVSV vaccine expressing soluble glycoprotein (sGP) of Zaire Ebola virus (ZEBOV) protected mice from lethal challenge (Garbutt et al., 2004). In a related study, single cycle VSV vaccines expressing ZEBOV GP and Marburg virus (Musoke strain) GP were shown to be safe, efficacious and protect cynomolgus macaques (Jones et al., 2005). In a related study, the Marburg VSV vaccine (Musoke strain) was shown to protect macaque against the Angola and Ravn strains of Marburg virus (Daddario-DiCaprio et al., 2006a; Daddario-DiCaprio et al., 2006b). Single cycle VSV expressing Lassa fever GP afforded immuneprotection in

Figure 1.6: VSV glycoprotein exchange vectors. VSV G glycoprotein is the only glycoprotein expressed on the virion surface. This is cleverly exploited in constructing recombinant VSV expressing the vaccine antigen. G glycoproteins for the VSV Indiana G(In) and the Chandipura G glycoprotein G(Ch) have approximately 40% sequence identity and antibodies generated against one G(In) do not readily neutralize rVSV expressing the G(Ch). Constructing two rVSV expressing the same WNV E glycoprotein and either G(In) or G(Ch) as priming and boosting vaccines therefore overcomes vector neutralization as the result of primary vaccination while still efficiently presenting the WNV E antigen to the immune system.







cynomolgus macaques against lethal challenge (Geisbert et al., 2005).

Mice vaccinated with VSV expressing SARS coronavirus spike (S) protein elicited long-term protective immune responses against challenge (Kapadia et al., 2005). VSV vaccine against Herpes Simplex Virus type 2 expressing the glycoprotein D was shown to protect mice and guinea pigs against vaginal challenge (Natuk et al., 2006).

Xing and Lichty have developed a rVSV expressing the interferon inducing antigen Ag85A for tuberculosis with the intent of using a heterologus prime-boost approach with recombinant adenovirus expressing the same antigen (Xing and Lichty, 2006). A VSV vaccine against the plague bacteria *Yersinia pestis* expressing LcrV protein induced long-term protection against challenge in mice (Palin et al., 2007).

VSV vectors have been most used for the development of vaccine against retroviruses, especially HIV. HIV-1 gag precursor, gag and Env or a chimeric Env with a VSVG cytoplasmic tail have been generated (Haglund et al., 2000; Johnson, Rodgers, and Rose, 1998; Johnson et al., 1997). Furthermore, multiple strains of rVSVs have also been used in oncolytic viral therapy (Fernandez et al., 2002; Lichty et al., 2004; Stojdl et al., 2003).

VSV-vectored vaccines have been administered via multiple routes including intranasal, intramuscular and subcutaneously and have been shown to elicit robust mucosal and systemic humoral and cellular immune responses (Brandsma et al., 2007a; Brandsma et al., 2007b; Daddario-DiCaprio et al., 2006a; Daddario-DiCaprio et al., 2006b; Ezelle, Markovic, and Barber, 2002; Garbutt et al., 2004; Geisbert et al., 2005; Grigera et al., 2000; Haglund et al., 2000; Johnson, Rodgers, and Rose, 1998; Johnson et al., 1997; Jones et al., 2005; Kahn et al., 2001;

Kapadia et al., 2005; Natuk et al., 2006; Roberts et al., 1999; Roberts et al., 1998; Schlereth et al., 2003; Schwartz et al., 2007).

Safety of rVSVs and Vector Attenuation: Any successful vaccine must also have a proven safety record before approval for human use. VSV causes a vesicular disease clinically similar to foot-and-mouth disease and afflicts cattle, pigs, and horses. The signs include vesicles and ulcers in mouth and oral mucosa, feet and teats of affected animals (OIE. Office international des épizooties (Paris), 2004). Natural human infections with wildtype VSV is generally self limiting. Serious illnesses are extremely rare with the exception of a single case of a three year old boy with viral encephalitis (Quiroz et al., 1988). Seroprevalence of VSV antibodies in human population is rare with small pockets in Georgia and Central America (Cline, 1976; Roberts et al., 1999). A rural population in Panama was reported to have 94% seroprevalence to VSV (Tesh, Peralta, and Johnson, 1969).

rVSVs have been extensively tested for safety in rodent models and non-human primates. Wildtype VSV have been shown to be neuropathogenic in mice following intranasal (i.n.) or intracerebral (i.c.) inoculation (Miyoshi, Harter, and Hsu, 1971). Schnell et al. showed that mice inoculated with rVSVs via the i.n. route resulted in significant weight loss among the animals but they quickly recovered by day 6 (Rose et al., 2000). rVSVs expressing green fluorescent protein (GFP) was shown to infect neuronal and non neuronal tissues after i.n. or i.c. inoculation. The fluorescence subsided by day eight in the intranasally infected mice (van den Pol, Dalton, and Rose, 2002). Over the years, more than a hundred and fifty non-human primates have been inoculated with rVSV (IN) via intranasal and intramuscular routes without adverse effects (Egan et al., 2004; Rose et al., 2001). More recently, studies on cynomolgus macaques inoculated with

high titer rVSVs via intranasal route established that the virus did not spread to the CNS (Johnson et al., 2007).

The rVSV can be attenuated by a number of ways to significantly reduce vector associated pathogenicity while still retaining immunogenicity. Rearrangement of genes is known to attenuate the virus. Moving the N gene to position 4 (between G and L genes) resulted in a rVSV that was 100% efficacious but attenuated (Wertz, Perepelitsa, and Ball, 1998). Rerrangement of VSV genes was also shown to eliminate clinical disease in pigs (Flanagan et al., 2001). A recent paper demonstrated that rVSV expressing HIV-1 gag that were attenuated by gene translocation or truncation of G gene exhibited enhanced immunogenicity and reduced neurovirulence in mice (Cooper et al., 2008). Similarly deletion of the C terminus of VSV G protein from 29 amino acids to either one or nine amino acid residues resulted in highly attenuated rVSVs while still retaining vaccine efficacy (Roberts et al., 1998; Schnell et al., 1998). A VSV G deleted virus (ΔG virus) was first described in 1997 (Schnell et al., 1997). The ΔG virus is recovered from cells that have the G glycoprotein provided in *trans* (Schnell et al., 1997). These ΔG viruses can only undergo a single round of replication and any progeny virus particle produced cannot infect fresh cells as they lack the G glycoprotein. Single cycle rVSV vaccines were shown to be efficacious and protective against influenza in mice but elicited an immune response of a lesser magnitude than replication competent rVSV vaccines (Roberts et al., 1999). A later study in non-human primates elegantly dissected the T and B cell immune responses to rVSV expressing HIV envelope glycoprotein. This study compared the G deficient recombinant with the rVSV containing the G glycoprotein and demonstrated that the ΔG virus elicited a better immune response if not equivalent to the replication competent rVSVs

(Publicover, Ramsburg, and Rose, 2005). Similar results have also been observed for single cycle rVSV vaccines against Hepatitis C virus (Majid et al., 2006).

Other methods of attenuation that could be used include mutations in the M gene and its two internal inframe start codons that reduce the cytopathogenic effects of the rVSV (Jayakar and Whitt, 2002). Another interesting method is the development of propagation defective G-stem rVSVs. These viruses have the most of the extracellular portion deleted but retain the transmembrane domain and ectodomain membrane proximal 42 amino acids (Robison and Whitt, 2000). Despite of the promise these newer methods hold, the practicality of safety, efficacy and large scale production may still have to be determined.

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CHAPTER 2

COMPLETE GENOME ANALYSIS AND VIRULENCE CHARACTERISTICS OF THE LOUISIANA WEST NILE VIRUS STRAIN LSU-AR01

INTRODUCTION

WNV is a member of the *Flaviviridae* family. It is an enveloped virus with a single-stranded, positive sense RNA genome of approximately 11 kb. The genome is translated as a single polyprotein, which is subsequently cleaved by host as well as virally encoded proteases to produce three structural and seven nonstructural proteins (Brinton, 2002; Chambers et al., 1990). The structural proteins include a capsid (C) protein, a pre-membrane (prM) protein, and an envelope (E) glycoprotein that mediates attachment, virus-induced membrane fusion and virion assembly (Mukhopadhyay et al., 2003; Mukhopadhyay, Kuhn, and Rossmann, 2005). The viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) regulate viral transcription and replication, as well as attenuating host antiviral responses (Best et al., 2005; Guo, Hayashi, and Seeger, 2005; Khromykh, Sedlak, and Westaway, 2000; Lin et al., 2006; Liu et al., 2005; Munoz-Jordan et al., 2005).

WNV was first isolated in 1937 from a febrile patient in the West Nile province of Uganda (Smithburn et al., 1940). The significance of WNV infection as an emerging infectious disease rapidly increased since its initial detection in New York in 1999. The rampant spread of the virus throughout North America resulted in a significant and unusual mortality among birds, specifically corvids. Moreover, WNV was also responsible for cases with severe neurological disease in humans. Approximately 80% of patients infected with WNV were found to be asymptomatic and 20% of the patients had West Nile fever. Typically, less than 1% of the patients suffered from West Nile neuroinvasive disease (WND) including West Nile meningitis (WNM), encephalitis (WNE) and acute flaccid paralysis (poliomyelitis- like syndrome) (WNP)

(Sejvar, 2007). Among WND cases, an estimated 55-60% of the patients had WNE resulting in an estimated 20% case fatality. Additionally, 10-50% of mortalities in humans could be attributed to WNP (Sejvar, 2007).

WNV has worldwide distribution ranging from Australia, India and China in the east to the Middle East, Africa, Europe and the United States in the west. Most phylogenetic analyses have utilized specific genomic regions, such as short regions of the E glycoprotein (Anderson et al., 2001; Lanciotti et al., 1999). Alternatively, entire WNV genomes have been used for phylogenetic analysis to provide more accurate information about relationships among different WNV strains (Grinev et al., 2008; Lanciotti et al., 2002). WNV strains from North America, Europe, Middle East, Africa, Australia and India belong to lineage I. The Australian (Kunjin), Indian, Czech (Rabensburg) and LEIV-Krnd88-190 virus (Russia) isolates form separate sub-lineages within lineage I (Bakonyi et al., 2006; Bondre et al., 2007; Coia et al., 1988; Lanciotti et al., 2002). While some researchers have assigned these viruses to subclades of lineage I (Briese et al., 2002; Lanciotti et al., 2002), others have proposed, based on the genetic distances, that the Rabensburg isolate, the Krnd88-190 Russian isolate and the Indian isolates, should be classified as lineages III, IV and V respectively (Bakonyi et al., 2005; Bakonyi et al., 2006; Bondre et al., 2007). In lineage I, the Israel 98/North American belongs to clade Ia and the Kunijn virus from Australia to clade Ib. Most likely, due to microevolution within clade Ia, the North American isolates have been subdivided into subclades differentiating early viruses and dominant genotypes that were established as they spread (Davis et al., 2005; Grinev et al., 2008). Lineage II WNV is mainly composed of isolates originating from the African continent and the island of Madagascar.

WNV surveillance was first established in Louisiana in the spring of 2000 and included surveillance of mosquitoes, dead birds, horses and sentinel chickens (CDC, 2002b). The first cases of WNV infection were reported in humans (CDC, 2001) and horses (CDC, 2002a) in 2001 and the virus was first isolated in Louisiana in 2001. The largest out-break in the state of Louisiana occurred in 2002 (CDC, 2002c) resulting in 329 human WN cases with 25 fatalities (Figure 2.1) (Ou and Ratard, 2005). The predominant mosquito vector in Louisiana is *Culex quinquefasciatus*, which appears to be linked to a high risk of WND (Gleiser et al., 2007; Godsey et al., 2005; Lindsey et al., 2008).

The LSU-AR01 strain representing a putative predecessor of the 2002 Louisiana outbreak was isolated from a kidney sample of a dead blue jay found in Jefferson Parish, Louisiana in 2001. The entire genome of this isolate was sequenced and phylogenetically analyzed using a database of 75 full-length WNV genomes deposited in GenBank. This analysis showed that the LSU-AR01 strain was closely related to a 1999 mosquito isolate from Connecticut and did not group with the New York-99 strain and its close-related strains, or with strains established later in various regions of the USA. Importantly, the LSU-AR01 strain exhibited increased neuroinvasiveness and neurovirulence in comparison to the New York-99 strain at low viral doses.

MATERIALS AND METHODS

Virus Isolation and Genome Sequencing

LSU-AR01 was isolated in 2001 from a dead blue jay (*Cyanocitta cristata*) at the Louisiana Animal Disease Diagnostic Laboratory (LADDL), LSU School of Veterinary Medicine (LSU SVM). The entire genome was sequenced and submitted to GenBank (accession number AY000000). Specifically, viral RNA was extracted using TRI reagent (Molecular

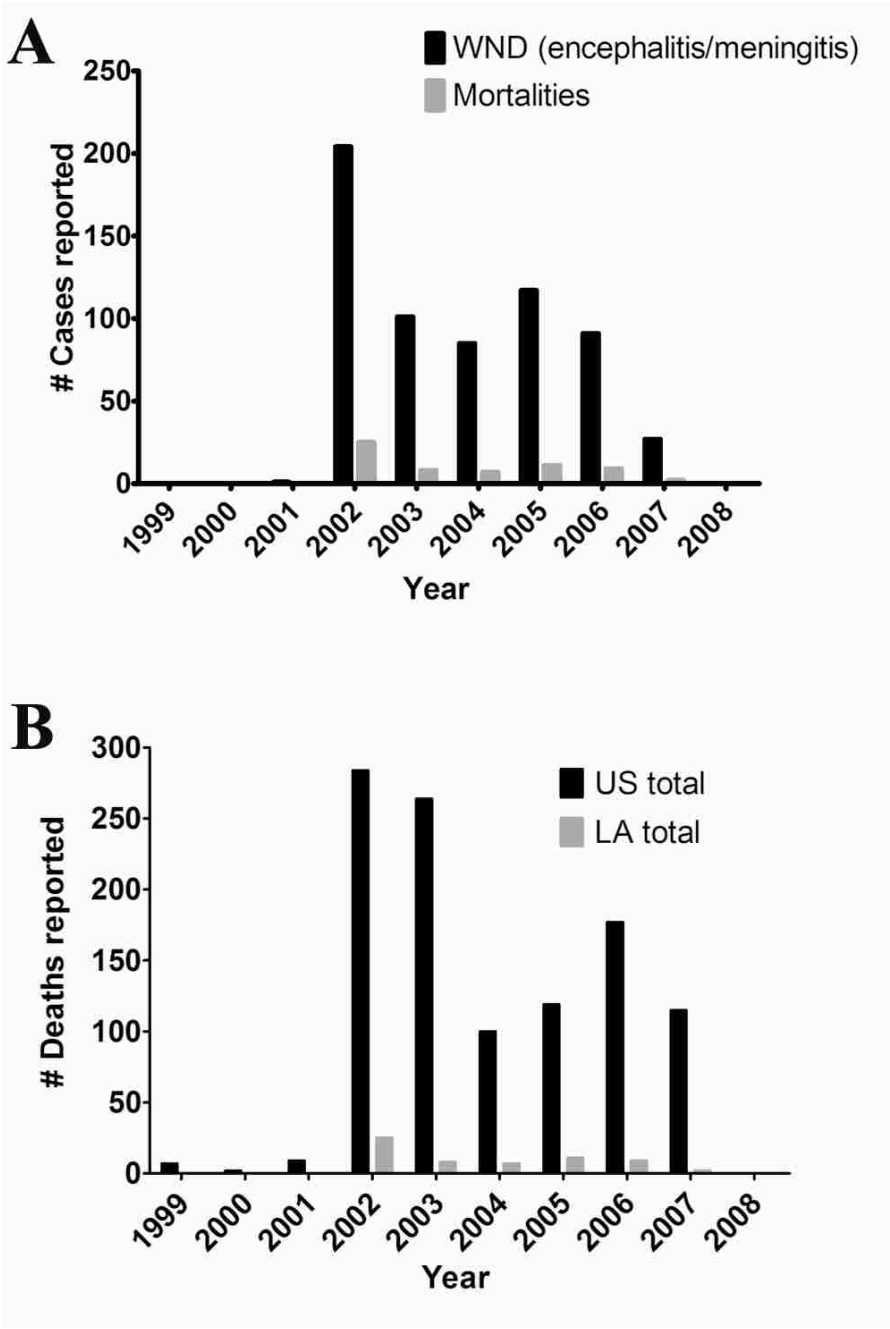
Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. Ready -To-Go-You-Prime First Strand Beads (Pharmacia Biotech, Uppsala, Sweden) were used for the cDNA construction.

Table 2.1: WNV Primers

Primer Name	Primer Sequence
WN-5'	5′-agtagttcgcctgtgtgagc-3′
WN-F1	5′-ccgtcattggttggatgc-3′
WN-F2	5′-agcattgacacatgcgcc-3′
WN-F3	5′-agcaacactgtcaagttg-3′
WN-F4	5′-cgctctaggagacacagc-3′
WN-F5	5′-cgatcagtttccagactg-3′
WN-F6	5′-tgaagtcaaatcatgtac-3′
WN-F7	5′-aagaggtggacagccaag-3′
WN-F8	5′-ataacattcacaacgacatc-3′
WN-F9	5′-ttgatgtgcggcttgatg-3′
WN-F10	5′-ttgcagcacaagtggaac-3′
WN-F11	5′-tgtggctgctgagatggc-3′
WN-F12	5′-ttgcctacaacgtgctgg-3′
WN-F13	5′-cgtgagaaggtatatacc-3′
WN-F14	5′-agcgcagtggcagccaac-3′
WN-F15	5′-cggacagcggccggaatc-3′
WN-F16	5′-atgtggaagaggcggttg-3′
WN-F17	5′-ggtgagtcgagcttcagg-3′
WN-F18	5′-accgccagaaggagtgaag-3′
WN-F19	5′-ctcaggaggaggtgtcgag-3′
WN-F20	5′-ccgcatggctgtcagtgg-3′
WN-F21	5′-cagaggacatgttggagg-3′
WN-3'	5′-agatcctgtgttctcgcacc-3′
WN-B5	5′-tacatcttcctcgtattg-3′
WN-B2	5′-ccgaccatcaacagcagtatc-3′

The entire virus genome (11029 bp) was divided into 3 segments and 3 different reverse primers: WN 3′ (bp 11029-11010), WNB5 (bp 8454-8437), and WNB2 (bp 4070-4049) were used to produce overlapping cDNAs spanning the entire virus genome.

Figure 2.1: West Nile incidence in Louisiana and United States. (A) Comparison of West Nile neuroinvasive disease (WND) and associated mortality rates in Louisiana. (B) Comparison of West Nile mortalities in the US and Louisiana. Case counts were obtained from CDC statistics.


A
WND (encephalitis/meningitis)
Mortalities
Cases reported
250
200
150
100
50
0
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year
B
US total
LA total
Deaths reported
300
250
200
150
100
50
0
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year

Primer pairs: WN 5′-WNB2, WN F7-WNB5, and WNF16-WN 3′ and Fail Safe DNA polymerase (Epicentre Biotechnologies, Madison, WI) were used to generate PCR overlapping DNA products. PCR products were directly sequenced in an automated DNA sequencer (Applied Biosystems) after column purification (Zymo Research Corp., Orange, CA). The list of all synthetic oligonucleotides used for PCR and sequencing is shown in Table 2.1.

Viral Replication Kinetics

All WNV experiments were conducted within a BSL-3 level biosafety laboratory according to CDC guidelines. Vero cell monolayers in 6-well plates were washed two times with ice-cold PBS and infected with LSU-AR01 or NY-99 at a MOI of 1. The cells were incubated at 4°C for an hour to allow for virus adsorption and then for one more hour at 37°C. The remaining virus inoculum was aspirated and cells were washed with phosphate buffered saline (PBS) followed by a wash with acidic PBS to eliminate unadsorbed virus particles. DMEM growth medium containing 2% FCS was added and cell cultures were incubated at 37°C. Individual plates were frozen at zero time point and every twelve hours after that for 72 hours. Virus titers for stocks prepared at each time point were determined using 10-fold dilutions.

Comparative Virulence and Pathogenicity Studies

Five-week-old female Balb/c mice were used. All experiments were approved by the LSU SVM Institutional Animal Care and Use Committee (IACUC), Institutional Biological and Recombinant Safety Committee (IBRDS) and BSL-3 bio-safety committees. Mice were divided into five groups of ten mice each. A 31-guage tuberculin syringe was used for tail vein injections. Animals were inoculated intravenously with either 100µl of the virus for the experiment group, or 100µl uninfected cell culture supernatant for the mock group. Group I and II received 10^3 PFU/ml and 10^6 PFU/ml of the WNV NY-99 and were designated as NY-99 low

and high groups, respectively. Groups III and IV received 10^3 PFU/ml and 10^6 PFU/ml of the WNV LSU-AR01 isolate and were designated as LSU-AR01 low and high groups, respectively. Group V animals served as the control group and received cell culture supernatant. All animals were observed three times daily for 14 days for clinical signs of WNV infection including ruffled fur, hunching, ataxia, lethargy and hind limb paralysis. At the end of the experiment all remaining animals were humanely euthanized and selected organs were collected from all animals for histopathological examination.

Histopathology

Tissues (brain, lung, liver, bilateral kidneys, heart, spleen, skull, and vertebra) were collected from the mice euthanized or after death and fixed by immersion in 10% neutral buffered formalin. The skull and vertebra were decalcified in 10% formic acid for three days. All sampled tissues were routinely processed into paraffin, and 3 to 4 micrometer sections were cut for hematoxylin and eosin staining (H&E). H&E sections of the nasal olfactory epithelium and bulb in the skull and four sections of the spinal cord including two consecutive anterior cervico-thoracic and two consecutive lumbar-sacral posterior sections in the vertebrae were examined under the light microscope.

Phylogenetic Analysis

Accession numbers for the complete genome sequences of 75 WNV isolates (Table 2.2) were obtained by BLAST search using the WNV LSU-AR01 sequence as well as various specific references. The complete genome sequences were obtained from GenBank. A Japanese Encephalitis virus (JE) (M18370) sequence was used as the out-group. Sequences were imported into and aligned using ClustalX 2.0 (Thompson et al., 1997). The alignment was iterated at the final step using ClustalX so as to improve the overall quality of the alignment. A distance matrix

was generated with the DNADIST program in the Phylogeny Inference Package (PHYLIP) version 3.6 (Felsenstein, 1989) and the Kimura 2-parameter (Kimura, 1980) evolutionary model was applied to the distances. The Neighbor program in PHYLIP was used to construct a Neighbor-Joining (NJ) tree using the method of Saitou and Nei (Saitou and Nei, 1987). The tree was rooted using the JE virus as out-group. Bootstrap analysis was done using one thousand replicates and the values were added to the NJ tree. The tree was edited using Dendroscope version 1.2.4 software (Huson et al., 2007). The alignment was also imported into BioEdit software version7.0.9.0 (Hall, 1999) to generate a sequence identity matrix.

Table 2.2: WNV Strains Used for Phylogenetic Analysis

Virus name	Accession number	Country	State	Year	Source
WNFCG	M12294	Uganda		1937	Human
Uganda B956	AY532665	Uganda		1937	Human
Egypt101	AF260968	Egypt		1951	Human
Kunjin strainMRM61C	D00246	Australia		1960	*Culex annulirostris*
PTRoxo	AM404308	Portugal		1971	*An. maculipennis*
Ethiopia strainEthAn4766	AY603654.1	Ethiopia		1976	Bird
Madagascar-AnMg798	DQ176636.2	Madagascar		1978	Parrot (*Coracopsis vasa*)
India804994	DQ256376	India		1980	Human
ArB3573/82	DQ318020.1	Central African Republic		1982	Tick
SPU116-89	EU068667.1	South Africa		1989	Human
ArD76104	DQ318019.1	Senegal		1990	Mosquito
Romania RO97-50	AF260969	RO97-50		1996	*Culex pipens*
Morocco 96-111	AY701412	Morocco		1996	Horse
PaH001	AY268133	Tunisia		1997	Human
Rabensburg isolate 97-103	AY765264	Czech Republic		1997	*Culex pipiens*
Italy-98-equine	AF404757	Italy		1998	Equine
IS-98 STD	AF481864	Israel		1998	Stork

TABLE 2.2 continued

KN3829	AY262283	Kenya		1998	*Culex univittatus*
LEIV-Krnd88-190	AY277251.1	Russia		1998	Tick (*Dermacentor marginatus*)
New York 99-flamingo382-99	AF196835	USA	NY	1999	Chilean flamingo
VLG-4	AF317203	Russia	Volgagrad	1999	Human
NY99-eqhs	AF260967	USA	NY	1999	Horse
LEIV-Vlg99-27889	AY277252.1	Russia	Volgagrad	1999	Human
HNY1999	AF202541	USA	NY	1999	Human
CT-mosquito2741	AF206518	USA	CT	1999	*Culex pipiens*
Ast99-901	AY278441	Russia	Astrakhan	1999	Human
PaAn001	AY268132	France		2000	Horse
MD 2000-crow265	AF404753	USA	MD	2000	Crow
NJ 2000 MQ5488	AF404754.1	USA	NJ	2000	*Culex pipiens* pool
NY 2000-grouse3282	AF404755.1	USA	NY	2000	Grouse
LEIV-Vlg00-27924	AY278442.1	Russia	Volgagrad	2000	Human
3356K VP2	EF657887.1	USA	NY	2000	American crow
NY 2001 Suffolk	DQ164194	USA	NY	2001	American crow
Chin-01	AY490240.2	China		2001	
FL232	DQ080072.1	USA	FL	2001	Catbird
LSU-AR01	AF000000	USA	LA	2001	Blue Jay
TX 2002 2	DQ164205.1	USA	TX	2002	Human
OH 2002	DQ164202.1	USA	OH	2002	Human
TX 2002 1	DQ164198.1	USA	TX	2002	Human
GA 2002 2	DQ164197.1	USA	GA	2002	Human
NY 2002 Nassau	DQ164195.1	USA	NY	2002	*Culex pipiens/restuans*
GA 2002 1	DQ164196.1	USA	GA	2002	Human
FL234	DQ080071.1	USA	FL	2002	Horse
TWN165	DQ080062	USA	LA	2002	Mosquito
ARC10	AY795965.1	USA	MI	2002	Human
Clinton02	DQ164193.1	USA	NY	2002	American crow
Broome02	DQ164187.1	USA	NY	2002	American crow
Queens02	DQ164186.1	USA	NY	2002	American crow
IN2002	DQ164200	USA	IN	2002	Human
2003 Rockland	DQ164192.1	USA	NY	2003	American crow
Mex03 strainTM171-03	AY660002.1	Mexico	Tabasco	2003	Raven
NY 2003 Albany	DQ164189	USA	NY	2003	American crow

TABLE 2.2 continued

Bird 1171	AY712946	USA	TX	2003	Blue jay
goose-Hungary/03	DQ118127.1	Hungary		2003	Goose
Morocco 4.05	AY701413	Morocco		2003	Horse
03-113FL	DQ431697	USA	FL	2003	Human
AZ-03-1799	DQ080053.1	USA	AZ	2003	*Culex tarsalis*
CA03GRLA-1260	DQ080054.1	USA	CA	2003	*Culex quinquefaciatus*
AZ-03-1681	DQ080052.1	USA	AZ	2003	*Culex tarsalis*
CA-03Arc	DQ080058.1	USA	CA	2003	Crow
CO03-2	DQ164203.1	USA	CO	2003	Magpie
CO03-1	DQ164204.1	USA	CO	2003	Red-tailed hawk
03-124FL	DQ431699.1	USA	FL	2003	Human
03-120FL	DQ431698.1	USA	FL	2003	Human
OK03	EU155484	USA	OK	2003	
TVP9220	DQ080066	Mexico	Baja California Norte	2003	Cormorant
TX 2004 Harris 4	DQ164206.1	USA	TX	2004	Blue jay
TWN496	DQ080061	USA	LA	2004	Cardinal
goshawk-Hungary/04	DQ116961	Hungary		2004	Goshawk
France 407/04	DQ786573.1	France		2004	Common magpie
France 405/04	DQ786572.1	France		2004	House Sparrow
WNVCc	DQ080060	Mexico	Baja Calfornia Norte	2004	Raven
Sarafend	AY688948	Unknown (lineage II)			
385-99 isolate hamster passage strain 9317A	AY848695	USA			Hamster
Japanese encephalitis virus (strain JaOArS982)	M18370				

RESULTS

Virus Isolation, Characterization and Genome Sequencing

The WNV-LSU-AR01 was isolated by direct incubation of a dead blue jay kidney sample on African monkey kidney cells (Vero). The virus was passed four times before larger stocks

were made for subsequent analyses. The entire LSU-AR01 genome was sequenced as described in Materials and Methods. Alignment of the LSU-AR01 genomic sequence to that of the NY-99 genome revealed that the two genomes differed by 26 nucleotides. Twenty (approximately 77%) of these nucleotide changes of the LSU-AR01 genome did not code for amino acid changes, while the remaining six (approximately 23%) resulted in amino acid changes (Table 2.3). Overall, there were 20 nucleotide transitions and 3 (15%) of these transitions caused an amino acid change. Six of the nucleotide changes were transversions with half of them resulting in amino acid changes. As compared to the NY99 sequence, there was one amino acid change within each of the NS2B, NS3, NS4A and NS4B coding sequences and two amino acid changes within NS5.

The LSU-AR01 strain produced viral plaques on Vero cells, which appeared to be similar to those produced by the NY-99 strain occasionally producing a "comet" like streaking appearance (Figure 2.2: A). Viral replication kinetics were determined in Vero cells. Both viruses appeared to replicate with similar kinetics yielding highest titers at 36-48 hours post infection with an MOI of 1 (Figure 2.2: B).

Comparative Pathogenicity of LSU-AR01 and NY-99 Strains in Mice

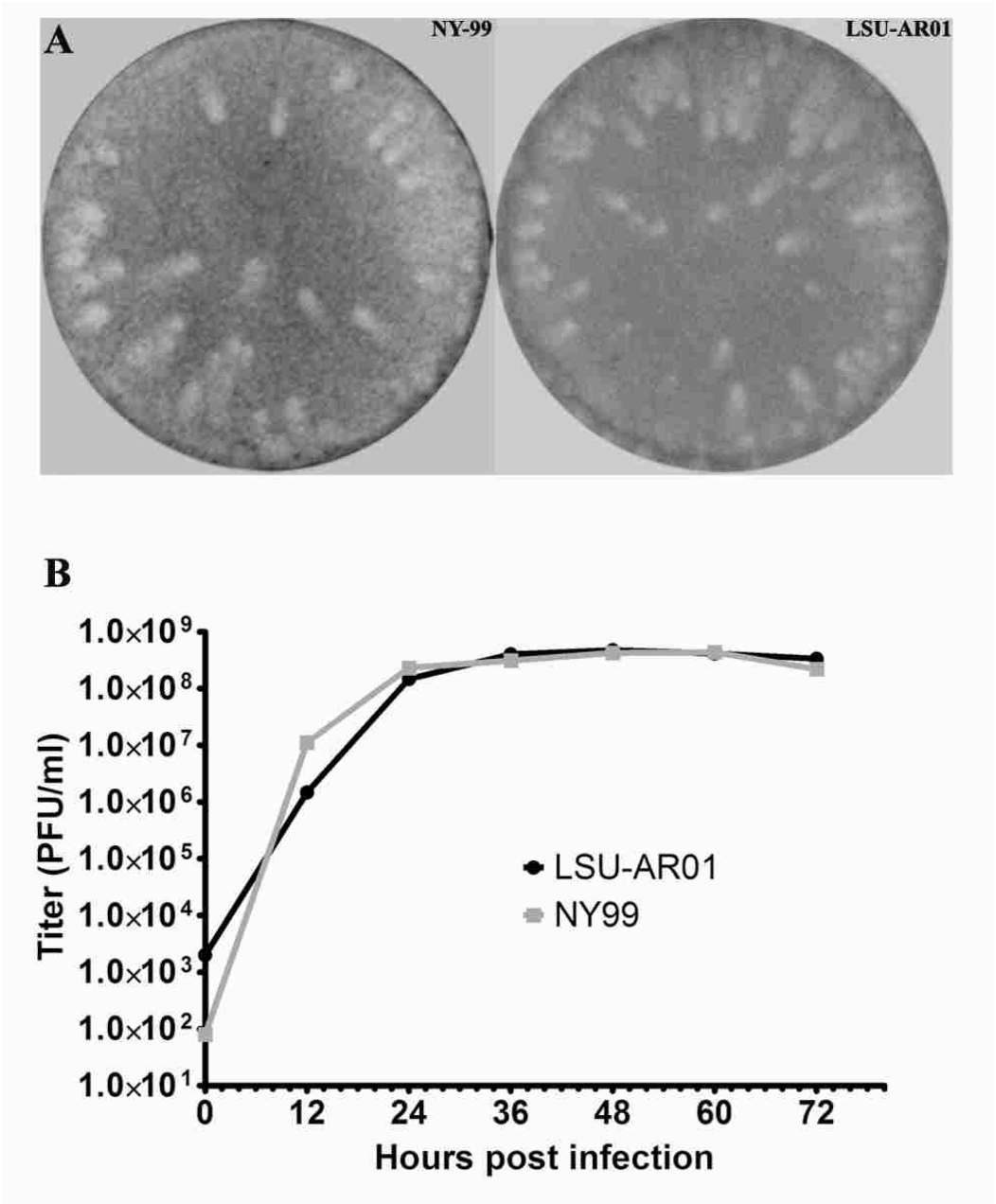
Mice were infected intravenously via direct injection of virus into the tail vein of Balb/c mice. Two different doses of viruses were used, a low dose consisting of 10^3 PFU, and a high dose of virus consisting of 10^6 PFU (see Materials and Methods). Mice were observed for a 14 days. Mice infected with either viral strain showed similar clinical symptoms including ruffled fur, hunched posture, ataxia, and lethargy.

Table 2.3: Genome-wide Comparison of the WNV NY-99 and LSU-AR01 Strains

Base#	NY99	LSU-AR01*	Amino acid	Region
969	C	T		E
2001	A	G		E
2289	T	C		E
2400	T	C		E
3240	A	G		NS1
3804	G	A		NS2A
3810	C	T		NS2A
4561	**A**	**G**	**Ile→Val**	**NS2B**
4743	T	C		NS3
5142	C	T		NS3
5709	C	T		NS3
5758	**G**	**A**	**Val→Ile**	**NS3**
5928	A	G		NS3
6591	**A**	**C**	**Arg→Ser**	**NS4A**
6678	C	T		NS4A
7015	T	C		NS4B
7551	**T**	**G**	**Asn→Lys**	**NS4B**
8415	T	C		NS5
8811	T	C		NS5
9751	**A**	**C**	**Lys→Gln**	**NS5**
10085	**T**	**C**	**Ile→Thr**	**NS5**
10551	T	A		UTR
10768	T	A		UTR
10851	A	G		UTR
10996	C	T		UTR
10998	A	T		UTR

*A total of 26 mutations were observed throughout the genome. Six of these mutations resulted amino acid changes.

Figure 2.2: Comparison of plaque morphologies and replication kinetics of LSU-AR01 and NY-99 strains. (A) photomicrographs of LSU-AR01 and NY-99 viral plaques formed in Vero cells stained with crystal violet. (B) Vero cells were infected at an MOI of 1 and viral titers at different time points post infection were obtained by viral plaque assay on Vero cells.


A
NY-99
LSU-AR01
B
Titer (PFU/ml)
1.0×10^9
1.0×10^8
1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
1.0×10^2
1.0×10^1
0
12
24
36
48
60
72
Hours post infection
LSU-AR01
NY99

However, 30% of mice (3 mice) infected with the LSU-AR01 strain appeared to develop hind-limb paralysis, while only 10% of mice (one mouse) per group appeared to exhibit similar clinical symptoms in the NY-99 infected mice (not shown). The median survival time for LSU-AR01 and NY-99 were 9.5 and 8 days, with 80% and 90% mortality, respectively, at the low viral dose (Figure 2.3: A). For the high virus dose, the median survival time for LSU-AR01 and NY-99 were 6 and 7 days, with 90% and 100% mortality, respectively (Figure 2.3: B). The LSU-AR01 high dose caused the death of 70% of mice on day 6 as compared to 40% of the mice in the NY-99 high group. Mice started to die earlier in the LSU-AR01 low group (10% on day 6) but slower than the NY-99 low group.

Histopathological Examination of Mice Infected with Either LSU-AR01 or NY-99 Strains

The most prominent histological changes were observed in the brain. Lesions were seen in 60% of the mice in the LSU-AR01 low infectious dose versus none of the mice in the NY-99 low dose group. In the high infectious dose groups, 60% of the LSU-AR01-infected mice versus 90% of the NY-99-infected mice exhibited brain lesions. Encephalitic lesions typically consisted of lymphoplasmacytic perivascular cuffing (Figure 2. 4: A), neuronal necrosis (Figure 2.4: B), and gliosis. Neutrophilic infiltration was occasionally present in perivascular areas and rarely extended into the neuropils. Lesions were highly variable ranging from focal and very mild to severe and diffuse. Meningitis was not noted in the mice of any groups. Neuronal necrosis was also present in the spinal cords of the mice in high dose groups (70% for LSU-AR01 and 20% for the NY-99 infected mice). Only the mice in high dose LSU-AR01 group (30%) had mild hemorrhage in the spinal cord. No overt lesions were present in the mice of both low dose groups. The control mice did not have any lesions in the brain and spinal cord.

Figure 2.3: Comparison of the West Nile Virus NY-99 and LSU-AR01 virulence in mice. (A) Mice were infected with a low viral dose (10^3 PFU) of either LSU-AR01 strain (black) or NY-99 strain (gray). (B) Mice were infected with a high viral dose (10^6 PFU) of either LSU-AR01 strain (black) or NY-99 strain (gray). Kaplan-Meier survival curves and statistical analysis were generated using GraphPad Prism software version 5.01.

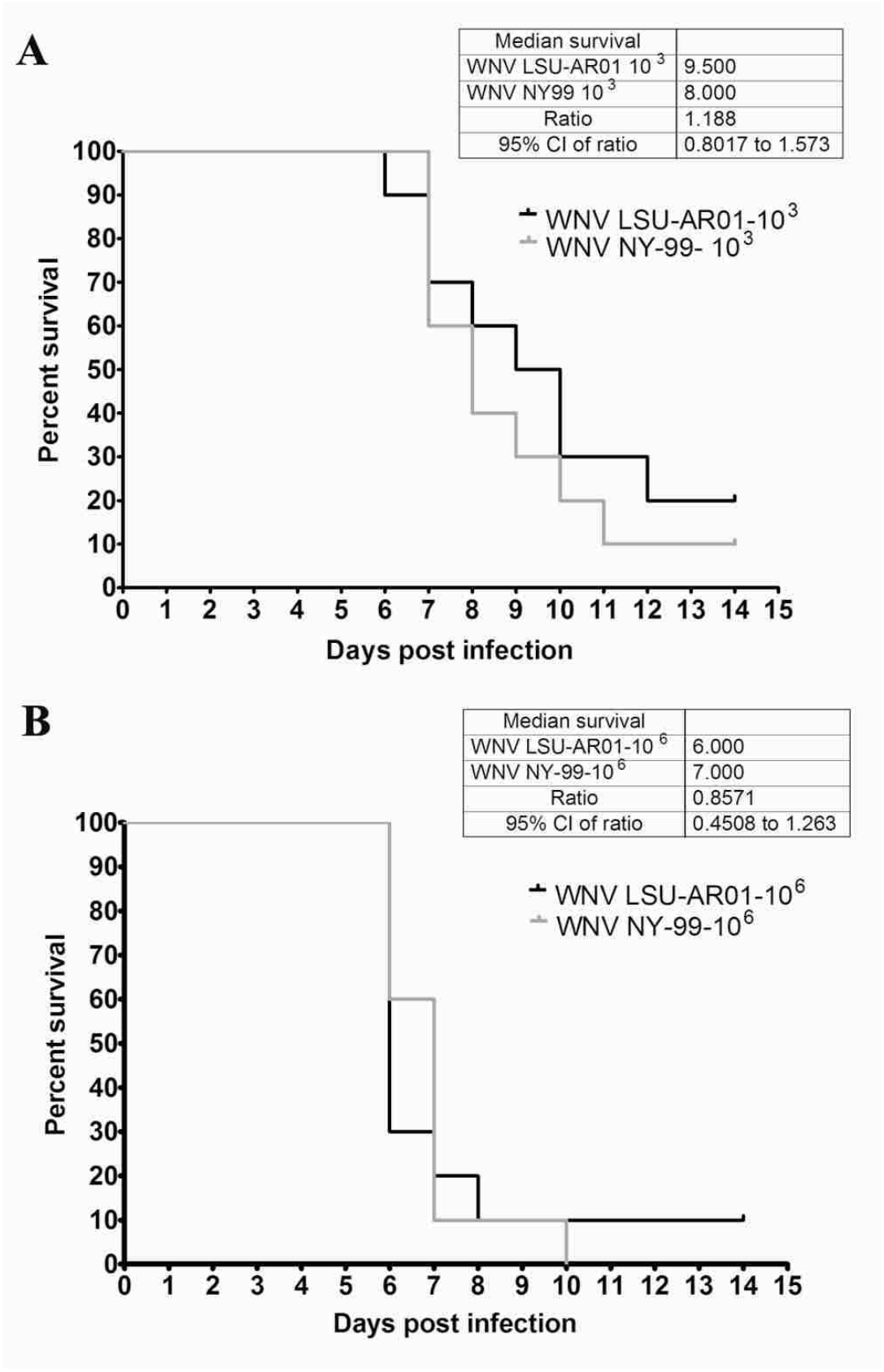
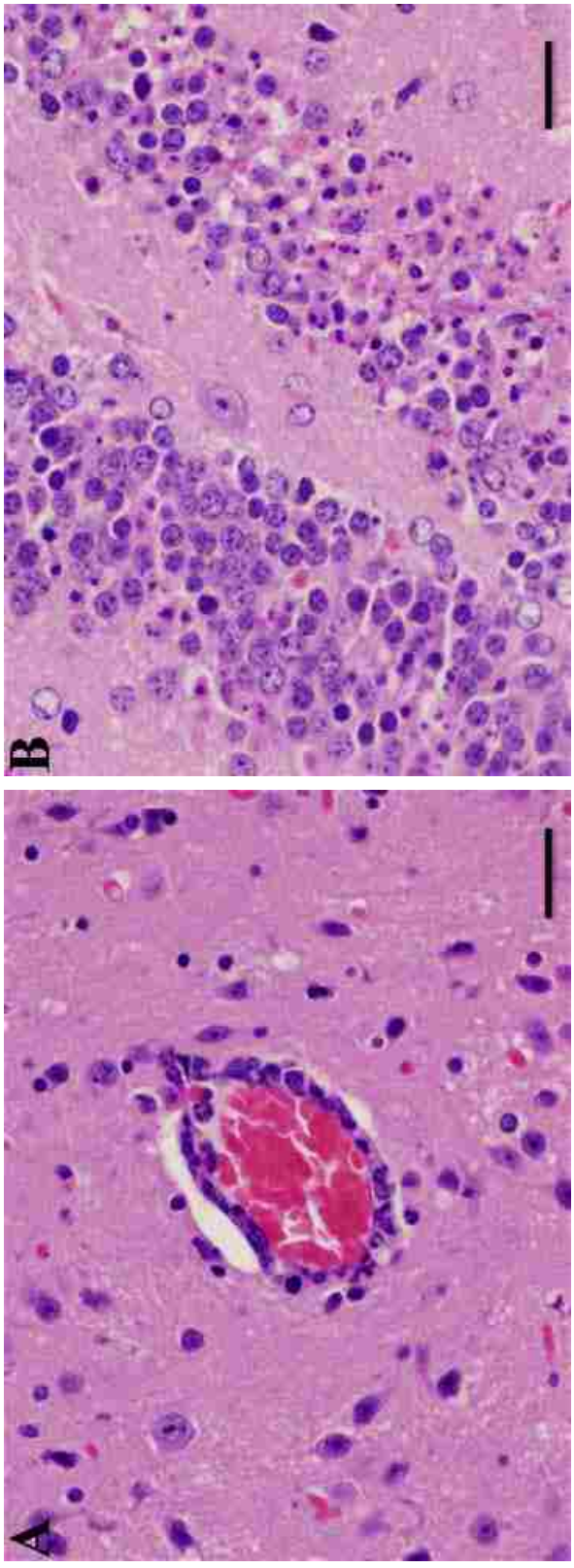

A
Median survival
WNV LSU-AR01 10 3
9.500
WNV NY99 10 3
8.000
Ratio
1.188
95% CI of ratio
0.8017 to 1.573
WNV LSU-AR01-10 3
WNV NY-99- 10 3
Percent survival
Days post infection
B
Median survival
WNV LSU-AR01-10 6
6.000
WNV NY-99-10 6
7.000
Ratio
0.8571
95% CI of ratio
0.4508 to 1.263
WNV LSU-AR01-10 6
WNV NY-99-10 6
Percent survival
Days post infection

Figure 2.4: Central nervous system changes in mice experimentally infected with high dose WNV LSU-AR01. (A) A perivascular cuff composed of lymphocytes and plasma cells in the cerebrum (H&E). Bar = 50 μm. (B) Severe neuronal necrosis in dentate gyrus of the hippocampus, characterized by densely eosinophilic, angular and shrunken cytoplasm and pyknosis and fragmentation of the nucleus (H&E). Bar = 50 μm.


B
A

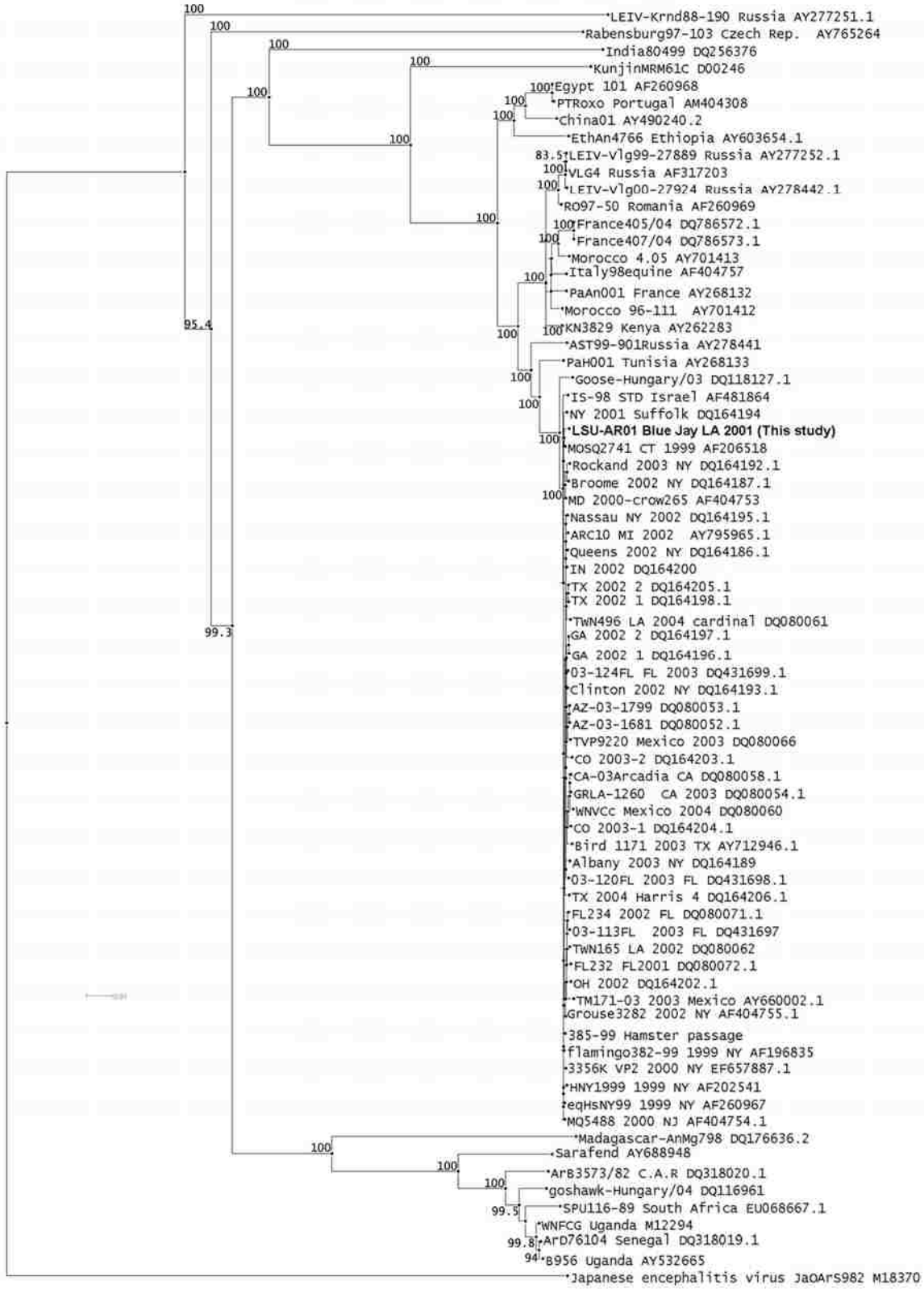
Increased numbers of tangible body macrophages were noted in the splenic white pulp of many infected mice, more commonly high dose (90% in both strains) than low dose (40% in LSU-AR01 and 30% in NY-99). The heart from both infected and control mice occasionally had mild to severe epicardial mineralization along with mixed inflammatory cell infiltration composed of macrophages, lymphocytes, plasma cells, and neutrophils, which are most consistent with non-viral induced background lesions seen in healthy mice. Hepatocytes had mild to severe cytoplasmic microvacuoles, interpreted as glycogen in the liver from infected mice (60% low dose LSU-AR01; 70% low dose NY-99; 20% high dose LSU-AR01; 90% high dose NY-99). Rarely, mild inflammatory cell infiltration composed of neutrophils or mixed inflammatory cells were present in both infected and control mice (0% low dose LSU-AR01; 10% low dose NY-99; 10% high dose LSU-AR01 ; 20% high dose NY-99; 10% control). No overt lesions were observed in the olfactory tissues, lungs, or kidneys.

Phylogenetic Analysis of the LSU-AR01 Strain Based on 75 WNV Genomes

Seventy-five WNV genomes available in GenBank were aligned using the multiple sequence alignment program CLUSTALX 2.0. The PHYLIP software package was utilized to generate a distance matrix using the Kimura 2-parameter evolutionary model. A neighbor-joining (NJ) phylogenetic tree was generated using the Japanese Encephalitis virus (M18370) genomic sequence as the out-group. Bootstrap values greater than 75% were added to the tree (see Materials and Methods) (Figures 2.5, 2.6).

The North American branch of Lineage I was extracted to obtain a better perspective of the relationship between the isolates (Figure 2.6). A nucleotide identity matrix was generated from the alignment using BioEdit software (Hall, 1999). The LSU-AR01 strain was found to

Figure 2.5: Neighbor-Joining (NJ) tree for WNV isolates. A NJ tree generated using complete genomes of 75 WNV isolates. The tree was rooted using the Japanese Encephalitis virus as the outgroup. Percent bootstrap values ≥ 75% are shown.


LEIV-Krnd88-190 Russia AY277251.1
Rabensburg97-103 Czech Rep. AY765264
India80499 DQ256376
KunjinMRM61C D00246
Egypt 101 AF260968
PTRoxo Portugal AM404308
china01 AY490240.2
EthAn4766 Ethiopia AY603654.1
LEIV-Vlg99-27889 Russia AY277252.1
VLG4 Russia AF317203
LEIV-Vlg00-27924 Russia AY278442.1
RO97-50 Romania AF260969
France405/04 DQ786572.1
France407/04 DQ786573.1
Morocco 4.05 AY701413
Italy98equine AF404757
PaAn001 France AY268132
Morocco 96-111 AY701412
KN3829 Kenya AY262283
AST99-901Russia AY278441
PaH001 Tunisia AY268133
Goose-Hungary/03 DQ118127.1
IS-98 STD Israel AF481864
NY 2001 Suffolk DQ164194
LSU-AR01 Blue Jay LA 2001 (This study)
MOSQ2741 CT 1999 AF206518
Rockand 2003 NY DQ164192.1
Broome 2002 NY DQ164187.1
MD 2000-crow265 AF404753
Nassau NY 2002 DQ164195.1
ARC10 MI 2002 AY795965.1
Queens 2002 NY DQ164186.1
IN 2002 DQ164200
TX 2002 2 DQ164205.1
TX 2002 1 DQ164198.1
TWN496 LA 2004 cardinal DQ080061
GA 2002 2 DQ164197.1
GA 2002 1 DQ164196.1
03-124FL FL 2003 DQ431699.1
Clinton 2002 NY DQ164193.1
AZ-03-1799 DQ080053.1
AZ-03-1681 DQ080052.1
TVP9220 Mexico 2003 DQ080066
CO 2003-2 DQ164203.1
CA-03Arcadia CA DQ080058.1
GRLA-1260 CA 2003 DQ080054.1
WNVCc Mexico 2004 DQ080060
CO 2003-1 DQ164204.1
Bird 1171 2003 TX AY712946.1
Albany 2003 NY DQ164189
03-120FL 2003 FL DQ431698.1
TX 2004 Harris 4 DQ164206.1
FL234 2002 FL DQ080071.1
03-113FL 2003 FL DQ431697
TWN165 LA 2002 DQ080062
FL232 FL2001 DQ080072.1
OH 2002 DQ164202.1
TM171-03 2003 Mexico AY660002.1
Grouse3282 2002 NY AF404755.1
385-99 Hamster passage
flamingo382-99 1999 NY AF196835
3356K VP2 2000 NY EF657887.1
HNY1999 1999 NY AF202541
eqHsNY99 1999 NY AF260967
MQ5488 2000 NJ AF404754.1
Madagascar-AnMg798 DQ176636.2
Sarafend AY688948
ArB3573/82 C.A.R DQ318020.1
goshawk-Hungary/04 DQ116961
SPU116-89 South Africa EU068667.1
WNFCG Uganda M12294
ArD76104 Senegal DQ318019.1
B956 Uganda AY532665
Japanese encephalitis virus JaOArS982 M18370
100
95.4
99.3
83.5
99.5
99.8
94

have 99.5% and 99.7% nucleotide identity with the Israel 98 (ISR-98) (AF481864) WNV and the NY-99 (AF196835) WNV respectively. The NY-99 and ISR-98 had 99.7% nucleotide identity and was close to the value (99.8%) obtained by Lanciotti et al. (Lanciotti et al., 2002). The % nucleotide identities are based solely on the alignment of sequences in ClustalX. To produce the phylogenetic tree a distance matrix was generated using the Kimura-2 parameter evolutionary model. The LSU-AR01 Isolate was closely related to a WNV strain isolated from Culex Spp.

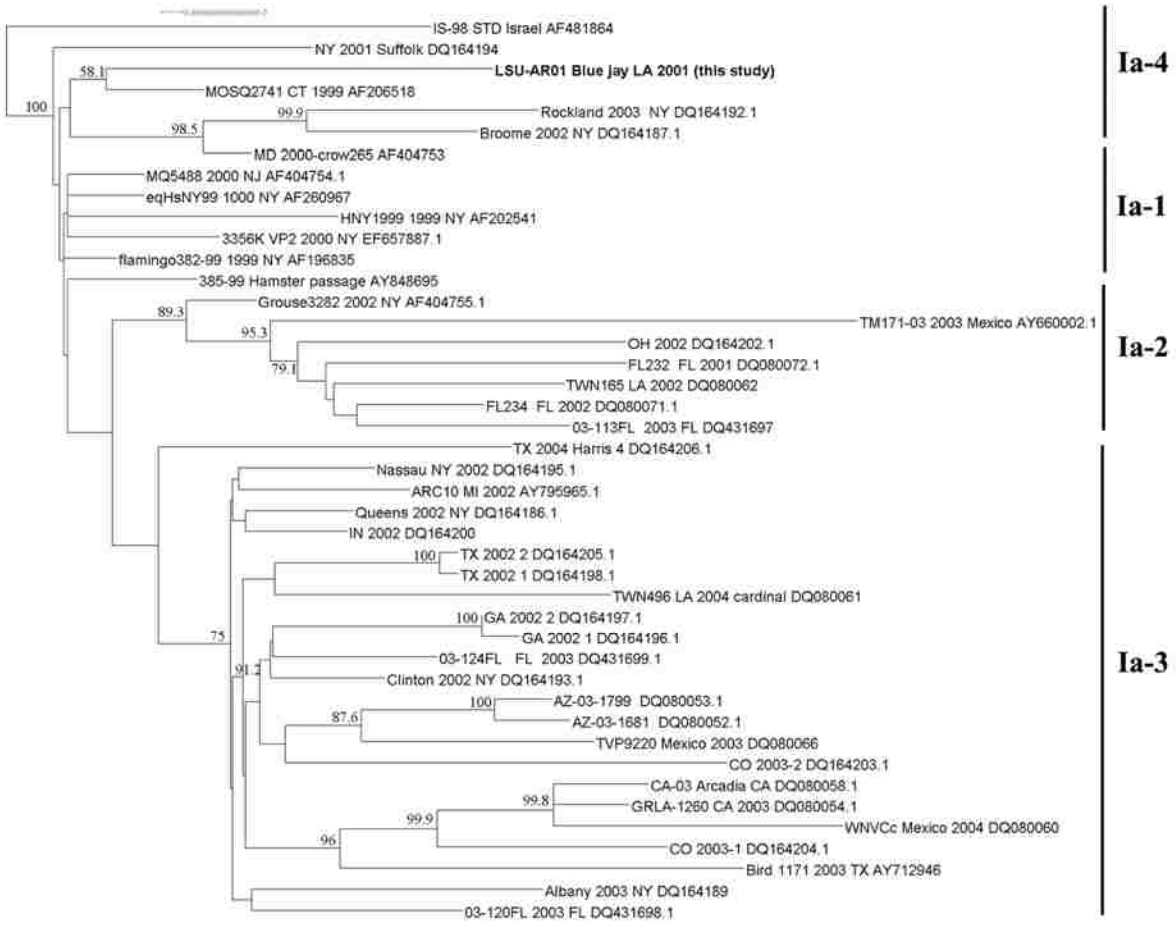


Figure 2.6: The Lineage Ia Sub-Tree. The lineage Ia sub-tree was extracted from the neighbor-joining tree shown in figure 5. This sub-tree contains the Israel-98 and the North American WNV isolates. Percent bootstrap values ≥ 75% are shown.

mosquitoes in 1999 from Connecticut (AF206518). This isolate shared 99.2% nucleotide identity with the LSU-AR01 strain.

DISCUSSION

In this paper, we present the entire genomic sequence of the WNV strain WNV-LSU-AR01 isolated from a blue jay during the early stages of the Louisiana-wide WNV surveillance program. This virus appeared to cause increased neuroinvasiveness and neurovirulence in mice in comparison to the prototypic NY-99 strain, especially at low infection doses. Phylogenetic analysis showed that the LSU-AR01 strain belonged to a distinct subclade among the North American isolates having a close relative strain previously identified in Connecticut in 1999. The results presented herein suggest that the Connecticut-related strains may contain highly neurovirulent strains such as the LSU-AR01 that have spread in North America.

The Louisiana WNV Outbreaks

The Louisiana WNV surveillance program was initiated in 2000. WNV incidence peaked in 2002 for both bird and human cases (CDC, 2002c). Blue jays are particularly susceptible to contact infection with WNV (Komar et al., 2003) rendering this bird species good sentinels for WNV. Consequently, 75% of all dead birds in Louisiana in 2002 were blue jays (Palmisano et al., 2005), while 91.5% of dead blue jays in the Texas 2002 outbreak were WNV positive (Lillibridge et al., 2004). The WNV-LSU-AR01 was isolated from a blue jay found dead in Jefferson Parish, Louisiana that borders with and is located immediately south of the St. Tammany Parish in which the epicenter of the 2002 WNV outbreak was recorded. In this well-reported outbreak, 36% of non-human primates housed outside at the Tulane National Primate Research Center (TNPRC) developed WNV antibodies (Ratterree et al., 2003). Towards the

end of the outbreak, 25% of the birds developed anti-WNV antibodies and the blue jay population was almost reduced by half (Komar et al., 2005).

Plaque morphologies and replication kinetics produced by the LSU-AR01 strain were generally similar to those produced by the prototypic NY-99 strain revealing that these viruses replicated and spread in cell culture with similar efficiencies. Genomic sequence comparison of the LSU-AR01 with the NY-99 strain revealed 26 nucleotide differences 6 of which coded for different amino acids. All the coding changes were located in non-structural proteins involved in regulation of viral replication and transcription. Three amino acid changes represented non-conservative changes with respect to size and polarity, and thus may affect the *in vivo* replication and pathogenic properties of the virus, although none of these changes appeared to affect viral replication and virus spread in cell culture. Two amino acid changes in LSU-AR01 in comparison to the NY-99 strain, asparagine-to-lysine and lysine-to-glutamine, were located immediately proximal or within known or predicted $CD8^+$T cell epitopes located within the nonstructural proteins NS4B and NS5, respectively (Brien, Uhrlaub, and Nikolich-Zugich, 2007; Purtha et al., 2007). Other CTL epitopes located within the E glycoprotein were not changed. The WNV E glycoprotein is known to elicit robust humoral and cellular immune responses that can protect mice from lethal WNV challenge (Dauphin and Zientara, 2007). The vaccine potential of the WNV E glycoprotein has been recently confirmed in our laboratory revealing that intranasal administration of the E glycoprotein induces robust $CD4^+$ and $CD8^+$ E-specific immune responses (Iyer and Kousoulas, unpublished). However, in these mice experiments, high levels of anti-E immune responses are generated to secure protection from WNV challenge. Low-dose infection in naïve animals may allow the virus to replicate in the brain before sufficient humoral and cellular immune responses are generated. Thus, it is possible that the

potential loss or otherwise altered recognition of the two LSU-AR01 CTL epitopes within the NS4B and NS5 proteins could allow increased replication of the virus in the brain contributing to the observed increased neurovirulence of the LSU-AR01 in comparison to the NY-99 strain.

Comparative Virulence in Mice

Intravenous infection of Balb/c mice with either the LSU-AR01 or NY-99 strain revealed substantial differences in brain pathologies, especially at the lower infectious dose of 10^3 plaque forming units (PFU). Specifically, 60% of the mice infected with the LSU-AR01 exhibited CNS pathology including neuronal necrosis, perivascular cuffing, and gliosis, while none of the mice infected with the NY-99 strain exhibited similar pathological profiles in the brain or spinal cord. Also, LSU-AR01 infected mice appeared to develop neurological signs such as hind limb paralysis more often than the NY-99 strain infected mice. It is possible that the six amino acid differences between the LSU-AR01 and NY-99 strain are responsible for the *in vivo* virulence differences between these two viral strains. Generally, mice are highly susceptible to most WNV strains. Two different infectious patterns have been reported, which are highly dependent on the initial viral dose. Infections with relatively low viral titers appeared to produce substantially increased neuronal inflammation due to immunopathogenesis. In contrast, high infectious doses caused direct brain damage due to high viral replication in the brain (Wang et al., 2003). Pathological examination of internal organs of infected mice did not reveal overall apparent histopathological differences between the LSU-AR01 and NY-99 at both high and lose infection doses. Therefore, it is unclear at this point, whether the LSU-AR01 strain was able to cross-the blood-brain barrier more efficiently than the NY-99 strain, or whether it was able to replicate more efficiently than the NY-99 strain either in the brain or at other tissues prior to spread to the central nervous system (CNS).

WNV-LSU-AR01 Phylogenetic Classification

A phylogenetic tree was derived based on alignment of 75 complete genomes using the NJ method. The overall topology of the NJ tree is in general agreement with previously published phylogenetic trees (Bakonyi et al., 2005; Bakonyi et al., 2006; Bondre et al., 2007; Charrel et al., 2003; Davis et al., 2005; Lanciotti et al., 2002; Parreira et al., 2007). A large majority of the clades in the NJ tree were supported by 100% bootstrap values. Values of 75% or more are generally considered to be significant. The LSU-AR01 strain was classified as a lineage I virus belonging to the North American Clade Ia. All other isolates in this subclade in this tree were isolated from American crows and appeared to belong to the eastern US isolates described by Davis et al.(Davis et al., 2005). The derived tree showed a close but distinct relationship between the subclade of these corvid isolates and the subclade containing the NY99 virus and closely-related strains. Therefore, these results suggest that the NY99 and the corvid subclade of LSU-AR01 shared a common ancestor. The tree appeared to branch out into four subclades: subclade Ia-1 consisting of NY-99 and its immediate relatives; Ia-2 containing mostly WNV strains from Florida and one strain from Mexico; Ia-3 containing multiple strains from different southern and western states; Ia-4 containing two strains from New York isolated in 2002-2003, one strain from Maryland isolated in 2000, a Connecticut mosquito strain isolated in 1999 and the LSU-AR01 isolated in 2001. Although not highly significant, the association of LSU-AR01 and the CT 1999 isolate is supported by a 58.1% bootstrap value.

WNV strains belonging to lineage I are known to be neurovirulent in mice. In agreement with these findings, the LSU-AR01 strain appears to be highly pathogenic in mice. The phylogenetic tree clearly indicates that the WNV strains that spread from New York to southern and western states shared a common ancestor closely related to the NY-99 strain. There are a

number of possible reasons why strains classified in subclade Ia-4 may not have spread as well as the NY-99 relatives. It is likely that these strains were highly virulent in birds causing rapid mortality of the host substantially reducing the chance of further vector-mediated transmission to migratory birds. This argument is supported by the fact that a large number of WNV strains in Connecticut originated from crows and blue jays suggesting that corvid mortality blocked further viral transmission (Anderson et al., 2001). It is also possible that these strains were not efficiently transmitted by mosquitoes to other birds. Alternatively, these strains may have spread to limited geographical areas, but remained undetected due to the overwhelming presence of the NY-99 derived strains.

The derived phylogenetic tree suggests that the LSU-AR01 strain could be derived from the Connecticut strain migrating to Louisiana in a similar pattern to the NY-99 strain. There are two major migratory flyways that overlap the state of LA (Gubler, 2007) and the location of these flyways is believed to be responsible for the abrupt appearance of WNV in FL, LA and TX in 2001. Alternatively, the newer Connecticut strains of this subclade could be derived from Louisiana strains during overwintering in Louisiana and subsequent reverse migration to the East. The LSU-AR01 strain may be closely-related to WNV strains, which caused the 2002 outbreak in Louisiana. Although there are many partial sequences in GenBank for other viral strains, generation of phylogenetic trees based on these partial sequences may lead to false conclusions (Charrel et al., 2003). Ideally, additional full genomes of Louisiana WNV strains are needed to provide more insight of how these viruses were spread and established in different geographic regions of the USA including Louisiana.

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CHAPTER 3

RECOMBINANT VESICULAR STOMATITIS VIRUS-BASED WEST NILE VACCINE ELICITS STRONG HUMORAL AND CELLULAR IMMUNE RESPONSES AND PROTECTS MICE AGAINST LETHAL CHALLENGE WITH THE VIRULENT WEST NILE VIRUS STRAIN LSU-AR01

INTRODUCTION

West Nile Virus (WNV)

West Nile virus (WNV) was first isolated more than seventy years ago from a febrile patient in the West Nile province of Uganda (Smithburn et al., 1940). WNV is a positive-sense RNA virus belonging to genus *Flavivirus* in the Falviviridae family (Lindenbach, Thiel, and Rice, 2007). The lipid-bilayer membrane of the nascent virus contains 180 molecules of the envelope (E) and premembrane (preM) proteins organized into 60 asymmetric trimeric spikes of preM-E heterodimers (Zhang et al., 2003; Zhang et al., 2004). The E glycoprotein is the major antigenic determinant and is involved in virus binding and fusion (Ledizet et al., 2007). WNV spread rapidly in North America after its initial introduction in New York (Lanciotti et al., 1999). WNV was transmitted via mosquito vectors and caused substantial morbidity and mortality in birds, horses and other animals including humans. Humans constitute a dead-end host because the virus does not efficiently replicate in humans. WNV can be transmitted by the intrauterine route (Monath et al., 2002), through breast milk (CDC, 2002b; Hayes and O'Leary, 2004), blood transfusion (CDC, 2002a; CDC, 2004b; Mukhopadhyay et al., 2003), bone-marrow transplant (Hiatt et al., 2003), organ transplantation (CDC, 2002c; Iwamoto et al., 2003) and through kidney dialysis (Cairoli, 2005; CDC, 2004a).

The human incubation period for West Nile is 2-14 days (Gea-Banacloche et al., 2004). WNV-infected persons may exhibit a variety of clinical symptoms including fever, headache,

muscle weakness, fatigue, nausea, vomiting, gastrointestinal manifestations, lymphadenopathy and non-pruritic maculopapular skin rash (Davis et al., 2006; Del Giudice et al., 2005; Ferguson et al., 2005). Additional non-neurological clinical manifestations include rhabdomyolysis (Jeha et al., 2003; Kulstad and Wichter, 2003), pancreatitis (Perelman and Stern, 1974), hepatitis (Sampson et al., 2000), myosistis, orchitis (Smith et al., 2004), chorioretinitis (Khairallah et al., 2004) and cardiac dysrythmias (Hayes et al., 2005). Typically, less than 1% of patients suffer from West Nile neuroinvasive disease (WND) including West Nile meningitis (WNM), encephalitis (WNE) and acute flaccid paralysis (poliomyelitis- like syndrome, WNP) (Sejvar, 2007). Among WND cases, an estimated 55-60% of the patients had WNE resulting in an estimated 20% case fatality. Additionally, 10-50% of mortalities in humans could be attributed to WNP (Sejvar, 2007).

WNV Vaccines

The absence of effective treatment against WNV infection has encouraged vaccine development. A variety of different approaches have been employed to produce WNV vaccines including inactivated virus, subunit and DNA-based vaccines. Most of these vaccines appeared to be highly immunogenic, and in some cases protected against WNV-infection in experimental animals (Dauphin and Zientara, 2007). Recently, recombinant viruses expressing WNV antigens have been shown to induce strong immune responses and protection against WNV challenge in animals. Specifically, a recombinant live canarypox-vectored vaccine expressing the preM protein and the E glycoprotein induced strong immune responses in horses and cats (Grosenbaugh et al., 2004; Karaca et al., 2005; Minke et al., 2004; Siger et al., 2004), that appeared to be partially protective (Siger et al., 2006). Other viral-vectored vaccines that elicited protective immune responses in mice include a lentivirus vector based vaccine (TRIP/sE_{WNV})

(Iglesias et al., 2006), and a measles virus-vectored vaccine (Despres et al., 2005). Recombinant yellow fever virus (YFV) has also been used to express WNV preM and E proteins based on the extensive safety record of the YFV attenuated vaccine (Monath, 2001a; Monath, 2001b). A YFV recombinant vaccine (ChimeriVax^{TM}) has shown good immune responses in hamster, mice, non-human primates and humans (Arroyo et al., 2004; Monath et al., 2006; Tesh et al., 2002). A Phase II clinical trial with ChimeriVax^{TM}-WNV is currently underway (Hall and Khromykh, 2007).

Vesicular Stomatitis-vectored Vaccines

VSV is an enveloped, negative strand RNA virus belonging to the Rhabdoviridae family. Natural VSV infections of humans is rare causing at most mild flu- like illness (Lichty et al., 2004). VSV infectious viruses can be efficiently recovered by a reverse genetic approach that utilizes multiple plasmids expressing VSV genes. This methodology has enabled the rapid construction of recombinant VSV viruses expressing a variety of viral and bacterial antigens for vaccine purposes including influenza virus, bovine diarrhea virus, cotton-tail papillomavirus, human immunodeficiency virus, simian immunodeficiency virus, respiratory syncytial virus, hepatitis C, measles virus, Ebola virus, Lassa fever virus, Marburg virus, severe acute respiratory syndrome virus (SARS), and herpes simplex type-2 virus (Brandsma et al., 2007a; Brandsma et al., 2007b; Daddario-DiCaprio et al., 2006a; Daddario-DiCaprio et al., 2006b; Ezelle, Markovic, and Barber, 2002; Garbutt et al., 2004; Geisbert et al., 2005; Grigera et al., 2000; Haglund et al., 2000; Johnson, Rodgers, and Rose, 1998; Johnson et al., 1997; Jones et al., 2005; Kahn et al., 2001; Kapadia et al., 2005; Natuk et al., 2006; Roberts et al., 1999; Roberts et al., 1998; Schlereth et al., 2003; Schwartz et al., 2007). Recombinant VSVs have been also constructed

and tested as vaccines for bacterial pathogens including *Mycobacterium tuberculosis* and *Yersinia pestis* (Palin et al., 2007; Xing and Lichty, 2006). VSV-vectored vaccines have been administered via intranasal, intramuscular and subcutaneous routes and have been shown to elicit robust mucosal and systemic humoral and cellular immune responses (Brandsma et al., 2007a; Brandsma et al., 2007b; Daddario-DiCaprio et al., 2006a; Daddario-DiCaprio et al., 2006b; Egan et al., 2004; Ezelle, Markovic, and Barber, 2002; Garbutt et al., 2004; Geisbert et al., 2005; Grigera et al., 2000; Haglund et al., 2000; Johnson, Rodgers, and Rose, 1998; Johnson et al., 1997; Jones et al., 2005; Kahn et al., 2001; Kapadia et al., 2005; Natuk et al., 2006; Ramsburg et al., 2004; Roberts et al., 1999; Roberts et al., 1998; Rose et al., 2001; Schlereth et al., 2003; Schwartz et al., 2007).

We constructed recombinant VSVs expressing the WNV E glycoprotein. A prime-boost approach was employed utilizing two different recombinant VSVs expressing either the Indiana or the Chandipura G glycoproteins for priming and boosting immunizations, respectively. Intranasal immunization of mice conferred high protection against lethal challenge with the virulent WNV strain WNV LSU-AR01 (Iyer et al., 2008). Neuronal necrosis was observed in mock-vaccinated but not in vaccinated mice. These results suggest that VSV recombinant vaccines expressing the WNV E glycoprotein may be efficacious intranasal vaccines for animal and human use.

MATERIALS AND METHODS

Cells and Plasmids

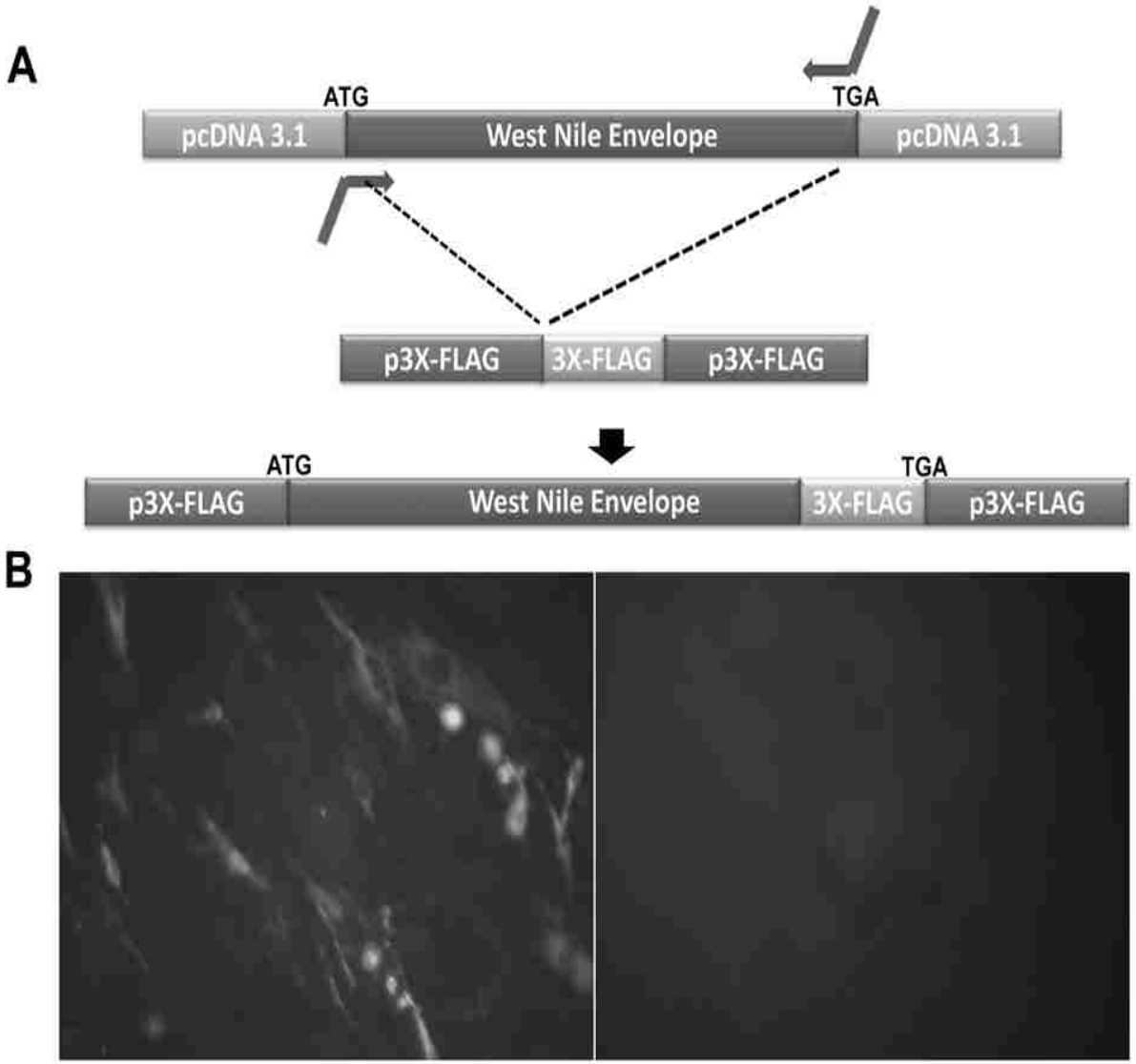
Baby hamster kidney cells (BHK-21) were obtained from the American Tissue Culture Collection (ATCC). These cells were grown using Dulbecco's modified minimal essential

media (DMEM) supplemented with 10% fetal bovine serum (FBS) and appropriate amounts of antibiotics. The West Nile virus envelope (E) gene was obtained by first producing a cDNA of the E gene from the WNV LSU-AR01 strain, and subsequently cloning this gene into the pcDNA3.1 plasmid (Invitrogen, Inc.) after PCR amplification. The E gene was further amplified by PCR from this plasmid using primers that introduced unique NotI and BamHI sites at the 5′ and 3′ (Table 3.1), and cloned into plasmid p3XFLAG-CMV-14 (Sigma) placing the FLAG epitope coding sequence downstream and in-frame with the E glycoprotein sequence (Figure 3.1: A). All recombinant plasmids were confirmed by restriction endonuclease digestion and DNA sequencing.

Transient Expression of the WNV E Gene

BHK-21 cells were transfected with the WNV E-p3XFLAG plasmid using Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. E glycoprotein was detected at 48 hours post transfection using anti-FLAG (Sigma) and anti-West Nile rabbit polyclonal antibody (Abcam). For IFA, cells were washed twice with phosphate buffered saline (PBS) and fixed with ice cold methanol. Cells were then washed with PBS and wells were blocked with 2% BSA and 5% goat serum in TBS (Tris-buffered saline) for one hour. Mouse anti-FLAG antibodies (Sigma) in blocking buffer and rabbit anti-WNV antibodies were added to respective wells at a 1:500 dilution and incubated for one hour at room temperature. Cells were then washed six times with TBS and the secondary antibody; Alexa Fluor® 488 goat anti-mouse IgG and goat anti-rabbit IgG (Invitrogen) was added to the respective wells at the same dilution. Cells were incubated in dark for one hour. Finally, cells were washed six times with TBS and observed under a fluorescence microscope.

Figure 3.1: Cloning and transient expression of the WNE-3XFLAG gene construct in BHK21 cells. (A) Insertion of the 3XFLAG coding sequence in-frame and at the 3′ terminus of the WNV E gene. (B) BHK 21 cells were transfected with the WNV E-3XFLAG expression plasmid (left panel) or mock-transfected (right panel). Transient expression of the WNV E gene was assayed using anti-FLAG antibodies.


A
ATG
TGA
pcDNA 3.1
West Nile Envelope
pcDNA 3.1
p3X-FLAG
3X-FLAG
p3X-FLAG
ATG
TGA
p3X-FLAG
West Nile Envelope
3X-FLAG
p3X-FLAG
B

Construction of Recombinant VSVs Expressing the WNV E Gene

Plasmid clones that efficiently expressed the WNV E gene were used as the template for PCR amplification of the gene, while at the same time introducing unique XhoI and NheI sites at the 5′ and 3′ ends (Table 3.1) of the gene fragment. This DNA fragment was cloned into the pVSV-XN2-IN and pVSV-XN2-CH transfer vectors. Cells were infected with recombinant vaccinia virus expressing T7 polymerase (vTF7-3) at a multiplicity of infection (MOI) of 10 for one hour. Subsequently, BHK 21 cells were co-transfected with pBS-N, pBS-P, pBS-L and pVSV-XN2 containing the WNV E gene and recombinant virus was recovered as described in detail previously (Schnell et al., 1996a; Schnell et al., 1996b). Control viruses having no exogenous inserted genes were also produced using pBS-N, pBS-P, pBS-L and the pVSV-XN2 (empty vector). Anti-FLAG and anti-WNV-E antibodies were used to detect expression of the E glycoprotein by immunofluorescence assay (IFA) in VSV-infected BHK cells. Viral isolates expressing high amounts of the WNV E glycoprotein were selected through multiple rounds of plaque purification. Viral titers were determined and stocks were stored at -80 °C for vaccination studies.

Table 3.1: List of Primers for rVSV Construction Expressing WNV E

Primer Name	Primer Sequence[a]
5′ WNE-FLAG-Not-I	5′-GACGAC*GCGGCCGC*ATGTTTAACTGCCTTGGAATGAGC-3′
3′ WNE-FLAG-BamHI	5′-GCAGCA*GGATCC*AGCGTGCACGTTCACGGAGAGG-3′
5′-XN2-Xho-I	5′-CCGCGG*CTCGAG*ATGTTTAACTGCCTTGGAATGAGC-3′
3′-XN2-Nhe-I	5′-GACGAC*GCTAGC*GGATCACTACTTGTCATCGTC-3′

[a] Enzyme restriction sites are italicized.

Vaccination Study

All animal studies were carried out after the appropriate approvals were obtained from

the LSU Institutional Animal Care and Use Committee (IACUC) and BSL3 Biosafety Committee. Four groups of ten four week-old female Balb/c mice (Harlan, IN, USA) were used in this study. Each individual mouse was identified with an ear tag (National Band and Tag Company, KY, USA). **Group I (vaccine group):** These animals were mildly anesthetized by inhalation of 2-3% isoflurane and 10 µl dose of vaccine containing 10^5 PFU of the vaccine (rVSV-IN-WNV E) was administered intranasally using a 10 µl pipette (5 µl per nostril). Animals were boosted with the rVSV-CH-WNV E at 21 days post vaccination using the same technique. One mouse from this group was not included in the fluorescence activated cell sorting (FACS) analysis due to sample preparation problems (n=9). **Group II (control for vaccine group):** Control group animals were vaccinated in the same way as described above with the exception that they were inoculated with 10 µl of uninfected cell culture supernatant. These animals were boosted at 21 days post vaccination with uninfected cell culture supernatant. Animals belonging to groups I and II were humanely euthanized at 14 days post boost. Spleens were collected in Eppendorf tubes containing RPMI and processed by flow cytometry for intracellular cytokines and cell surface markers associate with memory T cells, regulatory T cells and cytotoxic T cells among others. For serology, animals were bled by the sub-mandibular route (cheek bleed) using Golden-Rod lancets (Medipoint, NY). Animals were bled on 21 days post vaccination and 14 days post boost. Blood was collected in Becton Dickinson microtainers with serum separators (Becton Dickinson).

Challenge Studies: Group III (Challenge Group) and Group IV (Challenge Group Control)

These 20 animals were treated exactly in the same way as groups I and II until the boost stage. At 10 days post-boost, these animals were transported to the animal biosafety level-3 (ABSL-3) facility for acclimatization. Blood was collected at 14 days post boost (before

challenge). Animals were challenged intraperitonially with 10^5 PFU of WNV-LSU-AR01 and observed 2-3 times a day for 18 days. Animals showing severe neurological symptoms (like ataxia and hunching posture) were humanely euthanized and dead animals were surgically processed immediately (thoracic and abdominal cavities opened up and placed in 10% formalin jars) for pathological studies.

Plaque Reduction Neutralization Test ($PRNT_{90}$)

Serum samples were inactivated by incubation at 65°C for 30 minutes. Serial two-fold dilutions of the serum were incubated with equal volumes of 50 PFU WNV LSU-AR01 at 37°C for one hour. Serum-virus mixtures were then added to Vero cell monolayers in 12-well plates in triplicates and the plates were incubated for another hour. Plates were then overlaid with Dulbecco's modified minimum essential media (DMEM) containing 1% methyl cellulose and 2% fetal bovine serum. Plates were incubated at 37°C for 72 hours and then fixed with 10% formalin in phosphate buffered saline (PBS). Plates were washed three times with PBS and stained with 0.01% crystal violet. Plaques were counted and the highest dilution of serum resulting in reduction of 90% of the plaques was noted.

Polychromatic Flow Cytometric Staining and Analysis

Mouse splenocytes were adjusted to 10^7 cells/ml. One-hundred µl aliquots of splenocyte suspension was incubated with appropriately diluted concentrations of antibodies for 30 minutes at room temperature. Cells were washed once with PBS and fixed with 1X BD stabilizing fixative buffer (BD Biosciences) in distilled water. Cells were kept protected from light at 4°C and flow cytometric acquisition was completed within 24 hours of staining. Polychromatic (7 parameters) flow cytometric acquisition was performed on a LSR II Becton Dickinson instrument having three lasers (488nm blue laser, 633nm red laser and 407 violet laser) by using

FITC, PE-Texas red, APC, APC-Cy7 and Pacific Blue as the available fluorochrome parameters. Single-stained controls for each fluorochrome were used for setting flow cytometry compensation. Monoclonal antibodies including CD127 FITC (A7R34, ebiosciences), CD62L PE-Texas Red (MEL-14, Invitrogen), CD25 APC (3C7, BD Biosciences), CD4 APC-Cy7 (GK1.5, BD Biosciences) and CD8a Pacific Blue (53-6.7, BD Biosciences) were used. At-least 50,000 events were collected by gating on $CD4^+$ T cells and those data were analyzed using FlowJo software (TreeStar Inc.) version 8.7.1.

To test $CD4^+$ or $CD8^+$ T lymphocytes subsets for IFNγ production, intracellular cytokine flow cytometry (CFC) assay was employed in response to each WNV peptide pool stimulation as described previously (Pahar et al., 2007). Briefly, processed splenocytes were resuspended at $1x10^6$ cells/ml in complete RPMI-10 with 10% FCS, and stimulated with 2 different WNV peptide pools at a final concentration of 1µg/ml of each peptide pool. Peptide pools (15-19mers with 10-11 amino acids overlap) derived from the WNV E glycoprotein were based on the WNV-NY99 E amino acid sequence (NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH). The 67 peptide array was divided to generate two peptide pools. Peptide pool 1 (pp1) was made of peptides 1-34 and peptide pool 2 (pp2) was composed of peptides 35-67. For positive controls, PMA (50ng/ml, Sigma) and ionomycin (1µg/ml, Sigma) were used. Negative controls had no antigen or mitogen stimulation. Brefeldin A (10µg/ml, Sigma) was added to cultures after the first hour, in a 6 hour incubation period. Following stimulation, cells were stained for cell surface markers with directly conjugated mAbs to CD69 FITC (H1.2F3, BD Biosciences), CD62L PE-TR, CD4 APC-Cy7 and CD8a pacific blue for 30 minutes at room temperature and washed with dPBS/BSA wash buffer. Cells were then fixed and permeabilized by using Cytofix/Cytoperm (BD Biosciences), washed twice in

Perm Buffer (BD Biosciences), and stained with intracellular mAbs. IFNγ PE (XMG1.2, BD Biosciences) and/or CD154 APC (MR1, eBiosciences) were added to cells and incubated at room temperature for 30 minutes. Single color and isotype-matched control antibodies were used to confirm staining specificity. After washing, cells were resuspended in 1% paraformaldehyde in PBS and stored in the dark at 4°C. Data were acquired within 24 hours of staining using a LSR II instrument (BD Immunocytometry System) and FACSDiva software (BD Immunocytometry System). For each sample, 50,000 events were collected by gating on $CD4^+$ T cells. Data analysis was performed using FlowJo software. Gated $CD4^+$ and $CD8^+$ T cells were further analyzed for its cytokine production. Positive cytokine responses were determined based on the percentage of cytokine responses obtained above background responses (unstimulated medium control) in each experiment.

Histopathology

Tissues (brain, lung, liver, bilateral kidneys, heart, spleen, skull, and vertebra) were collected from the mice euthanized or after death and fixed by immersion in 10% neutral buffered formalin. The skull and vertebra were decalcified in 10% formic acid for three days. All sampled tissues were routinely processed into paraffin, and 3 to 4 micrometer sections were cut for hematoxylin and eosin staining (H&E). H&E sections of the nasal olfactory epithelium and bulb in the skull and four sections of the spinal cord including two consecutive anterior cervico-thoracic and two consecutive lumbar-sacral posterior sections in the vertebrae were examined under the light microscope.

RESULTS

Cloning and Transient Expression of the WNV E Glycoprotein

The WNV LSU-AR01 strain was isolated from a dead blue jay (*Cyanocitta cristata*) in Louisiana in 2001. Recently, the entire genome of this strain was sequenced and phylogenetically compared to 75 full WNV genomes deposited in GeneBank (Iyer et al., 2008). The E gene was amplified from viral RNA using specific primers as described in Materials and Methods and cloned into plasmid p3XFLAG (Sigma) placing the entire open reading frame of WNV E in-frame with the 3XFLAG coding sequence resulting in the addition of the 3XFLAG amino acid sequence immediately after the last carboxyl terminal amino acid of the E glycoprotein (Figure 3.1: A). The p3XFLAG-E plasmid was transfected into baby hamster kidney cells (BHK-21) and E glycoprotein expression was detected at 48 hours post transfection using anti-FLAG monoclonal antibody. The anti-FLAG antibody detected E glycoprotein expression in 3XFLAG-E transfected BHK cells, while mock-transfected BHK cells failed to react with the anti-FLAG antibody (Figure 3.1: B).

Construction of Recombinant Vesicular Stomatitis Virus (VSV) Expressing the WNV E Glycoprotein

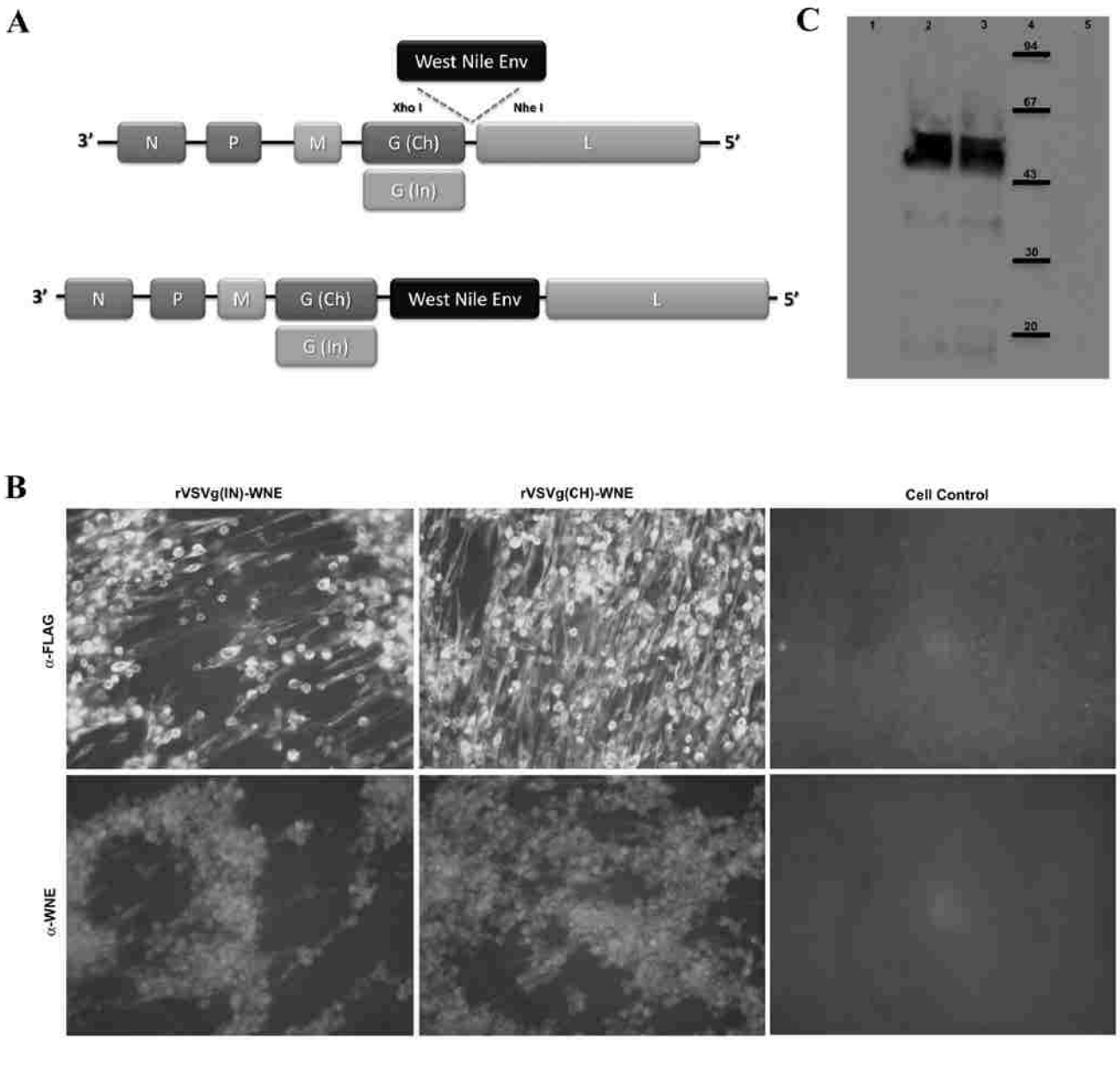
To construct recombinant VSVs expressing the E glycoprotein, the E gene was amplified with primers engineered to have unique XhoI and NheI restriction sites at the E 5′ and 3′ termini, respectively. The amplified E gene (with the 3XFLAG coding sequence) was cloned within the unique XhoI and NheI restriction sites of plasmids pVSV-XN2-IN and pVSV-XN2-CH containing the Indiana and Chandipura G glycoprotein gene, respectively (Figure 3.2: A). Recombinant VSV was recovered after co-transfection of pVSV-XN2-E with three other plasmids encoding the VSV polymerase subunits (P and L), and the nucleocapsid (N), purified

by filtration and extensively plaque-purified. The appropriate insertion of the WNV gene within the VSV genomes was confirmed by direct DNA sequencing of viral RNA after RT-PCR amplification of specific cDNA regions. WNV E expression was readily detected by indirect immunofluorescence assay (IFA) using anti-FLAG monoclonal antibody in recombinant VSV-infected BHK cells, while WNV E was not detected in mock-infected BHK cells (Figure 3.2: B). Cell lysates from BHK-21 cells infected with recombinant VSVs expressing the WNV E glycoprotein were tested for E glycoprotein expression in western immunoblots. Anti-FLAG antibody readily detected major protein species with apparent molecular masses of approximately 53-55 kDa, respectively in agreement with previous reports (Figure 3.2: C) (Davis et al., 2001; Minke et al., 2004).

Mouse Immunization and Challenge Schedule

Four groups of 4-week-old Balb/c mice (Harlan, IN, USA) were used for the vaccine-challenge experiments. All four groups of mice were vaccinated by intranasal administration of 10^5 PFU of pVSV-XN2-IN-E recombinant virus at day 0 and boosted with pVSV-XN2-CH-E (10^5 PFU) 21 days post vaccination (Figure 3.3: A). Mice in groups I and II were processed for immunological analyses (see Materials and Methods), while groups III and IV were challenged with 10^5 PFU of WNV LSU-AR01 administered intraperitoneally. Mice in the challenge groups were observed for 18 days for clinical signs including ruffled fur, ataxia, hunching posture, lethargy and mortality. VSV-E vaccinated and boosted animals exhibited 90% survival, while only 10% of the mock-vaccinated animals survived WNV LSU-AR01 challenge (P=0.004) (Figure 3.3: B). Vaccinated animals appeared to have mild clinical signs post challenge including mild fur ruffling, but recovered quickly to a full healthy status. In contrast, mock-vaccinated animals exhibited severe clinical signs post challenge including high degree of fur

Figure 3.2: Construction of rVSVs expressing the LSU-AR01 E glycoprotein. (A) The WNV LSU-AR01 E-FLAG fusion gene was cloned into the unique XhoI and NheI sites in pVSV-XN2. (B) IFA showing rVSV expression of WNV envelope glycoprotein after infection of BHK-21 cells. Expression of the WNV E glycoprotein was assayed using anti-FLAG and anti-West Nile antibodies. (C) Expression of the WNV E protein was with an apparent molecular mass of 53-55 kDa on a western immunoblot using anti-FLAG antibodies. Lane 1 is cell control, lanes 2 and 3 are rVSV-IN-WNE and rVSV-CH-WNE respectively, lane 4 is the molecular mass ladder and lane 5 is cell lysate from VSV-infected BHK-21 cells (control).


A
West Nile Env
Xho I
Nhe I
3'
N
P
M
G (Ch)
L
5'
G (In)
3'
N
P
M
G (Ch)
West Nile Env
L
5'
G (In)
C
1
2
3
4
5
94
67
43
30
20
B
rVSVg(IN)-WNE
rVSVg(CH)-WNE
Cell Control
α-FLAG
α-WNE

ruffling**,** ataxia, lethargy and eventually death. Post-mortem histopathological examination revealed that none of the vaccinated mice showed any central nervous system (CNS) pathology as compared to mock-vaccinated animals, which exhibited severe neuronal necrosis and lymphoplasmacytic perivascular cuffing (Figure 3.4: A, B). The single mouse in the vaccinated group that died at 12 days post challenge had suppurative rhinitis which may be suggestive of bacterial infection. Mild suppurative inflammation was also observed in the visceral pleura and subpleura of three mock-vaccinated mice that died before 11 days post challenge (not shown). There were no significant histopathological abnormalities within other tissues examined.

Induction of Strong Neutralizing Antibody Correlates with Protection

The ability of mouse sera to neutralize WNV-LSU-AR01 strain was tested in a standard plaque reduction neutralization test ($PRNT_{90}$). Vaccinated animals developed strong neutralizing antibody responses against the LSU-AR01 at 21 days after primary vaccination. Specifically, 9 of 10 mice developed $PRNT_{90}$ titers of 1:32 and one mouse had a titer of 1:64. Neutralizing antibody titers increased at 14 days post boost vaccination. Specifically, 9 of 10 mice had a $PRNT_{90}$ titer of 1:64, while the remaining mouse had a titer of 1:128.

CD154 expression in $CD4^+$ T cells is intimately involved in the polyclonal activation of immature B cells (Brines and Klaus, 1993). Therefore, we compared the expression of CD154 in both vaccinated and mock-vaccinated mice after *in vitro* stimulation with PMA/ionomycin followed by FACS analysis (see Materials and Methods). These experiments revealed the presence of a significantly higher population of $CD4^+CD154^+IFN\gamma^+$ T cells in vaccinated mice compared to mock-vaccinated mice (mean value = 1.73% versus 1.0% in vaccinated and mock-vaccinated mice respectively, $P<0.0001$, Figure 3.5: A, C), as also indicated by the observed

Figure 3.3: Vaccination and animal challenge (A) Schematic of the time line followed for vaccination, boost-vaccination and challenge with WNV-AR01. Mice in groups I and II were not challenged and were used for immunological evaluations. Mice in groups III and IV were challenged with WNV LSU-AR01 at 14 days post boost. (B) Kaplan-Meier Survival curves. Mice in challenge groups were challenged intraperitoneally with 10^5 PFU of WNV LSU-AR01 14 days post boost vaccination and observed for 18 days. Ninety percent of the WNV vaccinated animals survived, while 90% of the mock-vaccinated animals died. A statistically significant difference was observed between the WNV and mock-vaccinated groups (P=0.0004) using the Gehan-Breslow-Wilcoxin test. The Graphpad prism software version 5.01 was used to generate survival curves and statistical analysis. The one animal that died in the vaccinated group exhibited suppurative rhinitis of non-viral origin as revealed by histopathological examination (not shown).

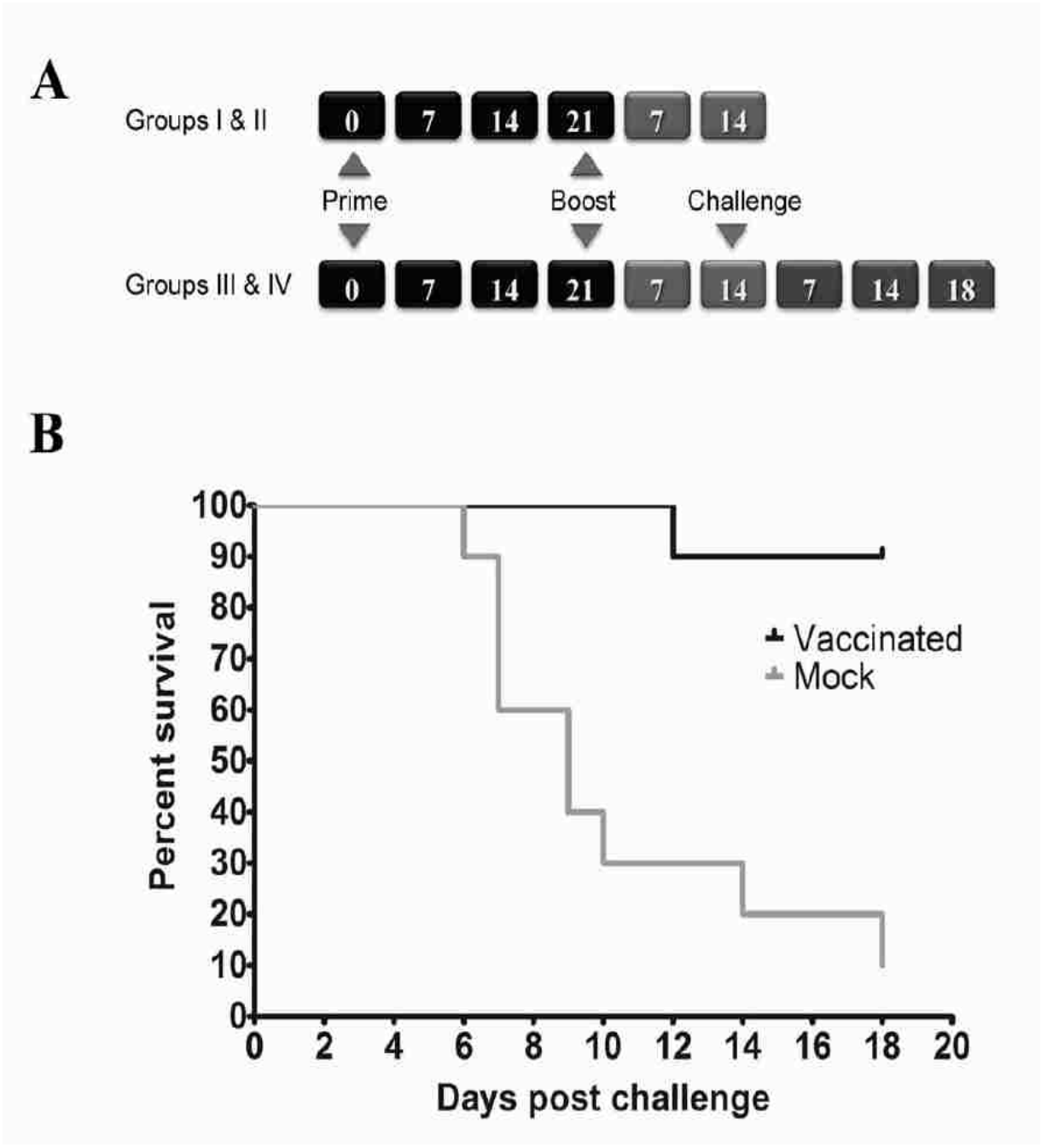
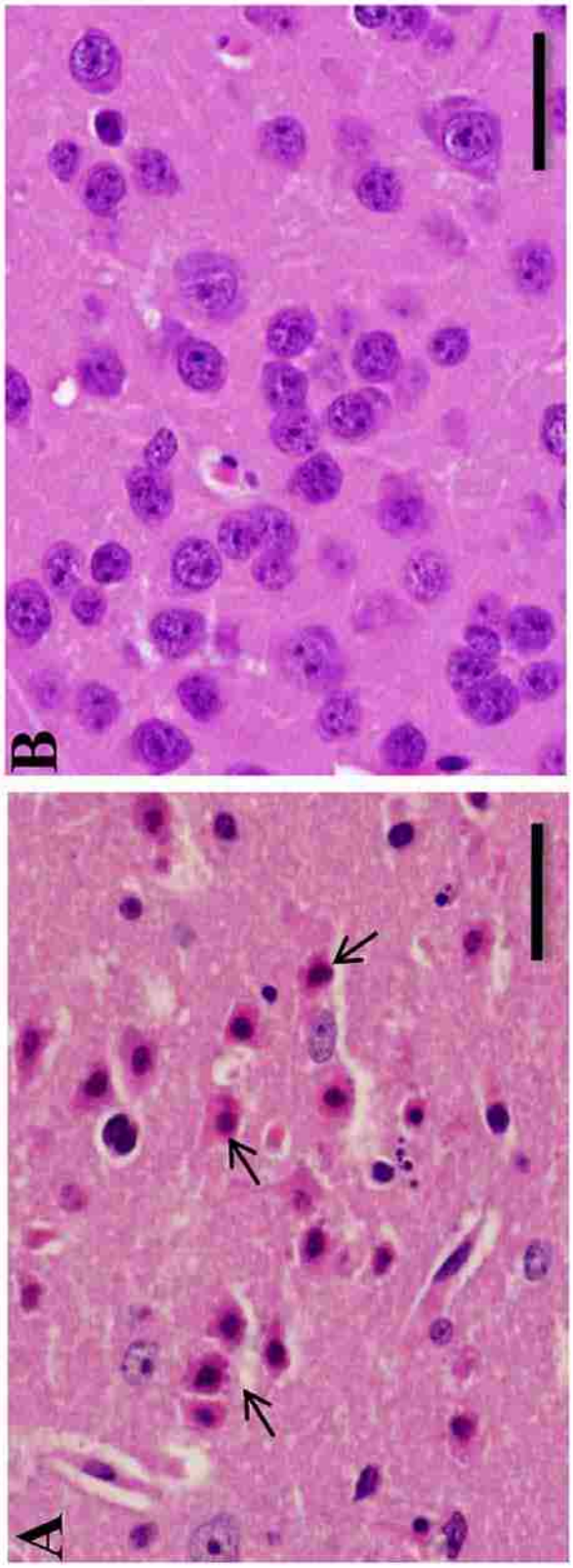

A
Groups I & II
0
7
14
21
7
14
Prime
Boost
Challenge
Groups III & IV
0
7
14
21
7
14
7
14
18
B
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Days post challenge
Vaccinated
Mock

Figure 3.4: Histopathology of cerebrum sections from mock-vaccinated and vaccinated mice after WNV challenge. (A) Mock-vaccinated group: Cerebral cortex showing large numbers of necrotic neurons (arrows), characterized by angular and shrunken cell bodies containing pyknotic nucleus and densely eosinophilic cytoplasm. (B) WNV vaccinated group: Cerebral cortex showing normal neurons. H&E stain, Bar = 50 μm.


B
A

differences in their mean fluorescence intensities (Figure 3.5: B, D).

Antigen-Specific Cellular Immune Responses

Antigen-specific cytokine responses were determined in all vaccinated and mock vaccinated mice. Specifically, WNV-E specific T cell responses were measured using cytokine flow cytometry (CFC) to determine IFNγ responses. Overall, 7 of 9 vaccinated mice had detectable IFNγ responses (ranged from 0.07-0.80%) in splenic $CD8^+$ T cells (Figure 3.6: A). $CD4^+$ T cell positive IFNγ responses were absent in any of the vaccinated mice. Both peptide pools 1(E amino acids 291-554) and 2 (E amino acids 544-791) appeared to contain T cell epitopes, however, peptide pool 1 contained dominant T cell epitopes. (Figure 3.6: B). One of the 9 mice developed antigen-specific IFNγ responses against both the WNV-E peptide pools. None of the mock-vaccinated mice had any detectable IFNγ responses above background levels.

Profiles of CD62L and CD69 Expression

CD62L is a lymphocyte homing marker that is generally associated with extravasation of activated T cells to peripheral sites of inflammation. Generally, increased percentages of $CD8^+$ T cells were present in the vaccinated mice compared to the mock-vaccinated mice (mean=18.3% and 15.3% for vaccinated and mock-vaccinated mice respectively, P=0.01) (Figure 3.7A). $CD8^+$ T cell subsets in all vaccinated mice had lower CD62L expression compared to mock-vaccinated mice (P=0.0003) (Figure 3.7: B). To further characterize the cells responsible for inducing cytokine responses, antigen-specific cytokine positive cells were determined. A significant population (0.73%) of the IFNγ positive cells was memory cells ($CD8^+ CD62L^-$) (Figure 3.7: C).

CD69 is an early activation marker indicative of the presence of antigen-specific stimulation

Figure 3.5: Correlates of T cell-mediated induction of humoral immune responses: (A) Representative contour plot showing increased percentage of $CD4^+CD154^+IFN\gamma^+$ T cells in vaccinated mice compared to mock-vaccinated mice after 6 hours of *in vitro* PMA/ION stimulation. (B) Histogram showing increased mean fluorescence intensity (MFI) of $CD4^+CD154^+IFN\gamma^+$ T cells in a vaccinated mouse compared to a mock-vaccinated mouse. (C) Increased MFI in $CD4^+CD154^+IFN\gamma^+$ T cells was observed in WNV mice compared to mock-vaccinated animals (P=0.01). (D) The increased MFI percentage of $CD4^+CD154^+IFN\gamma^+$ T cells suggests that activated $CD4^+$ T cells stimulated B cells with the help of co-stimulatory signals inducing humoral immune responses. A statistically significant difference was observed in the percentage of $CD4^+CD154^+IFN\gamma^+$ T cells between vaccinated and mock-vaccinated animals (P<0.001).

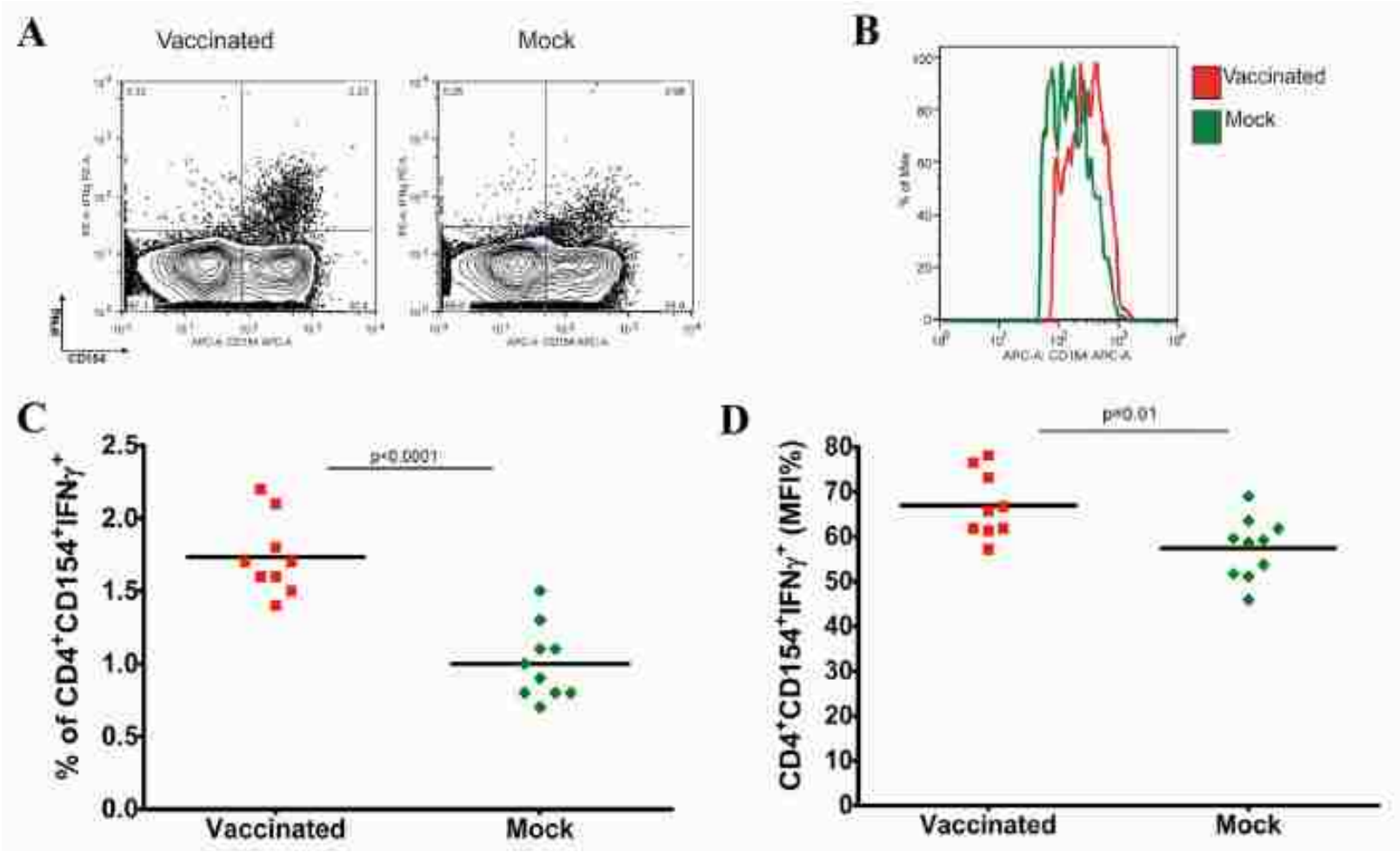
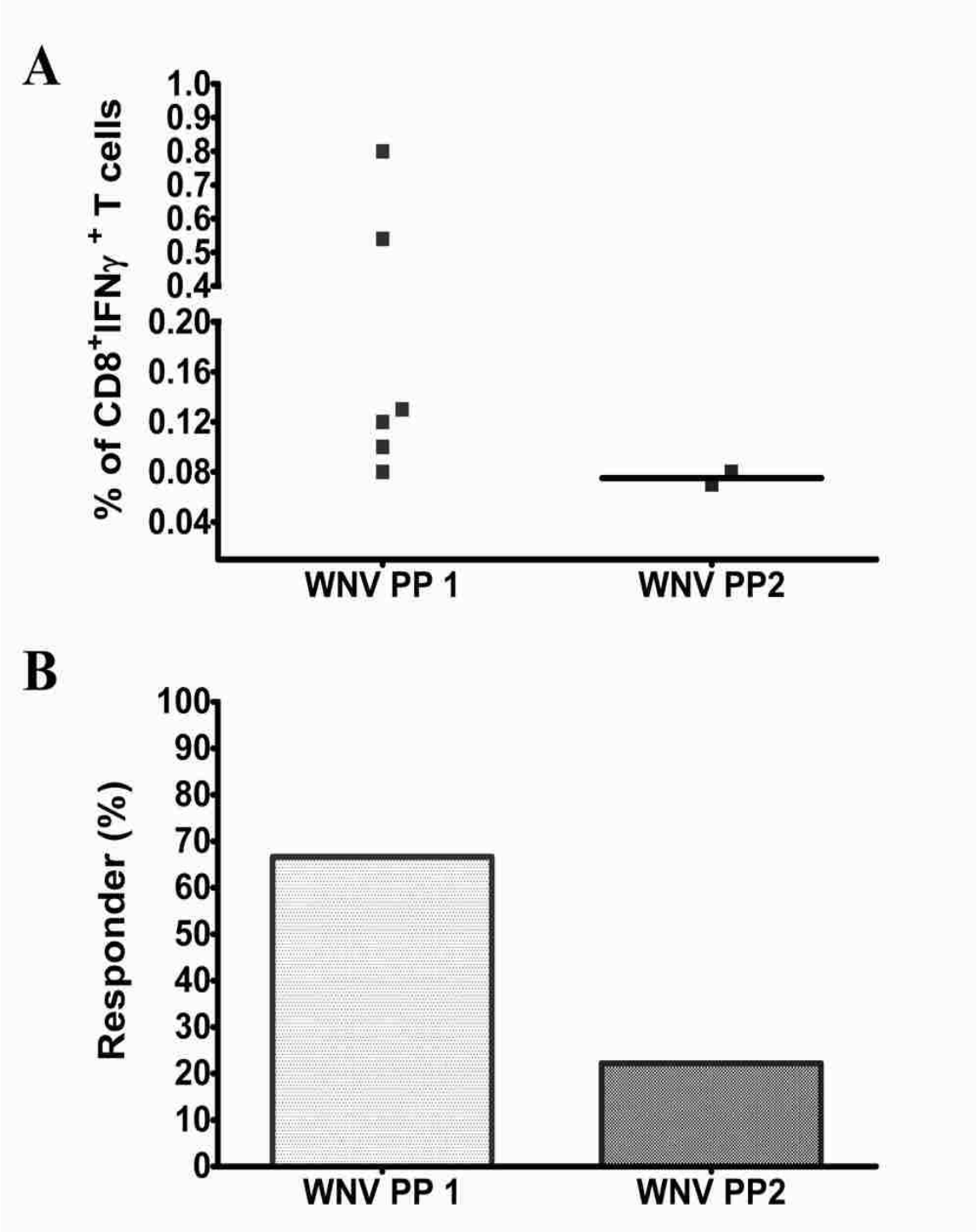

A
Vaccinated
Mock
CD154
B
Vaccinated
Mock
C
p<0.0001
% of CD4+CD154+IFNγ+
Vaccinated
Mock
D
p=0.01
CD4+CD154+IFNγ+ (MFI%)
Vaccinated
Mock

Figure 3.6: Cytokine responses to WNV E overlapping peptide pools in vaccinated animals. (A) Percentage of $CD8^+IFN\gamma^+$ T cells in WNV vaccinated animals with peptide pools 1 and 2, respectively, after 6 hours of *in vitro* stimulation. (B) Percentage of vaccinated mice responding to peptide pool 1 and 2 stimulation. Positive $CD8^+IFN\gamma^+$ T cell responses were detected in 7 out of 9 mice. Criteria for positive cutoff values was established at > 0.06% as compared to the negative control. The data shown on panels A and B were obtained after subtracting the negative control values obtained from antigen-specific responses.


A
% of CD8+IFNγ+ T cells
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.20
0.16
0.12
0.08
0.04
WNV PP 1
WNV PP2
B
Responder (%)
100
90
80
70
60
50
40
30
20
10
0
WNV PP 1
WNV PP2

of mature T cells (Sancho, Gomez, and Sanchez-Madrid, 2005). CD69 upregulation of activated $CD8^+$ T cells was detected in all the vaccinated mice following antigen stimulation compared to mock-vaccinated mice (mean=1.8% versus 0.8% in vaccinated and mock-vaccinated mice, *P*=0.012) indicating E-specific stimulation of mature T cells in vaccinated animals (Figure 3.7:D).

Profile of T reg Activation in Vaccinated Versus Mock-vaccinated Mice

Initial determination of $CD4^+$T cell percentages in splenocytes revealed no significant differences between vaccinated and mock-vaccinated mice (Figure 3.8: A). CD127, the α-chain of the IL7 receptor, in combination with CD25, the α-chain of the IL2 receptor, were used to define the relative abundance of T reg cells within the population of conventional T cells (Seddiki et al., 2006). Analysis of $CD4^+$ $CD25^+CD127^{low}$ cells revealed that vaccinated mice had a significantly lower population of these cells (mean 6.3%) in comparison to the mock-vaccinated mice (mean 7.3%) ($p<0.05$) (Figure 3. 8: B, C).

DISCUSSION

VSV-vectored vaccines have shown exceptional promise for protecting animals and humans against different viral and bacterial pathogens. A VSV-vectored vaccine expressing the WNV-E glycoprotein was constructed and found to efficiently protect mice after intranasal administration against lethal WNV challenge. The salient features of this vaccine study are: 1) A prime-boost intranasal vaccination approach with recombinant VSVs expressing the WNV E glycoprotein produced robust $CD8^+IFN\gamma^+$ T cell responses; 2) This vaccine approach produced strong neutralizing titers against the WNV; 3) Vaccinated mice were protected against lethal challenge and they were free of neuronal necrosis, while unvaccinated mice exhibited severe neuronal necrosis and inflammation in the brain. These results suggest that a prime-boost VSV-vectored

Figure 3.7: Induction of WNV E-specific CD8+ T cells: (A) A higher percentage of $CD8^+$ T cells was present in vaccinated animals compared to mock-vaccinated controls (*P*=0.01). (B) Down regulation of CD62L expression in $CD8^+$ T cells in vaccinated mice. A statistically significant difference was observed in the $CD8^+CD62L^+$ T cell populations (*P*=0.0003) in the WNV vaccinated versus the mock-vaccinated animals. (C) Representative contour plots showing WNV E-specific $CD8^+$ T cells. The majority of the IFNγ producing cells were $CD8^+CD62L^-$ T cells (black circle) indicating the presence of activated effector T cells. (D) The percentage of $CD8^+CD69^+$ T cell population was increased (*P*=0.012) in vaccinated mice versus mock-vaccinated mice indicating the presence of WNV E-specific stimulation of T cells.

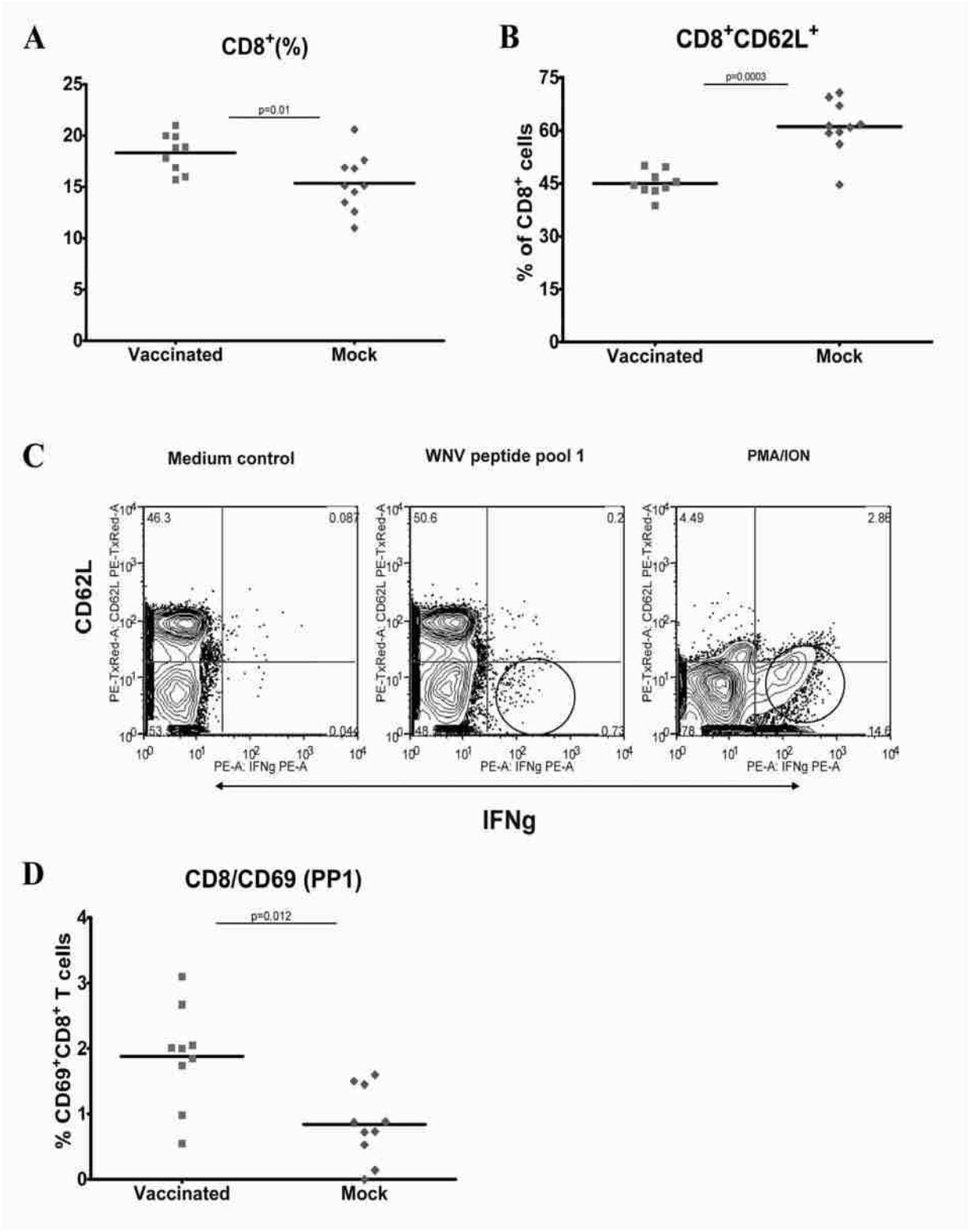
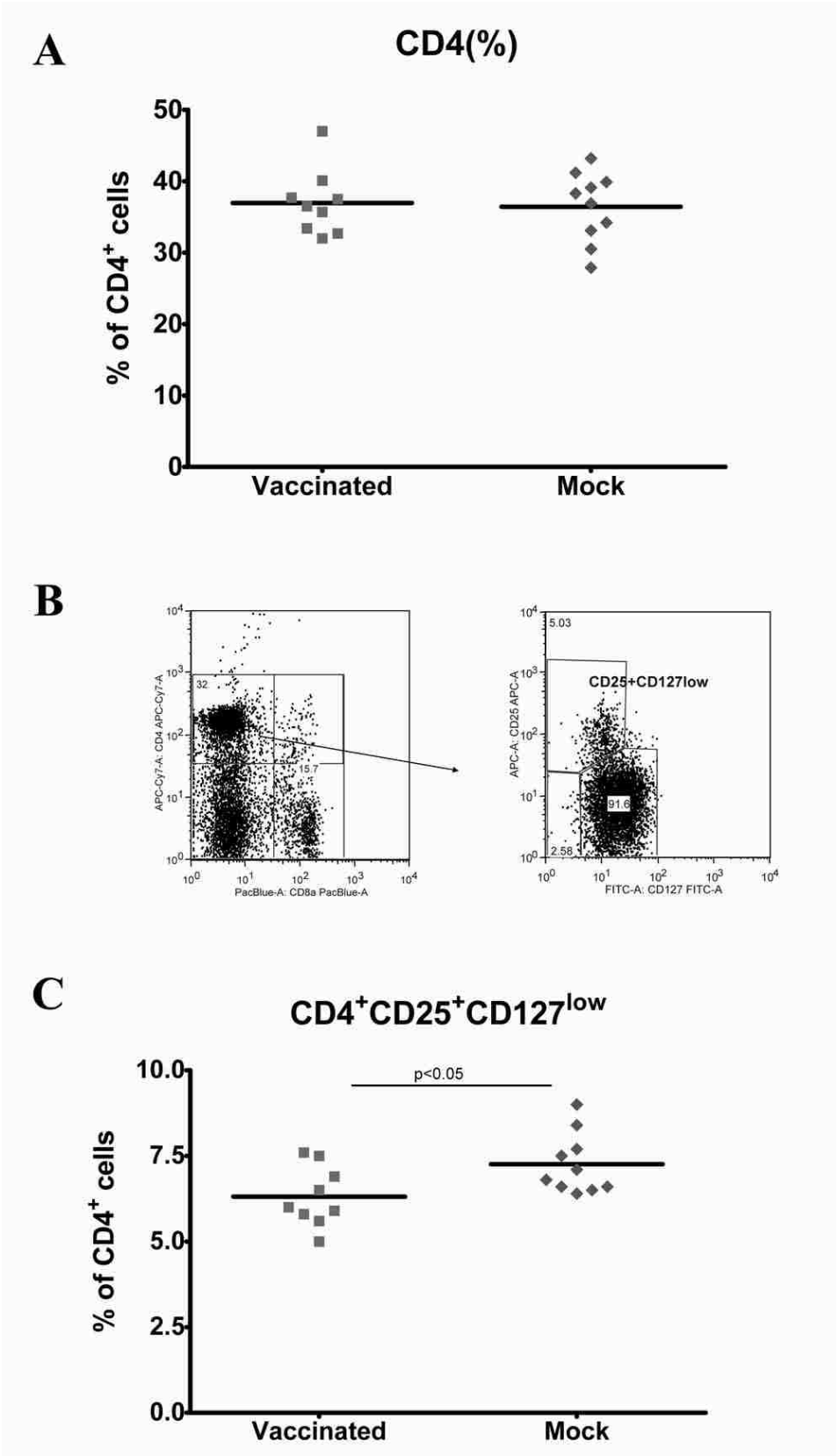

A
CD8+(%)
p=0.01
25
20
15
10
5
0
Vaccinated
Mock
B
CD8+CD62L+
p=0.0003
% of CD8+ cells
75
60
45
30
15
0
Vaccinated
Mock
C
Medium control
WNV peptide pool 1
PMA/ION
CD62L
PE-TxRed-A: CD62L PE-TxRed-A
PE-A: IFNg PE-A
46.3
0.087
53
0.044
50.6
0.2
48
0.73
4.49
2.8
78
14.6
IFNg
D
CD8/CD69 (PP1)
p=0.012
% CD69+CD8+ T cells
4
3
2
1
0
Vaccinated
Mock

Figure 3.8: Role of regulatory T cells in vaccinated animals: (A) Percentage of $CD4^+$ T cells in WNV vaccinated and mock-vaccinated animals. There was no statistically significant difference observed between these two groups. (B) Representative dot plots showing the gating strategy for T reg cells derived from spleenocytes. $CD4^+$ T cells were first gated and plotted for CD25 and CD127. $CD4^+$ $CD25^+CD127^{low}$ T cells were defined as T regs. (C) Percentage of $CD4^+CD25^+CD127^{low}$ T reg cells. A statistically significant difference ($P<0.05$) was observed between WNV-vaccinated and mock-vaccinated mice.


A
CD4(%)
% of CD4+ cells
50
40
30
20
10
0
Vaccinated
Mock
B
APC-Cy7-A: CD4 APC-Cy7-A
PacBlue-A: CD8a PacBlue-A
32
15.7
APC-A: CD25 APC-A
FITC-A: CD127 FITC-A
5.03
CD25+CD127low
91.6
2.58
C
CD4+CD25+CD127low
p<0.05
% of CD4+ cells
10.0
7.5
5.0
2.5
0.0
Vaccinated
Mock

intranasal vaccine approach induces strong humoral and cellular immune responses that protect mice against WNV-induced neuronal necrosis.

Mucosal surfaces constitute the natural route of VSV infections. VSV is primarily a veterinary viral pathogen that infects cattle, horses, sheep and other animals. VSV infects animals via transmucosal and transcutaneous routes (OIE. Office international des épizooties (Paris), 2004). VSV may also be transmitted through sandflies, blackflies and mosquitoes (Clarke et al., 2006; Lichty et al., 2004). The VSV G glycoprotein is a potent immunogen and also serves important functions in virus-entry and virus-induced cell fusion (Roche et al., 2007). Recombinant VSVs expressing a variety of viral and bacterial antigens have been constructed. Vaccine studies with these recombinant VSVs have showed that intranasal and intramuscular administration of the rVSVs were safe and efficient in inducing protective humoral and cellular immune responses against a variety of pathogens (Clarke et al., 2006). Of particular interest is the ability of the VSV-vector system to elicit strong humoral and cellular immune responses via the intranasal route (Grigera et al., 2000; Kahn et al., 2001; Kapadia et al., 2005; Natuk et al., 2006; Palin et al., 2007; Reuter et al., 2002) that can be substantially easier to administer than intramuscularly injected vaccines. In these vaccine studies, although the "empty" VSV vector elicited robust humoral and cellular immune responses against VSV, these responses did not contribute to protection against a variety of pathogens indicating that specific immune responses against the expressed transgene were primarily responsible for protection (Cooper et al., 2008; Geisbert et al., 2005; Kahn et al., 2001; Kapadia et al., 2005; Natuk et al., 2006; Publicover, Ramsburg, and Rose, 2005).

We constructed rVSVs that expressed the WNV-E glycoprotein and either the VSV Indiana G glycoprotein, or the Chandipura vesiculovirus G glycoprotein. This pair of rVSVs

was used in a prime-boost vaccination approach to maximize humoral immune responses against the WNV-E glycoprotein expressed by both viruses, while minimizing the anamnestic immune response against the VSV vector targeted predominantly against the G glycoprotein. This is largely accomplished because the Chandipura G and the VSV-Indiana G glycoproteins are approximately 60% different in their amino acid sequences (Rose et al., 2000). Recombinant VSVs are known to non-specifically incorporate certain other viral and cellular glycoproteins into their virions without adversely affecting viral infectivity (Schnell et al., 1996a). The insertion of the foreign E gene into the VSV genome did not adversely affect viral replication and infectivity, because rVSV containing the E gene replicated to similar titers with those of the VSV control virus that did not have a foreign gene inserted within their genomes (not shown). Moreover, rVSV-E isolates were stable, since multiple serial passages of virus stocks in BHK cells did not affect E glycoprotein expression and genomic stability (not shown). Although it is unclear whether the WNV E glycoprotein is inserted into VSV envelopes, these results suggested that rVSV-E were stable retaining wild-type levels of viral replication and infectivity. Recombinant VSV-E expressed WNV-E glycoprotein to high levels in BHK cells and the expressed E glycoprotein appeared to be fully glycosylated as evidenced by the apparent molecular mass of approximately 53-55 kDa in SDS-PAGE in agreement with published reports (Davis et al., 2001; Minke et al., 2004).

Based on the known strong immune responses generated by VSV, especially when administered via the intranasal route, we devised an experimental vaccine protocol to vaccinate mice through the intranasal route using a prime-boost strategy. This prime-boost vaccination approach resulted in 90% (9 of 10) of the mice surviving lethal challenge with the WNV LSU-AR01 virulent strain. The single mouse from the vaccinated group of mice that died late in the

experiment (12 days post challenge) appeared to die from WNV-unrelated causes, since histopathological examination showed severe suppurative rhinitis but no histological abnormality in the brain. Therefore, the rVSV-E prime boost vaccination protocol was highly efficacious in protecting mice against WNV infection.

Primary WNV infection is thought to result in local replication of the virus in peripheral organs and viremia that ultimately results in virus invading the CNS. WNV mortality is thought to be largely caused by replication of the virus in the CNS tissues of animals and the resultant immunopathological damage of CNS tissues. Accordingly, unvaccinated mice showed obvious clinical signs of neurological disease such as ataxia, hunching posture, lethargy and hindlimb paralysis. Histopathological examination of brain tissues showed neuronal necrosis, perivascular cuffing, and microgliosis. In contrast, only a few vaccinated mice developed mild clinical signs such as mild ruffled fur, but recovered quickly. Importantly, none of the vaccinated mice exhibited any neuronal necrosis.

The interaction of CD40 on B cells with CD154 (CD40L) on $CD4^+$ T cells results in T cell mediated activation of B cells resulting in immunoglobulin class switching, somatic hypermutation and proliferation (Grewal et al., 1996; Kawabe et al., 1994; O'Keefe, Nguyen, and Benveniste, 2002). Accordingly, $CD4^+$ $CD154^+$ $IFN\gamma^+$ T cells were upregulated in vaccinated but not control mice indicating generation of T-cell mediated B cell activation. The specificity of this response is not discernable, since it may be due to either or both VSV and WNV antigens. However, strong neutralizing antibody titers were also produced against WNV indicating the induction of E-specific humoral immune responses. This result is in agreement with previous reports showing that other VSV-vectored vaccines induced strong humoral immune responses

against different VSV-expressed antigens. Specifically, recombinant VSVs expressing either the respiratory syncytial virus F glycoprotein (Kahn et al., 2001), or rVSV expressing the severe acute respiratory syndrome (SARS) corona virus (SARS-CoV) produced high antibody titers against the F glycoprotein and SARC-CoV spike (S) glycoprotein, while strong immune responses against the VSV virus was noted (Kapadia et al., 2005).

The WNV E glycoprotein contains multiple predicted and experimentally verified cytotoxic T cell (CTL) epitopes (Brien, Uhrlaub, and Nikolich-Zugich, 2007; De Groot et al., 2001; McMurtrey et al., 2008; Purtha et al., 2007). The availability of a library of overlapping peptides derived from the WNV E glycoprotein allowed the elucidation of antigen-specific cellular immune responses. Peptide pool 1 composed of the first 34 peptides averaging 12-18 amino acids each generated stronger cellular $CD8^{+}IFN\gamma^{+}$ T cell responses in *in vitro* proliferation assays, in comparison to peptide pool 2, which represented the carboxyl terminus-half of the WNV E glycoprotein. Peptide pool 1 contains the experimentally verified CTL epitope RSYCYLAT (E 347-354) while peptide pool 2 contains the CTL epitope IALTFLAV (E771-778), both of which have been shown to confer protection against lethal WNV-challenge in mice (Brien, Uhrlaub, and Nikolich-Zugich, 2007; Purtha et al., 2007). *In vitro* stimulation of lymphocytes from vaccinated mice revealed the presence of antigen-specific IFNγ responses specifically in $CD8^{+}CD62L^{low}$ T cells. CD62L (L-selectin) mediates adhesion of resting lymphocytes to peripheral lymph nodes. Typically, high expression of CD62L ($CD62L^{hi}$) reveals entrapment of lymphocytes within lymph nodes, while low CD62L ($CD62L^{low}$) cell-surface expression (the result of T cell activation) is indicative of lymphocyte extravasation to sites of inflammation (Waters et al., 2003). Splenocytes from vaccinated mice had significantly lower expression of the CD62L marker on E-specific $IFN\gamma^{+}$ $CD8^{+}$T cells revealing activation and

extravasation of these cells to peripheral sites, potentially involved in killing virus-infected cells prior to transmission to the CNS.

CD69 is an early activation marker that is absent in resting lymphocytes (Sancho, Gomez, and Sanchez-Madrid, 2005). The upregulation of the $CD8^{+}CD69^{+}$ E-specific T cell responses in vaccinated versus mock-vaccinated mice provides additional evidence for the stimulation of T cells. Accordingly, $CD8^{+}CD69^{+}$ E-specific population of T cells was upregulated in vaccinated versus mock-vaccinated mice indicating the generation of activated memory $CD8^{+}$ T cells. It is unclear whether the observed $CD8^{+}$T cell memory responses confer long-term immunity against WNV infection. T regs are known to play important roles in down-regulation of anti-self immune responses (Zhang and Zhao, 2007), and to suppress proliferation and cytokine production of effector T cells (Banham, 2006). Typically, during viral infections, upregulation of humoral and cellular immune responses causes down-regulation of T reg activation. Typically, T-regs express the FoxP3 and CD25 markers. The IL-7 receptor CD127 marker expression is inversely correlated to FoxP3 expression and $CD127^{low}$ $CD25^{+}$ cells have been shown to be positive for FoxP3 (Banham, 2006; Liu et al., 2006). Consequently, the $CD25^{+}CD127^{low}$ population was used to define T regs. As expected, there was a negative correlation between the relative population of T reg cells ($CD4^{+}CD25^{+}CD127^{low}$) and antigen-specific CTL responses in the vaccinated mice. However, the specificity of this immune response cannot be discerned, since it most likely is caused by both VSV and E glycoprotein antigens.

A variety of experimental vaccine approaches have been reported to generate protective humoral and cellular immune responses against flaviviruses and specifically WNV. The relative

role of humoral versus cellular immune responses has been extensively debated in the literature. Certain studies have suggested that a strong humoral immune response evidenced by the production of high titer anti-WNV titers is necessary and sufficient to protect mice from CNS infection, while other reports have argued that a cellular immune response characterized by a robust anti-WNV $CD8^+$ T cell responses is necessary for protecting and clearing brain tissues from WNV (Purtha et al., 2007; Shrestha and Diamond, 2004; Wang et al., 2003). One report has argued that CTL-immune responses may result in exacerbated immunopathology in brain and CNS tissues at infections with low WNV titers (10^3 PFU) (Wang et al., 2003). In our experiments, 10^5 WNV PFU were inoculated intraperitoneally. Vaccinated mice had no evidence of neuronal necrosis suggesting the $CD8^+$T cell responses conferred protection and virus clearance. It is probable that both humoral and cellular immune responses generated against the WNV E glycoprotein prevented the virus from entering CNS, potentially arresting the virus at peripheral sites. Alternatively, if some virus escaped peripheral immune surveillance, it is possible that CTLs cleared the virus from brain tissues before it could cause significant damage and resultant immunopathological manifestations.

In summary, the VSV-E-vectored vaccine appeared to elicit robust humoral and cellular immune responses that efficiently protected mice from WNV lethal challenge. Intranasal vaccination is second only to oral vaccination with regard to the relative ease of administration and patient compliance issues rendering this approach attractive for human use. Recently, single-cycle VSV-vectored vaccines have been shown to generate robust immune responses against a number of viral pathogens including HIV, Ebola, Marburg, Lassa, influenza, avian influenza, hepatitis C and RSV viruses (Buonocore et al., 2002; Daddario-DiCaprio et al., 2006a;

Daddario-DiCaprio et al., 2006b; Garbutt et al., 2004; Geisbert et al., 2005; Jones et al., 2005; Kahn et al., 2001; Publicover, Ramsburg, and Rose, 2005; Roberts et al., 1999; Schwartz et al., 2007). Based on these results, it is expected that single cycle VSV-WNV vaccines would be also efficacious. Additional improvements in attenuating VSV can be made by providing more than one viral protein *in trans* through complementing cells, as well as engineering additional mutations that are known to attenuate VSV.

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CHAPTER 4

CONSTRUCTION OF rVSVs EXPRESSING SIMIAN RETROVIRUS 2 GAG AND ENV GLYCOPROTEIN AND rVSVs EXPRESSING HERPES B VIRUS GLYCOPROTEIN D FOR USE AS VACCINES IN NON-HUMAN PRIMATES.

INTRODUCTION

Type D simian retrovirus and Herpes B virus (Cercopithecine Herpes virus) are important pathogens of non-human primate (NHP) colonies in the United States. These pathogens pose a significant threat to the NHPs and to humans that come in contact with them. Simian retrovirus (SRV) causes an immunosuppressive disease in macaques and is the leading cause of fatalities in Asian macaques colonies in the United States (Gardner et al., 1988). The use of asymptomatic SRV infected animals in biomedical research for AIDS or other vaccine research severely affects the outcomes (Hara et al., 2005). Similarly, accidental transmission of herpes B virus to humans via bites, scratches, infected tissues or body fluids results in a disease that has a 70% fatality rate in humans (Bennett et al., 1999). The urgent need to test NHPs for these diseases and eliminate them would greatly help in developing specific pathogen free (SPF) NHP colonies for biomedical research. Additionally, it also improves safety for researchers and animal handlers.

Simian Retroviruses (SRVs)

Simian acquired immunodeficiency (SAIDS) of macaques is known to be caused by several independent type D retroviruses (betaretroviruses). These viruses predominantly infect Asian macaques. The Mason-Pfizer monkey virus (MPMV) also known as Simian retrovirus 3 (SRV-3) (Sonigo et al., 1986; Thayer et al., 1987) is the prototype of SRV group was shown to cause a non-oncogenic immunosuppressive disease (Bryant et al., 1986; Fine et al., 1972; Fine et al., 1975). SRV-1 isolated from the California National Primate Research Center (CNPRC) and

SRV-2 isolated from the Oregon National Primate Research Center (ONPRC) have been directly linked to SAIDS (Marx et al., 1985; Marx et al., 1984). SRV-4 (cynomolgus macaque isolate) and SRV-5 (rhesus macaque isolate) have been reported or partially sequenced (Hara et al., 2005; Li, Axthelm, and Machida, 2000). The partial sequence for SRV/D-6 isolated from the Hanuman langur has also been reported (Nandi et al., 2000; Nandi et al., 2003). In 2005 a Japanese group at Tsukuba primate center isolated a new SRV (SRV/D-T) from their cynomolgus macaque colony (Hara et al., 2007; Hara et al., 2005). Partial sequences of another isolate, SRV-7 from Indian rhesus macaques in Jaipur, India have also been deposited in GenBank (Nandi et al., 2006).

SRV-3 was found to be more closely related to SRV-1 than SRV-2 (Marx et al., 1985; Thayer et al., 1987). Moreover, the SU domain of SRV-1 and SRV-3 envelope shares 83% identity as compared to 58% identity shared between SRV-2 with SRV-1 and SRV-3 (Brody et al., 1992).

Typically, SAIDS results in an immunosuppressive disease that may at times be fatal. As the disease progresses, NHPs exhibit lymphadenopathy, splenomegaly, anemia, lymphoid depletion, bone-marrow hyperplasia, weight loss, persistent diarrhea, chronic opportunistic infections and malignant neoplasias (Hara et al., 2005; Tsai et al., 1986).

SRV-1 predominantly infects rhesus macaques, while SRV-2 infects cynomolgus and pig-tailed macaques (Lerche and Osborn, 2003). Rare cases of cutaneous fibromatosis have been associated with SRV-1 infections (Marx and Lowenstine, 1987). However, SRV-2 infections were frequently found to be associated with retroperitoneal fibromatosis (RF). RFs are multicentric lesions characterized by vascular fibroproliferative nature in an immunosuppressed

environment. This is reminiscent of AIDS related Kaposi's sarcoma (Giddens et al., 1985). The etiologic agent of RF is a gammaherpes virus which happens to be a macaque homolog of the human Kaposi's sarcoma virus (HHV-8) (Rose et al., 1997).

SRV Vaccines

Experimental infection of rhesus macaques with SRV-1 revealed that certain animals can overcome the disease with no clinical signs indicating the feasibility of developing successful vaccines (Kwang et al., 1987). Indeed, the first vaccine for SRV-1 was a formalin inactivated vaccine that protected rhesus macaques against lethal challenge (Marx et al., 1986). However, another experimental vaccine that used SRV-1 gp70 and gp20 expressed in yeast failed to elicit neutralizing antibodies (Kwang et al., 1988). Recombinant vaccinia virus expressing SRV-2 envelope glycoprotein protected pig-tailed macaques against lethal challenge (Benveniste et al., 1993; Hu et al., 1989). This protection was correlated to development of neutralizing antibodies. In another study, recombinant vaccinia virus expressing SRV-1 or SRV-3 gp70 and gp20 were shown to protect against homologous virus. Although a degree of cross-neutralization was observed among SRV-1 and SRV-3 vaccinated animals, they failed to cross neutralize the more distantly related SRV-2 (Brody et al., 1992).

Herpes B Virus/Cercopithecine Herpes

Herpes B virus is an alphaherpesvirus that is endemic to monkeys belonging to the genus *Macaca* that infects 90-100% of its host in primate breeding colonies (Cohen et al., 2002; Holmes et al., 1995; Weigler et al., 1993; Weigler et al., 1990). In macaques, the virus is asymptomatic causing recurrent oral and genital lesions which clear spontaneously (Weigler, 1992). Accidental transmission to humans via bites, scratches, infected tissues or body fluids

results in a disease that has a 70% fatality rate (Bennett et al., 1999). The first case of human B virus infection occurred in 1932 (Gay and Holden, 1933; Sabin and Wright, 1934). The human Herpes B virus disease is characterized by fatal encephalomyelitis and severe neurological disease (Holmes et al., 1995; Palmer, 1987; Weigler, 1992). The ease of transmission is exemplified by the fact that the virus has been shown to cause infection via mucosal exposure (CDC, 1998) and from person to person (Holmes et al., 1990).

Herpes B Virus Vaccines

Herpes virologists have typically used HSV glycoproteins gB and gD as vaccine candidates. HSV-1 gD has been shown to elicit protective immune reponses against HSV-1 and HSV-2 (Bennett, Harrington, and Kelly, 1992). In addition, recombinant vaccinia virus expressing HSV-1 gD have shown to protect mice from lethal challenge and establishment of latent HSV infections (Blacklaws et al., 1990; Cremer et al., 1985; Rooney et al., 1988).

Several approaches to developing vaccines for B virus have been tested including a formalin-inactivated B virus vaccine (Hull, 1971). This vaccine elicited very low antibody titers and required frequent boosters. A recombinant vaccinia virus expressing B virus glycoprotein D was shown to protect 91% of the vaccinated rabbits upon challenge (Bennett et al., 1999). One study immunized rabbits with B virus glycoproteins gB, gC, gD, gE and gG (Perelygina et al., 2002). This study showed that sera from animals immunized with gB, gC and gD DNA vaccines cross neutralized HSV antigens. But only the gB vaccine elicited B virus neutralizing antibodies. Several researchers have tested DNA vaccines using various B virus glycoprotein gB, and gD (Hirano et al., 2002; Loomis-Huff et al., 2001). B virus gD DNA vaccine elicited both humoral and cellular immune response.

Considering the seriousness of these two diseases especially at the Tulane National Primate Research Center (TNPRC), a study was designed with the goal of developing recombinant vesicular stomatitis virus (rVSV) based vaccines for these SRV-2 and B virus. rVSV vectored vaccine have been successfully used against a plethora of infectious viral diseases (see Chapters 1 and 3). Engineering rVSVs that express SRV-2 gag and Env glycoprotein and the herpes B virus gD glycoprotein could significantly reduce NHP and human fatalities.

MATERIALS AND METHODS

Cloning and Testing of Simian Retrovirus-2 gag Protein, Envelope Glycoprotein Constructs and Herpes B Virus gD in p3XFLAG Vector

SRV-2 (GenBank accession number M16605) gag gene was PCR amplified from the plasmid pSRV2 (provided by C. Apertei, TNPRC). NotI and BamHI sites were introduced at the 5′ and 3′ ends respectively using 5′-GAG-FLAG-NotI and 3′-GAG-FLAG-BamHI primers (Table 4.1). For the full length Env gene 5′-ENV-FLAG-NotI and 3′-ENV-FLAG-BamHI primers were used (Table 4.1). To construct the SRV-2 Env/VSV G fusion, portions of the individual genes were PCR amplified and the fragments were later fused by using PCR overlap extension. Briefly, C-terminus truncated portion of SRV-2 envelope gene was based on the 38 amino acid (aa) cytoplasmic domain of SRV-3 (Brody, Rhee, and Hunter, 1994) and also estimated using SOSUI software (Hirokawa, Boon-Chieng, and Mitaku, 1998; Mitaku and Hirokawa, 1999; Mitaku, Hirokawa, and Tsuji, 2002). The SRV-2 Env region (bases 1-1632) was PCR amplified using 5′-ENV-FLAG-NotI and 3′-SOLENV-FLAG-BamHI while the region encoding the last 29 amino acids of VSV G cytoplasmic tail (bases 1449 to 1533) (Rose and Gallione, 1981; Rose et al., 1980) was PCR amplified using 5′-G-CYT-ENV-JN and 3′-G-CYT-

BamHI. In a final PCR these two fragments were fused by PCR overlap extension technique using 5′-ENV-FLAG-NotI and 3′-XN2-NheI primers. A schematic describing the constructs is shown in Figure 4.1. The constructs encoding the truncated portion of ENV in frame with the 3X FLAG was termed ENVTrunc and the construct that had the VSV-G cytoplasmic tail fusion was termed Env-GCYT.

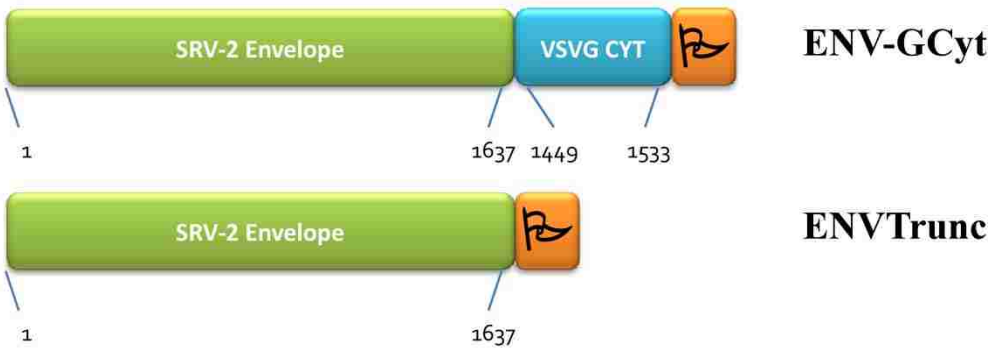


Figure 4.1: SRV-2 Env constructs. Schematic describing engineering the Env-GCYT construct which consist of the extracellular and transmembrane domains of SRV-2 Env glycoprotein fused inframe with the cytoplasmic tail (29 aa) of VSV G and the 3XFLAG epitope. The second schematic deptics the truncated portion of SRV-2 Env fused in frame with the 3XFLAG epitope.

Herpes B virus (GenBank accession number AF533768) glycoprotein D was a C-terminus truncated version encoded by 918 bases (306 aa). This region was PCR amplified so as to introduce NotI and BamHI sites at the 5′ and 3′ end respectively using 5′-MB-gD-FLAG-Not I and 3′-MB-gD-FLAG-Bam HI primers (Table 4.1). The DNA fragment was then transferred to a C-terminal 3X FLAG vector (p3XFLAG-CMV-14), a necessary step prior to the isolation of recombinant VSVs expressing either the Indiana or Chandipura G glycoprotein (Figure 4.2).

Transient Expression of the SRV-2 Full Length ENV and Herpes B Virus gD FLAG Fusion Proteins

The recombinant plasmid clones were confirmed by restriction endonuclease digestion

and sequencing. Transient expression of SRV-2 gag, SRV-2 Env and herpes B virus gD was confirmed after transfection of a BHK 21 cell monolayer. Controls included the use of mock transfections as well as transfection with the vector alone onto BHK 21 cells. Anti-FLAG antibody was also used to detect transient expression of SRV-2 gag, SRV-2 ENV and Herpes B gD. Transient transfections were not done to check for expression of the ENVTrunc or the ENV-GCYT constructs although they were checked and confirmed by DNA sequencing.

Table 4.1: Primer list: List of primers used for PCR amplification and cloning, engineering of gene fusions into p3XFLAG plasmid and pVSV-XN2 for SRV-2 env constructs, gag and herpes B glycoprotein D

Primer Name	Primer Sequence
5′-GAG-FLAG-NotI	5′-ATA TAT GCG GCC GCA TGG GAC AAG AAT TAA GCC AAC-3′
3′-GAG-FLAG-BamHI	5′-ATA TAT GGA TCC ATA CTG TGT GGG TGG TGG AAC AG-3′
5′-ENV-FLAG-NotI	5′ ATA TAT GCG GCC GCA TGT TTT CTT TGC TCA TAG AGA TGA CTG-3′
3′-ENV-FLAG-BAMHI	5′-ATA TAT GGA TCC CGA TAC ACG TAA GTA TAC ACC-3′
5′-G-CYT-ENV-JN	5′-ATT TTC AAT AAG ATC ATA CGA GTT GGT ATC CAT CTT TGC-3′
3′-SOLENV-FLAG-BamHI	5′-ATA TAT GGA TCC ATA GGG GAG GAG TCC ATG TAG ACC AGT-3′
3′-G-CYT-BamHI	5′-ATA TAT GGA TCC CTT TCC AAG TCG GTT CAT CTC-3′
3′-gD-FLAG-BamHI	5′-ATA TAA GGA TCC GTA GGG CGC GCC CCG CCG CGC-3′
5′-gD-FLAG-HindIII	5′-ATA TAT AAG CTT ATG GGG CCCGGC ATC GCC GCG-3′
5′-XN2-GAG-XhoI	5′-ATA TAT CTC GAG ATG GGA CAA GAA TTA AGC CAA CAT GAA C-3′
5′-XN2-ENV-SALI	5′-ATA TAT GTC GAC ATG TTT TCT TTG CTC ATA GAG ATG ACT G-3′
3′-gD-FLAG-BamHI-42	5′-ATA TAA GGA TCC GTA GGG CGC GCC CCG CCG CGC CGG CCC GTG-3′
3′-XN2-NheI	5′-GAC GAC GCT AGC GGA TCA CTA GTC ATC GTC ATC CTT-3′

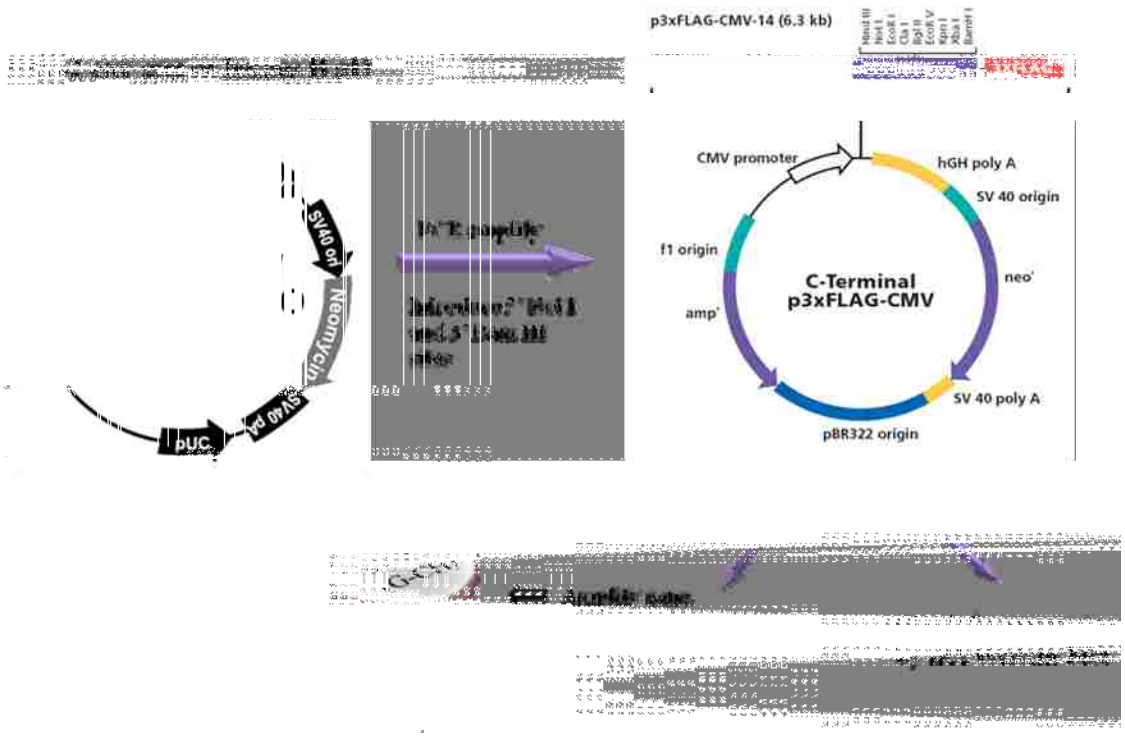


Figure 4.2: Cloning schematic. The gene-of-interest (SRV-2 gag/ env constructs or Herpes B gD) was PCR amplified so as to insert unique restriction sites and then cloned into p3X-FLAG-CMV-14 vector (Sigma). The resultant construct was then tested for expression after transient transfection of BHK-21 cells. Genes with the best expression are then cloned into pVSV-XN2 plasmids expressing the G glycoprotein from VSV Indiana (IN) or Chandipura vesiculovirus, the glycoprotein exchange vectors, to recover rVSVs.

Construction of Recombinant VSVs Expressing the SRV-2 gag, SRV-2 Env Constructs and Herpes B Virus gD Construct

For the SRV-2 gag, the plasmid clone that efficiently expressed the gag gene was used as the template for PCR amplification of the gene, while at the same time introducing unique XhoI and NheI sites at the 5′ and 3′ ends of the gene fragment. 5′-XN2-GAG-XhoI and 3′-XN2-NheI primers (Table 4.1) were used for this process. This DNA fragment was cloned into the pVSV-XN2 Indiana and pVSV-XN2 Chandipura transfer vectors. Similarly, the full length Env, EnvTrunc and the Env-GCYT were PCR amplified using 5′-XN2-ENV-SalI and 3′-XN2-NheI

primers (Table 4.1) before cloning into pVSV-XN2 Indiana and pVSV-XN2 Chandipura transfer vectors.

The plasmid clone that efficiently expressed Herpes B virus gD gene was used as the template for PCR amplification of the gene, while at the same time introducing unique XhoI and NheI sites at the 5′ and 3′ ends of the gene fragment using 5′-MB-gD-XN2-XhoI and 3′-XN2-NheI primers (Table 4.1). This DNA fragment was cloned into the pVSV-XN2 Indiana and pVSV-XN2 Chandipura transfer vectors.

Subsequently, BHK 21 cells were co-transfected with pBS-N, pBS-P, pBS-L and pVSV-XN2 with the insert using standard procedures (Figure 4.3, 4.4). A control using pBS-N, pBS-P, pBS-L and the pVSV-XN2 (empty vector) was also maintained.

Detection of SRV-2 gag, SRV-2 Env constructs and Herpes B virus gD Gene Expression by the Recombinant VSVs

The VSV-SRV-2 gag, VSV-SRV-2-ENVTrunc, VSV-SRV-2 EnvGCYT and Herpes B gD recombinants were tested for expression by infecting BHK 21 cells. Anti-FLAG antibodies were used to detect expression of gag, env constructs and gD by IFA. The best expressing viral isolates for the SRV-2 gag and Env constructs and the Herpes B gD were plaque-purified through multiple rounds. The titers were determined and stocks were stored at -80 °C.

RESULTS

Cloning and Testing of Simian Retrovirus-2 gag Protein, Envelope Glycoprotein Constructs and Herpes B Virus gD in p3XFLAG Vector

A schematic describing the cloning in the p3XFLAG-CMV14 vector is shown in Figure

Figure 4.3: Schematic representation of site of insertion of the gene-of-interest. The gene of interest was inserted into unique Xho I and Nhe I sites in pVSV-XN2.

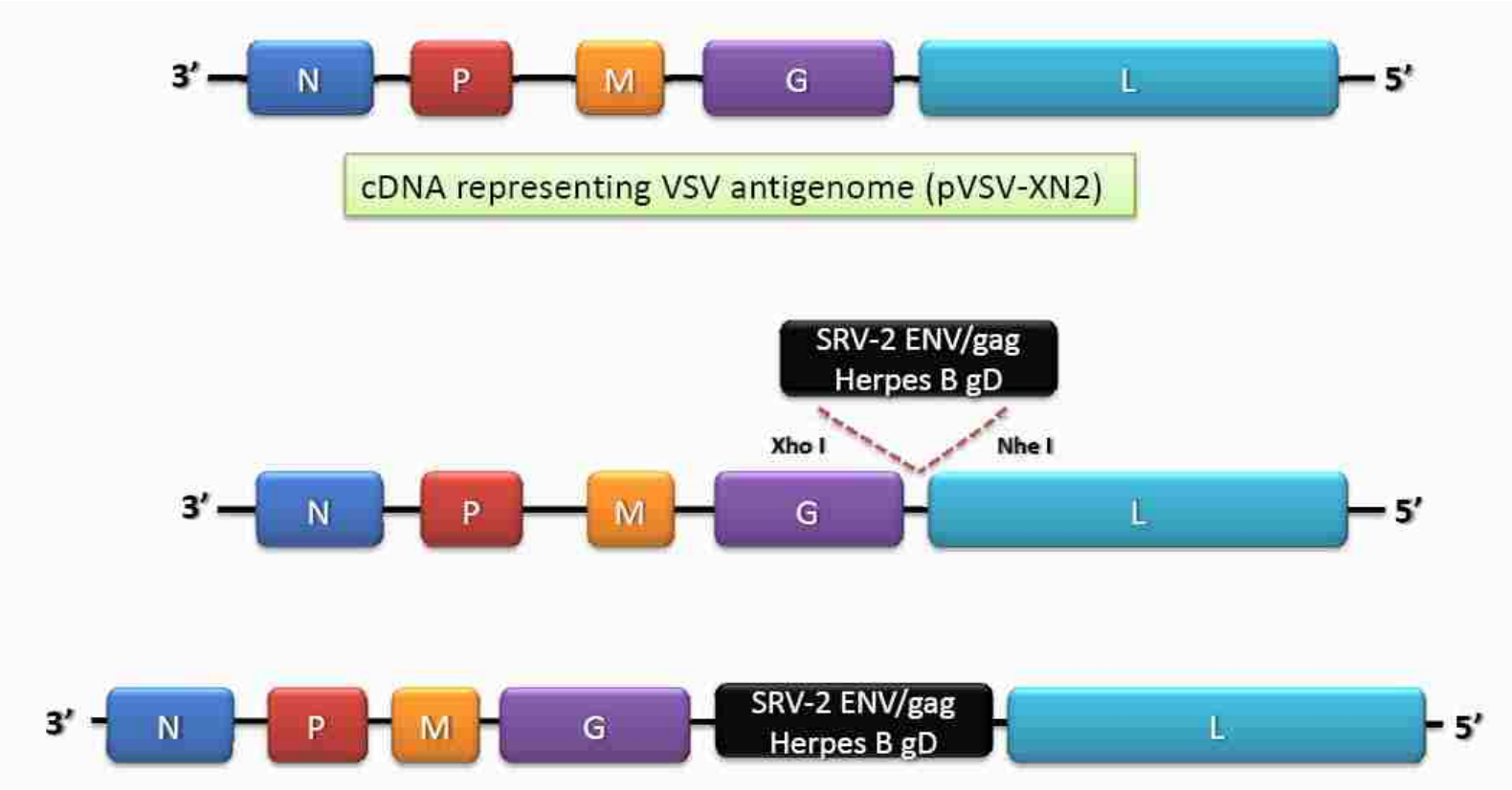
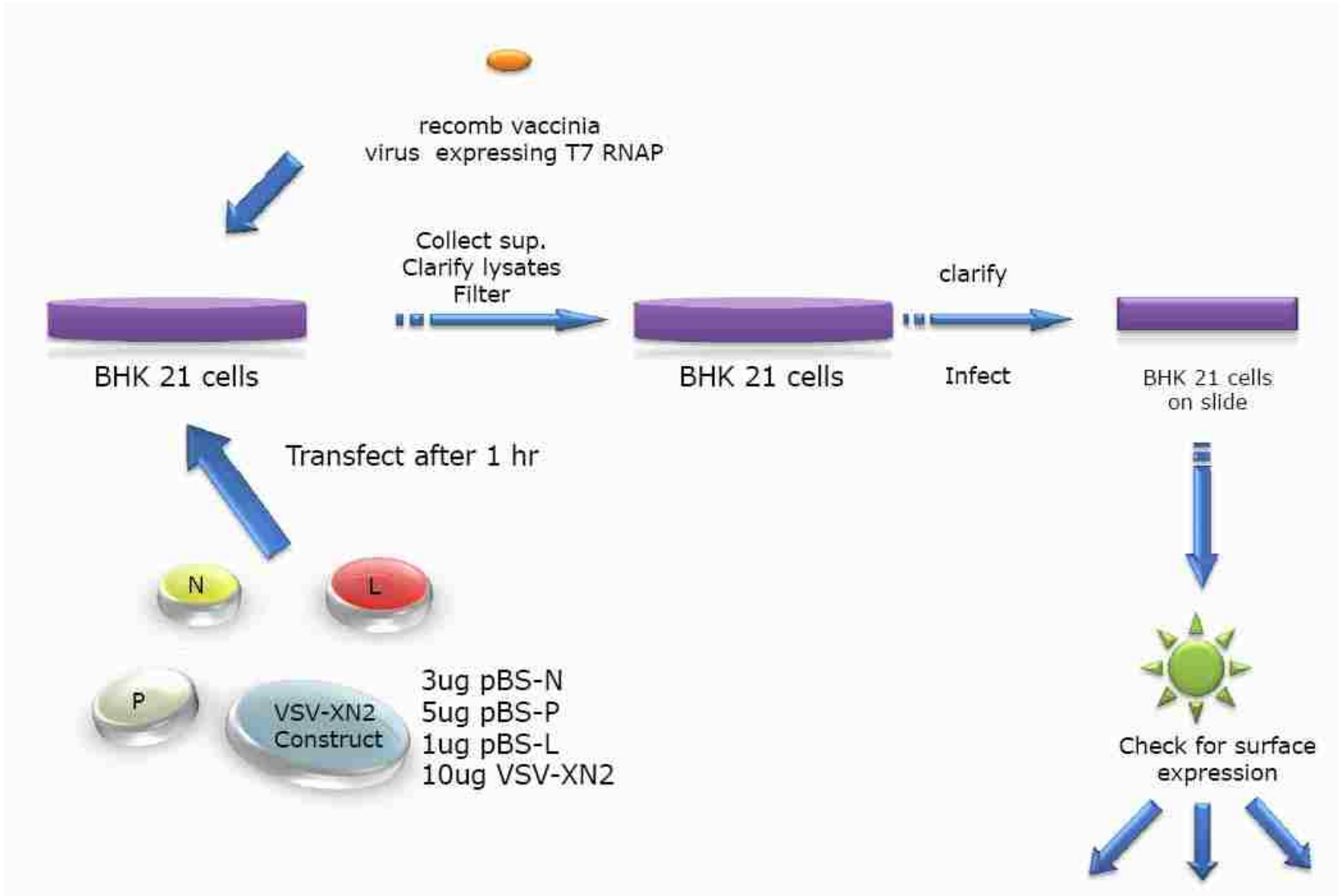

3'
N
P
M
G
L
5'
cDNA representing VSV antigenome (pVSV-XN2)
SRV-2 ENV/gag
Herpes B gD
Xho I
Nhe I
3'
N
P
M
G
L
5'
3'
N
P
M
G
SRV-2 ENV/gag
Herpes B gD
L
5'

Figure 4.4: Generalized scheme for recovery of recombinant VSVs (rVSVs). BHK 21 cells were infected with recombinant vaccina virus expressing T7 polymerase (vTF7-3) so as to facilitate expression of the individual viral genes under the T7 promoter. rVSVs were generated by co-transfecting BHK 21 cells with pVSV-XN2 along with plasmids encoding the VSV nucleoprotein (pBS-N), the phosphoprotein (pBS-P), and the large polymerase subunit (pBS-L) one hour post infection with vTF7-3 using standard procedures. Recombinant VSVs were then harvested by multiple rounds of filtration to eliminate the vaccine virus. The rVSVs were the checked for expression using Western immunoblot and/or IFA and plaque purified.


recomb vaccinia
virus expressing T7 RNAP
Collect sup.
Clarify lysates
Filter
clarify
BHK 21 cells
BHK 21 cells
Infect
BHK 21 cells
on slide
Transfect after 1 hr
N
L
P
VSV-XN2
Construct
3ug pBS-N
5ug pBS-P
1ug pBS-L
10ug VSV-XN2
Check for surface
expression

4.2. DNA Sequencing of the constructs confirmed that the 3X FLAG epitope was in frame with the genes.

Transient Expression of the SRV-2 Full Length ENV and Herpes B Virus gD FLAG Fusion Proteins

Transient expression SRV-2 full length Env, SRV-2 gag and herpes B gD was confirmed by IFA. Additionally, SRV-2 gag (Figure 4.5) and full length Env (Figure 4.5) were visualized by Western immunoblot. SRV-2 gag appeared as an approximately 70Kda molecule and the full length Env showed gp70 and gp20 bands. Similarly, transient expression of gD::FLAG was detected by IFA using anti-FLAG antibodies (Figure 4.6).

Construction of Recombinant VSVs Expressing the SRV-2 gag, SRV-2 Env Constructs and Herpes B Virus gD Construct

All clones were checked by DNA sequencing revealing that the constructs were cloned in frame with the 3X FLAG epitope and between the G and L genes in pVSV-XN2 plasmid. A schematic for recovery of rVSV is shown in Figure 4.4 and has been described in Chapter 3 of this dissertation.

Detection of SRV-2 gag, SRV-2 Env Constructs and Herpes B Virus gD Gene Expression by the Recombinant VSVs

BHK-21 cells were infected with the recombinant viruses and the infected cells were used to check for protein expression using IFA and Western immunoblot. The antibodies readily detected expression of the gag (Figure 4.7, 4.8), env (Figure 4.9) constructs and gD (Figure 4.10, 4.11) in VSV-infected cells, while cell controls and VSV empty vector controls produced no reaction with the antibodies.

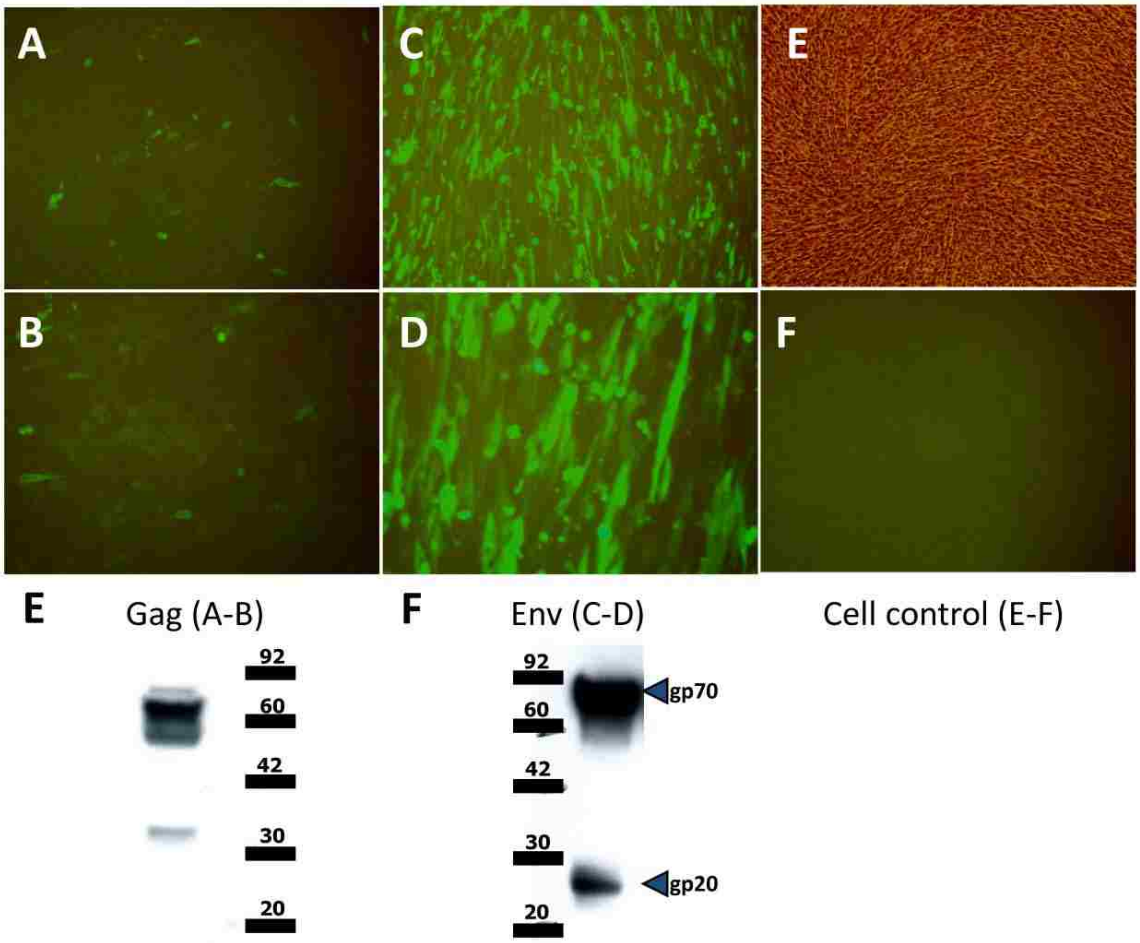


Figure 4.5: IFA and Western blots for transiently expressed SRV-2 gag and Envelope protein. BHK 21 cells were transfected with SRV-2 gag-3X FLAG plasmid (Panel A-B) and SRV-2 Env-3XFLAG plasmid (Panel C-D). Transient expression of gag and Env proteins were detected by using anti-FLAG antibodies. Appropriate cell controls (panel E-F) were maintained.

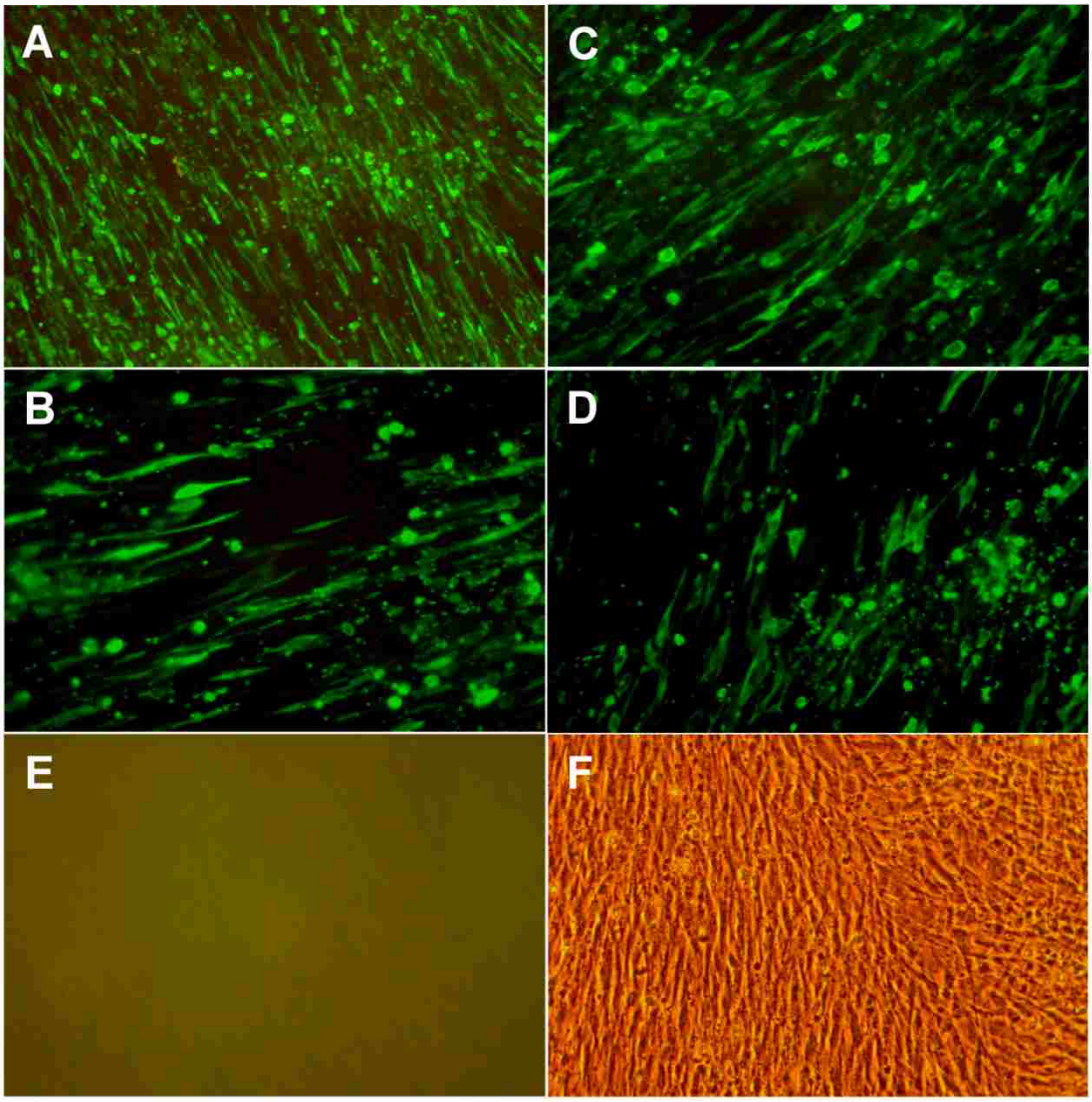


Figure 4.6: IFA and Western blots for transiently expressed Herpes B gD. BHK 21 cells were transfected with Herpes B virus gD::3X FLAG plasmid (Panel A-D). Appropriate cell controls (panel E-F). Transient expression was detected by using anti-FLAG antibodies.

Figure 4.7: Plaque purified rVSV expressing SRV-2 gag protein. BHK 21 cells were infected with rVSV glycoprotein exchange vaccine vectors expressing SRV-2 gag protein. Protein expression was assayed using anti-FLAG antibodies. Appropriate cell and empty virus vector controls were maintained. Panels A-C are clones of the rVSV-gag-IN priming vector, panels CD-E are clones of the rVSV-gag-CH boosting vector, pane; D is the empty vector rVSV without any insert and panels H and I are cell controls under UV and visible light.

rVSV-gag-IN
rVSV-gag-CH
Empty vector
A
B
C
D
E
F
G
H
I
CC
CC

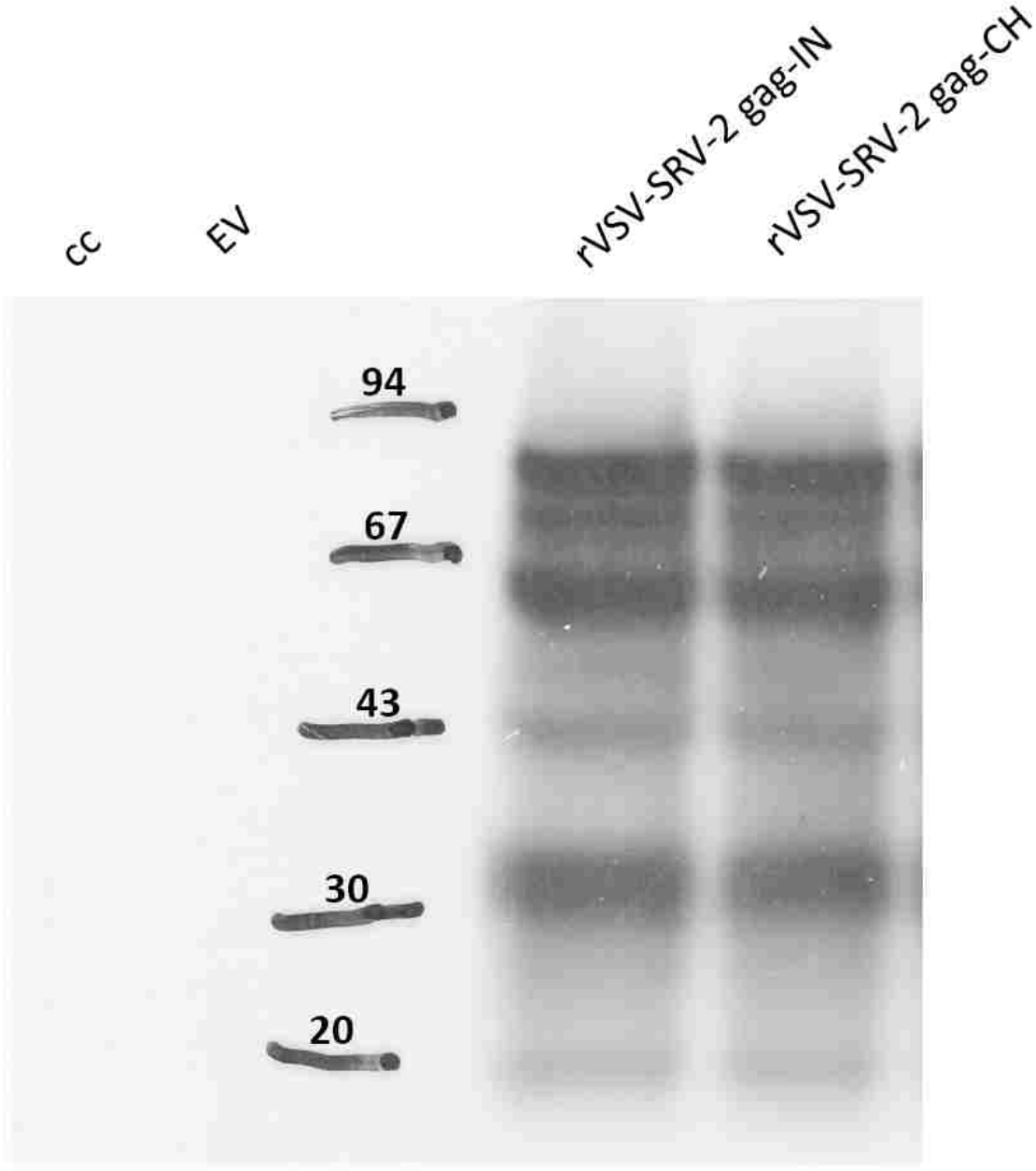


Figure 4.8: Plaque purified rVSV expressing SRV-2 gag protein. rVSV glycoprotein exchange vaccine vectors expressing SRV-2 gag protein were assayed using Western immunoblot. Anti-FLAG antibodies was used to detect the expression. Appropriate cell (cc) and empty virus vector controls (EV) were maintained

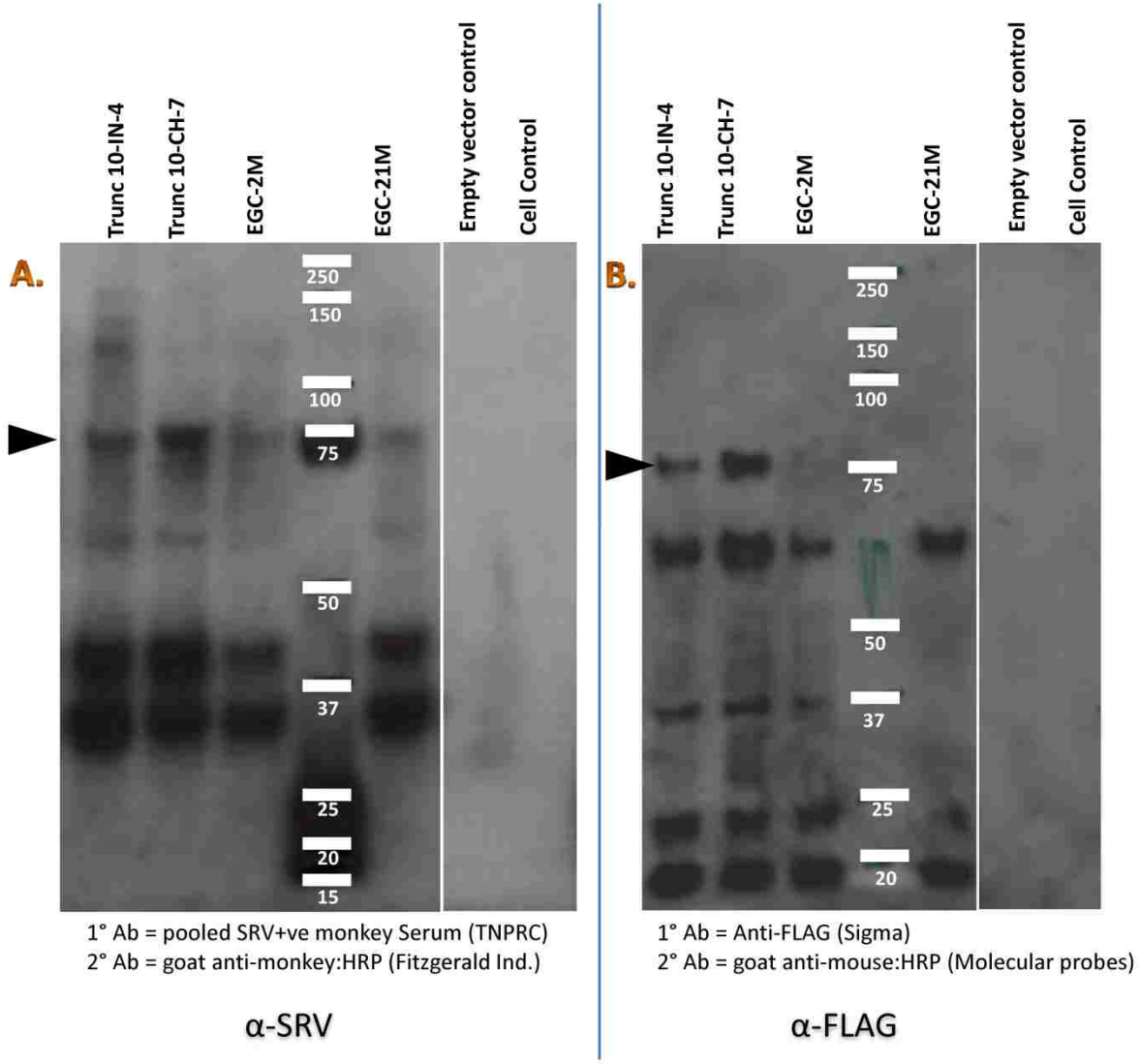
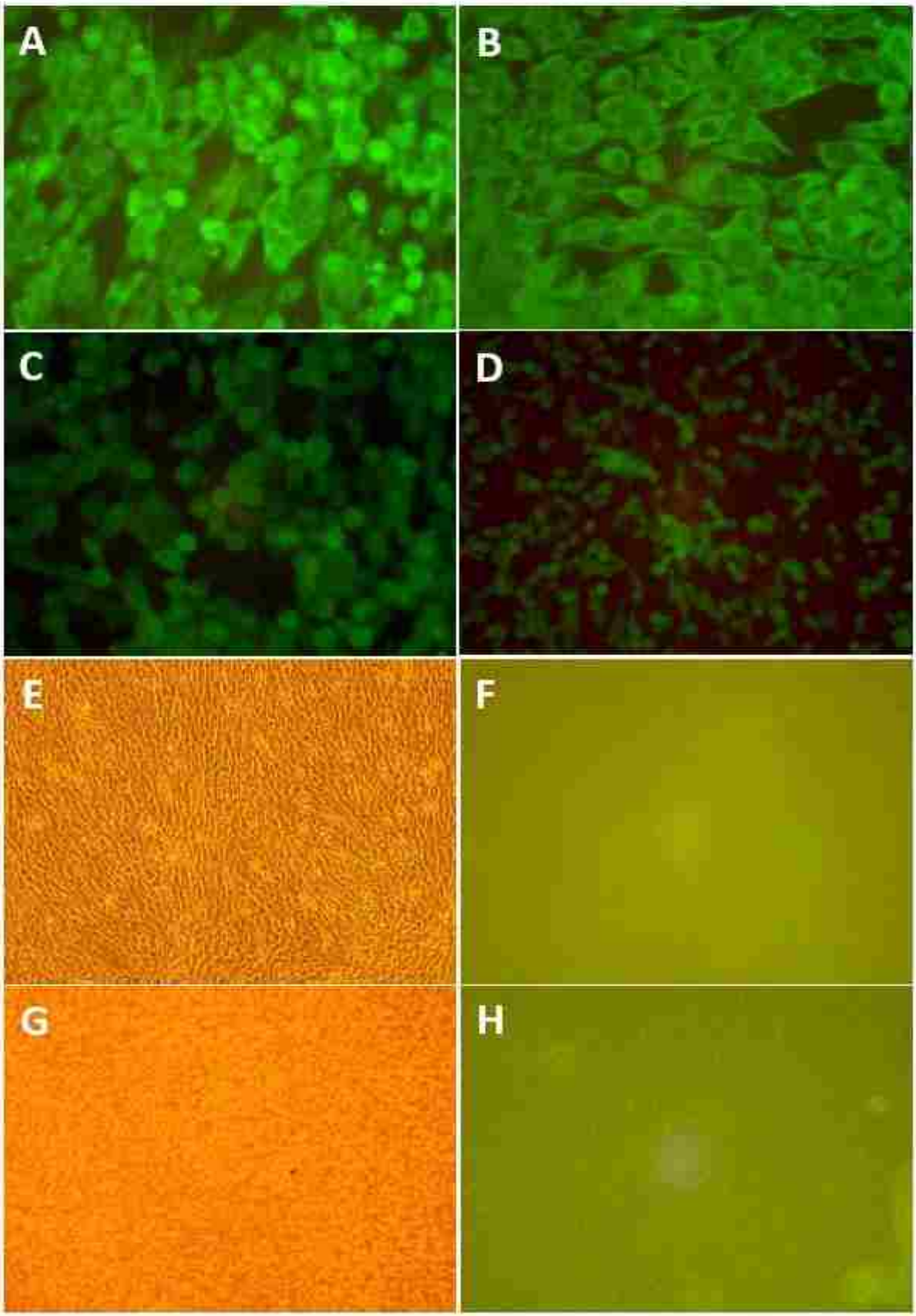


Figure 4.9: Plaque purified rVSV expressing SRV-2 Env contruct proteins. Panel A. rVSV glycoprotein exchange vaccine vectors expressing SRV-2 EnvTrunc proteins (Trunc 10-IN-4 and Trunc 10-CH-7) and EnvGCYT proteins (EGC 2M and EGC 21M). The expressed proteins were detected using pooled sera from five known SRV positive monkeys at the TNPRC. Goat anti-monkey HRP conjugate was used as the secondary antibody. **Panel B.** The same proteins were also probed using anti-FLAG antibodies to detect expression of the env constructs. Appropriate cell and empty virus vector controls were maintained.

Figure 4.10: Plaque purified rVSV expressing Herpes B virus glycoprotein D (gD). BHK 21 cells were infected with rVSV glycoprotein exchange vaccine vectors G Chandipura (A-B), G-Indiana (C-D) expressing Herpes B virus glycoprotein D. Protein expression was assayed using anti-FLAG antibodies. Appropriate cell (E-F) and empty virus vector controls (G-H) were also maintained.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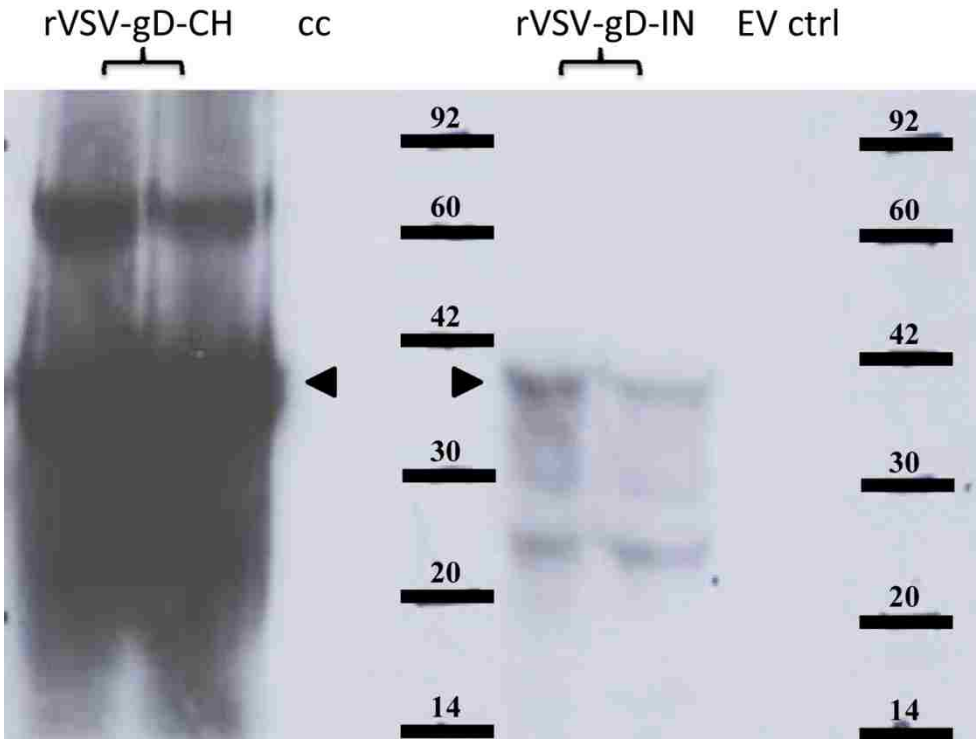

Figure 4.11: Plaque purified rVSV expressing Herpes B virus glycoprotein D (gD). rVSV glycoprotein exchange vaccine vectors expressing Herpes B virus glycoprotein D were assayed using Western immunoblot. Anti-FLAG antibodies was used to detect the expression. Appropriate cell (cc) and empty virus (EV) vector controls were also maintained.

DISCUSSION

The goal of this work was to construct stable recombinant VSVs that could express the SRV-2 gag and Env genes and the extracellular portion of the Herpes B glycoprotein D. These antigens are known to be targets of protective host immune responses by their respective viruses. Therefore, it was hypothesized that their mucosal delivery via a replicating rVSV-vectored vaccine could provide protection against these viruses.

rVSV-SRV-2 gag recombinant viruses were easily recovered and propagated indicating that the gag gene did not adversely affect rVSV replication and infectivity. However, recovering

rVSVs expressing full length SRV-2 Env was a rather difficult task. A similar issue was also encountered by other researchers, while trying to recover rVSV expressing HIV-1 envelope and may be attributed to toxicity associated with the cytoplasmic terminus of the Env glycoprotein. Previously, it was shown that the HIV-1 Env was not incorporated into VSV or rabies virus unless its cytoplasmic domain was substituted with that of VSV G (Johnson et al., 1997; Mebatsion and Conzelmann, 1996; Owens and Rose, 1993). Studies with the 29aa cytoplasmic tail of VSV G indicated that it may be required for high-level incorporation of foreign glycoproteins or the VSV G itself into VSV virions (Kretzschmar et al., 1997; Schnell et al., 1998; Schnell et al., 1996a; Schnell et al., 1996b; Schnell et al., 1997). However, Schnell et al also showed that a short VSV G cytoplasmic tail was required for virion budding but there is no specific requirement in the tail sequence (Schnell et al., 1998). HIV-1 Env is therefore the only protein that required the G cytoplasmic tail for efficient incorporation into VSV virions. In a series of elegant experiments, Johnson et al showed that rather than providing a positive signal, the substitution of the HIV-1 cytoplasmic tail with the VSV G cytoplasmic tail eliminated the negative signal in the HIV-1 Env. This negative signal was traced to the membrane proximal 3-10 amino acids in the cytoplasmic tail of HIV-1 Env (Johnson, Rodgers, and Rose, 1998).

Since SRV-2 envelope sequences were not analyzed for the presence or absence of such a signal, this may or may not be true for SRV-2 Env. Nevertheless, to increase the efficiency of recovering the recombinant VSVs, the SRV-2 Env gene was truncated to eliminate the last 38 amino acids. This was done on the basis of published sequences of SRV-3 indicating that these last three amino acids coded for the cytoplasmic tail of the Env glycoprotein (Brody, Rhee, and Hunter, 1994). Two constructs were generated. One construct had the truncated Env glycoprotein moiety fused in frame with the 29 amino acid VSV G glycoprotein cytoplasmic tail

region (Rose and Gallione, 1981; Rose et al., 1980) and 3XFLAG epitope. This construct was termed ENV-GCYT. The other construct only had the truncated SRV-2 Env and 3XFLAG epitope termed ENVTrunc. Both recombinants were efficiently recovered, propagated and plaque purified. The constructs did not affect the two T cell epitopes and the T and B cell epitopes encoded by the gp70 portion of the Env and retained the transmembrane region (gp20) of the Env intact (Philipp-Staheli et al., 2006).

Monkeys were vaccinated and boosted with rVSV-EnvTrunc, rVSV-EnvGCYT and rVSV-gag recombinant at TNPRC. Preliminary results indicate that the vaccine efficiently protected vaccinated animals upon lethal challenge (Preston Marx, personal communication). Moreover, all vaccinated animals were clinically normal. The disease onset was rapid in the control animals. Two of the four unvaccinated control animals died of fatal anemia as a result of SRV-2 infection while a third animal was expected to go for necropsy (Preston Marx, personal communication).

For the Herpes B virus vaccines, the truncated version of gD was chosen based on its known high immunogenicity and the previous work with the HSV-1 gD showed partial protection against HSV-1 and HSV-2 (Bennett, Harrington, and Kelly, 1992; Blacklaws et al., 1990; Cremer et al., 1985; Rooney et al., 1988). Herpes B virus is a select agent causing fatal disease in humans. Challenging NHPs for vaccine efficacy therefore requires a BSL-4 containment facility. Animals may be subjected to contact challenge and additional work is expected to be carried out at the TNPRC. Currently we have an approved Institutional Animal Care and Use Committee (IACUC) for testing B virus vaccines in mice at LSU to evaluate its immunogenicity. While the animals cannot be challenged, detailed serological work and

evaluation of T and B cell responses will be carried out at the LSU School of Veterinary Medicine.

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CHAPTER 5

CONCLUDING REMARKS AND FUTURE CHALLENGES

The overall goal of these investigations was to explore the use of recombinant VSV for the construction of VSV-vectored vaccines that could protect animals and humans against other potentially lethal viral infections. This goal was largely accomplished by the construction of VSV-based vaccines for WNV, SRV-2 and Herpes B. VSV-vaccines against the WNV virus were extensively studied in mouse experiments and showed high levels of protection against highly neurovirulent strains of WNV. In this thesis, particular attention was devoted to dissecting the humoral and cellular immune responses that were responsible for protecting mice against lethal WNV challenge. It was rewarding to find that the VSV-vectored vaccines expressing the WNV E glycoprotein generated robust humoral and cellular immune responses as evidenced by multiple immune parameters detailed in this thesis.

In the process of characterizing WNV strains isolated in Louisiana, and in an attempt to select a highly virulent WNV strains for the necessary vaccine-challenge experiments, it was found that one Louisiana WNV strain was more virulent than the prototypic strain WNV NY99 (Chapter 2). This virus strain, LSU-AR01 was isolated from a dead blue jay (*Cyanocitta cristata*) in Jefferson Parish in 2001 (Alma Roy, personal communication). This finding prompted a detailed characterization of the phenotypic and virulence characteristics of this virus as well as extensive phylogenetic analysis to understand its relationship to other known WNV viruses. The phylogenetic work generated some potentially highly important findings with regard to the epidemiology of WNV in North America. The important finding was that the LSU-AR01 strain was closely related to a 1999 mosquito isolate from Connecticut and belongs to a

subclade that was distinct from the prototypic NY99 virus clade. This finding coupled with the fact that the WNV LSU-AR01 strain appeared to be more neurovirulent in mice than the NY99 strain posed the question whether highly virulent strains not directly related to the NY99 strain could be circulating in birds or other animal hosts that could potentially evolve in the future to infect humans and cause disease. Unfortunately, the phylogenetic data, although it utilized entire WNV genomes, was dependent on only one Louisiana-based WNV strain, the LSU AR01 strain. Additional strains need to be sequenced and incorporated into full genome phylogenetic analysis to further strengthen the findings presented in this thesis. In this regard, I have obtained a number of isolates from 2001-2006 and 2008 from Dr. Alma Roy. These strains were isolated from a variety of birds and from horses and alligators and will be sequenced and characterized. Phylogenetic trees generated from this data using a number of different algorithms is expected to yield clues about the introduction, spread and microevolution of WNV in Louisiana over the years.

The rVSV-vectored vaccines for WN produced robust humoral and cellular immune responses. Furthermore, the vaccines protected mice against lethal WNV challenge. Dissection of the immune responses was largely enabled by the use of polychromatic fluorescence cytometry to track the expression of multiple markers associated with the activation of specific lymphocyte populations. It was found that the VSV-vectored WNV E vaccine elicited anti-WNV E specific cells $CD8^+$ IFN γ^+ T cell responses and downregulated T reg cells, as a result of the strong enhancement of effector T cell functions. This was the first rVSV-vectored vaccine study where elaborate immunological markers were utilized to dissect the immune response against the vaccine.

WNV virulence is intimately coupled to immunological responses against the virus. It is largely thought that strong neutralizing responses against the virus may protect subsequent dissemination to neuronal tissues. However, cellular immune responses and specifically $CD8^{+}$T cells against the virus may ultimately contain the virus in neuronal tissues and prevent virus dissemination in the brain. Although, we did not generate direct evidence that cellular immune responses in the brain were elicited and prevented WNV lethality, it is highly suspected that these responses were elicited and were primarily responsible for the observed protection from WNV infection. Additional immunopathological studies are needed to confirm this hypothesis. In this regard, the differences in the comparative virulence characteristics of the NY99 strain in comparison to the LSU AR01 strain may be due to amino acid differences within CTL epitopes contained in different genes (Chapter 2).

An important point to be made about these vaccination experiments is that the VSV-based vaccines were administered via the intranasal route. This route of immunization provides direct access to mucosal tissues and would be highly preferred for human use largely due to the relative ease of administration. The current work did not specifically address mucosal immune parameters such as secretory IgA and IgM which could potentially be highly important in containing viral infections locally. This may especially be important for infections that are primarily transmitted via aerosol and mucosal surfaces such as the influenza viruses.

A potential drawback with the rVSV-vectored vaccine approach detailed in this thesis is that the VSV viruses that were generated were replication competent. rVSVs have been extensively tested for safety in rodent models and non-human primates. Wildtype VSV have been shown to be neuropathogenic in mice following intranasal (i.n.) or intracerebral (i.c.) inoculation (Miyoshi, Harter, and Hsu, 1971). Recent studies with VSV-vectored vaccines

expressing the HIV env gene revealed that live replicating virus inoculated via the intanasal or intramuscular route were not neuroinvasive. However, rVSV-IN when incoulated intrathalamically into rhesus macaques caused moderate to sever neuropathology comparable to wild type VSV (Johnson et al., 2007). Neuroinvasiveness of viral vectors is a substantial obstacle for the approved use of these viruses in humans. However, addditional studies have shown that replication defective viruses can generate similar robust immune responses to replication competent viruses (Majid et al., 2006; Publicover, Ramsburg, and Rose, 2005; Roberts et al., 1999; Schnell et al., 1997). Therefore, the logical next-step of this work is to generate replication defective viruses as vehicles for vaccine production. In this regard, genes essential for viral infectivity and replication could be provided in trans through the use of complementing cells that could provide the missing gene. This has already been achieved by providing the VSV G gene in trans through cellular complementation (replication defective virus). Additional safeguards could include the development of cell lines that could provide more than one gene such as a combination of the G and M proteins and the introduction of mutations within the M gene that are known to attenuate viral replication in the host (Jayakar and Whitt, 2002). Furthermore, the development of propagation defective G-stem rVSVs, wherein most of the extracellular portion of the VSV G is deleted, retaining the transmembrane domain and ectodomain membrane proximal 42 amino acids, should also be considered (Robison and Whitt, 2000).

Vaccines remain the most important defense strategy to combat important infectious diseases of humans and animals. In general, the use of recombinant viruses that naturally elicited robust immune responses provide important new avenues for the construct of robust vaccines. These vaccines can be made extremely safe for human use provided that

multiple safeguards are carefully engineered and that these vaccines are extensively studied in animal models prior to use in humans. I remain convinced that viral vectored vaccines will provide new avenues to combat highly virulent bacterial and viral pathogens. Currently, I am engineering rVSV expressing *Mycobacterium tuberculosis* antigens for Dr. Alistar Ramsay at the LSU Health Science Center, New Orleans. These antigens are fusion combinations of highly immunogenic ESAT-6, Ag85A, Ag85B and heat shock protein HSP (Alderson et al., 2000; Brandt et al., 2000; Dietrich, Weldingh, and Andersen, 2006; Hoft, 2008; Kamath et al., 1999; Langermans et al., 2005; Li et al., 1999; McShane et al., 2004; Stewart et al., 2002).

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APPENDIX A

ADDITIONAL WORK

INTRODUCTION

To complement the Neighbor Joining tree for the 75 WNV genomes in Chapter 2, I carried out additional phylogenetic analyis using Maximum Likelihood (ML) and Bayesian analysis. The ML analysis is extremely memory intensive could not be completed even with the help of powerful computers at the LSU Center for Computation and Technology (CCT). However, the Bayesian analysis was carried out and the results are presented in this appendix.

MATERIALS AND METHODS

Phylogenetic tree was also constructed using Mr. Bayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) using Markov chain Monte Carlo algorithm (MCMC). The general time reversible (GTR) evolutionary model was selected with gamma-distributed rate variation across sites and a proportion of invariable sites. The analysis was run for 1100000 generations till the standard deviation of split frequencies was <0.01. The potential scale reduction factor values (PSRF) were found to be very close to 1.0 indicating a good sample for posterior probability distribution. The consensus tree was edited in Dendroscope software (Huson et al., 2007).

RESULTS

The complete tree is presented in the form of a rectangular cladogram in Figure A.1. The clades representing the North American isolates was extracted and is shown as a phylogram in Figure A.2. The values at the nodes indicate posterior probability of the clades.

CONCLUSION

This tree further bolstered the relationship between LSU-AR01 and the 1999 Connecticut isolate. The relationship had a 66% posterior probability support as compared to a

Figure A.1. Rectangular Cladogram using Bayesian Analyis: A Bayesian analysis of LSU-AR01 (in bold) along with seventy-five WNV whole genomes was carried out using the Markov chain Monte Carlo alogrithm in Mr. Bayes. The closest relative of LSU-AR01 was a 1999 mosquito isolate from Connecticut. This branching had a 66% posterior probability as per the analysis.

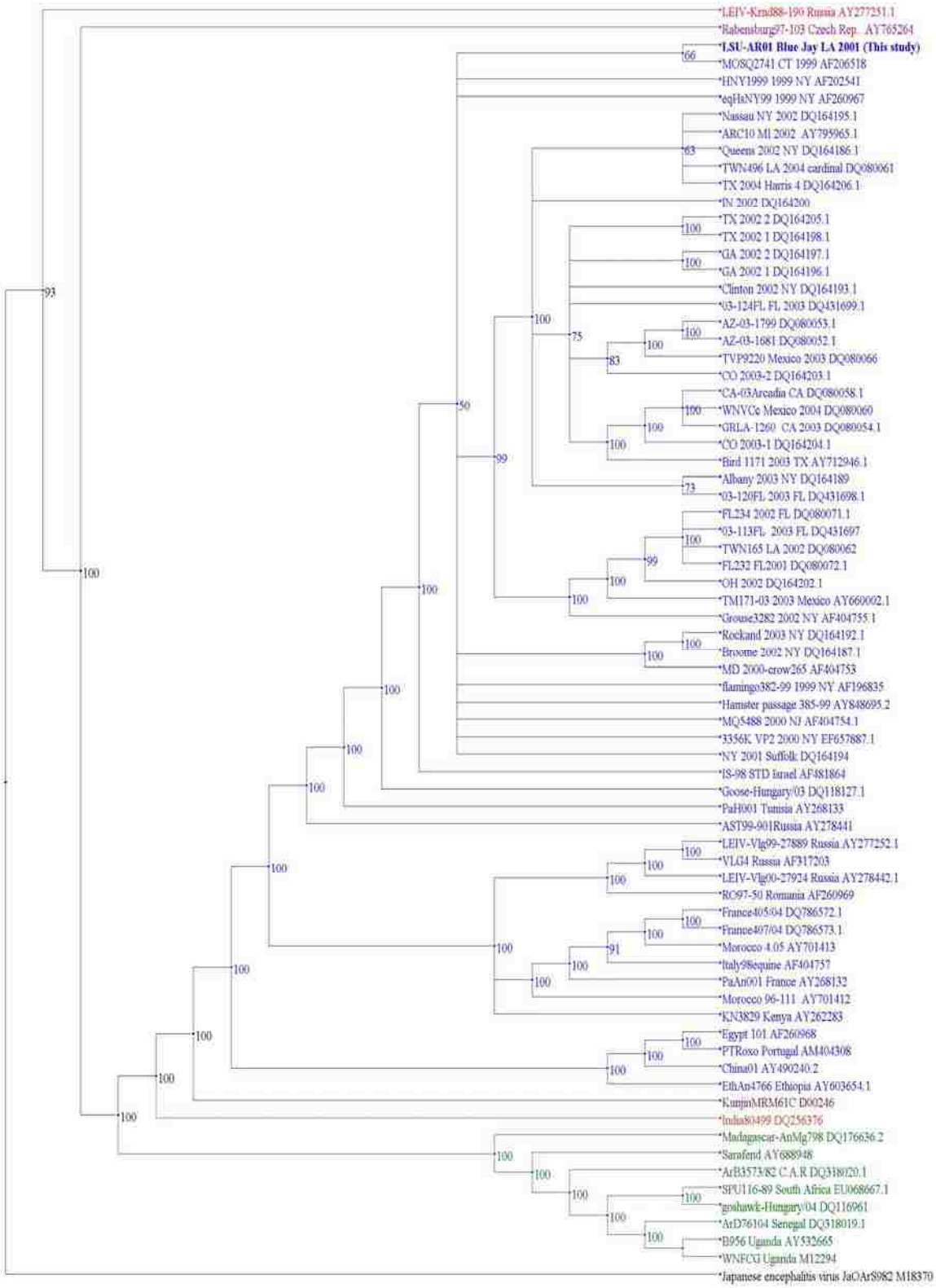
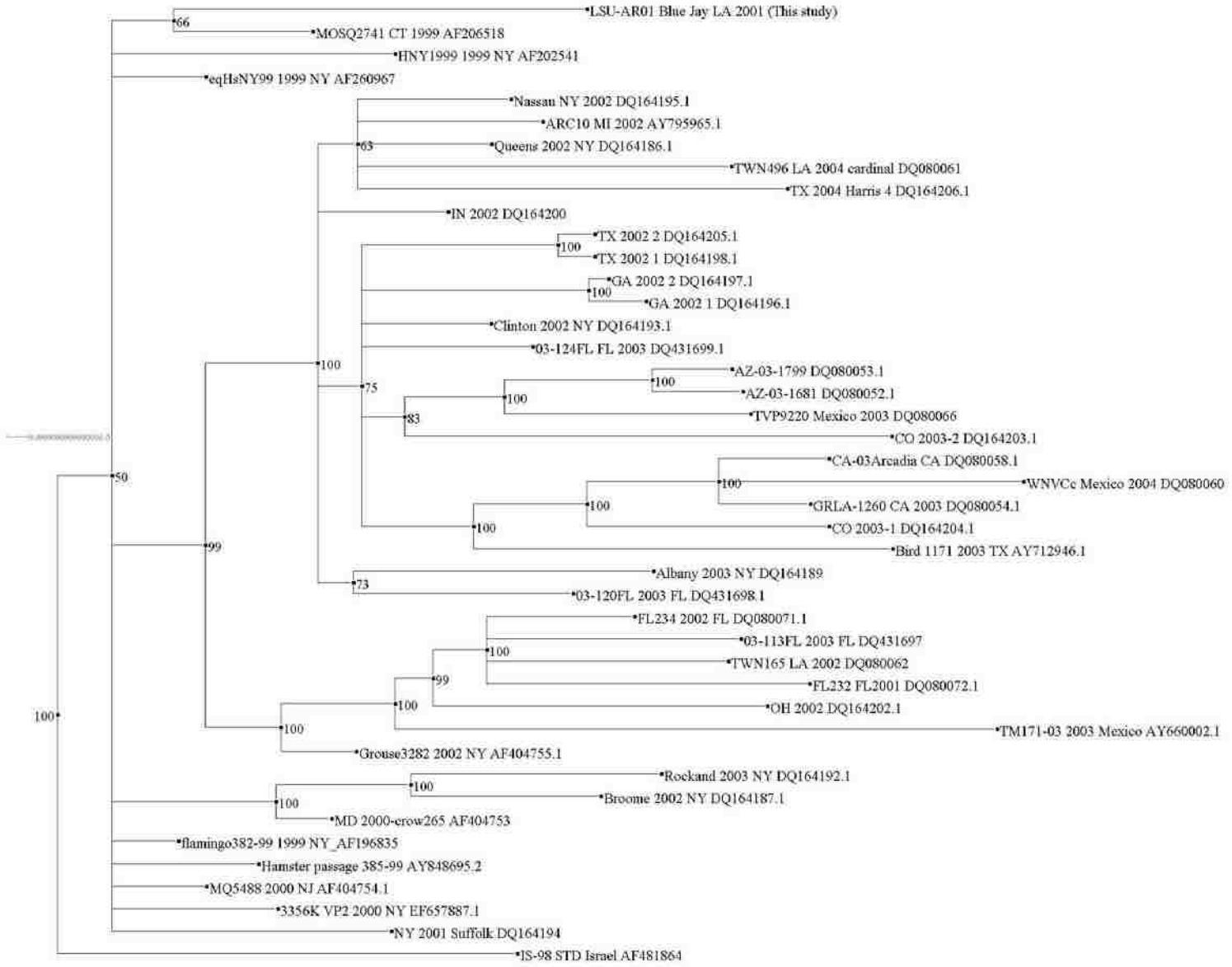

LEIV-Krnd88-190 Russia AY277251.1
Rabensburg97-103 Czech Rep. AY765264
LSU-AR01 Blue Jay LA 2001 (This study)
MOSQ2741 CT 1999 AF206518
HNY1999 1999 NY AF202541
eqHaNY99 1999 NY AF260967
Nassau NY 2002 DQ164195.1
ARC10 MI 2002 AY795965.1
Queens 2002 NY DQ164186.1
TWN496 LA 2004 cardinal DQ080061
TX 2004 Harris 4 DQ164206.1
IN 2002 DQ164200
TX 2002 2 DQ164205.1
TX 2002 1 DQ164198.1
GA 2002 2 DQ164197.1
GA 2002 1 DQ164196.1
Clinton 2002 NY DQ164193.1
03-124FL FL 2003 DQ431699.1
AZ-03-1799 DQ080053.1
AZ-03-1681 DQ080052.1
TVP9220 Mexico 2003 DQ080066
CO 2003-2 DQ164203.1
CA-03Arcadia CA DQ080058.1
WNVCo Mexico 2004 DQ080060
GRLA-1260 CA 2003 DQ080054.1
CO 2003-1 DQ164204.1
Bird 1171 2003 TX AY712946.1
Albany 2003 NY DQ164189
03-120FL 2003 FL DQ431698.1
FL234 2002 FL DQ080071.1
03-113FL 2003 FL DQ431697
TWN165 LA 2002 DQ080062
FL232 FL2001 DQ080072.1
OH 2002 DQ164202.1
TM171-03 2003 Mexico AY660002.1
Grouse3282 2002 NY AF404755.1
Rockand 2003 NY DQ164192.1
Broome 2002 NY DQ164187.1
MD 2000-crow265 AF404753
flamingo382-99 1999 NY AF196835
Hamster passage 385-99 AY848695.2
MQ5488 2000 NJ AF404754.1
3356K VP2 2000 NY EF657887.1
NY 2001 Suffolk DQ164194
IS-98 STD Israel AF481864
Goose-Hungary/03 DQ118127.1
PaH001 Tunisia AY268133
AST99-901Russia AY278441
LEIV-Vlg09-27889 Russia AY277252.1
VLG4 Russia AF317203
LEIV-Vlg00-27924 Russia AY278442.1
RC97-50 Romania AF260969
France405/04 DQ786572.1
France407/04 DQ786573.1
Morocco 4.05 AY701413
Italy98equine AF404757
PaAn001 France AY268132
Morocco 96-111 AY701412
KN3829 Kenya AY262283
Egypt 101 AF260968
PTRoxo Portugal AM404308
China01 AY490240.2
EthAn4766 Ethiopia AY603654.1
KunjinMRM61C D00246
India80499 DQ256376
Madagascar-AnMg798 DQ176636.2
Sarafend AY688948
ArB3573/82 C.A.R DQ318020.1
SPU116-89 South Africa EU068667.1
goshawk-Hungary/04 DQ116961
ArD76104 Senegal DQ318019.1
B956 Uganda AY532665
WNFCG Uganda M12294
Japanese encephalitis virus JaOArS982 M18370

Figure A.2. Extracted Phylogram using Bayesian Analyis: The lineage Ia sub-tree (Chapter 2) was extracted from the Bayes tree shown in Figure A.1. This sub-tree contains the Israel-98 and the North American WNV isolates.


LSU-AR01 Blue Jay LA 2001 (This study)
MOSQ2741 CT 1999 AF206518
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TX 2004 Harris 4 DQ164206.1
IN 2002 DQ164200
TX 2002 2 DQ164205.1
TX 2002 1 DQ164198.1
GA 2002 2 DQ164197.1
GA 2002 1 DQ164196.1
Clinton 2002 NY DQ164193.1
03-124FL FL 2003 DQ431699.1
AZ-03-1799 DQ080053.1
AZ-03-1681 DQ080052.1
TVP9220 Mexico 2003 DQ080066
CO 2003-2 DQ164203.1
CA-03Arcadia CA DQ080058.1
WNVCc Mexico 2004 DQ080060
GRLA-1260 CA 2003 DQ080054.1
CO 2003-1 DQ164204.1
Bird 1171 2003 TX AY712946.1
Albany 2003 NY DQ164189
03-120FL 2003 FL DQ431698.1
FL234 2002 FL DQ080071.1
03-113FL 2003 FL DQ431697
TWN165 LA 2002 DQ080062
FL232 FL2001 DQ080072.1
OH 2002 DQ164202.1
TM171-03 2003 Mexico AY660002.1
Grouse3282 2002 NY AF404755.1
Rockand 2003 NY DQ164192.1
Broome 2002 NY DQ164187.1
MD 2000-crow265 AF404753
flamingo382-99 1999 NY_AF196835
Hamster passage 385-99 AY848695.2
MQ5488 2000 NJ AF404754.1
3356K VP2 2000 NY EF657887.1
NY 2001 Suffolk DQ164194
IS-98 STD Israel AF481864
66
63
100
100
100
75
100
83
100
100
50
99
73
100
99
100
100
100
100
100
100

58% bootstrap value support for the two isolates in the Neighbor joining tree (Chapter 2).

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VITA

Arun Iyer was born in March 1975 to Kamakshi and Venkatesh Iyer. He earned a bachelor's degree from the University of Mumbai (Bombay), India, and majored in microbiology. He pursued his master's degree (microbiology) at the Haffkine Insitute, University of Mumbai, and developed novel oil adjuvant vaccines for foot-and-mouth disease virus. He briefly worked at Hoechst Roussel Vet. (Intervet), Pune, India. He then decided to pursue higher education in the United States. He was awarded the Deans Excellence award and earned a second master's degree (molecular genetics and microbiology) at the University of Texas at Austin. His work focused on computational biology and phylogeny. After graduation in 2004, Arun joined the laboratory of Dr. K. G. Kousoulas to pursue a doctoral degree, once again focusing on vaccine development for infectious diseases. Upon graduation, Arun will work as a post-doctoral researcher for a year at Dr. Kousoulas' laboratory and work on newer methods towards developing efficacious vaccines. Arun plans to work in the vaccine industry thereafter and aims to develop vaccines against emerging viral disease agents of arboviral and rodent-borne origins.