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CHARACTERIZATION OF SINGLE-CYCLE FLAVIVIRUS PARTICLES FOR USE AS A VACCINE TO PREVENT WEST NILE DISEASE AND TO EXAMINE IMMUNE RESPONSES TO FLAVIVIRUS INFECTION

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by

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Dedication

To my parents, who never gave up on me and did everything in their power to help me achieve my dream. This is the culmination of a long hard road, and I am so grateful to you for being there every step of the way with me.

To two very special people in my life; Grandma and Grandma Freeman, for being such an important part of who I am today. You will always be with me Grandma Freeman.

To Kasey, who never let me get too down on myself but always kept me grounded. This would not be possible without you and I look forward to the adventures that await us together.

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Perhaps one of the most significant events in our recent history, Hurricane Ike, left its indelible mark on this dissertation. The completion of this work in the face of a 2 month shutdown and the utter devastation of the university is a tribute to Peter, Nigel, Gregg and countless others at UTMB that I wish could all be mentioned. This significant moment in our lives will never be forgotten.

CHARACTERIZATION OF SINGLE-CYCLE FLAVIVIRUS PARTICLES FOR USE AS A VACCINE TO PREVENT WEST NILE DISEASE AND TO EXAMINE IMMUNE RESPONSES TO FLAVIVIRUS INFECTION

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Supervisor: Peter W. Mason

West Nile virus (WNV) is responsible for the largest outbreak of viral encephalitis in the history of North America, yet there are no vaccines available to prevent this disease. To address these needs we have developed RepliVAX WN, a single-cycle flavivirus (SCFV)-based vaccine to prevent West Nile disease. RepliVAX WN contains a C-deleted WNV genome, and is produced in trans-complementing cell lines that express WNV C. When used for vaccination, RepliVAX WN infects a single cell where the genome replicates and drives the production of highly antigenic subviral particles (SVPs) and NS1 without producing infectious virions. Thus, RepliVAX is expected to be highly potent yet exhibit a safety profile superior to traditional live-attenuated viral vaccines.

Here we demonstrate that RepliVAX WN can be safely passaged in C-expressing cell lines and that this blind passage selected for mutations used to engineer a second-

generation RepliVAX WN with an enhanced in vitro growth phenotype. When evaluated in mouse and hamster models of WN disease, this second-generation RepliVAX was safe, exhibited 100% protective efficacy, and induced significantly higher antibody levels than the parental virus. Furthermore, we observed that RepliVAX WN-induced antibody levels remain steadily at high levels for at least 6 months after vaccination of hamsters, and all animals were protected from lethal WNV challenge at this time. Evaluation in non-human primates indicated that one or two doses of RepliVAX WN was safe, induced WNV-specific antibody responses, and protected animals from WNV viremia.

Having demonstrated the usefulness of RepliVAX WN as a vaccine to prevent WN disease, we were interested in the immunological mechanisms underlying vaccine immunity. We observed that although RepliVAX WN vaccination induces high levels of interferon alpha (IFNα), the ability to respond to either type-I or type-II IFNs was not required for the development of activated B cells, IgG, IgM, or neutralizing antibody titers. Type-I IFN signaling did, however, play a role in viral gene expression, as in vivo imaging of animals inoculated with luciferase-expressing SCFVs revealed 1000-fold greater bioluminescence in the absence of a type-I IFN response. The affect of this IFN response on gene expression was dramatic, but short lived and did not appear to play a role in SCFV persistence, as SCFV gene expression was detectable for at least 18 days after SCFV inoculation. Taken together these results demonstrate the usefulness of SCFVs like RepliVAX WN as vaccines to prevent flavivirus disease, and tools with which to examine immune responses to viral infection.

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List of Abbreviations

2'5'-OAS	2’5’-oligoadenylate synthetase
2-ME	2-mercaptoethanol
aa	Amino acid
ABSL	Animal biosafety level
AnchC	Anchor of capsid
ANOVA	Analysis of variance
BAC	Bacterial artificial chromosome
BVDV	Bovine viral diarrheal virus
C	Capsid
cDNA	Complementary deoxyribonucleic acid
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
CS	Cyclization sequence
CSFV	Classical swine fever virus
Cx	Culex
DC	Dendritic Cell
DCSIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DCSIGNR	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin related protein
DENV	Dengue virus
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
dpi	Days post-infection
EDTA	Ethylenediaminetetraacetic acid
EF1A	Eukaryotic translation elongation factor 1 alpha
ELISA	Enzyme-linked immunosorbency assay
ELISA	Envelope
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FFU	Focus-forming units
FLUC	Firefly luciferase
HAI	Hemagglutination inhibition assay
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	Hours post-infection
hr	Hour
HSV	Herpes simplex virus
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INV	Inactivated viral vaccine

IP	Intraperitoneally
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISGF	Interferon-stimulated gene factor
IU	Infectious units
JAK	Janus-activated kinase
JEV	Japanese encephalitis virus
kb	Kilobases
KO	Knockout
KUNV	Kunjin virus
LAV	Live attenuated vaccine
M	Membrane
MDA5	Melanoma-differentiation-associated gene 5
MDC	Myeloid dendritic cells
MEM	Minimal essential medium
min	Minute
ml	Milliliters
MOI	Multiplicity of infection
MVEV	Murray Valley encephalitis virus
Neut	Neutralizing antibody
NHP	Non-human primate
NIH	National Institutes of Health
nm	Nanometer
NS	Non-structural
nt	Nucleotide
NY	New York
NYC	New York City
OD	Optical density
ORF	Open reading frame
Pac	Puromycin n-acetyl transferase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PDC	Plasmacytoid dendritic cell
PKR	Double-stranded RNA-activated protein kinase
prM	Precursor membrane
PRR	Pattern recognition receptor
Pur	Puromycin n-acetyl transferase
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RTPase	RNA triphosphatase
SC	Subcutaneously
SCFV	Single-cycle flavivirus
SFBR	Southwest Foundation for Biomedical Research
SFM	Serum-free medium
SLEV	St. Louis encephalitis virus
SP	Signal peptidase

SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
SVP	Subviral particle
TBEV	Tick-borne encephalitis virus
TCID50	50% Tissue culture infectious dose
TIAR	T-cell restricted intracellular antigen related protein
TLR	Toll-like receptor
trC	Truncated capsid
trE	Truncated envelope
TYK	Tyrosine kinase
Ubi	Ubiquitin
US	United States
UTR	Untranslated region
VEE	Venezuelan equine encephalitis virus
VEErep	Venezuelan equine encephalitis virus replicon
VLP	Virus-like particle
VRP	Virus replicon particle
WNE	West Nile encephalitis
WNF	West Nile fever
WNV	West Nile virus
WNVLP	West Nile virus-like particle
WT	Wild type
YFV	Yellow fever virus
YFV-17D	Yellow fever virus strain 17D

CHAPTER 1 : INTRODUCTION[1]

Family Flaviviridae

There are over 70 distinct viruses that comprise the Flaviviridae family. Members of this virus family are able to infect a wide variety of vertebrate and invertebrate hosts, and are responsible for a great number of human diseases. While sharing many common characteristics including virion morphology, genomic structure and organization, and replication strategy, worldwide distribution and disease manifestations differ widely from virus to virus. The Flaviviridae family is subdivided into three genera: *Hepacivirus*, *Pestivirus*, and *Flavivirus*. Two yet unclassified viruses, GB virus A and GB virus C, which share similar nucleotide homology with the hepacivirus genus (Lindenbach, Thiel, and Rice, 2007) are also placed within the family. Flaviviridae virions are spherical in shape and range in diameter from 30 to 80nm. They are composed of a nucleocapsid core containing the positive-sense single-stranded RNA genome coding for a single open reading frame (ORF), surrounded by a host-derived lipid bilayer coated with viral glycoproteins. Members of the *Flaviviridae* family are believed to infect cells through a shared mechanism of receptor-mediated endocytosis, and their genomes are capable of acting as mRNA once in the cell. Genome replication occurs cytoplasmically and in association with perinuclear membranes, and mature virions are assembled and released in the cellular exocytic pathway. Despite these similarities, however, the distinct members of the flaviviridae family differ greatly in their disease pathology, transmission, and geographic distribution.

[1]A portion of this chapter has been previously published in Advances in Virus Research (AVR). AVR does not require copyright permission as long as proper citation is given. The citation for this article is: Widman, D. G., Frolov, I., and Mason, P. W. (2008). Third generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. *Advances in Virus Research.* **72**:77-126.

GENUS HEPACIVIRUS

The lone disease-causing member of the *Hepacivirus* genus is hepatitis C virus (HCV), which was first recognized in 1975 (Feinstone et al., 1975), but not actually identified until 1989 as the causative agent of non-A non-B hepatitis (Choo et al., 1989). It is estimated that 2.2% of the world's population, corresponding to 130 million individuals, are infected with HCV (Alter, 2007). Transmission of HCV occurs through percutaneous exposure to blood or serum, and is most commonly associated with intravenous drug use, contaminated health care injections and high-risk sexual behavior. Disease progression of HCV infection generally occurs in two phases; an acute phase early after exposure that is typically asymptomatic (although can be accompanied by symptoms of liver dysfunction), and a chronic phase into which 70-85% of infected individuals enter. Patients with chronic HCV infection are at an increased risk for serious liver dysfunction including steatosis, progressive fibrosis, cirrhosis, and hepatocellular carcinoma, the onset of which usually occur decades after initial infection (Lemon et al., 2007). The current standard of care for treatment of HCV infection is a combination therapy of pegylated IFN-α and ribavirin, however this is only effective in 40-50% of infected individuals.

While HCV research has been slowed by the lack of a viable cell culture system to propagate HCV, necessitating the use of replicon helper systems to examine the virus in vitro (Lohmann et al., 1999), the identification of an HCV strain capable of completing a full replication cycle in cell culture (Wakita et al., 2005) has advanced the field tremendously. The HCV genome is about 9.6kb in length and codes for 3 structural proteins (C, E1, and E2) required for virion production, 5 non-structural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) required for genome replication, and 2 proteins (p7 and NS2) of still unknown function (Lemon et al., 2007). Translation of the viral genome is

cap-independent, and occurs via an internal ribosome entry site (IRES) located in the 5' UTR. As the name would imply, the primary site of HCV infection occur in the liver, and while the pathology associated with HCV infection is generally regarded as immune-mediated, there is still little understanding of the relative role and contribution of various arms of the immune system in the pathogenesis of HCV.

GENUS PESTIVIRUS

The *Pestivirus* genus contains three well-characterized members: bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus, which are all responsible for severe disease in livestock, along with a number of newly discovered viruses that infect giraffes and other wild ruminants. Members of the pestivirus genus are of particular economic importance, since they cause highly contagious and fatal disease in domesticated livestock. In particular, CSFV is responsible for acute and chronic hemorrhagic syndromes in swine, while BVDV is capable of a wide range of disease in cattle that has made its prevention and eradication from infected herds difficult. Fetuses exposed to BVDV in the womb go on to develop either a rapidly fatal acute disease, birth defects, or a persistent infection that leaves the animal as a highly infectious carrier for life. Incidence of persistent infection is estimated to be at about 1% (Brock, 2003), and these animals are capable of shedding extremely large amounts of infectious virus (as high as 10^7 $TCID_{50}$ per ml) (Houe, 1999), making them the primary means of transmission among herds. Persistent infections often progress to fatal mucosal disease, and this is correlated with a shift in the viral population from those with a non-cytopathic phenotype to those with a cytopathic phenotype (Lindenbach, Thiel, and Rice, 2007). While a live-attenuated vaccine does exist to prevent BVDV, vaccination of persistently infected cattle can result in the development of fatal mucosal disease, complicating eradication strategies.

While the life cycle of pestiviruses closely resembles that of all other flaviviridae members, the genome organization and replication strategies of pestiviruses are more similar to hepaciviruses than to flaviviruses. The pestivirus genome is 12.3kb in length, and codes for 4 structural proteins (C, E^{rns}, E1 and E2), 7 non-structural proteins (N^{pro}, NS2, NS3, NS4A, NS4B, NS5A and NS5B), and p7 which similar to HCV has an unknown function in the pestivirus life cycle. The N^{pro} protein, unique to pestiviruses among the flaviviridae family, serves as an autoprotease that cleaves itself from the N-terminus of the C protein and acts as an IFN antagonist (Ruggli et al., 2003; Ruggli et al., 2005) at least in part by inhibiting the transcription of IRF-3 (La Rocca et al., 2005). As with other flaviviridae, the pestivirus NS3 has both protease and helicase activities. Interestingly, the shift in BVDV phenotype from non-cytopathic to cytopathic (and the corresponding development of fatal mucosal disease) is associated with increased expression of NS3 (Lindenbach, Thiel, and Rice, 2007).

GENUS FLAVIVIRUS

The *Flavivirus* genus contains the greatest number of viruses in the *Flaviviridae* family, with 53 viral species. Unique among the flaviviridae, most flaviviruses are vector borne, with the vast majority being transmitted by hematophagous arthropods such as ticks and mosquitoes. Geographic distribution of flaviviruses is ubiquitous, with viruses from the genus present on all continents of the world except Antarctica. A number of flaviviruses are responsible for severe and debilitating disease in humans, and thus a great deal of research is focused on a better understanding of these pathogens. In particular, yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV) are of particular interest because of the severe disease they cause in humans.

Physical structure

Mature flavivirus particles are roughly spherical in shape, 30-50nm in diameter, and consist of a nucleocapsid structure surrounded by a lipid bilayer studded with viral proteins (Fig. 1.1). The nucleocapsid is composed of multiple copies of the highly basic capsid (C) protein in association with the RNA genome of the virus. The host cell-derived lipid bilayer contains two viral proteins, the envelope glycoprotein (E) and the glycosylated membrane protein (M), which is produced in cells as a precursor (prM). Interestingly, observations using cryoelectron microscopy have failed to detect interactions between the lipid membrane or the surface proteins with the nucleocapsid (Kuhn et al., 2002; Zhang et al., 2003a). Unlike the structure of many other viruses, the surface glycoproteins are not oriented as "spikes" protruding from the lipid envelope, but instead lie flat along the virion surface (Rey et al., 1995).

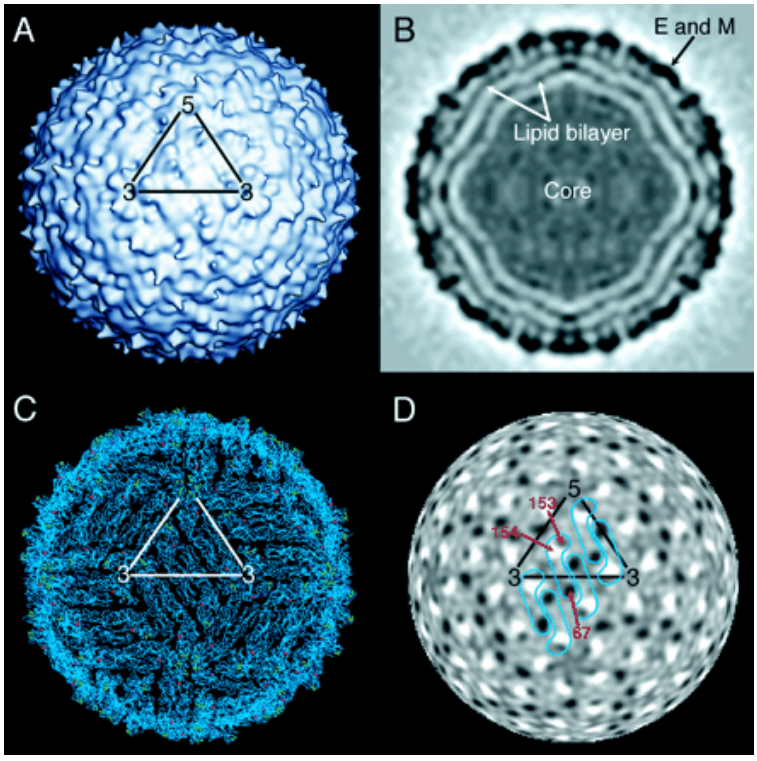

Figure 1.1: Flavivirus structure. A. Cryo-electron microscopic reconstruction of WNV with 5-fold and 3-fold axes of symmetry indicated. **B.** Cross-section of viral particle showing electron-dense layers. **C.** Virion structure showing the placement and arrangement of E proteins on the surface. **D.** Electron density differences are highlighted by overlay of WNV electron densities (white) on DENV densities (black). Outlines of 3 E homodimers are shown in blue. Reprinted with permission (Mukhopadhyay et al., 2003)

Genome organization

The genomic structure of flaviviruses is similar to that of hepaciviruses and pestiviruses in that it consists of a positive sense single-stranded RNA genome about 11kb in length. The genome contains a type-I 7-methyl-guanine cap ($m^7GpppAmpN_2$) at its 5' terminus (Cleaves and Dubin, 1979), but is not polyadenylated (Wengler and Gross, 1978). Thus, unlike pestiviruses and hepaciviruses that are translated in a cap-independent manner via an IRES, initiation of flavivirus translation is cap-dependent and is initiated by ribosomal scanning. The ORF of flaviviruses (Fig. 1.2) codes for 3 structural (C, prM and E) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins that are responsible for virion structure and genome replication. Flanking the ORF, at the 5' and 3' termini of the genome, are untranslated regions (UTRs) that play important roles in genome replication, positive-strand synthesis and translation of the viral genome. The 5'UTR is about 100nt in length, and contains conserved stem-loop RNA secondary structures that are important for efficient translation (Brinton and Dispoto, 1988; Thurner et al., 2004). The 5'UTR of some mosquito-borne flaviviruses have been shown to interact with cellular proteins such as La (Yocupicio-Monroy et al., 2003) and TIAR (Li et al., 2002), however the exact implication of these interactions on

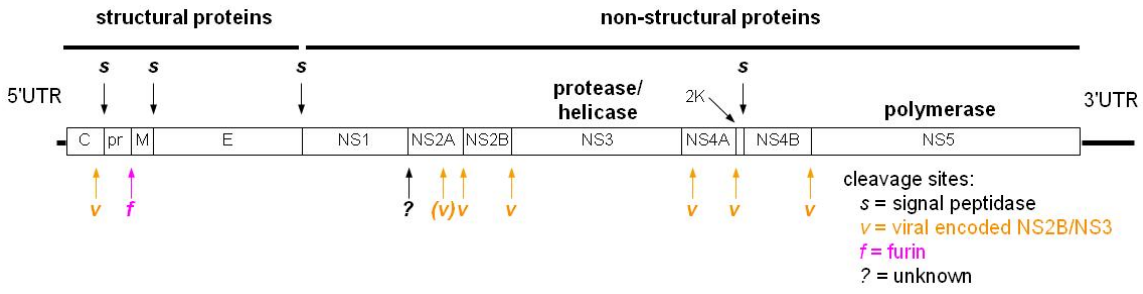

Figure 1.2: Flavivirus genome. Structural genes (C, prM, E) are clustered at the 5' end of the genome while the non-structural components (NS1-5) are located at the 3' end of the genome. Proteolytic cleavage sites are indicated by arrows color-coded to correspond to the enzyme responsible for cleavage. Reprinted with permission (Widman, Frolov, and Mason, 2008)

viral replication or pathogenesis are not well understood. Furthermore, the 5' cyclization sequence (CS; discussed with genome replication below) of tick-borne flaviviruses is located in the 5'UTR. The UTR at the 3' terminus of the flavivirus genome ranges from 400-700nt, and similar to the 5'UTR contains elements important in genome replication, translation and negative-strand synthesis. A highly conserved stem-loop structure is found in the 3'UTR, as is the 3'CS for all flaviviruses (Lindenbach, Thiel, and Rice, 2007). The 3'UTR of WNV and DENV have been shown to bind to the translation elongation factor EF1A (Blackwell and Brinton, 1997; De Nova-Ocampo, Villegas-Sepulveda, and del Angel, 2002), and this is thought to play an important role in genome replication.

Proteins

Structural proteins

The three flaviviral structural protein genes are located at the 5' end of the viral genome, and are thus the first to be translated. The first protein is the highly basic 11 kilodalton (kd) capsid (C) protein, multiple copies of which associate with the viral RNA genome to form the nucleocapsid structure. This association of C with the viral RNA is believed to be facilitated by positively charged residues clustered at each terminus of the protein. An internal hydrophobic region is believed to mediate interaction with the lipid envelope of the viral particle (Ma et al., 2004), although this interaction has yet to be observed. C is cleaved at its C-terminus from the polypeptide by the viral NS2B/NS3 protease at its junction with anchor of C (anchC). AnchC is a short transmembrane domain between C and prM that serves as the signal sequence for translocation of prM into the lumen of the endoplasmic reticulum (ER).

The next protein produced, precursor of membrane (prM), is a 26kd glycoprotein inserted into the ER lumen upon translation. Here, it is cleaved from anchC by the host

signal peptidase. There is strong evidence to indicate that in order to produce infectious virions the cleavage of prM must be controlled, presumably to first allow cleavage of C by NS2B/NS3 (Lee et al., 2000). Upon cleavage from its signal sequence by signal peptidase, prM remains anchored to the luminal side of the ER membrane which eventually become the outer surface of the virion. Flavivirus particles exit infected cells via the secretory pathway, an environment with relatively low pH. A key function of prM is to prevent the rearrangement of E residues on the virion surface in this low pH environment that would confer fusogenic activity upon the virus (Guirakhoo, Bolin, and Roehrig, 1992; Heinz et al., 1994). Upon virion transit through the Golgi apparatus, maturation occurs when prM is cleaved by furin to form M. This cleavage of prM results in a rearrangement of the viral glycoproteins in such a way as to confer infectivity (see below), and prevention of this cleavage results in the production of noninfectious particles (Guirakhoo, Bolin, and Roehrig, 1992; Elshuber et al., 2003).

The 53kd envelope (E) glycoprotein is the final structural protein translated in the flavivirus ORF. The E protein is the major surface component of flavivirus virions, and plays important roles in receptor binding and uncoating of virus particles. Cellular tropism is also affected by determinants in the E protein, and it has been shown that the glycosylation status of the WNV E protein plays a role in the development of neuroinvasive disease (Shirato et al., 2004; Beasley et al., 2005). Following translation, the E protein also translocates to the ER lumen where it is cleaved at its N-terminus by the host signal peptidase, but remains attached to the ER membrane at its C-terminus and is thus found on the virion surface. Proper folding, incorporation into virus particles (Konishi and Mason, 1993), and stabilization in the low pH environment of the secretory pathway (Heinz et al., 1994) of E require the co-expression of prM.

The envelope (E) protein is involved in cell binding and fusion. During assembly E associates with prM to form provirions covered with 60 trimers of prM/E heterodimers (Zhang et al., 2003b); however, upon maturation in the Golgi, these heterodimers dissociate and E forms 90 homodimers that lie flat on the surface of the mature virion (Kuhn et al., 2002). Upon infection, these homodimers undergo a pH-dependent conformational change in the endosome that results in the formation of homotrimers that facilitate fusion of the viral lipid envelope with the endosome membrane, resulting in release of the nucleocapsid into the cytoplasm (Modis et al., 2004).

Nonstructural proteins

The seven flavivirus non-structural proteins are involved in genome replication and polyprotein processing, as well as modulating host cell function and immune response. NS1 is a 46kd glycoprotein that is translocated to the ER lumen upon translation, and cleaved at its N-terminus by the host signal peptidase and at its C-terminus by an unknown protease in the ER. NS1 has a wide distribution in infected cells, being found in the cytoplasm, on the cell surface and in supernatants collected from infected cells. While briefly existing as a hydrophilic monomer, NS1 molecules form hydrophobic homodimers with an affinity for lipid membranes within 60 minutes of synthesis (Winkler et al., 1988; Winkler et al., 1989). These homodimers have been observed bound to the surface of infected cells (Schlesinger et al., 1990) and are secreted into the cellular milieu, the immunological consequences of which are discussed below with other flavivirus antigenic components.

The intracellular form of NS1 appears to play a role in RNA replication (Lindenbach and Rice, 1997), and it has been observed to localize to areas containing replicative intermediates (Westaway et al., 1997). Furthermore, mutational analyses demonstrated that ablation of NS1 glycosylation sites (Muylaert et al., 1996) or

dimerization (Hall et al., 1999) had dramatic effects on RNA replication and virus production, and interaction between NS1 and NS4A may be necessary for replicase function (Lindenbach and Rice, 1999). The function of extracellular NS1 in the viral life cycle is still not understood, however the secretion of this protein from infected cells has profound implications on the immunological responses to flaviviral infections (see below).

NS2A is a small (22kd) hydrophobic protein with transmembrane topology. It is cleaved from the polyprotein at its N-terminus by an unknown ER protease, and at its C-terminus on the cytosolic side of the ER membrane by the viral NS2B/NS3 protease. Similar to NS1, NS2A in infected cells has been observed co-localized with known replicase components NS3, NS5 and dsRNA, and was shown to interact with the 3'UTR of the viral genome (Mackenzie et al., 1998). Thus NS2A is believed to play a role in genome replication, although this is not its only purported role in the flavivirus life cycle. Analyses of mutations found in the NS2A genes from YFV and KUNV have demonstrated a role for NS2A in the assembly of viral particles (Kummerer and Rice, 2002; Liu, Chen, and Khromykh, 2003). Mutation of a single amino acid in either case resulted in the loss of infectious particle production; however the production of SVPs was not impaired in either case. These results indicate NS2A plays a role in packaging of the viral genome into viral particles. Recently it has been shown that the NS2A mutation in KUNV also prevents the formation of virus-induced membrane structures, but that compensating mutations that increase the hydrophobicity of the protein restore membrane formation and infectious particle production, suggesting that the ability of NS2A to interact with membranes is critical for its role in virus assembly. Based on the role of NS2A in both RNA replication and viral assembly, a role for NS2A in the shift from RNA replication to RNA packaging has been postulated (Khromykh et al., 2001b).

Interestingly, NS2A has also been shown to act as an interferon (IFN) antagonist in some mosquito-borne (Munoz-Jordan et al., 2003; Liu et al., 2005), but not tick-borne (Best et al., 2005) flaviviruses.

NS2B is also a small (14kd) ER membrane-associated protein that is cleaved at both termini by the viral NS2B/NS3 protease. As the name of the protease would imply, NS2B acts as a required cofactor for the serine protease function of NS3 (Falgout et al., 1991), which is responsible for the cleavage of C and all non-structural proteins except NS1. Functional analyses of NS2B have indicated a critical role for a central hydrophilic region in the activity of the NS3 protease (Falgout, Miller, and Lai, 1993). Hydrophobic membrane-interacting regions of the protein, however, while not essential for protease function have been shown to enhance its activity (Clum, Ebner, and Padmanabhan, 1997).

The next protein translated in the flavivirus ORF is the large (70kd) multifunctional NS3 protein, which plays critical roles in RNA replication and polyprotein processing. Contained within the N-terminal third of the protein is the catalytic domain for the serine protease activity of NS3 (Bazan and Fletterick, 1989; Gorbalenya et al., 1989a; Chambers et al., 1990). As described above, the NS3 protease requires the cofactor NS2B to function, and when active is responsible for the cleavage of C and nonstructural proteins.

In addition to its well-characterized function as a serine protease essential for the production of mature viral proteins, recent studies have indicated that NS3 functions in a number of important ways in the replication of the viral genome. NS3 and NS5 of JEV have been shown to bind to the 3'UTR of the genome in infected cells, an interaction believed to form the basis of the replicase complex (Chen et al., 1997a). In addition to the N-terminal serine protease domain, NS3 also contains a C-terminal RNA helicase domain

(Gorbalenya et al., 1989b). This domain is a functionally active helicase (Warrener, Tamura, and Collett, 1993), and this function is essential for viral replication (Matusan et al., 2001). The NS3 helicase likely serves to unwind RNA secondary structures during the process of genome replication, and also releases newly synthesized complementary strands following replication. Yet another function of NS3 is that of an RNA triphosphatase (RTPase), which is thought to dephosphorylate the 5' end of the genome in order to allow addition of the m^7G cap (Wengler, 1993). NS3 has also been implicated in the pathogenesis of flaviviruses, as expression of NS3 results in cellular apoptosis in a caspase 8-dependent manner (Prikhod'ko et al., 2002; Shafee and AbuBakar, 2003; Ramanathan et al., 2006).

NS3 is followed in the polyprotein by three small membrane-associated proteins: NS4A, NS4B, and 2K, whose functions are not well understood. NS4A (16kd) is cleaved from the polyprotein by the viral NS2B/NS3 protease, and is thought to be involved in RNA replication. Based on its observed interactions with NS1 (Lindenbach and Rice, 1999) and NS2A, NS3, NS5, and dsRNA (Mackenzie et al., 1998), NS4A is likely a component of the replicase complex. Lying between the NS4A and NS4B proteins in the flavivirus ORF is a 2kd transmembrane signal peptide that is cleaved on the cytosolic side (NS4A/2K junction) by the viral protease and on the ER luminal side (2K/NS4B junction) by the host signal peptidase (Lindenbach, Thiel, and Rice, 2007). There is evidence to suggest that, similar to the processing of the C protein, these cleavage events are coordinated to ensure that the viral protease releases NS4A from 2k before NS4B is cleaved by signal peptidase (Preugschat and Strauss, 1991; Lin et al., 1993), and that this coordination is important for the formation of viral-induced membrane structures important for viral replication (Roosendaal et al., 2006). As has been observed for NS2A,

both NS4A and NS4B act as IFN antagonists (Munoz-Jordan et al., 2003), with NS4B doing so by blocking the activation of STAT-1 (Munoz-Jordan et al., 2005).

The final protein produced in the flavivirus polypeptide is NS5, a 103kd multi-functional protein that contains the core subunit of the viral RNA-dependent RNA polymerase (RdRP), as well as a methyltransferase that serves in capping of the viral RNA (Lindenbach, Thiel, and Rice, 2007). Located at the C-terminus of the protein, the RdRP domain of NS5 shares significant sequence homology to the RdRPs of other positive-strand RNA viruses (Rice et al., 1985), and the polymerase activity of NS5 has been repeatedly demonstrated (Tan et al., 1996; Ackermann and Padmanabhan, 2001; Guyatt, Westaway, and Khromykh, 2001). Mutations made to the active site of the NS5 polymerase ablate viral replication, however, it was shown that NS5 is capable of functioning in trans (Khromykh, Kenney, and Westaway, 1998).

Although it is widely accepted that NS5 acts as the viral polymerase and thus must localize to the sites of viral RNA replication, this has yet to be observed. NS5 has been shown to interact with NS3 (Kapoor et al., 1995; Johansson et al., 2001) and the 3'UTR of viral genomic RNA (Chen et al., 1997a), and NS5 appears to play a role in the activation and regulation of the nucleoside triphosphatase and RTPase activity of NS3 (Cui et al., 1998; Yon et al., 2005). As has been observed for a number of other flavivirus nonstructural proteins, NS5 plays a role in immune evasion and pathogenesis. The NS5 of TBEV and JEV block signaling of IFN-α/β (and IFN-γ in the case of TBEV NS5) by inhibiting phosphorylation of JAK1 and TYK1, and thus preventing downstream activation of STAT-1 (Best et al., 2005; Lin et al., 2006).

Life Cycle

Binding and entry

Flaviviruses enter cells through the process of receptor-mediated endocytosis, and based on the wide range of cell types and host species infected appear to be capable of using a number of different receptors to gain entry (Lindenbach, Thiel, and Rice, 2007), or a surface molecule ubiquitous across many species. While the identification of a flavivirus internalization receptor molecule has proven elusive, a number of different attachment receptors, or "tethers" have been identified and appear to play a key role in flavivirus infection. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DCSIGN), a C-type lectin expressed by dendritic cells, has been identified as a key component for infection of human cells by DENV (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003), and the related DC-SIGNR is an important component of WNV infection (Davis et al., 2006). The demonstration that internalization of DC-SIGN was not necessary for DENV infection, however, seems to indicate a role for DC-SIGN in virus attachment, not internalization (Lozach et al., 2005).

Heparin sulfate (HS), a highly sulfated glycosaminoglycan is also an important attachment molecule involved in flavivirus infection. Studies have demonstrated that the interaction between flavivirus particles and heparin sulfate molecules on target cells is a major determinant of infectivity (Su et al., 2001). A number of different flaviviruses have been shown to bind to HS, and treatment of flavivirus virions with HS prevents viral infection by blocking cellular attachment. (Chen et al., 1997b; Lee and Lobigs, 2000; Su et al., 2001; Germi et al., 2002). Interestingly, mutant strains of Murray Valley encephalitis virus (MVEV) that demonstrated variations in their ability to bind HS also were found to have altered host cell tropisms (Lee and Lobigs, 2000).

A number of other proteins including αvβ3 integrin (Chu and Ng, 2004b) and various heat shock proteins (Reyes-Del Valle et al., 2005; Ren et al., 2007) have also been implicated in flavivirus infection, demonstrating the plasticity of flaviviruses in infecting host cells. Antibody-enhanced infection, a process by which opsonization of antibody-complexed viral particles facilitates infection of F_c receptor-bearing cells, has

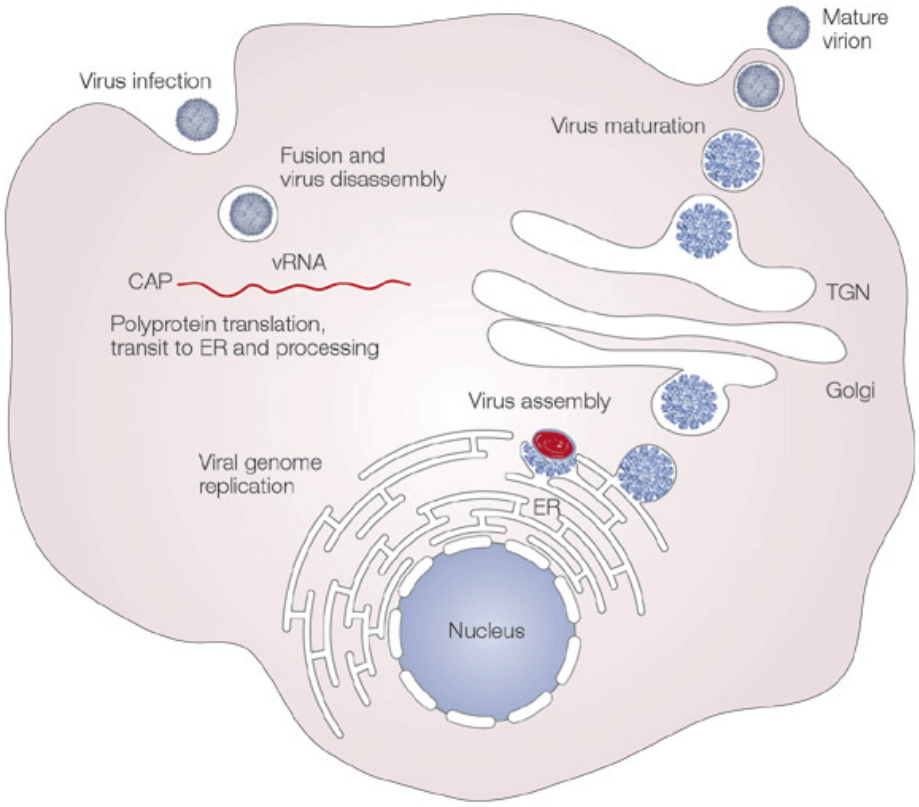

Figure 1.3: Flavivirus life cycle. Flaviviruses enter host cells through receptor-mediated endocytosis and fusion of the virion lipid envelope with the endosomal membrane in the low pH environment of the late endosome releases the nucleocapsid into the cytoplasm. Translation, genome replication and viral particle assembly all take place cytoplasmically and in association with perinuclear membranes and virions are released from infected cells after maturation in the exocytic pathway. Reprinted with permission (Mukhopadhyay, Kuhn, and Rossmann, 2005).

been demonstrated to enhance flavivirus infection (Peiris and Porterfield, 1979; Halstead, 2003), however the role for these phenomena in the pathogenesis of flavivirus infection remains unclear. Upon binding of the virion with the internalization receptor, flaviviruses enter cells (Fig. 1.3) through clatharin coated pits (Chu and Ng, 2004a) and are quickly trafficked to pre-lysosomal endocytic vesicles within the cell (Gollins and Porterfield, 1985). Here, the low pH environment induces a conformational change in the surface components of the virion, and trimers of the E protein fusion loop cross the endosomal membrane in a process that creates a channel to allow release of the viral nucleocapsid into the cell cytoplasm (Gollins and Porterfield, 1986; Modis et al., 2004).

Translation, polyprotein processing and genome replication

Flavivirus RNA is directly infectious, and upon release from the endosome can be translated in the host cell in the absence of any viral proteins. Translation is primarily cap-dependent, and initiates by ribosome scanning to the AUG start codon. There is evidence to suggest that RNA secondary structures in the 5'UTR of mosquito-borne flaviviruses act to pause ribosome scanning at the authentic start codon, initiating translation of the ORF (Clyde and Harris, 2006). The translated polyprotein localizes to the ER membrane, where it is co- and post-translationally cleaved by viral and host cell proteases (see Proteins).

RNA replication involves the formation of membrane-associated replicase complexes, which contain the small hydrophobic nonstructural proteins, viral RNA, and likely some host factors (Lindenbach, Thiel, and Rice, 2007). Negative-sense RNA is synthesized from the WNV genome, and this is subsequently used as a template for replication of the positive strand. Critical for viral RNA replication is the presence of complementary cyclization sequences (CS) in the 5’ end of C (and 5'UTR for tick-borne flaviviruses) and 3’UTR of the flavivirus genome. The base pairing of these 11nt regions

facilitates the circularization of the genome, and has been shown to be essential for replication of flaviviruses (Khromykh et al., 2001a; Lo et al., 2003).

Assembly and release

The flavivirus nucleocapsid forms in the cytoplasm of infected cells in a process that is not well understood, and is composed of a single viral RNA molecule associated with multiple copies of C dimers (Kiermayr et al., 2004). These nucleocapsids bud into the lumen of the ER of the infected cell, where they acquire a lipid envelope containing prM and E. Noninfectious particles, known as slowly sedimenting hemagglutinating particles, but referred to in more recent literature as sub-viral particles (SVPs) composed of host cell membranes and prM and E (but lacking the viral nucleocapsid) are also produced in flavivirus-infected cells (Stevens and Schlesinger, 1965; Brandt, Cardiff, and Russell, 1970; Smith et al., 1970). Viral particles accumulate in the exocytic pathway, at which point these provirions are poorly infectious and contain prM on their surface. Upon virion maturation in the Golgi apparatus, prM is cleaved to M by the host cell protease furin. Additionally the surface proteins of viral particles are glycosylated and post-translationally modified in the exocytic pathway (Mason, 1989; Courageot et al., 2000), and these maturation steps confer fusion activity and infectivity upon the virus particles (Zhang et al., 2003b; Hanna et al., 2005). The mature viral particles are then released from the cell via exocytosis.

West Nile Virus

West Nile virus (WNV), first isolated in 1937, was observed circulating in the Eastern hemisphere for nearly 75 years while causing little to no serious illness and garnering minimal attention from researchers and health professionals. This changed

dramatically, however, with the sharp increase in severe disease associated with WNV infection in Europe in the 1990s, and the subsequent introduction of WNV to North America in 1999. Today, just 10 years after this introduction to the Western Hemisphere, WNV is considered endemic in North America, and is rapidly spreading throughout the Caribbean and South America.

EPIDEMIOLOGY

West Nile virus was first isolated in 1937 from a febrile woman in the West Nile province of Uganda, by researchers conducting yellow fever surveillance (Smithburn et al., 1940). Although no information on the disease associated with WNV was provided by this patient, WNV outbreaks in Israel in the 1950s provided the first picture of West Nile disease in humans. Disease severity, pathology and susceptible populations (discussed below) were identified as a result of this outbreak, in which more than 1/3 of a small village became infected and four people died, the first deaths attributed to naturally acquired WNV infection (Hayes, 2001b). Following the Israeli outbreaks of the 1950s, WNV was responsible for only sporadic human (Hayes, 2001a) and equine (Castillo-Olivares and Wood, 2004) cases. From the time of its isolation in 1939 until 1999, WNV was found only in the Mediterranean regions of Africa and Europe, West Asia and the Middle East, and the disease that was associated with infection was generally mild and self-limiting (Gubler, Kuno, and Markoff, 2007).

In 1996 a large outbreak of moderate to severe WN disease in the southeastern region of Romania was the seminal event that began to change the way in which WNV was viewed. Of the 393 confirmed cases of WN disease in Romania, 352 were classified as acute central nervous system (CNS) infections, and there were 17 fatalities. As was seen in the Israeli outbreaks, elderly individuals were disproportionately afflicted with severe neurologic disease (Tsai et al., 1998). The Romanian outbreak was followed

closely by a large outbreak of human WN disease in the Volgograd region of southern Russia in 1998. In a 2 month period, over 800 patients were admitted with acute febrile and encephalitic diseases. Of these, 22% were confirmed to be acute WNV infections, and all told nearly 500 cases were considered to be a result of WNV infection, with 40 fatalities (Platonov et al., 2001).

In August, 1999 New York State officials began investigating a rash of bird deaths stretching from Buffalo to New York City (NYC). Simultaneously, an unusually high number of encephalitic disease cases (now thought to be the first human cases of WN disease in the US) were being investigated at a hospital in Queens, NY (United States General Accounting Office, 2000). Originally thought to be St. Louis encephalitis virus (SLEV) infection based on broad serological diagnoses, an army of researchers soon concluded that the bird deaths and human encephalitides were being caused by West Nile virus (Anderson et al., 1999; Lanciotti et al., 1999; Eidson et al., 2001; Komar et al., 2001). By the end of 1999, 61 laboratory-confirmed cases of human WN disease were reported in the NYC area, with 7 fatalities (Asnis et al., 2000). This marked the first report of WNV activity in the Western Hemisphere, and rapidly thrust a great deal of attention on understanding the virus and associated disease.

In the years following the 1999 NYC outbreak, WNV activity in the US would increase dramatically. In 2000, after the virus successfully survived the North American winter (Nasci et al., 2001), another, albeit smaller, outbreak occurred in NY, and WNV was detected in birds throughout the Mid-Atlantic region of the US (Fig. 1.4). Throughout the next several years, WNV activity steadily expanded westward through North America eventually reaching California in 2002 (Gubler, 2007). The peak of human WN disease in the US occurred in 2002, when nearly 10,000 cases of WN disease were reported, 2800 of which were severe meningoencephalitis, and 264 individuals died

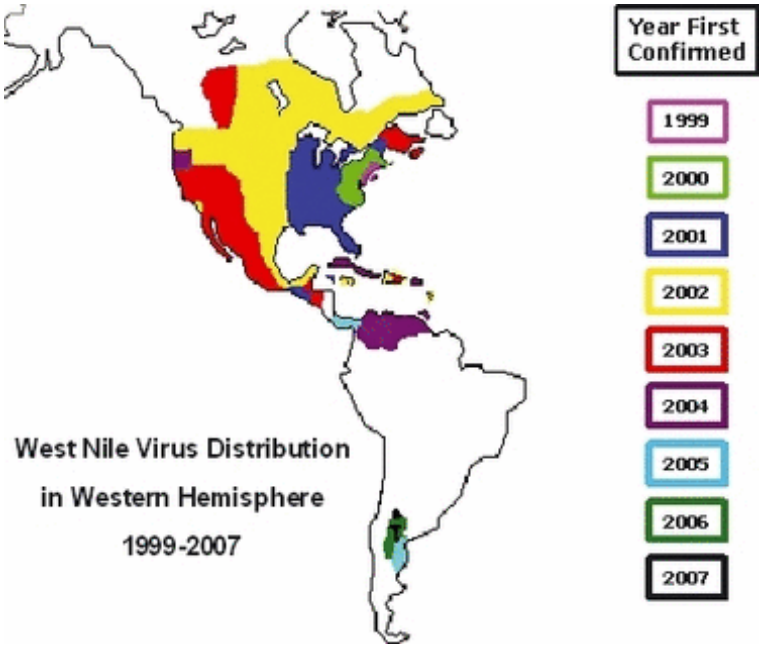

Figure 1.4: WNV distribution. Distribution of WNV in the Western Hemisphere. Colors indicate the year in which WNV was first identified in a particular region. Reprinted with permission (Artsob et al., 2009).

(CDC, 2009). WNV has now been detected in every contiguous US state, southern Canada (Weir, 2000), Mexico (Blitvich et al., 2003), the Caribbean (Dupuis, Marra, and Kramer, 2003), and South America (Mattar et al., 2005). WNV is now considered endemic in the US, with low-level transmission and sporadic illness occurring every summer. To date there have been over 28,000 confirmed cases of WN disease in the US since 1999, and this has led to 1130 deaths (CDC, 2009). The recent emergence of WNV in the western hemisphere has highlighted the ability of flaviviruses to gain footholds in new environments and increased interest in the development of treatments and vaccines against this and other emergent flavivirus diseases.

TAXONOMY

The flavivirus genus is subdivided into complexes based on similarities in serological activity (as determined by E protein amino acid homology), and as such WNV is found in the Japanese encephalitis serocomplex, along with JEV, SLEV and MVEV. Sequence analysis of WNV isolates collected worldwide since 1937 has revealed

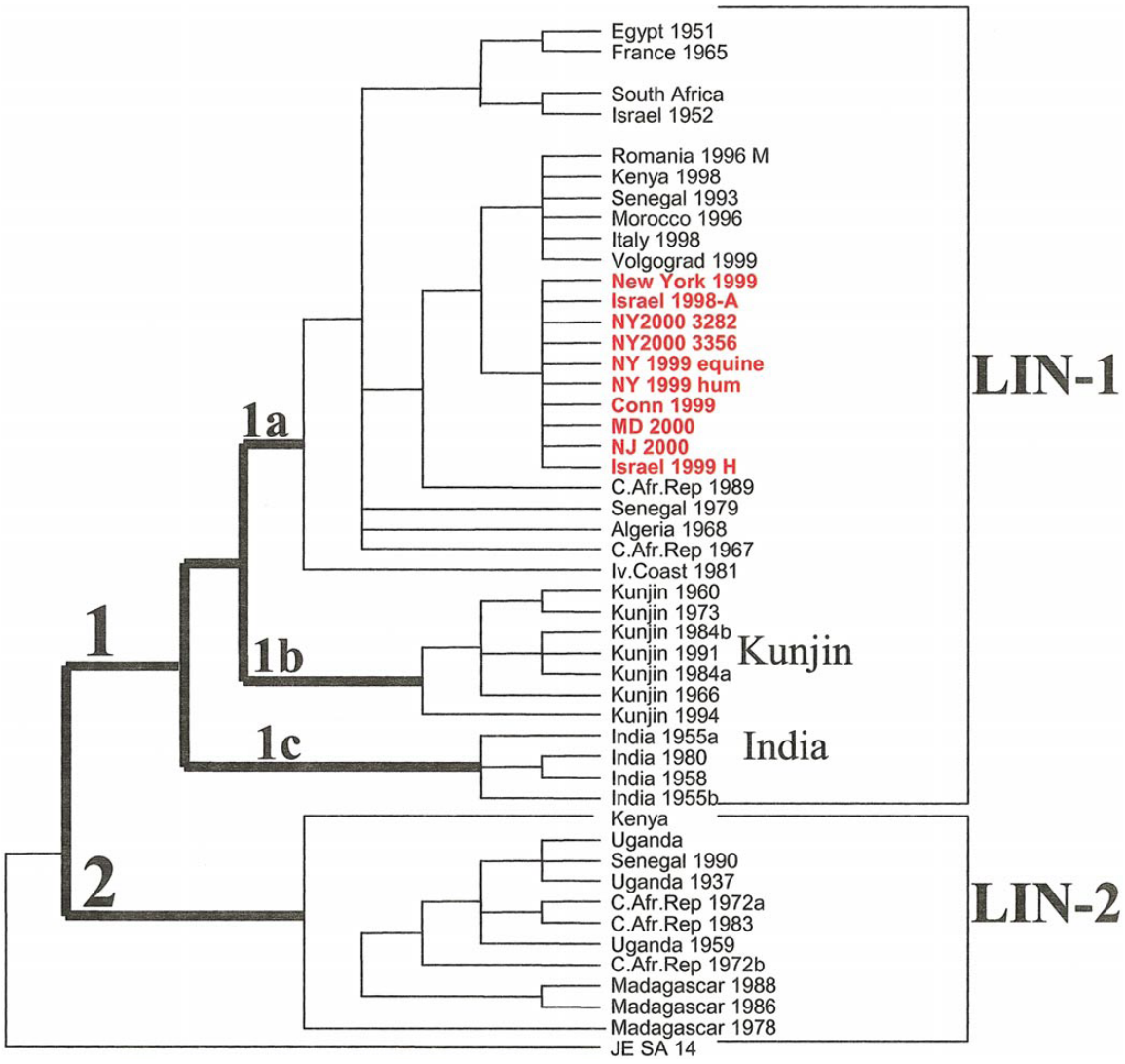

Figure 1.5: Phylogenetic analysis of WNV. Phylogenetic tree created by parsimony analysis of aligned nucleotide sequences from 47 WNV isolates. JEV SA14 was used as an outlier, and the closely related members of the US/Israel clade are highlighted in red. Reprinted with permission (Lanciotti et al., 2002).

two distinct lineages of WNV (Fig. 1.5). Lineage I viruses have typically been isolated from human epidemics where severe disease and mortality was observed. Within Lineage I, viruses isolated from the 1950s Israeli, the 1996 Romanian, the 1998 Russian, and the 1999 NY outbreaks cluster together into the Lineage Ia subgroup, while isolates of Kunjin virus from Australia and WNV from India are more divergent and are grouped

into Lineage Ib and Ic respectively (Fig. 1.5) (Lanciotti et al., 2002). Interestingly, an isolate of WNV from NYC in 1999 is most closely related to WNV from a goose found dead in Israel in 1998, indicating that the virus that emerged in the Western Hemisphere likely originated in the Mediterranean (Lanciotti et al., 1999). The virus introduced in NY in 1999 has changed little over the last 10 years, however isolates from Mexico (Beasley et al., 2004a) and Texas (Davis et al., 2004) in 2003 appear to have accumulated genetic changes that attenuate neuroinvasiveness. Lineage II viruses have been isolated only from Africa, and these were associated with mild or asymptomatic disease and were often found while conducting surveillance for other disease-causing agents, as was the case with the original 1937 isolation of WNV.

TRANSMISSION

WNV transmission occurs in an enzootic cycle in which avian species serve as amplifying hosts and mosquito species act as vectors (Fig. 1.6). WNV has an incredibly large host and vector range; it has been isolated from over 300 species of birds and is transmitted by mosquitoes with a worldwide distribution. Studies indicate that a circulating WNV concentration of about 10^7 pfu/ml of serum is necessary to infect a naive mosquito taking a bloodmeal (Goddard et al., 2002). Many avian species develop a circulating viremia in excess of 10^7 pfu/ml (Komar et al., 2003a), thus making them important if not essential in the maintenance of WNV in nature. In particular, it is believed that the house sparrow is a key component of the urban transmission cycle of WNV in North America (Komar et al., 2003b; Godsey et al., 2005). Infected humans, in contrast, are thought to develop viremia of only about 10^3 pfu/ml (Hayes et al., 2005a), far below the level at which transmission to naive mosquitoes has been observed. In addition to the cycle of transmission between birds and mosquitoes, a number of other

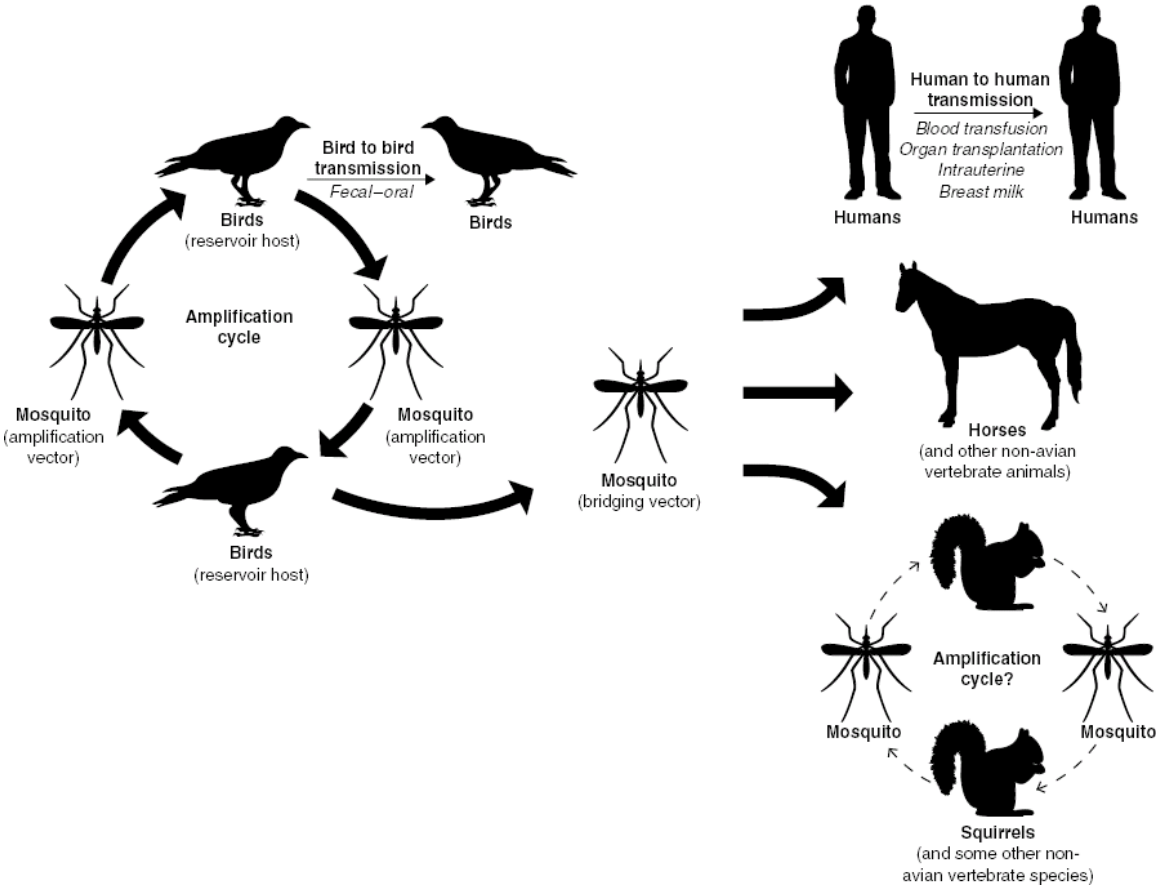

Figure 1.6: Transmission cycle of WNV. Thick arrows indicate common routes of transmission, thin arrows represent rare transmission events, and dashed arrows indicate routes of transmission yet to be confirmed in nature. Reprinted with permission (Blitvich, 2008).

vertebrates have been shown to be capable of supporting WNV infection, many of which develop some form of disease. WN disease has been observed in horses, dogs, cats, rodents, bats, and alligators (Komar, 2003). Interestingly it is thought that young alligators may develop high enough viremia to infect naive mosquitoes, thus aiding in WNV transmission and maintenance (Klenk et al., 2004).

Mosquito

Mosquitoes are considered far and away the primary vector responsible for transmission of WNV. Worldwide, *Culex* species of mosquitoes serve as the principal vector for WNV transmission. Although WNV has been found in nearly 60 unique species of mosquitoes in North America alone, less than 10 of these are considered to be important for WNV transmission (Hayes et al., 2005a). In North America, the principal WNV vectors are *Cx. pipiens pipiens* in the Northeast, *Cx. quinquefasciatus* in the Southeast, and *Cx. tarsalis* in the west (Gubler, Kuno, and Markoff, 2007). Other North American species including *Aedes* and *Ochlerotatus* (Sardelis et al., 2002; Turell et al., 2005) mosquitoes are competent vectors, however, due to their feeding habits they have been proposed to serve as bridge vectors for the infection of mammalian hosts and not integral for maintenance of WNV in nature.

Following uptake of WNV from the bloodmeal of an infected bird, WNV enters the midgut of the mosquito. Here it infects midgut epithelial cells and quickly disseminates to the posterior midgut by day 2 post-infection and is observed in the anterior midgut by days 3-5. By day 6 WNV is well-disseminated in the mosquito, with viral antigen detectable in tissues of the heart, abdomen, thorax, and head (Girard, Klingler, and Higgs, 2004). WNV is first observed in the salivary glands on day 8, and thus it is believed that at this point the mosquitoes are capable of transmitting WNV to naive hosts. Virus persists in the salivary glands for up to 21 days, although this infection is associated with cell death that makes it likely that WNV transmission efficiency decreases the longer a mosquito is infected (Girard et al., 2005). WNV antigen is also transiently observed in the ovaries of infected mosquitoes (Girard, Klingler, and Higgs, 2004), supporting the hypothesis that WNV can be transmitted transovarially in mosquitoes (Baqar et al., 1993; Miller et al., 2000; Anderson and Main, 2006).

Upon encountering a naive host, the female WNV-infected mosquito probes with its proboscis through the dermis and capillary bed until it finds a suitable blood vessel from which to extract blood. During this probing, WNV-containing saliva is deposited into the dermis and bloodstream, with an estimated 10^5 pfu being inoculated and as much as 10^2 pfu directly entering the bloodstream (Styer et al., 2007). It is believed that mosquito saliva is an important factor in the inoculation of WNV from infected mosquitoes, and enhancement of infection using mosquitoes instead of needles has been observed in mice (Schneider et al., 2006) and chickens (Styer, Bernard, and Kramer, 2006). It is thought that molecules contained in the saliva that alter the host haemostatic response act to inhibit inflammation at the site of inoculation, and this allows enhanced WNV replication and spread early in infection (Schneider and Higgs, 2008).

Non-vectored

Transmission in the absence of a hematophagous vector has been observed for WNV, although this is believed to play a minor role in the overall transmission cycle and maintenance of the virus. In humans this phenomena was first observed during a particularly large WNV outbreak in Michigan in 2002, when more than 600 individuals developed WN disease and there were 51 fatalities (CDC, 2009). During this time, WNV transmission via blood and organs transfused from an infected individual led to the development of WN encephalitis (WNE) in one recipient and WN fever (WNF) in a woman who had recently given birth (MMWR, 2002b). Later that month, it was reported that WNV was detectable in the woman's breast milk, and that she had transmitted the virus to her newborn child, although the infant did not develop clinical disease (MMWR, 2002a). These events marked the first time human to human transmission for WNV had been observed, and led to universal screening of blood and tissue donations for WNV.

In addition to these rare human events, non-vectored transmission of WNV has been observed in avian populations, and is thought to be possible in other vertebrates as well. In February of 2000, WNV was isolated from the brain of a red-tailed hawk that died in NY (Garmendia et al., 2000). This was particularly unusual due to the time of year, and it was hypothesized that this animal did not become infected with WNV through the bite of a mosquito (nearly none are active in February in NY) but instead through preying on an infected rodent. Indeed, oral transmission of WNV to birds feeding on infected mosquitoes and rodents has been observed in laboratory settings (Komar et al., 2003a). Oral transmission of WNV between rodents has also been observed (Odelola and Oduye, 1977). In addition to oral transmission, several species of birds, including American crows, blue jays, black-billed magpies, ring-billed gulls and domestic geese (Langevin et al., 2001; Swayne et al., 2001; McLean et al., 2002; Komar et al., 2003a), were shown to become infected with WNV simply by being in close contact with infected birds, although in some cases oral transmission could not be ruled out (Komar et al., 2003a). Infectious WNV has been detected in other animals including fox squirrels (Root et al., 2006), chipmunks (Platt et al., 2007), and hamsters (Xiao et al., 2001; Tesh et al., 2005; Tonry et al., 2005), although direct transmission has not been observed in these animals. Thus while a plethora of experimental evidence exists for the ability of WNV to be transmitted in the absence of a vector, the overall contribution of this phenomena to WNV transmission and maintenance in nature is not well understood and likely minor.

DISEASE AND PATHOLOGY

Although the first recognized cases of WN disease occurred in Israel in the 1950's, little was known about clinical WNV disease until recently. A rise in serious WNV disease in the 1990s followed by the introduction in 1999 and subsequent epidemic of WNV in North American is believed to be correlated with an increase in the

prevalence of the more neurotropic Lineage I WNV. This recent increase in severe disease has led to an increasingly clearer clinical picture of WN disease; however large gaps in our understanding still remain. Although avians are believed to be key maintenance hosts for WNV, many bird species, particularly in North America, appear to be susceptible to fatal disease from Lineage I WNV. In addition to birds and humans, reptiles, equines and rodents all develop fatal disease from WNV infection. A number of animal model systems have been developed to examine details of WNV pathogenesis and disease, and to evaluate novel therapeutics and vaccines. Parenterally inoculated rodents, in particular mice and hamsters, are the most commonly used models of WN disease. Additionally, a number of recent vaccine candidates have been evaluated using a non-human primate (NHP) model of WNV infection (Arroyo et al., 2004; Pletnev et al., 2006).

Avian

Not long after the isolation of WNV from humans, scientists began obtaining WNV isolates from a number of different bird species (Work, Hurlbut, and Taylor, 1953; Schmidt and Said, 1964) and it was proposed that birds play a role as a reservoir for WNV (Work, Hurlbut, and Taylor, 1955). Although important for WNV transmission and spread (via their migratory patterns), birds were not generally observed with symptomatic WN infection, and thus little study was done on avian disease. In the 1990s, the rise in prevalence of Lineage I WNV and its spread into the Western Hemisphere led to increased disease in birds (Savage et al., 1999; Bin et al., 2001) that expanded our knowledge of avian WNV pathology. It was, in fact, the unusual rash of bird deaths in NY state that prompted the initial investigations that first identified WNV in North America (United States General Accounting Office, 2000).

While WNV is capable of infecting hundreds of different bird species, symptomatic disease is not a hallmark of all avian infections. In one study, about 1/3 of the species experimentally infected developed signs of illness, most notably corvids and gulls (Komar et al., 2003a). The outbreak of WNV in NY in 1999 provided an unprecedented opportunity to examine the disease progression and post-mortem pathology of WNV in birds, as a number of highly prized species at the Bronx Zoo became infected and were treated by veterinarians (Steele et al., 2000). These birds demonstrated symptoms of encephalitis including weakness, ataxia, tremors, difficulty walking/standing and convulsions. Specific pathology varied amongst bird species, but WNV was found disseminated throughout the body with the myocardium and tissues of the central and peripheral nervous systems heavily infiltrated. Lesions were observed in multiple organs; however the cerebellum appeared to be a specific target of WNV, with severe lesions and hemorrhage observed. The liver, heart, and kidneys were also consistently found to be sites of WNV infection (Steele et al., 2000; Kramer and Bernard, 2001). WNV infects avian monocytic cells, and it is speculated that infection of these cells facilitates viral dissemination and cellular dysfunction that could be responsible for the hemorrhagic pathology observed. It is believed that most avian species die from a combination of neural and extraneural complications caused by WNV infection.

Rodent

Although not believed to play an important role in WNV transmission in nature, the laboratory use of rodent models to study WNV infections and pathogenesis has advanced our understanding of the mechanisms of WN disease. The two most widely utilized rodent models to study WN disease are parenterally inoculated mice and hamsters. Both species develop clinical disease consistent with meningoencephalitis in most inoculated animals, and mice and hamsters develop measurable innate and adaptive

immune responses to infection. Thus, most of our knowledge of WNV infection and pathogenesis comes from these rodent models.

Mice are most commonly inoculated with WNV by either the IP or SC route. While the IP route generally produces less variability in disease pathology and immune responses from animal to animal, the SC route, in particular in the footpad, is considered to be the most biologically relevant as it most closely mimics the bite of an infected mosquito. Even still, by most accounts SC needle inoculations are far more damaging to host tissues and inoculate virus much deeper than is a mosquito bite. Infection of mice produces disease similar to the most severe cases in humans. Viral encephalitis is observed in most infected animals, and this disease is often fatal. As in humans, animal to animal transmission is rarely if ever observed (Kramer and Bernard, 2001). Following SC inoculation of WNV, virus is detectable in the blood of infected mice for the first 3-4 days, and in the spleen, heart and kidneys by 1-4 days after infection (Garcia-Tapia et al., 2007). WNV is especially prevalent in lymphoid tissues early in infection, and these are believed to play a key role in the dissemination of virus to distant tissues. At 4-6 days, WNV is detectable in the brains of infected mice, and this correlates with the onset of clinical disease with symptoms including ataxia, weakness and bilateral hind limb paralysis (Kramer and Bernard, 2001). At this time infectious WNV is no longer present in the bloodstream, and WNV-specific IgM is detectable (Diamond et al., 2003). Days 7-9 are characterized by high viral load ($>10^5$ pfu/gram of tissue) and lymphocyte infiltration in neuronal tissues. As is observed in birds, the cerebellum is a primary target in WNV infection, and cells here and elsewhere in the CNS and peripheral nervous system (PNS) undergo virus-induced apoptosis (Diamond et al., 2003; Shrestha, Gottlieb, and Diamond, 2003; Samuel, Morrey, and Diamond, 2007). Also by day 7 WNV-specific IgG levels begin to rise, and around this time the interplay between the immune response

and viral infection determines disease outcome. In animals unable to clear infection, clinical signs of encephalitis become rapidly evident, and mice begin to die around day 7. Animals that succumb usually do so by 12-14 days after infection, and surviving mice demonstrate high antibody titers and resistance to re-infection.

Recently a hamster model of WN disease has been developed (Xiao et al., 2001) and used to evaluate vaccine candidates. The disease pathology and clinical presentation of WNV infection in hamsters is very similar to that observed in mice; however disease progression is slightly protracted and thought to be more similar to human and equine infection. SC inoculation of 10^4 $TCID_{50}$ of WNV was shown to cause productive infection in 100% of inoculated hamsters, and doses of 10^6 consistently produce clinical disease in 80-100% of hamsters and mortality rates of 40-100% (Xiao et al., 2001). Following SC inoculation with WNV, circulating viremia peaks at about 10^5 pfu/ml, and persists for about 6 days. Neutralizing antibody is detectable in the blood by day 6, and infectious virus is no longer detectable at this point. Also on day 6 infected hamsters begin to show clinical signs of CNS injury (weakness, tremors, paralysis) similar to mice and animals that succumb to illness do so from day 7 to 14. Virus can be detected during the second week of infection in the brain at levels exceeding 10^6 $TCID_{50}$. Virus distribution is similar to that observed in mice, and again neural tissues are heavily infiltrated. Surviving hamsters develop high antibody titers, making them useful for vaccine evaluation.

Non-human primate

The clinical presentation of WNV infection in non-human primates (NHPs) is similar to that of humans in that the vast majority of infections result in subclinical disease. The largest and best studied outbreak of WNV in NHPs occurred in 2002 at the Tulane National Primate Research Center in Louisiana, where an estimated 1,500

primates (36% of the colony) were infected (Ratterree et al., 2003). Rhesus macaques, pigtail macaques, and baboons were all found to be seropositive for WNV infection, although no clinical signs of illness were observed in any of the animals. A subsequent study by the same group examined the pathogenesis of WNV in rhesus macaques in a laboratory setting following SC inoculation (Ratterree et al., 2004a). NHPs inoculated with WNV NY99 develop low but detectable levels (peaking at 10^2-10^3 pfu/ml) of infectious virus in the serum during the first 5-6 days of infection. IgM responses are detectable by day 7 correlating with the loss of infectious virus in the serum, and peak at 14-21 days post-infection. Neutralizing IgG antibodies are detectable by 10 days, and these titers peak at about 1:320-1:640 by 21-45 days post-inoculation. Interestingly, although no animals developed neurological illness, WNV RNA was detected in the spinal fluid of one macaque, indicating the virus is capable of entering the central nervous system of NHPs. In addition to this parenteral challenge model, others have utilized intracranial inoculation of WNV directly into the brain of NHPs (Arroyo et al., 2004). While this route of inoculation does cause clinically detectable encephalitis in NHPs, it is unable to replicate early peripheral events in WNV infection and does not simulate infection from mosquito inoculation.

Human

The emergence of Lineage I WNV in Europe in the 1990s and North America in the 2000s led to a sharp rise in human disease, and thus our knowledge of the pathogenesis of WNV in humans has increased vastly during this time. It is estimated that 80% of all human WNV infections are asymptomatic (Mostashari et al., 2001). Of the 20% of individuals that develop symptomatic WN disease, most develop West Nile fever (WNF), a flu-like febrile illness that is often accompanied by muscle weakness and mild cognitive impairment that lasts up to 60 days. Severe disease is observed in <1% of all

WNV infections. In these individuals meningitis and encephalitis (WNE) are commonly observed, and in about 13% of these seriously ill patients acute flaccid paralysis occurs. In some cases this paralysis results in respiratory failure leading to death (Hayes et al., 2005b), although it is thought most patients die as a result of massive neural necrosis. Severe WN disease disproportionately affects the elderly, with a mean age at death of 78 years (Mostashari et al., 2001; Murray et al., 2006). Persons between 50-59 years of age have a 10-times higher incidence of severe disease, while persons aged 80 years or older have a 43-times higher incidence of severe disease compared to adults between 20-40 years of age (Nash et al., 2001; Murray et al., 2006). The overall mortality of the elderly in the first year post-infection is also significantly increased compared to age-matched controls (Murray et al., 2006), further demonstrating the impact of infection on this vulnerable population.

Following inoculation with WNV from the bite of an infected mosquito, it is thought that WNV replicates at the site of inoculation (perhaps in dendritic cells) and spreads to the lymph nodes and bloodstream. There is typically a 2-14 day incubation period from the time of infection to the onset of clinical illness. (Mackenzie, Gubler, and Petersen, 2004). During this time, WNV is often cleared resulting in asymptomatic infection, however in more severe disease cases WNV crosses the blood brain barrier to gain access to the CNS through a yet unknown mechanism. Infectious virus is occasionally detectable in the blood early after infection, but disappears with the onset of neurological symptoms or resolution of infection. Fatal cases have provided our only understanding of WNV neurotropism, and in these individuals WNV is found throughout the CNS, with the brain stem and anterior horns being particular targets. Pathology is a consequence of cell death, although is it not known the relative contributions of viral replication and host immune responses to this loss of neurons. The deep gray nuclei,

spinal cord, and brainstem appear to be most affected by this necrosis (Hayes et al., 2005b). The acute flaccid paralysis observed in some patients is associated with lymphocyte infiltration to and inflammation of the spinal cord and a loss of anterior horn cells. In survivors of WN disease, long-term neurological sequaela is commonly observed, with cognitive impairment persisting for over 1.5 years after even mild cases of acute infection (Sejvar, 2007)

Flavivirus Vaccine Development

While the development of vaccines to prevent disease associated with infection by YFV and JEV have demonstrated that flavivirus diseases can be prevented by vaccination, these vaccines are not without drawbacks. Furthermore, no vaccines exist for DENV and WNV. Thus a great deal of effort has been put into the development of new flavivirus vaccines. Despite many promising candidates, however, there have been no new human vaccines licensed to prevent flavivirus disease in humans, and there remains a need for new vaccines.

ANTIGENIC STRUCTURES

There are a number of flavivirus components that have been shown to elicit antibody production. The 3-dimensional structure for the E protein of TBEV has been solved, and it has been shown to closely resemble that of other flaviviruses (Rey et al., 1995; Zhang et al., 2003a). These studies have revealed that the E protein of flaviviruses lays flat against the surface of the virion. In addition to this, E was shown to be comprised of three domains. Of particular interest, domain III has been studied in great detail and has been shown to contain regions critical for virus neutralization, receptor binding (and therefore cellular tropism), and infectivity (Lindenbach, Thiel, and Rice,

2007). Additionally, truncated forms of the E protein (trE) have been engineered by removal of the carboxy-terminal transmembrane region of E facilitating secretion as a soluble product (Men, Bray, and Lai, 1991; Jan et al., 1993; Delenda, Frenkiel, and Deubel, 1994; Allison et al., 1995). These trE proteins have also been the primary antigenic component of a number of flavivirus subunit vaccine candidates, although as described below, side-by-side studies have suggested that SVPs are more effective than trE in eliciting protective immune responses in vivo.

In order for the proper folding, membrane association, and assembly of the flavivirus E protein to occur, the prM protein must be co-expressed with E (Konishi and Mason, 1993). During flavivirus infection, sub-viral particles (SVPs) composed of the prM and E proteins but lacking the nucleocapsid are released in addition to infectious virions (Mason et al., 1991a). Recombinant forms of SVPs have been produced in a number of different expression systems, and these have been studied extensively for their ability to protect against lethal flavivirus challenge and induce high levels of neutralizing antibodies in animal model systems (Mason et al., 1991a; Konishi et al., 1992; Qiao et al., 2004). They have been shown to elicit strong protective immune responses that involve humoral and cell-mediated activity (Konishi et al., 1997), and it is these characteristics that have made the use of SVPs in flavivirus vaccines an attractive approach.

In addition to infectious virions and SVPs, flavivirus-infected cells also secrete the NS1 protein (Brandt et al., 1970), and antibodies against NS1 can be detected in sera from infected humans (Falkler, Diwan, and Halstead, 1973). It has been shown that secreted NS1 exists as a homodimer (Winkler et al., 1988) and in addition to secretion into the surrounding milieu, NS1 can also remain associated with the surface of infected cells (Stohlman et al., 1975; Cardiff and Lund, 1976). These findings have led to more

recent observations that immune responses directed against NS1 play an important role in viral clearance (Schlesinger et al., 1990; Chung et al., 2007). Vaccination with NS1 in the absence of all other viral proteins has been shown to protect non-human primates (NHPs) from lethal challenge (Schlesinger et al., 1986) and based on this NS1 has been included in a number of promising vaccine candidates (Lin et al., 1998; Lieberman et al., 2007; Schepp-Berglind et al., 2007)

LICENSED HUMAN VACCINES EXIST TO PREVENT FLAVIVIRUS DISEASES

The existence of efficacious vaccines for a handful of flavivirus diseases has demonstrated that flavivirus diseases can be prevented by vaccination. Licensed vaccines are commercially available for three flavivirus diseases: YF, JE, and TBE although not all are universally accepted throughout the world. The YF vaccine, based on the attenuated YFV-17D strain of YFV, has been credited with preventing outbreaks of urban YF, and until recently, this vaccine was considered to be one of the safest and most efficacious live-attenuated vaccines (LAVs) in use. However, the recent observation that acute viscerotropic disease (YEL-AVD) can be caused by this vaccine has become a concern. A formalin-inactivated JEV vaccine produced in suckling mouse brain (SMB) has been in use in Japan for over 40 years; however its safety profile has recently come under scrutiny and this vaccine is no longer being manufactured. An LAV for JE is currently licensed for use in a few select nations. However, due to its production in a cell line unacceptable for vaccine generation in developed countries, it is not used worldwide. An inactivated viral vaccine (INV) is also available for TBE, and has demonstrated an excellent safety and efficacy record in Central and Eastern Europe, but is not widely used. Both the TBE and JE INVs suffer from high production costs and the requirement for multiple immunizations that make their use practical only in affluent regions of the world.

Thus despite the availability of these vaccines, there is a tremendous need for new flavivirus vaccines. The development of vaccines against dengue, arguably the most medically important of all flavivirus diseases, is complicated by the fact that low levels of cross-reactive immunity to individual DENV serotypes have been shown to be a risk factor for severe forms of the disease. Thus, there is widespread agreement that dengue vaccines must be tetravalent, and must be able to simultaneously induce strong immune responses to all four serotypes of DENV, and development of products to accomplish this has been difficult. Furthermore, the recent emergence of WNV in the Western hemisphere has highlighted the ability of flaviviruses to gain footholds in new environments and increased the urgency of development of treatments and vaccines against this and other emergent flavivirus diseases.

WNV VACCINES IN DEVELOPMENT FOR HUMAN USE

In order to address the lack of vaccines for WN diseases, research has focused on the development of new products based on live-attenuated (LAV), inactivated (INV), viral-vectored, genetically engineered live-attenuated, DNA, and subunit formulations. ChimeriVax WN, a vaccine based on the chimerization of the YFV-17D genome with structural genes of WNV, has been aggressively evaluated in preclinical and clinical tests. A DNA vaccine against WNV has begun clinical evaluation, while other vaccines based on subunit, live-vectored and replicon-based technologies have performed well in preclinical testing, and some appear poised for clinical trials. Based on these encouraging results, it seems possible that the next decade will see the licensure of a new vaccine to prevent WN disease in humans.

Subunit vaccines

The development of subunit vaccines containing purified antigenic components to prevent WN disease has been investigated for many years. Early studies using Kunjin

virus demonstrated that vaccination with purified SVPs or fragments of E purified from virus-infected cells was capable of inducing a humoral immune response in rabbits, although contamination of the SVPs with infectious virus complicated interpretation of these data (Della-Porta and Westaway, 1977). More recently, recombinant baculoviruses have been utilized to express the structural proteins of WNV with favorable results (Qiao et al., 2004), although this study highlighted the difficulty of obtaining purified flavivirus antigens from insect cells and the requirement of multiple vaccinations or vaccination with large amounts of antigen in order to elicit useful levels of efficacy. One of the most promising subunit vaccine candidates is a WNV product containing purified trE and NS1 proteins that is being developed by Hawaii Biotech. This product is based on combining a drosophila cell-expressed trE with a drosophila cell-expressed NS1 protein and an experimental adjuvant (Lieberman et al., 2007). The high levels of proteins produced by these cells (as high as 25mg per liter of culture) indicate that this product can be economically manufactured (Lieberman et al., 2007), and evaluation of this vaccine candidate in a hamster model for WNE has shown protection from lethal WNV challenge for up to one year after immunization (Watts et al., 2007). Subunit vaccine candidates such as this could prove useful in populations such as the elderly and immunocompromised for which application of LAVs is contraindicated.

Virus-vectored vaccines

A number of different viral vectors have been explored for flavivirus vaccine development. While these products have generally demonstrated high levels of efficacy and safety, prior immunity to the viral vector that prevents immunization to the flavivirus antigens has prevented these vaccine platforms from becoming widely accepted. The choice of viral vector is an important consideration when designing these vaccines, as they need to demonstrate tolerance for the insertion of foreign genes and the vectors

themselves are the primary determinants of infectivity, attenuation, and modulation of the host immune response. A recombinant virus vector based on the measles virus (MV) vaccine Schwarz strain was developed to express trE of WNV, and preclinical evaluation in immunocompromised mice demonstrated detectable $PRNT_{90}$ titers after a single immunization and robust titers after two doses (Despres et al., 2005). Furthermore, all mice receiving two doses survived lethal WNV challenge. Previous work from this group using a recombinant MV-vectored HIV vaccine in NHPs indicated that pre-existing immunity to measles did not impair the immune response to HIV (Lorin et al., 2004). The encouraging results of these studies warrant further evaluation of this MV-based vaccine vector, to demonstrate that vector immunity does not prevent immunization in the context of an MV-vectored flavivirus vaccine.

DNA vaccines

Research has recently focused on the development of DNA-based vaccine candidates for flavivirus diseases, due to both the inherent safety of such products and to circumvent vector immunity. Progress has been slowed by low levels of immunogenicity that have required lengthy immunization schedules using extremely large amounts of DNA. A potential step forward in addressing this limited immunogenicity came when it was shown that simultaneous administration of plasmid-encoded prM and E of WNV along with purified formalin-inactivated WNV particles induced a higher neutralizing antibody response than administration of the individual components (Ishikawa et al., 2007). These results lend the possibility of using increasingly smaller doses of DNA and/or protein in future vaccine formulations, making such vaccines more cost effective.

Shortly after the introduction of WNV to North America, a DNA vaccine encoding the WNV prM and E proteins was developed and shown to be efficacious in mouse and horse models of WNV disease (Davis et al., 2001) In 2005 this product

became the first DNA vaccine licensed for use in animals when it was approved for use in horses (USDA, 2005). Following these encouraging developments, a human Phase I clinical trial was initiated using a plasmid construct analogous to the licensed equine vaccine. While all subjects receiving a two- or three-dose schedule of the vaccine (administered at 4mg per dose) demonstrated seroconversion, an individual receiving a single vaccination did not (Martin et al., 2007). Two subjects who were given two doses had barely detectable $PRNT_{50}$ titers of 1:2 and 1:4 respectively, and most of the remaining 12 volunteers who received the full three-dose schedule displayed a decrease in antibody titer at 32 weeks post-vaccination (Martin et al., 2007). While this is an important step forward in flavivirus DNA vaccine development, these results highlight the need for long-term studies that will examine long-lasting immunity, and for further research into optimized formulations that will increase immunogenicity in order to decrease the amount of material required for eliciting protective immunity.

Chimeric live-attenuated vaccines

One of the interesting features of flavivirus biology is the ability to swap structural genes of one virus with those of another within the genus. This has led to the development of a number of chimeric flavivirus vaccine candidates, in which the structural genes of the virus for which the vaccine is being developed are placed into the backbone of an attenuated flavivirus. While the replicational machinery is comprised of components of the backbone virus, the antigenic structures on the surface of the chimeric virions are those of the virus of interest and participate in the induction of immunity. In most cases, this act of chimerization serves to attenuate the phenotype of the resulting virus, and has provided an attractive option for vaccine development. Based on this, the WNV prM/E genes were used to create a chimera with the genetic backbone of DENV-4 (Pletnev et al., 2002). This WN/DEN4 chimeric virus demonstrated an attenuated

phenotype and has been shown to confer complete protection in mice and NHPs at doses as low as 100,000 ffu (Pletnev et al., 2002; Pletnev et al., 2003). The simultaneous development of a WN vaccine candidate using a rationally attenuated DENV-4 backbone containing a 30nt deletion in the 3'UTR that has demonstrated similar efficacy with a higher degree of attenuation (Pletnev et al., 2003; Pletnev et al., 2006), however, make it unlikely that the chimera based on the wt DENV-4 backbone will be evaluated further. A similar chimera, using the backbone of the well-characterized attenuated DENV-2 PDK53 strain and the prM and E genes of WNV (known as WN/DEN-2 PDK53) is safe and efficacious in a murine model of WNV disease (Huang et al., 2005), indicating that chimeras based on the DEN-2 PDK53 may be useful as vaccine candidates for other flavivirus diseases.

Perhaps the most promising vaccine candidate for WN disease has been built on the ChimeriVax vaccine platform that is based on chimeras produced by insertion of various flavivirus structural genes (specifically prM and E) into the YFV-17D backbone. The long-standing history of safety and efficacy of YFV-17D made it an attractive option for use in chimera construction. The ChimeriVax vaccine platform has been used to develop a vaccine candidate for WN known as ChimeriVax-WN. Initial studies were performed using a chimeric YFV-17D virus containing the prM-E of WNV NY99 which displayed a measurable level of neurovirulence in mice, albeit lower than the parental YFV-17D virus (Monath, 2001). To further attenuate this chimeric LAV, three mutations corresponding to those distinguishing the E protein of the SA14-14-2 strain of JEV from its parental virus were engineered into the WNV E. This mutated ChimeriVax-WN (ChimeriVax-WN02) was selected for all subsequent NHP and human studies. These NHP studies confirmed the safety of ChimeriVax-WN02 by demonstrating that intracranial inoculation of NHPs with this product resulted in significantly less

neurovirulence than YFV-17D, and that viremia from ChimeriVax-WN02 was within WHO specifications outlined for YFV-17D (Arroyo et al., 2004). ChimeriVax-WN02 also demonstrated efficacy, as all immunized monkeys were completely protected from WNV viremia following intracranial challenge (Arroyo et al., 2004). Based on these findings ChimeriVax-WN02 was evaluated in Phase I clinical trials where it stimulated robust neutralizing antibody titers and T cell responses against WNV after a single vaccination (Monath et al., 2006). Initial reports of results from Phase 2 trials have indicated a 97% seroconversion rate from one dose (Anonymous, 2006), however these data have yet to be peer-reviewed. Thus, similar to its sister vaccines for JE and DEN, ChimeriVax-WN02 has demonstrated an acceptable safety and potency profile through all preclinical and clinical studies, and warrants further investigation as a potential LAV for WNV.

REPLICATION-DEFECTIVE AND SINGLE-CYCLE VIRUS VACCINES

Replication-defective virus vaccines, which utilize mutant virus strains that contain defects that render them incapable of replicating their viral genomes, or single-cycle virus vaccines (also referred to as single-round infectious particles and pseudoinfectious viruses) which utilize mutant virus strains that are unable to assemble and release progeny virus particles, are a new area of vaccine development being explored for a number of diseases. These classes of vaccines (particularly single-cycle viruses) combine the replicative capacity of LAVs with the safety of INV products without the concern for residual virulence and/or reversion to virulence associated with traditional LAVs, or the difficult production methods (requiring large-scale production and removal of contaminants that can produce adverse reactions) and low potency of INVs. Both replication defective and single-cycle viruses are propagated in complementing or packaging cell lines designed to express the defective gene(s) of the

deliberately mutated virus, allowing for efficient genome replication and packaging, respectively. When these viruses infect normal cells (in a vaccinee for instance) genome replication (replication-defective) or production of infectious progeny (single-cycle) does not occur, but instead the intact functions of the virus serve to drive gene expression. When used as vaccines this gene expression results in the production of viral antigens that can induce protective immune responses without cell-to-cell spread of the virus.

REPLICATION-DEFECTIVE AND SINGLE-CYCLE VIRUS VACCINES FOR OTHER VIRAL FAMILIES

Replication-defective and single-cycle virus vaccines have been developed from herpes simplex viruses (HSV). Initial studies with these DNA viruses demonstrated that vaccination with replication-defective HSV-1 viruses could safely protect mice from lethal challenge (Nguyen, Knipe, and Finberg, 1992; Morrison and Knipe, 1994). Studies using a single-cycle HSV-1 that was unable to synthesize a surface-exposed glycoprotein necessary for infectivity demonstrated that it was possible to propagate such a virus in packaging cell lines (Forrester et al., 1992) and these defective viruses could be used as vaccines (Farrell et al., 1994). Replication-defective adenoviruses have also been explored for their utility as vectors to produce vaccines against diseases caused by several agents (Dudek and Knipe, 2006) including flaviviruses.

It is likely that in the case of positive-stranded RNA viruses, single-cycle virus vaccines would be advantageous over replication-defective products because multiple copies of the viral genome produced by single-cycle viruses in infected cells would lead to high-level expression of viral proteins needed to elicit a protective immune response (the small number of genome copies and associated viral proteins produced by cells infected with replication defective positive-strand viruses would be unlikely to elicit strong immune responses). Single-cycle viruses composed of packaged replicons encoding the intact nonstructural polyprotein of alphaviruses as well as the antigenic

proteins of heterologous pathogens under control of the alphavirus subgenomic promoter have been extensively studied. Current state-of-the-art in packaging of these replicon genomes requires simultaneous electroporation of three different RNA species (the replicon genome encoding the foreign antigen, a helper RNA to supply the alphavirus capsid protein, and a helper RNA supplying the alphavirus glycoproteins) into the same cells. This strategy has been used to develop vaccine candidates for influenza virus (Pushko et al., 1997), Marburg virus (Hevey et al., 1998; Lee et al., 2006), respiratory syncytial virus (Elliott et al., 2007; Mok et al., 2007), and DENV (White et al., 2007). The technology is currently being developed by several companies including Alphavax, which recently reported results from Phase 1 influenza trials (Anonymous, 2007). Although elegant, the method is not without drawbacks that may make these types of vaccines technically difficult to produce and expensive. These problems include the fact that the vaccines are prepared directly from cultures of electroporated cells. Thus there is no amplification step of the type used to produce traditional LAVs; all vaccine is recovered from cells that have been directly electroporated with three synthetic RNAs that need to be repeatedly synthesized following the very high manufacturing standards needed to produce a product for use in humans. Furthermore, the simultaneous replication of three alphavirus RNAs within the same cell has led to concerns about recombination between the genomes that could produce a disease-causing virus capable of developing a spreading infection, requiring additional quality control steps. Finally, repeated vaccinations with packaged alphavirus replicons can lead to the development of adaptive immune response to the alphavirus envelope (Davis et al., 2000). Thus, problems with vector-immunity observed with other types of viral-vectored flavivirus vaccines could have a significant impact on the utility of alphavirus replicon particles as vaccines.

FLAVIVIRUS SINGLE-CYCLE NUCLEIC ACID VACCINE CANDIDATES

Because the non-structural components of flaviviruses are required for efficient genome replication and the preservation of prM and E is crucial for production and secretion of highly immunogenic SVPs from infected cells, the capsid gene was targeted for development of single-cycle flavivirus mutants. The utility of this approach was first demonstrated by Kofler et al. using TBEV (Kofler, Heinz, and Mandl, 2002). These authors reported that deletion of a large internal portion of the C gene completely ablated production of infectious progeny without significantly impacting RNA replication or translation. Cells transfected with in vitro-synthesized RNA with several of these deletions in the C gene (in some cases augmented by specific mutations added to optimize the signal sequence at the start of prM) released large amounts of E protein structurally and antigenically identical to recombinant SVPs. Furthermore, synthetic TBEV RNAs containing these C gene-deletions were found to be highly attenuated in suckling mice (no infectious progeny or disease were detected after intracranial inoculation) yet still capable of eliciting protective immune responses in adult mice that were comparable to those produced by the licensed inactivated TBEV vaccine (Kofler et al., 2004; Aberle et al., 2005). In addition, the RNA vaccine induced strong cellular immune responses not produced by vaccination with the INV (Aberle et al., 2005). Although this RNA-based vaccine candidate was ground-breaking, since it was the first report of a single-cycle flavivirus, the challenges that all RNA-based vaccines face [including stability, production, and limited potency (Cannon and Weissman, 2002)] may prevent single-cycle C gene-deleted RNAs from being developed into a viable vaccine candidates. A similar approach has been taken by creating a C gene-deleted genome of WNV for delivery in a DNA vaccine format (Seregin et al., 2006). When taken into cells, this DNA drives the production of viral RNA that initiates the infectious cycle and leads

to genome replication and secretion of SVPs and NS1. This vaccine candidate was capable of eliciting detectable neutralizing antibody titers and protecting mice from lethal WNV challenge, although protection was only examined after two DNA injections (Seregin et al., 2006), highlighting another challenge (limited potency) in nucleic acid vaccine development.

REPLIVAX: A PARTICLE-BASED, SINGLE-CYCLE FLAVIVIRUS VACCINE CANDIDATE

Since replicons can be packaged into particles, a logical extension of these C gene-deleted nucleic acid vaccines was the trans-complementation of their C gene-deleted genomes using a packaging technology similar to that utilized to create single-cycle flavivirus particles [SCFVs; also known as virus-like particles (VLPs) or pseudoinfectious particles (PIPs)] that have been used to study aspects of flavivirus assembly and/or infection (Khromykh, Varnavski, and Westaway, 1998; Gehrke et al., 2003; Scholle et al., 2004; Jones, Patkar, and Kuhn, 2005; Bourne et al., 2007; Yoshii et al., 2008). RepliVAX, the name we have given to our version of this type of vaccine, is a single-cycle flavivirus that consists of a replicationally active, C gene-deleted genome that is encapsidated into an infectious particle by packaging cells producing the missing C protein (Fig. 2A). Our initial studies demonstrated that the genomes of YFV or WNV engineered to lack most of the C gene (a portion the C gene was retained to maintain the 5'CS) could be readily packaged into high-titer stocks of infectious particles in stable cell lines expressing the missing C protein from a non-cytopathic Venezuelan equine encephalitis virus replicon (VEErep) (Mason, Shustov, and Frolov, 2006). The distinguishing feature of this packaging technology is that in the packaging cells, the C-deficient virus develops productive, spreading infection and the titers of infectious particles, containing defective genomes, approach the levels of wt virus grown in similar cell lines. Therefore, unlike other technologies (notably the alphavirus replicon particles),

RepliVAX vaccine production does not require repeated RNA transfections, can be easily standardized, and since the single-cycle virus is severely attenuated, the high biocontainment conditions required to propagate virus (such as those needed to produce INVs) are also not needed.

As shown in Fig. 1.7 (top), gene-deleted RepliVAX genomes can be packaged into infectious particles by transencapsidation in cells lines that continuously express the missing C protein in the context of a noncytopathic VEErep (Mason, Shustov, and Frolov, 2006). RepliVAX particles released from C-expressing cells are infectious, but are capable of performing only a single round of infection in cells that do not express C (Fig. 1.7, bottom). However, infection of these cells results in extensive vaccine genome replication and the efficient release of SVPs and NS1, products that are known to be potent and efficacious vaccine components when delivered by other methods (see above). RepliVAX has been shown to be highly attenuated in suckling mice (at least 1,000,000-fold less virulent than wt viruses, with no death detected in pups given as much as two million infectious units (IU)) (Mason, Shustov, and Frolov, 2006). Thus, as expected, RepliVAX WN was shown to be safe in adult mice and was also capable of eliciting neutralizing antibody responses and protecting these animals from lethal WNV challenge after a single dose (Mason, Shustov, and Frolov, 2006).

The studies presented in this dissertation document the evaluation of RepliVAX WN for its potential use as a vaccine to prevent WN disease. We hypothesized that blind passaging of RepliVAX WN in C-expressing cells would select for a better-growing variant, that could demonstrate increased potency in animals. Furthermore, we expected that robust IFN stimulation, along with localization of infected cells to immunologically important sites in vivo are largely responsible for the strong protective immune responses

observed in animals. We thus set out to test these hypotheses using a variety of animal model systems.

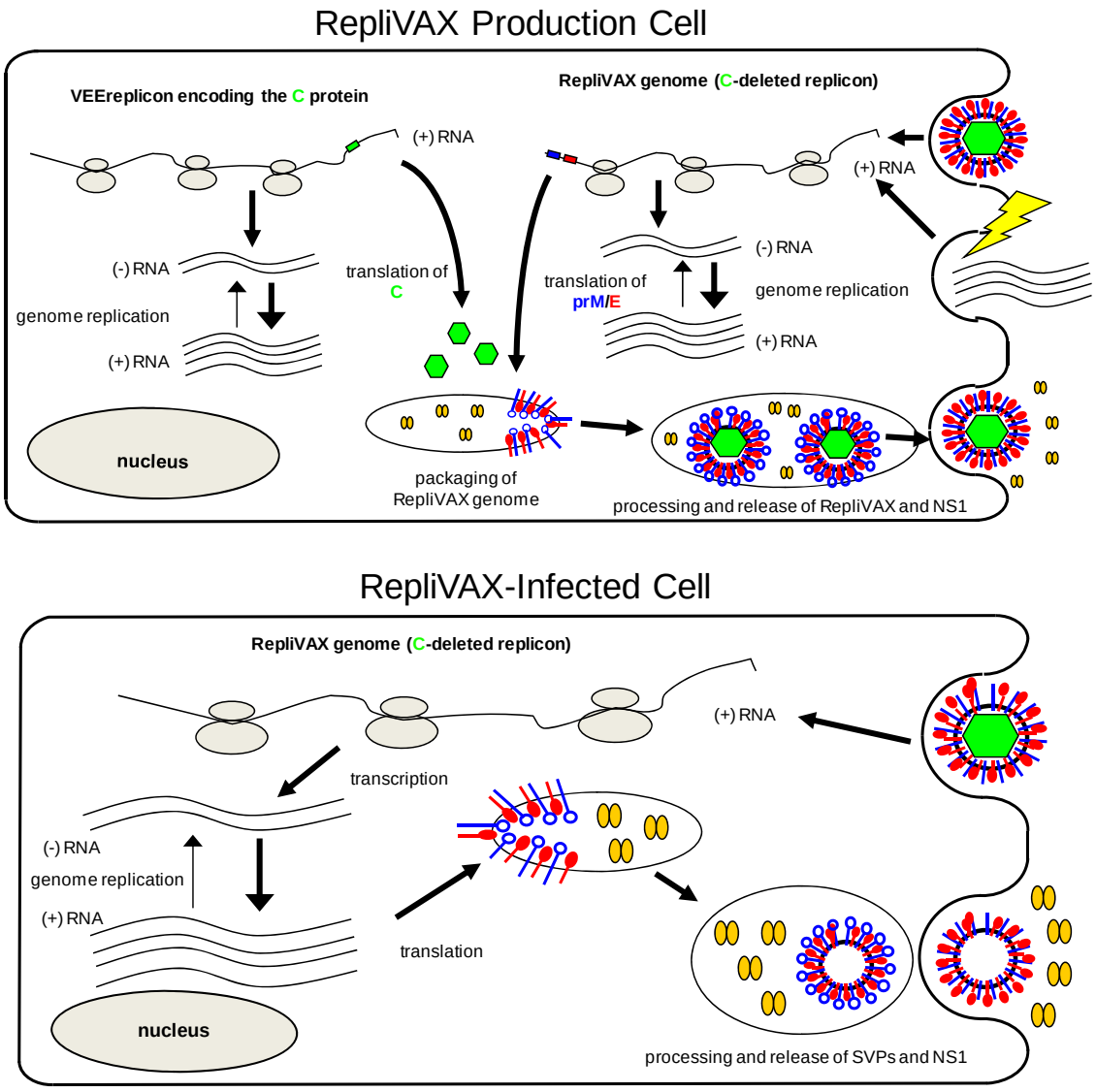

Figure 1.7: RepliVAX WN production and use. TOP: RepliVAX production in C-expressing cells. C-deleted RepliVAX genomes are introduced by electroporation into cells expressing the missing C gene from a VEErep. RepliVAX produces all components necessary for genome replication and expression of the C gene by the VEErep allows for packaging of RepliVAX genomes into infectious particles that are released along with NS1 and SVPs (not shown). Mature RepliVAX infectious particles can also be used to initiate the infection process for large-scale cultivation. **BOTTOM: RepliVAX infection of normal cells in culture or in a vaccinated individual.** Infection of cells with RepliVAX mimics the early events of natural flavivirus infection. The RepliVAX genome is replicated in the cell, and antigenic components are produced. The lack of C prevents RepliVAX genomes from being packaged into infectious particles, but does not interfere with secretion of SVPs and NS1 from RepliVAX-infected cells.

CHAPTER 2 :CONSTRUCTION AND CHARACTERIZATION OF A SECOND-GENERATION SINGLE-CYCLE WEST NILE VIRUS VACCINE PROPAGATED USING A NEW CULTIVATION SYSTEM[2]

ABSTRACT

Safer vaccines are needed to prevent flavivirus diseases. To help develop these products we have produced a single-cycle West Nile virus (WNV) lacking a functional C gene which we have named RepliVAX WN. Here we demonstrate that RepliVAX WN can be safely propagated at high titer in BHK cells and vaccine-certified Vero cells engineered to stably express the C protein needed to trans-complement RepliVAX WN growth. Using these BHK cells we selected a better growing mutant RepliVAX WN population and used this to generate a second-generation RepliVAX WN (RepliVAX WN.2). RepliVAX WN.2 grown in these C-expressing cell lines safely elicit strong protective immunity against WNV disease in mice and hamsters. Taken together, these results indicate the clinical utility of RepliVAX WN.2 as a vaccine candidate against West Nile encephalitis.

[2]A significant portion of this chapter has been previously published in the journal Vaccine. Vaccine does not require copyright permission as long as proper citation is given. The citation for this article is: Widman, D. G., Ishikawa, T., Fayzulin, R., Bourne, N., and Mason, P. W. (2008). Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. *Vaccine* **26**(22): 2762-71.

INTRODUCTION

It has been previously demonstrated that a WNV genome containing a large deletion in the C-coding region (RepliVAX WN) can be efficiently packaged into infectious particles by trans-complementation with functional C protein produced in packaging cell lines (Mason, Shustov, and Frolov, 2006). Furthermore, these particles are unable to spread cell-to-cell when they are used to infect cells that do not express the WNV C protein (Mason, Shustov, and Frolov, 2006). However, these studies propagated RepliVAX WN in cell lines expressing large portions of the WNV structural protein genes from a VEErep (Mason, Shustov, and Frolov, 2006), a strategy that could result in intergenomic recombination producing a viable infectious WNV genome. Furthermore, it was noted that some VEErep genomes can undergo intragenomic recombination, eliminating foreign genes (Fayzulin et al., 2006), which could compromise long-term propagation of packaging cells needed to produce clinically useful vaccines. We thus set out to produce a genetically stable cell line capable of producing high titers of RepliVAX WN over many generations with reduced possibility of intergenomic recombination.

Here we describe an improved system for replication of RepliVAX, develop an improved second-generation RepliVAX WN, and further characterize its utility as a vaccine to prevent WNE. The new propagation system consists of packaging cell lines expressing a form of the WNV C protein engineered for long-term stable expression and an inability to recombine with the RepliVAX genome in a way that could produce a live pathogenic virus. While BHK cells were used for initial propagation of RepliVAX WN, a packaging cell line from Vero cells approved for the production of human vaccines has also been developed. These latter cell lines were capable of producing RepliVAX WN at titers exceeding 10^7 IU/ml. Extensive blind passaging experiments in C-expressing BHK

cells failed to detect production of any infectious progeny that could have arisen by recombination, confirming the utility of packaging cell lines expressing this modified C gene for safe production of RepliVAX WN vaccines. Additionally, these blind passaging experiments selected RepliVAX WN mutants that demonstrate enhanced growth phenotypes. The mutations responsible for increased growth were identified and incorporated into the original RepliVAX WN genome to produce several second-generation RepliVAX WN. One of them, RepliVAX WN.2 SP, demonstrated the enhanced growth phenotype and was able to protect mice and hamsters from WNE. These animal studies also showed that this RepliVAX WN.2 was more potent than our original RepliVAX WN in eliciting antibodies against clinically relevant WNV epitopes, demonstrating that RepliVAX WN generated to have enhanced growth characteristics in our trans-encapsidation system are more effective vaccines, likely a result of enhanced SVP formation in vivo.

RESULTS

PRODUCTION OF REPLIVAX WN IN C-EXPRESSING CELL LINES

To enhance both the genetic stability and safety of our RepliVAX packaging cells, we generated a new VEErep-based construct. Specifically, a plasmid DNA encoding a non-cytopathic VEE replicon expressing the selectable marker puromycin n-acetyl transferase (*Pac*) was engineered so that its subgenomic promoter drove the expression of a *Pac/ubiquitin* fusion protein followed by the WNV C gene (VEErep/Pac-Ubi-C*) (Fig. 2.1). In order to reduce sequence homology (and thus reduce homologous recombination potential) between the truncated C gene in the RepliVAX WN genome and the functional WNV C gene in the VEErep, thirty-six silent mutations (Fayzulin et

al., 2006) were incorporated into the 5' region of the C gene (C*) contained in VEErep/Pac-Ubi-C* (Fig. 2.1). These changes were also designed to disrupt the CS, producing a sequence that was not complementary to the 3'CS of the RepliVAX WN genome and thereby preventing replication of a recombinant genome that had acquired the intact C protein encoded by the VEErep. Previous studies have indicated that packaging of flavivirus particles is performed more efficiently by VEE replicons that contain at least a portion of the prM signal sequence and gene (Mason, Shustov, and Frolov, 2006). To further minimize the amount of homology between RepliVAX WN and the VEErep, we engineered VEErep/Pac-Ubi-C* to contain a stop codon at the NS2B/NS3 cleavage site upstream of the prM signal sequence (Fig. 2.1). While a small amount of homology still exists between RepliVAX and the VEErep (Fig. 2.1), the modifications we made to our new construct were expected to eliminate the potential for recombination between VEErep/Pac-Ubi-C* and RepliVAX WN that could produce a genome capable of spreading infection between normal cells lacking C. A clonal cell line, BHK(VEErep/Pac-Ubi-C*), was derived from BHK cells electroporated with synthetic RNAs of VEErep/Pac-Ubi-C* and used for propagation of RepliVAX WN and for focus formation assays. RepliVAX WN infections in this clonal cell line routinely produced titers of $>10^7$ infectious units per milliliter (IU/ml), while three other clones produced RepliVAX WN at 0.5-1$\log_{10}$ lower levels (results not shown). As expected from the fusion of *Pac* to the C gene through *ubiquitin*, this clonally derived cell line was extraordinarily stable. Specifically, we found that RepliVAX WN infection of BHK(VEErep/Pac-Ubi-C*) cells at passage 10 and passage 74 produced foci that were indistinguishable in size, and yields of RepliVAX WN that were indistinguishable in titer (results not shown).

To demonstrate that RepliVAX WN could be produced in a cell line acceptable

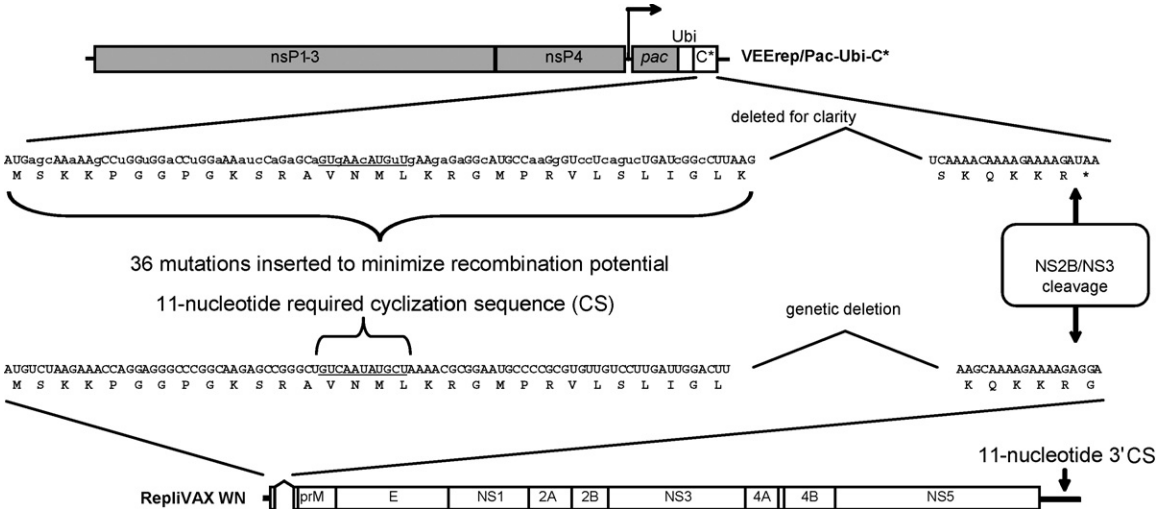

Figure 2.1: Schematic representation of the RepliVAX WN genome and the VEE replicon used to supply the C protein in packaging cells. **Top:** Non-cytopathic VEE replicon (VEErep/Pac-Ubi-C*) encoding the complete C of WNV along with the *Pac* selectable marker expressed as a fusion protein with ubiquitin. This replicon contains a C gene (C*) harboring 36 silent mutations designed to ablate homologous recombination. Among these mutations are included three changes to the CS sequence that must be 100% complementary to the 3'CS of RepliVAX WN to permit genome replication (see text). **Bottom:** RepliVAX WN genome showing the region of overlap with VEErep/Pac-Ubi-C* and the C gene deletion.

for human vaccine production, a Vero cell line approved for human vaccine manufacture was obtained from the NIH (S. Whitehead) and cultured in serum-free conditions. In vitro transcribed VEErep/Pac-Ubi-C* RNA was electroporated into these cells and replicon-expressing cells were selected by addition of Pur to the culture medium. Of three clonally-derived Vero(VEErep/Pac-Ubi-C*) cell lines, one was chosen for all subsequent analyses based on its ability to produce high titer stocks of RepliVAX WN.2. RepliVAX WN.2 infectious titers from Vero(VEErep/Pac-Ubi-C*) cells did not reach peak levels equivalent to those produced by BHK(VEErep/Pac-Ubi-C*) cells (Fig. 2.2), however RepliVAX WN-producing Vero cell monolayers remained intact for over 200 hours after infection while BHK cells displayed severe CPE by 96 hours post-infection. The ability

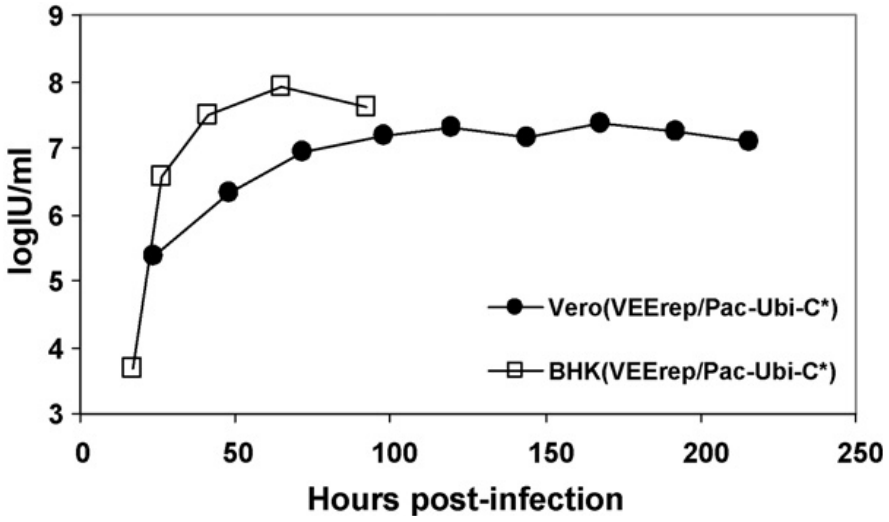

Figure 2.2: RepliVAX growth analyses. Growth curve analyses showing yield of RepliVAX WN.2 SP from clonally derived cultures of either BHK(VEErep/Pac-Ubi-C*) cells (open squares) or Vero(VEErep/Pac-Ubi-C*) (closed circles) infected at an MOI of 0.05.

to collect high titer (>10^7 IU/ml) stocks of RepliVAX WN over a 6 to 7 day period from these vaccine-certified Vero cells could be useful for large-scale RepliVAX WN production where cell viability may be an important factor.

REPEATED PASSAGING OF REPLIVAX WN THROUGH PACKAGING CELLS WAS UNABLE TO FORCE PRODUCTIVE RECOMBINATION

In order to examine the unlikely possibility that the RepliVAX genome could undergo recombination with the C-expressing VEErep producing an infectious WNV, RepliVAX WN was repeatedly passaged in BHK(VEErep/Pac-Ubi-C*) cells, and the passaged material was tested for its ability to produce a spreading infection in cells that did not express C. WT Vero cells were used to assess spreading infections since they are highly susceptible to infection by WNV and RepliVAX WN, typically exhibiting 10 to 50-fold higher infection rates than BHK cells (results not shown). To facilitate detection of recombinant viruses in RepliVAX WN preparations, RepliVAX WN passage 10, 20,

and 30 obtained from BHK(VEErep/Pac-Ubi-C*) cells were subjected to 3 successive passages on WT Vero cells. At the end of the 3rd passage, no immunopositive cells were detected after fixing and staining the entire Vero cell monolayer with an anti-WNV MHIAF, indicating that no viable progeny capable of causing a spreading infection in the absence of the C gene had been produced during RepliVAX WN propagation in C-expressing cells.

To examine the genetic stability of RepliVAX WN, total RNA from cells that were infected with passage 10, 20, or 30 RepliVAX WN was isolated, reverse-transcribed and amplified with oligonucleotides flanking the deleted region of C. In each passage level tested, the size of the amplified truncated C (trC) region was equal to that of the parental RepliVAX genome in accordance with the deletion engineered into RepliVAX WN. Sequence analyses of these amplicons also failed to detect any evidence of recombination that would restore a functional C gene to RepliVAX WN, and attempts to amplify their cDNA preparations with oligonucleotides derived from the missing region of C failed to produce any products (results not shown).

SERIAL PASSAGING OF REPLIVAX WN IN PACKAGING CELLS SELECTS FOR GENOMES WITH MUTATIONS IN PROTEOLYTIC CLEAVAGE SITES

In the process of demonstrating that RepliVAX WN could be safely passed in BHK(VEErep/Pac-Ubi-C*) cells we noted that the passage 10 (p10) RepliVAX WN produced larger and more heterogeneous foci than the passage 0 RepliVAX WN (Fig. 2.3A). Additionally, harvests of p10 RepliVAX WN had titers up to 5-fold higher than that of p0. Sequencing of the entire genome of this p10 population revealed that only two non-synonymous changes had been acquired during serial passaging. One change detected was an adenosine (A) to uracil (U) substitution in the codon at the +3 position to the NS2B/NS3 protease cleavage site that separates the trC gene from the prM signal peptide. This change, which was only observed in a portion of the population sequenced

by this method, resulted in a lysine (K) to methionine (M) substitution (QKKR | GGKT → QKKR | GGmT) in the polyprotein (Fig. 2.3B). The second change detected was an A to U change observed in a majority of the sequenced population at the codon 4 positions upstream of the host signalase cleavage site preceding the mature prM protein gene. This change resulted in a serine (S) to cystiene (C) substitution (SVGA | VTLS → cVGA | VTLS). Interestingly, both mutations were located within the region of the flavivirus genome that is known to affect production of C and prM, and in the case of RepliVAX WN it appears that only the latter product (namely prM) is essential for particle formation. No other non-synonymous sequence changes were detected in the structural gene region (trC through codon 145 of E) in the p20 or p30 RepliVAX WN populations.

Individual members of the RepliVAX WN p10 population were isolated using a standard 96-well-based limiting dilution purification method. The foci of infection produced by 10 of 12 independent isolates were observed to be of comparable size to the large foci produced by the RepliVAX WN p10 (four representatives shown in Fig. 2.3B). The structural genes of four of these isolates were sequenced as described above, and three of these were found to contain only the S → C substitution proximal to the host signalase cleavage site. The remaining isolate contained the K → M substitution at the NS2B/NS3 cleavage site, and none of the four isolates examined contained both mutations (Fig. 2.3B). The abundance of the signalase cleavage site mutant in the population of RepliVAX WN p10 suggests that it was slightly more fit than the NS2B/NS3 cleavage site mutation under these blind passage conditions.

INCORPORATION OF PROTEOLYTIC CLEAVAGE SITE MUTATIONS INTO THE REPLIVAX WN GENOME

To demonstrate that the selected mutations were responsible for the enhanced growth properties of the p10 RepliVAX WN, parental RepliVAX WN genomes containing the mutations were engineered. Second-generation RepliVAX WN

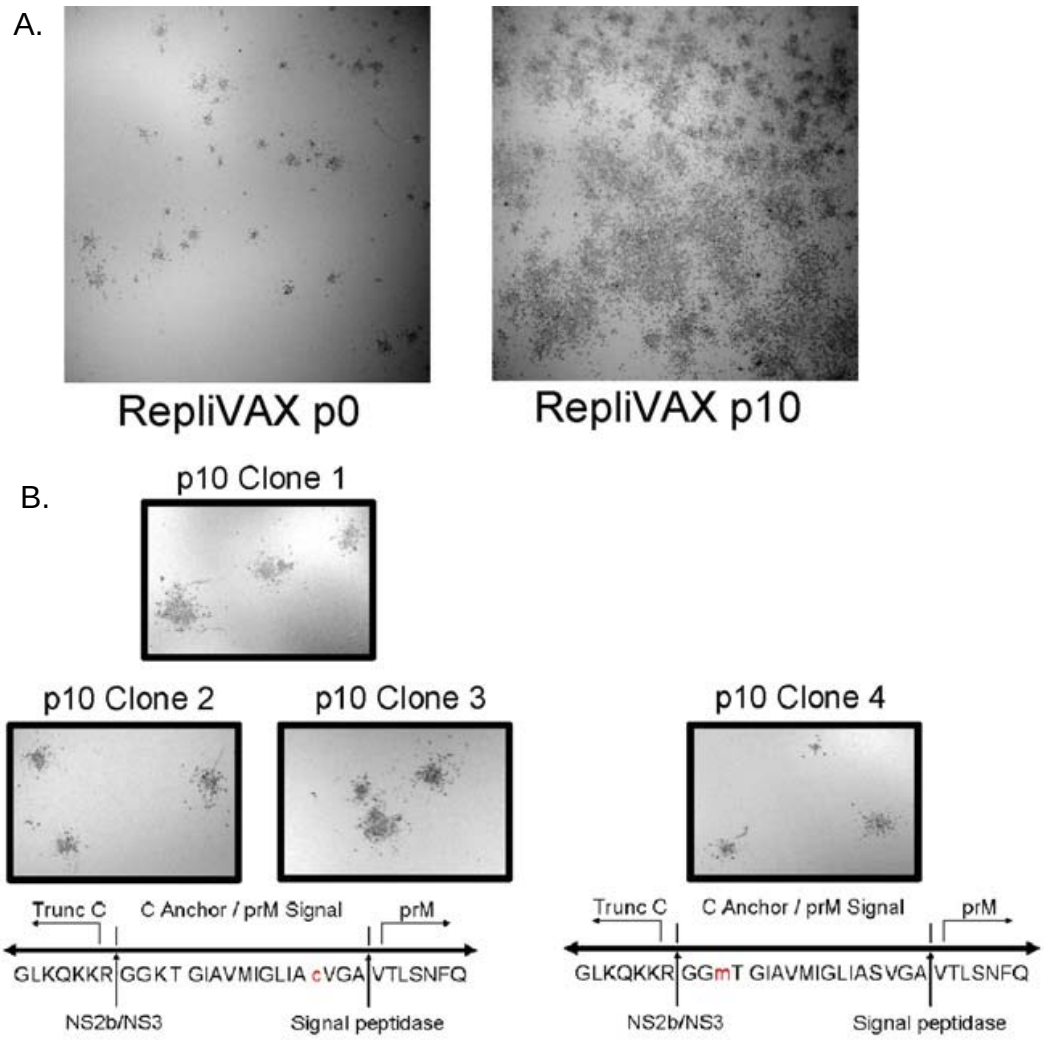

Figure 2.3: Characteristics of RepliVAX WN p10. A. Immunostained foci of infection produced by infection of BHK(VEErep/Pac-Ubi-C*) with p0 and p10 RepliVAX WN, showing that RepliVAX WN p10 produces larger and more heterogeneous foci than the p0 RepliVAX WN. **B.** Foci formed by four isolated clones from the RepliVAX WN p10 population on BHK(VEErep/Pac-Ubi-C*). The individual clones, which produced foci similar in size to the large foci in the RepliVAX WN p10 population, are shown above the sequences of their prM signal peptides, with their unique codon changes shown in lower case.

(RepliVAX WN.2) plasmids were constructed by inserting the signalase cleavage site mutation (RepliVAX WN.2 SP), viral NS2B/NS3 cleavage site mutation (RepliVAX WN.2 NS), or both (RepliVAX WN.2 SP-NS) into BAC plasmids encoding the RepliVAX WN genome. High titers (10^7-10^8 IU/ml) of RepliVAX particles were obtained from electroporation of BHK(VEErep/Pac-Ubi-C*) cells with in vitro transcribed RNAs obtained from each of the second-generation RepliVAX WN plasmids. After transfection, cells were infected with p0 RepliVAX WN.2 in order to verify the genetic sequence of trC, prM, and 30% of E (see Materials and Methods) which demonstrated that all constructs had only the desired mutations and that there was no evidence of change from the cDNA-encoded sequences at this passage level.

REPLIVAX WN.2 DEMONSTRATE ENHANCED GROWTH KINETICS WHEN PROPAGATED IN C-EXPRESSING CELLS

To determine the role of each mutation in growth phenotype, RepliVAX WN.2 particles harvested from electroporated cells were subjected to focus formation assays and one-step growth curves. Each of the three different RepliVAX WN.2 exhibited similar homogeneous foci sizes on BHK(VEErep/Pac-Ubi-C*) cells, all of which were larger than those produced by the original RepliVAX WN (Fig. 2.4A). These data suggest that both mutations (as well as the double mutant) enhanced the growth properties of RepliVAX WN, suggesting that the alteration of the cleavage sites influenced RepliVAX WN particle formation.

When the growth profile of the three RepliVAX WN.2 were compared to the parental RepliVAX WN in two independent one-step growth experiments, enhanced infectious yield was observed for only a brief period of time early after infection (Fig 2.4B). All of these RepliVAX WN.2 infections produced significantly higher yields ($p<0.05$) at 18 and 26 hours post-infection than original RepliVAX WN; however by 49 hours IU production had reached comparable levels in all four infections and remained

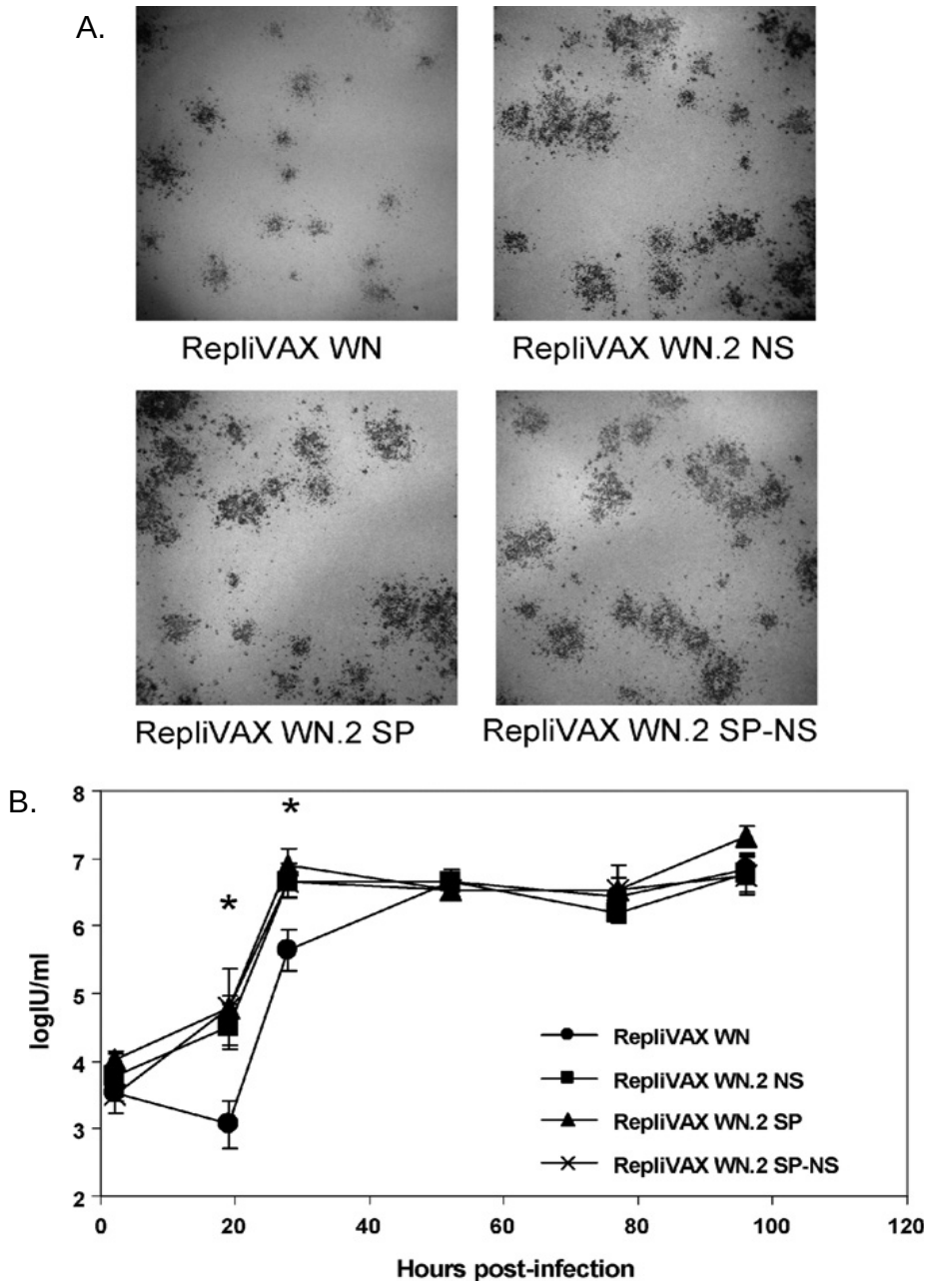

Figure 2.4: Characterization of RepliVAX WN.2. **A.** Immunostained foci produced on BHK(VEErep/Pac-Ubi-C*) cells infected with RepliVAX WN, RepliVAX WN.2 NS, RepliVAX WN.2 SP, and RepliVAX WN.2 SP-NS. **B.** One-step growth curves showing yield of RepliVAX WN.2 NS (closed square), RepliVAX WN.2 SP (closed triangle), RepliVAX WN.2 SP/NS (×), and RepliVAX WN (closed circle) from cultures of BHK(VEErep/Pac-Ubi-C*) cells infected at an MOI of 1. Values represent averages of two individual experiments. Extended bars indicate standard deviation and ‘‘*’’ denotes significance (p<0.05).

consistent throughout subsequent harvests, indicating the phenotype conferred by the mutations was only evident early in the one-step growth cycle.

MUTATIONS SELECTED FOR IN THE REPLIVAX GENOME DID NOT ENHANCE THE GROWTH CHARACTERISTICS OF WNV CONTAINING A FUNCTIONAL C GENE

Previous studies have indicated that the a.a. sequence surrounding the site of protease cleavage in flavivirus polypeptides have an impact on the production of viral particles (Stocks and Lobigs, 1998; Lee et al., 2000; Shiryaev et al., 2007). Using oligonucleotide mutagenesis similar to that used to engineer RepliVAX WN.2, substitutions were made into a BAC plasmid encoding the wt WNV genome and these plasmids were used to create viruses with mutations corresponding to those located within the genomes of the three RepliVAX WN.2 constructs. Virus containing the A → T substitution at the NS2B/NS3 cleavage site were designated WNV.2 NS, those containing the A → T change at the signalase cleavage site were designated WNV.2 SP, and virus containing both mutations simultaneously was termed WNV.2 SP/NS. The DNA plasmids were in vitro transcribed, and the synthetic RNA was electroporated into wt BHK cells. Infectious virus was collected from the cell culture supernatant and verified to contain the desired mutations by sequence analyses of genome region containing C, prM, and the N-terminus 30% of E.

To examine the effects the RepliVAX cleavage site mutations had on a WNV genome where a functional C gene is present, the three WNV mutants (WNV.2 SP, WNV.2 NS, and WNV.2 SP/NS) were compared side-by-side with the parental WNV derived from an infectious cDNA clone developed from a 2002 human isolate (Rossi et al., 2005). As shown in Fig. 2.5A, the three WNV mutants produced infectious foci indistinguishable from the parental wt virus. Furthermore, the growth profiles of all four viruses were indistinguishable (Fig. 2.5B), indicating that we were unable to detect the growth-enhancing phenotype observed in RepliVAX in the context of the wt WNV

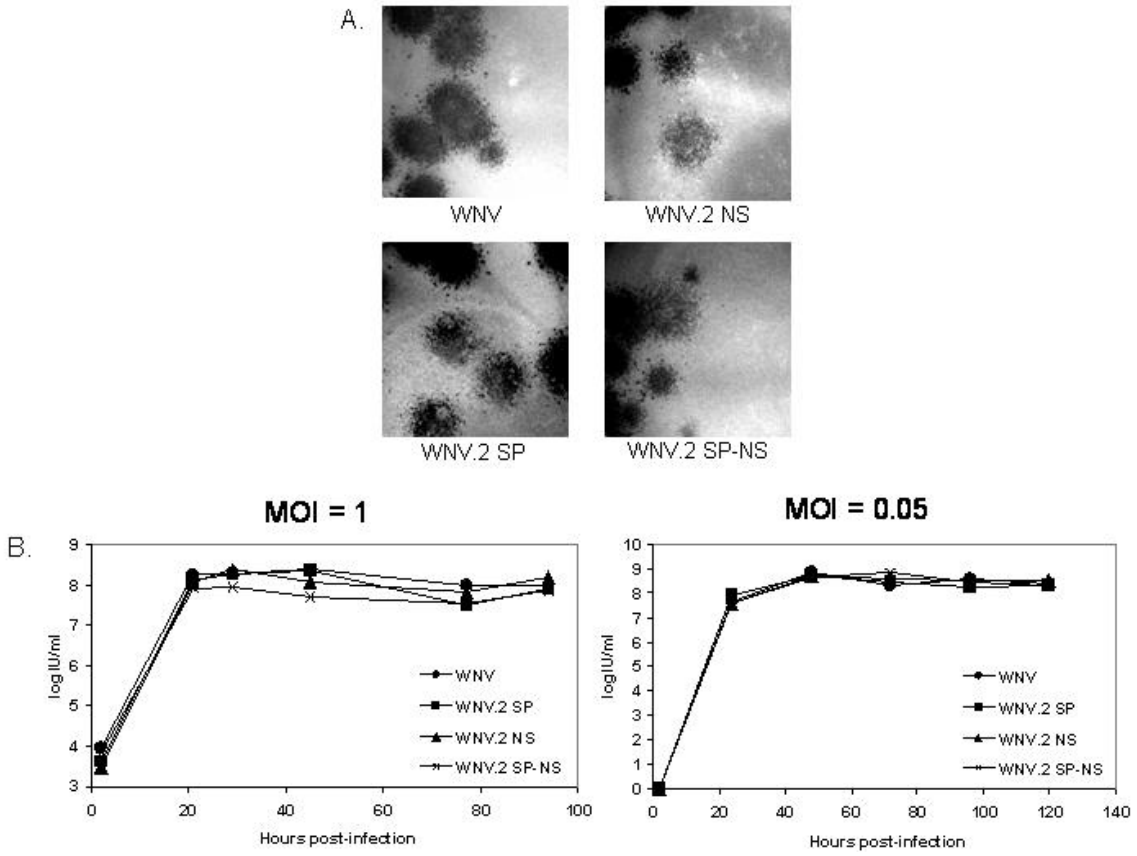

Figure 2.5: Characterization of WNV.2 full length infectious clone mutants containing base pair substitutions corresponding to RepliVAX WN.2. **A.** Immunostained foci produced on Vero cells infected with the parental WNV infectious clone, WNV.2 SP, WNV.2 NS, and WNV.2 SP/NS. All four infectious clones produce similar foci of infection. **B.** Growth curve analyses performed in Vero cells infected with WNV.2 NS (closed triangle), WNV.2 SP (closed square), WNV.2 SP-NS (X), and wt WNV (closed circle) at an MOI of 1 and 0.05.

backbone. Based on these findings, we conclude that the mutations selected for in the RepliVAX genome do not have an obvious influence on the growth of viruses in which a functional C protein is supplied in *cis*.

REPLIVAX WN.2 SAFETY AND EFFICACY IN ANIMALS

The safety, potency and efficacy of RepliVAX WN.2 were evaluated using murine and hamster models of WNE. We have previously demonstrated the safety of

RepliVAX WN in the highly sensitive suckling mouse brain model (Mason, Shustov, and Frolov, 2006). In those studies, doses as high as $2x10^6$ IU were safe when delivered intracranially (Mason, Shustov, and Frolov, 2006). Furthermore, adult mice were completely protected from lethal WN challenge after vaccination with as little as 30,000 IU of RepliVAX WN. To further evaluate our single-cycle flavivirus vaccine strategy, RepliVAX WN.2 SP (referred to from this point on as RepliVAX WN.2 for simplicity) was chosen for vaccination based on the frequency with which it was seen in the naturally occurring population (an indication of its selective advantage) and its enhanced growth phenotype *in vitro*. In addition to mouse studies the golden hamster, a well characterized model for WNV infection (Xiao et al., 2001), was also used.

Swiss Webster mice were immunized with escalating doses of RepliVAX WN.2 ($4x10^4$ IU, $2x10^5$ IU, or $1x10^6$ IU in 100ul inoculum), original RepliVAX ($4x10^4$ IU), or diluent alone. All doses were examined using the IP vaccination route which was previously shown to be potent and efficacious (Mason, Shustov, and Frolov, 2006). In addition, since live-attenuated flavivirus vaccines have been tested in non-human primates (Guirakhoo et al., 2000; Monath et al., 2000a; Pletnev et al., 2003; Arroyo et al., 2004; Monath et al., 2006; Pletnev et al., 2006) and man (Monath et al., 2002a; Durbin et al., 2006; Monath et al., 2006) using a SC route, two groups of mice were vaccinated SC with $1x10^6$ IU or $4x10^4$ IU of RepliVAX WN.2. None of the animals immunized with either RepliVAX WN construct by any route showed adverse effects as a result of vaccination. Three weeks after vaccination the humoral immune response to immunization was examined by ELISA for presence of IgG antibodies against WNV E and NS1. All vaccinated animals demonstrated seroconversion by this test and among the IP-immunized animals, these analyses revealed a dose-dependent response to RepliVAX WN.2 vaccination, with animals receiving the highest doses exhibiting the greatest

ELISA antibody titers against both proteins (Fig. 2.6). By one way ANOVA using Dunnett's post-test, RepliVAX WN.2 administered IP at a dose of $1x10^6$ IU elicited a significantly greater IgG response ($p<0.02$) to WNV E than IP doses of $2x10^5$ IU and $4x10^4$ IU of RepliVAX WN.2 (Fig. 2.6). Interestingly, at the lowest dose tested, IP RepliVAX WN.2 vaccination induced a significantly greater ELISA antibody titer against E ($p<0.02$) and NS1 ($p<0.02$) than original RepliVAX WN administered by the same route (Fig. 2.6). This suggests that the enhanced growth characteristics observed for second generation RepliVAX WN *in vitro* may correlate to an enhanced immune response *in vivo*. SC. immunization with RepliVAX WN.2 induced readily detectable antibody responses to E and NS1, but at both doses tested these responses were lower than those elicited by IP administration (Fig. 2.6). In the case of the high dose ($1x10^6$ IU),

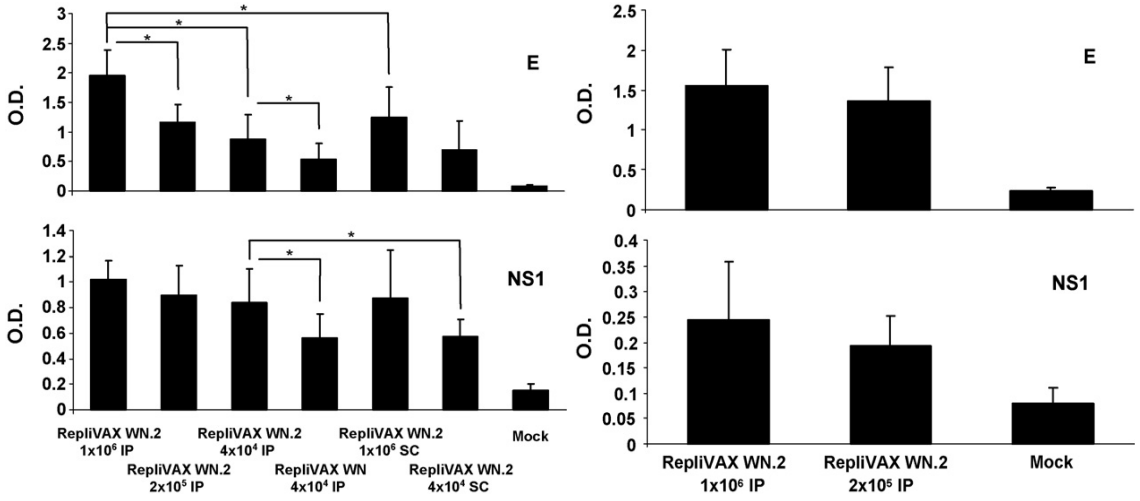

Figure 2.6: RepliVAX WN-induced antibodies in mice and hamsters. LEFT: ELISA antibody levels specific for WNV E and NS1 proteins in sera from vaccinated mice. Bars show average OD values for the indicated antigens from pre-challenge sera obtained from animals vaccinated as indicated. Extended bars indicate standard deviation. ''*'' indicates significant difference. **RIGHT:** ELISA antibody levels specific for WNV E and NS1 proteins in sera from vaccinated hamsters. Bars show average OD values for the indicated antigens from pre-challenge sera obtained from animals vaccinated as indicated. Extended bars indicate standard deviation.

these differences were statistically significant ($p<0.05$) for E but not NS1, however the difference observed at the lowest dose tested ($4x10^4$ IU) was statistically significant ($p<0.05$) for NS1 but not E. Ninety percent neutralizing antibody titers were detected by a luciferase encoding WNVLP assay and titers ranging from 1:80 to 1:160 were observed in pooled sera from each group of vaccinated animals (Table 2.1). Interestingly, despite the slightly lower ELISA titers detected in animals among some groups (see above and Fig. 2.6), the neut titers from pooled sera were indistinguishable (Table 2.1).

Following this single vaccination, mice were challenged with $10LD_{50}$ of WNV NY99 at 28 days post-vaccination and monitored for signs of illness. After challenge, 6 out of 15 control animals developed clinical signs of WNE and succumbed to infection, however none of the mice that received RepliVAX WN or RepliVAX WN.2 died ($p<0.02$ for each group relative to control group) (Table 2.1). The WNV-induced mortality in the control animals was lower than anticipated with the challenge inoculum used. We cannot explain this result since in an earlier study challenge using the same dose of this virus stock killed over 50% of mock-vaccinated mice, and LD_{50} titrations indicated that this dose correlated to 10 LD_{50} in mice of similar age (results not shown).

Table 2.1 Potency and Efficacy of RepliVAX WN.2 in mice

Inoculum	Dose (IU)	Pre-challenge neut Titer[a]	% Survival[b]	% Protection[c]
RepliVAX WN.2	$1x10^6$ i.p.	1:160	100 (15/15)	100 (15/15)
RepliVAX WN.2	$2x10^5$ i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN.2	$4x10^4$ i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN	$4x10^4$ i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN.2	$1x10^6$ s.c.	1:160	100 (15/15)	100 (15/15)[d]
RepliVAX WN.2	$4x10^4$ s.c.	1:80	100 (15/15)	100 (15/15)
Diluent	0	<1:40	60 (9/15)	60 (9/15)

[a] Neutralizing antibody titer of pooled sera collected from all animals 21 days post-vaccination (titer shown is the highest dilution of sera giving a 90% reduction of luciferase activity from Vero cells cultured with antibody-treated luciferase-bearing WNVLPs).
[b] Survival at 14 days post-inoculation with 100LD50 WNV NY99.
[c] Protection from illness (defined by a greater than 10% weight loss during the 14-day post-challenge observation period).
[d] One animal in this group demonstrated a weight loss of 10% on days 3-5 post-challenge, remained at this weight until day 10, when it began to gain weight. Since this animal did not show any signs of infection consistent with encephalitis, and none of the diluent-vaccinated animals that eventually died of infection showed any weight loss before day 7, we scored this RepliVAX WN.2-vaccinated animal as protected from illness.

All of the mice in the vaccine groups were protected from weight loss indicative of WNV-induced illness (>10% loss from weight at day of challenge) except for one animal in the $1x10^6$ IU RepliVAX WN.2 SC group (Table 2.1). This animal's sharp decline in body weight on days 3-5 post-challenge was likely not a result of WNV-induced illness, as diluent-vaccinated animals did not demonstrate weight loss until day 7 post challenge (results not shown) None of the vaccinated animals developed outward signs of illness (lethargy or signs of hind-limb paralysis) as a result of the challenge (Table 2.1). These results are consistent with previous protection seen in IP-vaccinated mice (Mason, Shustov, and Frolov, 2006) and demonstrate the efficacy of RepliVAX WN.2 SC vaccination in the murine model for WNE.

Following immunization of five-week-old female hamsters IP with $1x10^6$ or $2x10^5$ IU of RepliVAX WN.2, none of the vaccinated animals developed adverse effects. Animals in both vaccination groups developed similar neutralizing antibody titers of 1:160 (Table 2.2), and as was observed in mice there was a dose-dependent IgG response to E and NS1 (Figure 2.6). The hamsters were then challenged IP at 28 days post-vaccination with $1x10^6$ ffu of WNV NY99 and monitored for signs of illness. $1x10^6$ ffu was chosen as the challenge dose for hamsters even though a lower dose ($1x10^4$ IU) has been used by others for vaccine (Tesh et al., 2002; Watts et al., 2007) and pathogenicity (Xiao et al., 2001) studies. However this higher dose, which is approximately 10,000

Table 2.2 Potency and Efficacy of RepliVAX WN.2 in hamsters

Inoculum	Dose (IU)	Pre-challenge neut Titer[a]	% Survival[b]	% Protection[c]
RepliVAX WN.2	$1x10^6$ i.p.	1:160	100 (10/10)	100 (10/10)
RepliVAX WN.2	$2x10^5$ i.p.	1:160	100 (10/10)	100 (10/10)
Diluent	0	<1:40	40 (4/10)	10 (1/10)

[a] Neutralizing antibody titer of pooled sera collected from all animals 21 days post-vaccination (titer shown is the highest dilution giving a 90% reduction of luciferase activity from Vero cells cultured with antibody-treated luciferase-bearing WNVLPs)
[b] Survival at 21 days post-inoculation with 100 LD50 WNV NY99
[c] Protection from illness (defined by a greater than 10% weight loss during 14-day post-challenge observation period).

times higher than the minimal infectious dose (Xiao et al., 2001), causes clinically apparent disease in most animals ((Xiao et al., 2001) and R.B. Tesh, personal communication). At this challenge dose, 60% of the control animals succumbed to WNV infection and 90% displayed weight loss (>10% loss from weight at day of challenge) indicative of severe illness. In contrast, none of the RepliVAX WN.2 vaccinated animals died or displayed weight loss indicative of illness during the 3 weeks following challenge (Table 2.2). Collectively these results demonstrate the safety and efficacy of RepliVAX WN.2 as a vaccine against WNE in two rodent model systems.

DISCUSSION

The need for effective and safe vaccines to prevent flavivirus diseases has been the focus of a great deal of research. In order to combine the replicative capacity of LAV with the safety of INV, we engineered a new type of vaccine candidate consisting of a single-cycle flavivirus (RepliVAX WN) that contains a large deletion in a key structural protein, C (Mason, Shustov, and Frolov, 2006). RepliVAX WN grows well in C-expressing cells, and does not produce infectious progeny in normal cells. However, when RepliVAX WN infects normal cells, it produces an extracellular form of E (SVPs) that is known to be an effective immunogen (Mason et al., 1991b).

In this study, we demonstrated the ability of RepliVAX WN to be safely propagated in two WNV C-expressing cell lines, BHK(VEErep/Pac-Ubi-C*) and Vero(VEErep/Pac-Ubi-C*) derived from a vaccine-certified Vero cell line. The C-expression cassette used to create these cells was designed to ablate the possibility of homologous recombination between the cell line-encoded C gene and the RepliVAX WN genome. RepliVAX WN titers produced by BHK(VEErep/Pac-Ubi-C*) cells exceeded 10^7 IU/ml, although this is approximately 1 log lower than those produced by BHK cells

expressing WNV C, prM, and E proteins ((Mason, Shustov, and Frolov, 2006) and R. Suzuki, personal communication). It is possible that increased availability of prM and E from the VEErep, or the *cis* expression of C with prM and E facilitates more efficient packaging of RepliVAX WN in these cells. Although the boost in growth by cis-expression of prM and E could aid in vaccine production, the deletion of the glycoprotein genes from our new cell line minimizes the potential for productive intergenomic recombination with the RepliVAX WN genome, permitting safer production of RepliVAX WN. To document that C-only cell lines would not recombine with RepliVAX WN, an aggressive blind-passage procedure was performed in BHK(VEErep/Pac-Ubi-C*) cells to demonstrate a lack of productive recombination. Specifically, we demonstrated that after 30 low MOI serial passes in these cells there was no genetic (RT-PCR) or phenotypic (recovery of live virus) evidence of recombination. Furthermore even after 74 passages, BHK cells expressing this cassette were indistinguishable from a passage 10 population of these cells in their phenotype and ability to propagate RepliVAX WN. Because BHK cells are not currently approved for human vaccine production, we developed a stable line of cells expressing VEErep/Pac-Ubi-C* from Vero cells certified by the WHO for human vaccine manufacture. These C-expressing cells were also capable of producing RepliVAX WN.2 at high titers and unlike BHK cells, repeated harvests could be obtained from these Vero cells. These data demonstrate that stable cell lines suitable for human vaccine production can be engineered and safely used to prepare RepliVAX WN vaccines.

Our studies also showed that natural selection of better growing variants is a powerful tool for improvement of RepliVAX WN. Serial passaging of RepliVAX WN particles in a C-expressing BHK cell line resulted in the selection of genomes containing one of two mutations affecting the short polypeptide sequence between the truncated C

gene and prM of the RepliVAX WN genome. Isolation and propagation of individual members of this population suggested that these mutations allowed RepliVAX WN to produce larger, more homogenous foci of infection than the RepliVAX WN p0 population. When the proteolytic cleavage site mutations were incorporated into the original RepliVAX WN genome, the presence of either mutation, or surprisingly a combination of both, conferred an enhanced growth phenotype. These second-generation RepliVAX WN (RepliVAX WN.2) grew to similar titers, and produced larger foci of infection than the parental RepliVAX WN. Based on these results, and the frequency with which the signalase cleavage site mutation was observed in the naturally selected RepliVAX WN population, RepliVAX WN.2 SP was chosen for future studies.

The position of these growth-enhancing mutations within the RepliVAX genome was not unexpected since it has been shown that the sequences of the NS2B/NS3 and signalase cleavage sites between C and prM coding regions in the flavivirus genome are important determinants for formation of both SVPs and infectious particles. Early studies documented that prM/E-expressing constructs lacking C, but containing the prM signal peptide, efficiently produced SVPs, however the inclusion of C in these constructs prevented SVP formation (Konishi et al., 1991). Subsequently it was demonstrated that trans-expression of NS2B/NS3 and a functional NS2B/NS3 cleavage site were required for efficient production of SVPs from the C-prM/E constructs (Yamshchikov and Compans, 1993). Later studies documented the importance of the NS2B/NS3 cleavage site in the production of infectious YFV progeny (Amberg and Rice, 1999) and further studies using infectious YFV pointed out that the natural signalase cleavage site at the start of prM is suboptimal and went on to demonstrate that mutations that increased the efficiency of processing at this site blocked the production of infectious virus (Lee et al., 2000). Based on the idea that this signalase site was suboptimal, Kofler et al. rationally

altered their C-deleted TBEV RNA vaccine (see Chapter 1) to enhance signalase cleavage at this site, and achieved the expected increase in SVP production in vitro and immunogenicity in vivo (Kofler et al., 2004; Aberle et al., 2005). Although the results of all the studies cited above which utilized complete C genes are consistent with cleavage at the NS2B/NS3 site prior to signalase cleavage, work by Lee and Lobigs provided additional data to indicate that coordination of these two cleavage events is essential for efficient encapsidation of the nucleocapsid (Lobigs and Lee, 2004). Finally, further information on the importance of cleavage site utilization in virus viability has come from studies to produce chimeric flaviviruses designed to express foreign prM/E cassettes which demonstrated that certain synthetic NS2B/NS3 sites could not be used to produce viable chimeric viruses (Pletnev and Men, 1998; Pletnev et al., 2002). Although the lack of viability of some of these chimeric constructs could have resulted from altered ability of the chimera's NS2B/NS3 protease to recognize the newly created cleavage site, it is interesting to note that in one case, alteration of the +3 position of the NS2B/NS3 cleavage site was found to be particularly beneficial for growth of a WNV/DENV4 chimera (Pletnev et al., 2002).

Taken together, these published data suggest that the selective pressure we applied to RepliVAX [as well as a chimeric RepliVAX WN expressing the JEV prM/E gene cassette in place of the corresponding WNV genes (Ishikawa et al., 2008)] to produce better-growing variants could have selected mutations at the +3 position of the NS2B/NS3 cleavage site [RepliVAX WN.2 NS or RepliVAX JE.2 (Ishikawa et al., 2008)] that enhanced NS2B/NS3 cleavage or selected a mutation resulting in the replacement of the β-turn-promoting serine at the signalase cleavage site (RepliVAX WN.2 SP) reducing cleavage by signalase. Alternatively, the mutations selected at these two positions could have allowed for the signal peptide to shift within the lipid bilayer,

altering cleavage activity of these proteases at either face of the ER membrane. To better understand the role these mutations play in the production of infectious particles, they were incorporated into the wt WNV backbone. These experiments were unable to detect a growth-enhancing effect of these mutations in a live virus background, suggesting that these mutations were only effective in enhancing prM and E for particle formation in cells where C is supplied in *trans*.

Animal studies were performed to determine if the mutation that was incorporated into RepliVAX WN.2 altered its potency in mice, and to extend our RepliVAX efficacy data to a second animal model for WNE, the golden hamster. The incorporation of a single amino acid change at the signalase cleavage site of RepliVAX WN.2 SP did not alter the efficacy of RepliVAX WN; all vaccinated animals survived lethal challenge regardless of vaccine dose. All outbred mice and hamsters were protected by RepliVAX WN.2 vaccination, and antibody titers detected by ELISA were dose-dependent. Interestingly, RepliVAX WN.2 produced significantly greater pre-challenge ELISA antibody titers than RepliVAX WN when 40,000 IU was administered by the IP route. We believe that the increased potency of RepliVAX WN.2 contributed to its efficacy when delivered by the SC route, which is likely the most relevant route for future non-human primate studies and clinical trials of this type of flavivirus vaccine product.

We expected that the change in the genome of RepliVAX WN.2 that enhanced its growth characteristics *in vitro* would alter the ability of these genomes to produce SVPs in normal cells (see above), but we were unable to predict if these growth-enhancing mutations would increase or decrease SVP production. Work from our group on the chimeric JEV prM/E-expressing RepliVAX WN demonstrated a large increase in both infectious titer in C-expressing cells and SVP production in normal cells following introduction of a similar mutation at the +3 position of its NS2B/NS3 cleavage site

(Ishikawa, et al.). However, in the case of RepliVAX WN and RepliVAX WN.2 discussed in this paper, we were unable to detect a difference in SVP production from cells infected in vitro with RepliVAX WN.2 or the parental RepliVAX (results not shown). Furthermore, there was no evidence that any of the RepliVAX WN.2 constructs displayed increased levels of replication within infected cells (determined by Western blot analyses of NS3 antigen accumulation in infected cells; results not shown). Despite the lack of demonstrable enhancement of the RepliVAX WN.2 mutation on intracellular replication or production of SVPs in vitro, RepliVAX WN.2 displayed a statistically significant enhancement of *in vivo* IgG responses to WNV NS1 relative to the parental RepliVAX WN. This curious effect could be an indication that by enhancing the release of SVPs, that this mutation also facilitated the enhanced release of NS1.

Taken together, these results provide additional support for the utility of RepliVAX WN as a vaccine for WNE. We have demonstrated that this product can be safely passaged without recombination, we have produced and characterized a stable propagation system in cells suitable for human vaccine manufacture, and we have produced a better growing and more potent RepliVAX WN derivative. We have also confirmed our previous efficacy and potency results in mice and extended these studies to a vaccine route compatible with human use. In addition, we have provided efficacy and potency data in a second animal model. The results of our animal studies demonstrate that RepliVAX WN vaccination is capable of inducing 90% neutralizing antibody titers of ≥1:80, in excess of the 1:10 titers reported as sufficient to afford protection in humans against JE (WHO, 1998; Kurane and Takasaki, 2000). Furthermore, a 3-dose schedule of the JE INV vaccine was found to elicit neutralizing titers of 1:65 in humans (Aihara et al., 2000), while a similar study demonstrated 91% clinical efficacy when administering only two doses of this vaccine that appears to elicit neut titers of >1:10. (Hoke et al., 1988).

Thus the RepliVAX WN.2 potency measured in our studies exceed potency that has correlated with clinical efficacy for a vaccine for a closely related disease. These findings demonstrate the utility of RepliVAX as safe, economical, and efficacious vaccine to prevent flavivirus disease.

MATERIALS AND METHODS

CELL CULTURES AND VIRUSES

The baby hamster kidney (BHK) cells used for all studies and Vero cells used for titration and blind passaging studies have been previously described (Mason, Shustov, and Frolov, 2006). Vero cells certified to be acceptable for the production of human vaccines were a kind gift of S. Whitehead at the NIH (Bethesda, MD). These Vero cells were maintained in OptiPro serum-free medium (SFM) (Gibco/Invitrogen, Carlsbad, CA), in some case supplemented with 10μg/ml puromycin (Pur) (see below). Packaging cell lines were produced by electroporation of *in vitro* transcribed VEErep/Pac-Ubi-C* RNA into BHK cells or the certified Vero cells. Packaging cell lines were maintained in growth media supplemented with Pur at a concentration of 10 μg/ml to select for cells harboring the VEE replicon. Single-cell cloning was used to establish the BHK(VEErep/Pac-Ubi-C*) cell line from a stock of electroporated cells. Medium used for BHK packaging cells consisted of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 10mM HEPES buffer, and antibiotics/antimycotic. RepliVAX infections were performed in Eagle's minimum essential medium (MEM) supplemented with 1% FBS, 10mM HEPES, and antibiotics/antimycotic.

Animal challenge studies were performed using a snowy owl isolate of WNV NY99 (Xiao et al., 2001) kindly provided by R.B. Tesh (UTMB). For murine challenge, $10LD_{50}$ (determined by titration in 8-week old mice), corresponding to 1000 ffu of virus passed once in Vero cells was used while hamsters were challenged with $1x10^6$ ffu of the same WNV strain passed twice in Vero cells. WNV mutants were derived from and compared to an infectious cDNA clone developed from a 2002 human isolate (Rossi et al., 2005).

PLASMID CONSTRUCTS, RNA TRANSCRIPTION AND TRANSFECTIONS

DNA plasmid constructions were performed using standard recombinant DNA techniques. The original RepliVAX genome containing a 70-codon deletion in the C gene has been previously described and is outlined in Fig. 2.1 (Mason, Shustov, and Frolov, 2006). In order to increase stability, the RepliVAX genome was introduced into a bacterial artificial chromosome (BAC). Second generation RepliVAX genomes (RepliVAX WN.2 SP, RepliVAX WN.2 NS, and RepliVAX WN.2 SP-NS) containing single base changes at the NS2B/NS3 cleavage site, signalase cleavage site, or both were incorporated into the BAC-propagated cDNAs by PCR-based mutagenesis using synthetic oligonucleotides followed by fusion PCR using high fidelity Phusion *Taq* polymerase (NEB, Ipswich, MA). Sequences of these BAC plasmids and RepliVAX recovered from transfected cells were determined by standard techniques (see below). WNV infectious clone work was conducted using a previously described BAC plasmid cDNA encoding the full-length genome of a 2002 isolate of WNV (Rossi et al., 2005).

Fig. 2.1 shows the schematic diagram of the VEE replicon used to package RepliVAX WN. The techniques used to develop this replicon, including the generation of the mutated C gene (C*), are similar to those previously described (Fayzulin et al., 2006). Briefly, an expression cassette was engineered to contain the WNV C gene downstream

of a fusion of the puromycin N-acetyl transferase (*Pac*) gene with the ubiquitin gene. This cassette was inserted into a plasmid harboring a noncytopathic VEErep under the control of the subgenomic promoter, and *in vitro* transcription reactions obtained from this plasmid were prepared as previously described (Fayzulin et al., 2006).

In order to obtain WNV ELISA antigens, cell lines expressing WNV NS1 protein or soluble trE protein were generated from non-cytopathic VEE replicons (Petrakova et al., 2005). To obtain NS1, a VEErep (VEErep/Pac-Ubi-WNNS1NS2A) containing the *Pac*/*ubiquitin* fusion, 31 codons of the N terminus of WNV C gene, 30 codons of the C terminus of WNV E gene which serve as the signal sequence of WNV NS1, and the WNV NS1-NS2A genes (followed by a stop codon) was constructed. For production of WNV E antigen, a VEErep (VEErep/WNprMtrE-Pac) encoding WNV prM followed by the first 421 codons of the E gene (80% of full-length E) fused to a 6x histidine tag at its C-terminus was constructed.

PASSAGING OF REPLIVAX WN

RepliVAX WN particles obtained from electroporation of *in vitro* transcribed RepliVAX RNA into BHK(VEErep/Pac-Ubi-C*) cells were used for subsequent infection into these packaging cells. RepliVAX WN infections were carried out in small volumes at an approximate multiplicity of infection (MOI) of 0.01 in MEM containing 1% FBS, 10mM HEPES buffer, and antibiotics. After a 1 hour adsorption time, RepliVAX was removed and replaced with fresh medium. Culture medium was changed at 24 hour intervals post-infection, and the 72 hour collection of each generation was used to infect subsequent packaging cells at a MOI = 0.01. This infectious process was carried out to obtain 30 consecutive passages of RepliVAX WN. Genetic sequences of regions of the RepliVAX WN genome were analyzed for passage 10, 20, and 30 RepliVAX WN harvests as described below.

ANALYSES OF REPLIVAX WN

Sequence analyses of RepliVAX were performed by standard methods. Briefly, Vero cell monolayers were infected with undiluted RepliVAX harvests, allowed to adsorb for 1 hour and thoroughly washed to remove any unbound particles before being overlaid with fresh medium. 24 hours post-infection the culture media was removed and TRIzol (Invitrogen, Carlsbad, CA) was used to isolate the total RNA from the cells. This RNA was converted to cDNA using Improm-II reverse transcriptase (Promega, Madison, WI) with random hexamer primers, and specific oligonucleotide primers were used to amplify truncated C (trC), prM, and the N-terminal 145 codons (corresponding to the first 30%) of E in the RepliVAX genome. For passage 10 RepliVAX WN, the complete sequence of the genome was analyzed using similar methods. The resulting amplicons were gel purified and the sequence data were obtained from the purified fragments by standard techniques (Protein Chemistry Core Laboratory, UTMB).

The techniques used to characterize and enumerate RepliVAX WN have been described previously (Rossi et al., 2005; Mason, Shustov, and Frolov, 2006). Briefly, infectious titer determination for all RepliVAX WN harvest and growth curves was performed by infecting Vero cell monolayers with serial dilutions of virus, fixing the cells after a 28 hour incubation period with a 1:1 (v/v) solution of acetone/methanol, and immunohistochemically staining the cells with a polyclonal mouse hyperimmune ascites fluid (MHIAF) specific for WNV. Infected cells were enumerated and used to determine titer in infectious units (IU). To assess the ability of different RepliVAX WN species to form foci of infection, BHK(VEErep/Pac-Ubi-C*) were infected with serial dilutions of RepliVAX WN, overlaid with a 0.6% semisolid tragacanth solution, incubated at 37°C for 72 hours, fixed, and stained using the MHIAF as described above.

Growth curve analyses were performed in 12-well plates using BHK(VEErep/Pac-Ubi-C*) cell monolayers. Cells were infected at an MOI=1 and virus was allowed to adsorb for 2 hours at which time the monolayers were thoroughly washed and incubated at 37°C in MEM with 1% FBS, 10mM HEPES, and antibiotics. Cell culture fluid was completely removed and replaced at 12 to 24 hour intervals, and this fluid was titrated to determine the IU released from the monolayer at each time point.

REPLIVAX WN.2 ANIMAL STUDIES

Groups of 15 five-week-old female outbred Swiss-Webster mice (Harlan Sprague Dawley, Indianapolis, IN) were immunized intraperitoneally (IP) with the following: RepliVAX WN.2 at $1x10^6$ IU, $2x10^5$ IU, $4x10^4$ IU, original RepliVAX WN at $4x10^4$ IU, or diluent alone (mock). Two additional groups of 15 animals were immunized with $1x10^6$ IU or $4x10^4$ IU of RepliVAX WN.2 by the subcutaneous (SC) route. All inoculations were delivered in 100 µl of Leibovitz L-15 medium containing 10mM HEPES buffer and 0.5% FBS. Animals were monitored for vaccine-induced side effects including lethargy and hind-limb paralysis that correlate very well with less subjective criteria such as weight loss or death observed in response to live-virus challenge. At 21 days post-vaccination serum was collected from all of the animals by retro-orbital bleed. Seven days later, the animals were challenged IP with 10 LD_{50} of WNV NY99 and were monitored for changes in their weight or health status for 14 days. Animals scored moribund were humanely euthanized in compliance with UTMB Animal Care and Use requirements and scored as "dead" the following day. At 28 days post-challenge the surviving animals were humanely euthanized and serum was collected for subsequent analyses.

Groups of 10 four-week-old female golden Syrian hamsters (Harlan Sprague Dawley, Indianapolis, IN) were immunized IP with 100 µl containing $1x10^6$ IU or $2x10^5$

IU of RepliVAX WN.2 diluted in MEM containing 1% FBS, 10mM HEPES buffer, and antibiotics. A group of 10 control animals received diluent only (mock). Animals were monitored for three weeks for vaccine-induced side effects. On day 21 post-vaccination sera was obtained from all animals by retro-orbital bleed. Seven days later, the hamsters were challenged IP with $1x10^6$ ffu of WNV NY99 diluted in 100 μl PBS + 10% FBS. The animals' weights and health were monitored for three weeks following challenge. Animals scored moribund were humanely euthanized in compliance with UTMB Animal Care and Use requirements and scored as "dead" the following day. At 21 days post-challenge all surviving animals were humanely euthanized and bled for serum samples by cardiac puncture.

ELISAS AND NEUTRALIZATION ASSAYS

Serum antibody titers to WNV E and NS1 were measured using an enzyme-linked immunosorbent assay (ELISA). Immulon 2HB microtiter plates (Thermo Labsystems, Franklin, MA) were sensitized overnight with carbonate buffer containing soluble NS1 or truncated E protein present in serum-free media cultures of the VEE replicon-bearing cell line described above. Sera samples at a dilution of 1:100 were incubated with the antigen-sensitized wells for 1 hour at room temperature. After thorough washing, HRP-conjugated goat anti-mouse IgG antibody (KPL, Gaithersburg, MD) was added to the plates for 1 hour at room temperature. After a thorough washing, a soluble HRP substrate (TMB, Sigma) was allowed to react with bound enzyme for 2 minutes at room temperature, at which time the reaction was halted by the addition of 1M HCl. The reaction product was quantitated spectrophotometrically at 450nm. Values are corrected for background activity detected from wells that received diluent containing no animal sera samples.

Neutralizing antibody (neut) titers were determined by measuring the dilution of serum necessary to produce a 90% reduction of luciferase activity from Vero cells infected with a firefly luciferase-containing WNV VLP (WNVLP). This assay has been shown to be comparable with classical focus reduction assays (Ishikawa et al., 2008). Serial two-fold dilutions of heat-inactivated (30 minutes at 56°C) pooled serum samples were made in cell culture medium and incubated for 1 hour at 37°C with a fixed amount of WNVLPs containing a WNV replicon encoding the full C gene and NS1-5 along with the firefly luciferase gene (Fayzulin et al., 2006). These VLP/serum mixtures were used to infect Vero cell monolayers in black 96-well plates (Greiner Bio-One, Monroe, NC) for 2 hours at which time the infection medium was replaced with MEM containing 1% FBS, 10mM HEPES buffer, and antibiotic and allowed to incubate for 24 hours at 37°C. A solution containing cell lysis buffer supplemented with 25% Steady-Glo luciferase substrate (Promega, Madison, WI) was added to each well in a 1:1 (v/v) ratio to the culture medium. The plates were shaken at room temperature for 5 minutes and read on a TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA). Light output was measured and expressed as the percent activity obtained from lysates prepared from monolayers infected with WNVLPs incubated at 37°C in diluent only.

STATISTICAL ANALYSES

To compare potency and demonstrate efficacy of RepliVAX WN, serological data were compared by one way ANOVA using appropriate post-tests. Growth kinetics were compared by one way ANOVA using Dunnet's post-test. Survival incidence data were compared by Fisher's exact test. All comparisons were two-tailed.

CHAPTER 3 : REPLIVAX WN, A SINGLE-CYCLE FLAVIVIRUS VACCINE TO PREVENT WEST NILE DISEASE, ELICITS DURABLE PROTECTIVE IMMUNITY IN HAMSTERS[3]

ABSTRACT

West Nile virus (WNV) causes significant disease, yet no vaccines exist to prevent WN disease in humans. We have previously reported that RepliVAX WN is a safe and efficacious vaccine in mouse and hamster models of WN disease. Here, we report that vaccination of hamsters with RepliVAX WN induces antibody responses that remain stable for at least 6 months. Furthermore, animals challenged with virulent WNV 6 months after vaccination were protected from disease as well as those challenged 2 months post-vaccination, with no vaccinated animals succumbing to WNV challenge. These results indicate that RepliVAX is capable of inducing durable protective immunity after a single dose.

[3]A significant portion of this chapter has been previously published in the journal Vaccine. Vaccine does not require copyright permission as long as proper citation is given. The citation for this article is: Widman, D.G., Ishikawa, T., Winkelmann, E.R., Infante, E., Bourne, N. and Mason, P.W. (2009). RepliVAX WN, a single-cycle flavivirus vaccine to prevent West Nile disease, elicits durable protective immunity in hamsters. *Vaccine*. **27**(41):5550-3.

INTRODUCTION

Previously we have reported the development of RepliVAX WN, a single-cycle flavivirus vaccine to prevent WN disease (Mason, Shustov, and Frolov, 2006; Widman et al., 2008). RepliVAX WN consists of a WNV genome containing a deletion in the capsid (C) protein gene, which is packaged into infectious particles by transcomplementation in packaging cell lines developed to express WNV C (Widman et al., 2008). RepliVAX WN can be produced in packaging cells that have been generated from approved vaccine substrates. When RepliVAX WN infects normal cells, it produces all of the major WNV antigens that elicit humoral immunity including subviral particles (SVPs) and NS1, and a full complement of viral T cell epitopes (Nikolich-Zugich et al., unpublished data), however the absence of C precludes production of infectious progeny. RepliVAX WN has been shown to be safe and efficacious in rodent (Widman et al., 2008) of WN infection.

Here we extend these observations to describe the kinetics of antibody response and durable protective immunity following RepliVAX WN vaccination in a hamster model of WNE (Xiao et al., 2001; Watts et al., 2007). A single vaccination with as little as 40,000 infectious units (IU) of RepliVAX WN elicited robust antibody responses that increased for 2 months following immunization and remained stable for an additional 4 months. When challenged at 2 or 6 months post-vaccination, all hamsters were protected from lethal WNV challenge, and none displayed the clinical signs of illness observed in mock-vaccinated animals. Taken together, these results further confirm the utility of RepliVAX WN as a safe and efficacious vaccine to prevent WN disease.

RESULTS

VACCINATION WITH A SINGLE DOSE OF REPLIVAX WN INDUCES ROBUST ANTIBODY RESPONSES IN HAMSTERS

A total of 100 hamsters were divided equally into two groups: group 1 to be challenged at 9 weeks post-vaccination, and group 2 to be challenged 25 weeks post-vaccination. Groups of 10 animals from each challenge group were inoculated with 40,000 or 200,000 IU of RepliVAX WN by either IP or SC route, or vaccine diluent (mock). As expected from previous work in this model (Widman et al., 2008), vaccination was well-tolerated, and all vaccinated animals developed neut titers ranging from 110 to 684 four weeks post-vaccination (Fig. 3.1A). Interestingly, these neut titers increased in 5 of the 8 vaccinated groups by 8 weeks post-vaccination (Fig. 3.1A). A similar trend was observed among the total WNV-specific IgG levels measured by ELISA (Fig. 3.1B). In particular, antibody levels against WNV NS1 increased in all vaccinated groups between weeks 4 and 8 post-vaccination (Fig. 3.1B, bottom). Taken together, these results further demonstrate that vaccination with RepliVAX WN induces useful antibody responses in hamsters after a single immunization.

ANTIBODY RESPONSES ELICITED BY REPLIVAX WN VACCINATION REMAIN STEADY FOR AT LEAST 6 MONTHS

Antibody levels at 16 and 24 weeks post-vaccination were measured in group 2 animals to evaluate the durability of RepliVAX WN-induced immunity. Animals in this group receiving SC inoculations of RepliVAX WN demonstrated peak neut titers of 135 and 268 8 weeks after immunization, and these levels remained relatively constant throughout the remainder of the 24 week monitoring period (Fig. 3.1A). Higher peak neut titers were observed in animals receiving IP immunizations, with titers reaching 363 and

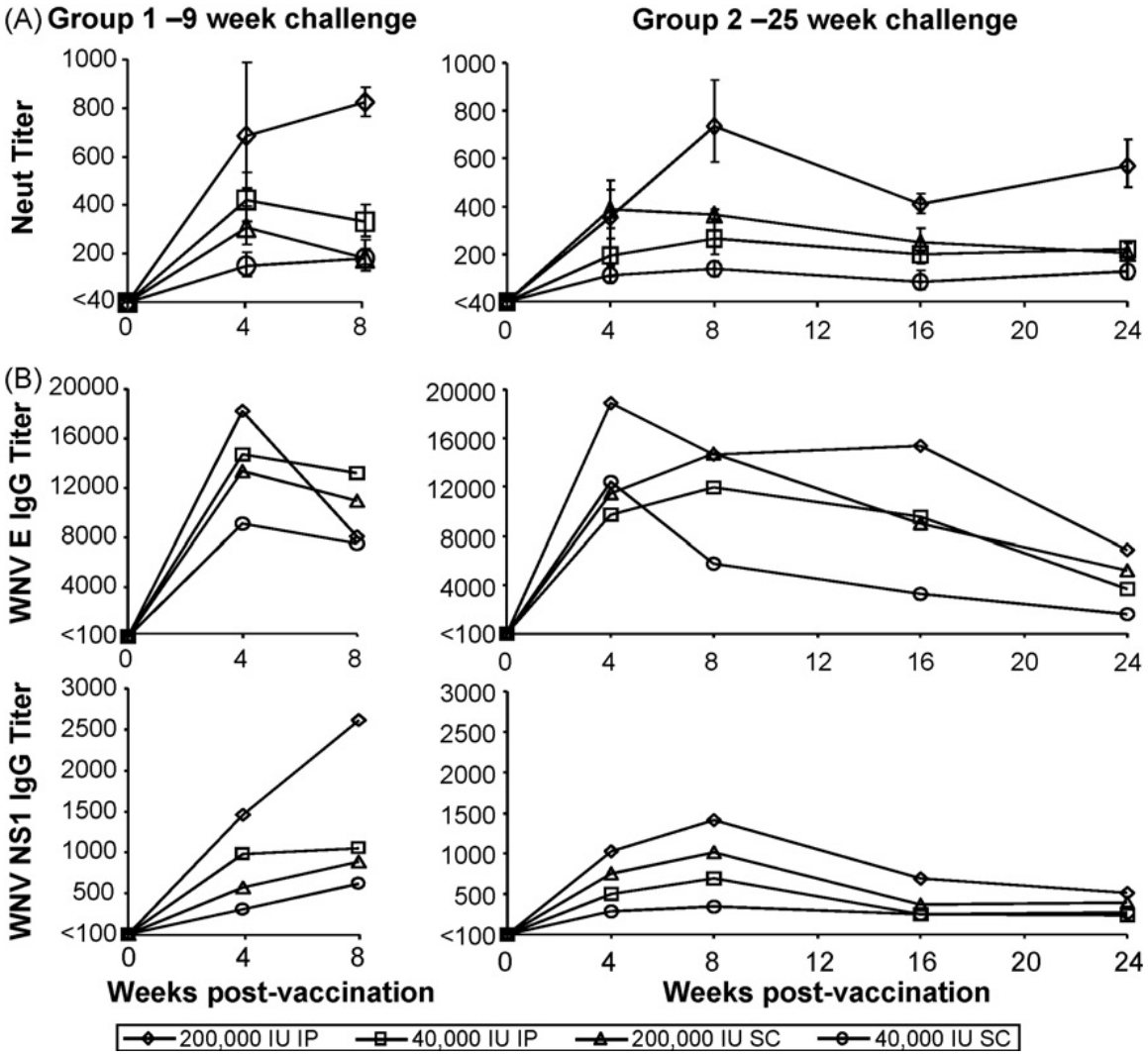

Figure 3.1: Antibody titers induced by single doses of RepliVAX WN. A. Neutralizing antibody levels following vaccination with 200,000 IU IP (diamond), 40,000 IU IP (square), 200,000 IU SC (triangle), or 40,000 SC (circle) of RepliVAX WN for hamsters challenged at 9 weeks (left) or 25 weeks (right) after vaccination. Values represent the reciprocal of the highest dilution of pooled sera that resulted in a 90% reduction of luciferase activity from Vero cells cultured with serum-treated luciferase-expressing WNVLPs, as determined by non-linear regression analysis. Mock-vaccinated animals (not shown) demonstrated neut titers of <40 throughout the study. Error bars represent the 95% confidence interval. **B.** Levels of IgG specific for WNV E (top) and NS1 (bottom) antigen in serum of vaccinated hamsters. Titers shown are the reciprocal of the highest dilution of pooled sera that produced an OD reading ≥3 standard deviations above the OD produced from sera of mock-vaccinated animals as determined by non-linear regression analysis. All OD curves fit with an R^2>0.99, and thus error on these data sets were smaller than the symbols and were hence excluded from the figure.

737 (Fig. 3.1A). Antibody levels in these animals decreased slightly at 16 weeks to 251 and 408 respectively, and at 24 weeks post-vaccination these titers remained steady with values of 205 and 571. Total IgG levels against WNV E rose sharply and reached a titer in excess of 9000 in all vaccinated groups, and although this decreased slightly by 24 weeks (Fig. 3.1B top right), all titers remained above 1600. NS1 antibody levels remained remarkably constant for 24 weeks (Fig. 3.1B bottom right), with titers ranging from 1400 (200,000 IU IP at 8 weeks) to 270 (40,000 IU SC group at 24 weeks) in group 2 during the 24 week observation period. These data indicate that immunization with a single dose of RepliVAX WN induces robust antibody responses in hamsters that are sustained for at least 24 weeks.

HAMSTERS VACCINATED WITH REPLIVAX WN ARE PROTECTED FROM WNV CHALLENGE 2 AND 6 MONTHS POST-VACCINATION

Although WNV-specific antibody levels appeared to be quite stable, it was important to correlate these observed antibody responses with protection from WN disease. As previously (Ishikawa et al., 2008; Widman et al., 2008), hamsters were challenged (group 1 at 9 weeks post-vaccination and group 2 at 25 weeks) IP with $1x10^6$ FFU WNV, and monitored for 28 days. All vaccinated hamsters in group 1 survived challenge as whereas 50% of mock animals succumbed to infection ($p<0.001$) (Fig. 3.2A, left). Furthermore, none of the vaccinated animals experienced a drop in body weight, while 80% of mock animals displayed this and other clinical signs (see methods) of WN disease ($p<0.0001$) (Fig. 3.2B, left). Similar results were observed in the group 2 animals. No vaccinated hamsters succumbed to WNV infection compared to 40% mortality in mock animals ($p<0.0001$) (Fig. 3.2A, right). In addition, by 10 days post-challenge, 100% of mock animals in group 2 demonstrated clinical signs of WNE whereas only 2 vaccinated animals developed weight loss of >10% ($p<.0.01$) (Fig. 3.2B, right). This

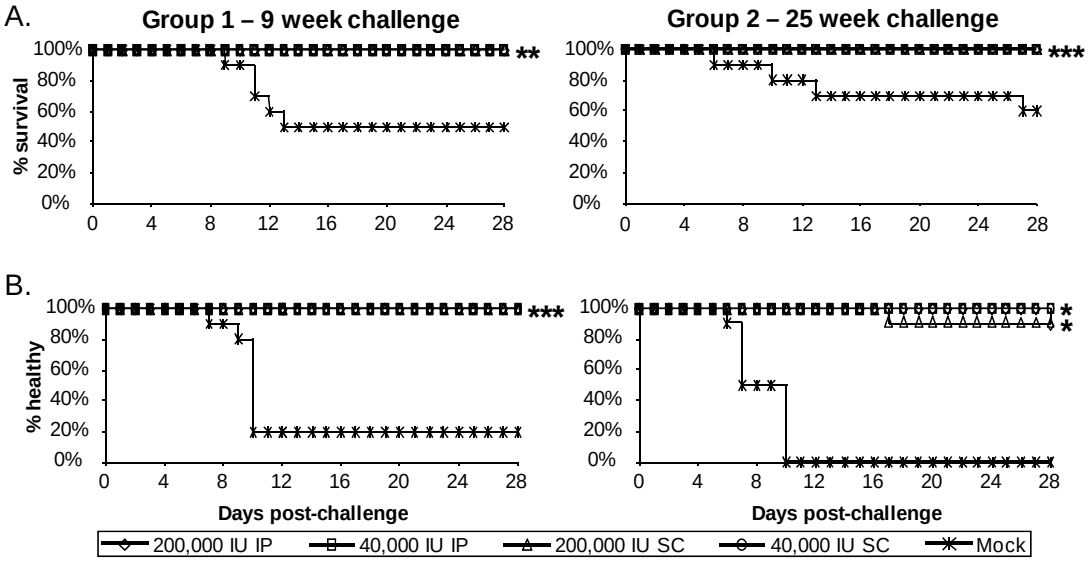

Figure 3.2: Survival and health of hamsters challenged with WNV at 9 and 25 weeks post-RepliVAX WN vaccination. A. Percentage of animals that survived WNV challenge at 9 weeks (left) and 25 weeks (right) post-vaccination. **B.** Percentage of animals that remained healthy following WNV challenge at 9 (left) and 25 (right) weeks post-vaccination. Animals demonstrating a >10% loss of body weight from day of challenge were classified as sick. Two vaccinated animals in group 2 that were scored as sick based on a >10% weight loss during the observation period did not display any of the other clinical signs of illness observed in mock-vaccinated animals. * denotes significant differences between vaccinated and unvaccinated groups (*p<0.01; **p<0.001; ***p<0.0001).

weight loss occurred late in the observation period (day 17 and day 28) and neither animal displayed the other signs of clinical illness that were observed in most of the mock animals. These results indicate that a single vaccination with RepliVAX WN is capable of eliciting immune responses in hamsters that remain protective up to 6 months after administration.

DISCUSSION

Humoral immune responses are believed to play a key role in the prevention and clearance of flavivirus infections (Kreil et al., 1998b; Beasley et al., 2004b; Shrestha et al., 2008), thus persistence of antibody after immunization is a necessary characteristic of vaccines. We have previously demonstrated that RepliVAX WN, a single-cycle flavivirus vaccine is both safe and efficacious in preventing WN disease in mice and hamsters (Widman et al., 2008). In these previous studies, WNV challenge was administered 4 weeks after vaccination. The hamster model of WNE has been utilized previously to examine duration of vaccine efficacy (Watts et al., 2007), and in the study presented here the kinetics of antibody response to RepliVAX WN vaccination and the duration of protection were analyzed for a much longer period.

Vaccinated hamsters demonstrated neut titers ranging from 110 to 684 by 4 weeks post-vaccination, and interestingly these levels increased by 8 weeks in most vaccination groups. This increase was also reflected in total WNV-specific IgG titers, where the trend was particularly evident with antibodies specific for NS1. This pattern of antibody response suggests durable antigen presentation by RepliVAX WN-infected cells, and/or the formation of a class of long-lived plasma cells (Manz, Thiel, and Radbruch, 1997) that continue to secrete antibodies long after the RepliVAX WN infection has been cleared, as has been observed following acute viral infection (Slifka, Matloubian, and Ahmed, 1995). These results indicate that vaccination with RepliVAX WN results in a prolonged period of increasing antibody production in hamsters, followed by a long period during which antibody levels against major WNV antigens remain at high levels.

Although the stability of antibody levels following RepliVAX WN vaccination was encouraging, it was important to correlate these immune responses to protection

from disease. Thus, groups of hamsters were challenged with WNV at 9 and 25 weeks post-vaccination. All vaccinated hamsters regardless of dose or route of administration survived challenge at both times. In contrast, 50% and 40% of naive hamsters from the 9 week and 25 week challenge groups respectively succumbed to WNE. Furthermore, all but 2 animals from the mock-vaccinated groups demonstrated clinical signs of encephalitic illness, while none of the vaccinated animals (including the 2 classified as sick by our weight loss criteria) demonstrated other clinical signs consistent with WN disease. While it cannot be ruled out that these 2 vaccinated challenged animals that demonstrated weight loss harbored a low-level infection that was mostly asymptomatic, these animals had outcomes clearly better than unvaccinated challenged hamsters. Thus a single vaccination with RepliVAX WN was sufficient to protect hamsters from lethal WNV challenge up to 25 weeks after administration.

In conclusion, a single dose of RepliVAX WN results in the induction of humoral immune responses in hamsters that generally increase in magnitude in the 8 weeks following vaccination. Antibodies persisted at high levels in vaccinated hamsters for up to 25 weeks after immunization, and these hamsters were protected from WNV challenge. Taken together, these results indicate that a single vaccination with RepliVAX WN is capable of eliciting durable protective immunity against WN disease.

MATERIALS AND METHODS

VIRUSES AND CELL LINES

Vero cells for RepliVAX WN enumeration and for virus neutralization assays have been previously described (Mason, Shustov, and Frolov, 2006). The RepliVAX WN vaccine stock was produced in BHK(VEErep/Pac-Ubi-C*) cells and has been previously

described (RepliVAX WN.2 SP in (Widman et al., 2008)). Enumeration of RepliVAX WN was performed by immunostaining fixed Vero cells for WNV antigen following an overnight incubation with serial dilutions of RepliVAX WN as previously described (Widman et al., 2008). The WNV NY99 used for challenge (Xiao et al., 2001) was passaged twice in Vero cells before animal inoculation and the infectious titer was determined by focus formation assay on Vero cells (Rossi et al., 2005).

ANIMAL STUDIES

A total of 100 five-week-old female Syrian hamsters (Harlan Sprague-Dawley, Indianapolis, IN) were divided into 2 groups of 50 animals (group 1 and group 2) and sets of 10 animals from each group were immunized with 40,000 or 200,000 IU RepliVAX WN by either subcutaneous (SC) or intraperitoneal (IP) injection. The remaining 10 animals in each group received an IP injection of cell culture medium (mock). Sera were obtained from group 1 animals 4 and 8 weeks post-vaccination, and the animals were challenged IP with $1x10^6$ focus-forming units (FFU) WNV NY99 at 9 weeks. Group 2 animals were bled for sera 4, 8, 16 and 24 weeks post-vaccination, and then challenged with an identical dose of WNV NY 99 25 weeks after vaccination. Following challenge, all animals were monitored daily for 28 days. Animals that demonstrated a >10% loss of body weight from day of challenge were scored as "sick". Animals that in addition to weight loss displayed at least three signs of disease- changes in grooming, hind-limb paralysis, tremors, difficulty walking, or eye discharge- were scored moribund and humanely euthanized in compliance with UTMB Animal Care and Use requirements and scored as ‘‘dead’’ the following day. In addition to animals that died during blood collection, three hamsters from each challenge group died prior to challenge, however there was no statistical correlation between treatment and death, and none of these animals displayed signs of encephalitis prior to death.

WNV E AND NS1 ELISAS

WNV-specific IgG ELISA titers were determined by a modification of a previously described protocol (Widman et al., 2008). Serial 2-fold dilutions of pooled sera from each treatment group were added to ELISA plates coated with WNV E or NS1 antigen, and after washing, bound IgG was detected using an HRP-conjugated anti-hamster IgG antibody (KPL, Gaithersburg, MD). Normalized optical density readings were curve fit using non-linear regression analysis, and only curves with an R^2 value >0.99 were used for analysis (all vaccinated groups). Titers presented were determined using these curves, and are the reciprocal of the highest dilution of serum at which ELISA OD readings are greater than 3 standard deviations above the OD reading produced by pooled sera from mock-vaccinated animals.

NEUTRALIZATION ASSAYS

Assays used to determine WNV-neutralizing antibody (neut) titers have been previously described (Ishikawa et al., 2008; Widman et al., 2008). Results were analyzed using non-linear regression analysis, and only curves with an R^2 value >0.90 were used for analysis (all vaccinated groups). Titers represent the reciprocal dilution of pooled sera producing in a 90% reduction in luciferase activity from Vero cells cultured with a serum-treated firefly luciferase-expressing WNV-like particle (WNVLP).

STATISTICAL ANALYSES

All statistical analyses were performed using Graphpad Prism 5.0 software. Survival and health curves were analyzed using log-rank tests. Neutralizing antibody and ELISA antibody titers were determined by non-linear regression analysis (see above).

CHAPTER 4 : EVALUATION OF REPLIVAX WN, A SINGLE-CYCLE FLAVIVIRUS VACCINE, IN A NON-HUMAN PRIMATE MODEL OF WEST NILE VIRUS INFECTION

ABSTRACT

West Nile virus (WNV) causes serious neurologic disease, but no licensed vaccines are available to prevent this disease in humans. We have developed RepliVAX WN, a single-cycle flavivirus with an expected safety profile superior to other types of live-attenuated viral vaccines. Here we describe studies examining RepliVAX WN safety, potency and efficacy in a non-human primate (NHP) model of WNV infection. A single immunization of four rhesus macaques with RepliVAX WN was safe and elicited detectable neutralizing antibody titers and IgM and IgG responses, which were boosted in two animals receiving a second immunization. Following challenge with WNV, three of four immunized animals were completely protected from viremia, and the remaining animal showed minimal viremia on a single day. In contrast, the unvaccinated animal developed viremia lasting 6 days. These results demonstrate RepliVAX WN safety and efficacy in this primate model of WNV infection.

INTRODUCTION

We have previously described the construction of RepliVAX WN, a rationally attenuated, single-cycle virus vaccine to prevent WNE (Mason, Shustov, and Frolov, 2006; Widman et al., 2008). The RepliVAX WN genome contains a large deletion in the gene encoding C in an otherwise complete WNV genome. RepliVAX WN can be propagated in cells expressing the WNV C protein (Widman et al., 2008) and when used for immunization each RepliVAX WN particle infects a single cell where the genome undergoes multiple rounds of replication, resulting in the sustained production of WNV antigens (SVPs and NS1) without producing infectious progeny. Thus RepliVAX WN demonstrates remarkable safety by producing a limited infection in vaccinees, yet is surprisingly potent. Vaccination with as little as 40,000 infectious units (IU) completely protected mice (Widman et al., 2008) and hamsters (Widman et al., 2009) from WN disease. T-cell responses in RepliVAX WN-vaccinated mice are similar to those induced by WNV infection, and initial studies indicate immunization with RepliVAX WN can protect immunocompromised mice from lethal WNV challenge (Nikolich-Zugich et al., unpublished data), indicating that RepliVAX WN has the potential to be safe and effective in high risk populations.

Here we report the initial evaluation of safety, potency and efficacy of RepliVAX WN in a non-human primate (NHP) model of WNV infection. A single immunization with 10^6 IU of RepliVAX WN was well-tolerated and induced antibody responses at levels known to correlate with protective immunity against flavivirus disease in humans (Hoke et al., 1988; WHO, 1998; Halstead and Tsai, 2004). A second dose administered to half of the animals produced an enhanced WNV-specific antibody response, and upon challenge with WNV 3 of 4 vaccinated animals were completely protected from WNV

viremia. Following challenge, immunized animals demonstrated a robust recall antibody response, and all animals displayed increased levels of activated dendritic cells and T cells. Taken together, these results demonstrate RepliVAX WN safety and efficacy in this NHP model of WNV infection.

RESULTS

REPLIVAX WN VACCINATION IS SAFE AND WELL TOLERATED BY NON-HUMAN PRIMATES

The rhesus macaque model of WNV infection (Arroyo et al., 2004; Ratterree et al., 2004b; Pletnev et al., 2006) was chosen to assess the safety and efficacy of RepliVAX WN in NHPs. While this model does not produce clinically apparent disease, sustained levels of WNV viremia are detectable in the sera of infected animals for up to 5 days post-challenge (Ratterree et al., 2004b), and animals develop detectable immune responses to vaccination and challenge. Five male rhesus macaques (30 to 42 months old) were chosen for study based on preliminary HAI serology confirming lack of previous exposure to medically important mosquito-borne flaviviruses. Four of these animals

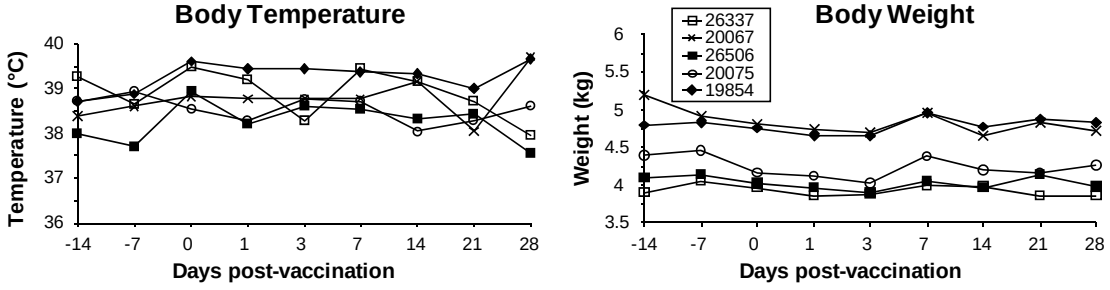

Figure 4.1: NHP vital signs following RepliVAX WN vaccination. Body temperature and weight of primates following primary vaccination with RepliVAX WN. Animals were monitored for fluctuations in body temperature (left panel) and weight (right panel) following primary vaccination (on day 0).

(26337, 20067, 20075, 26506) were injected SC in the upper arm with 10^6 IU of RepliVAX WN while one animal (19854) received a mock injection by the same route. No significant fluctuations in body weight or temperature were observed in any of the animals as a result of vaccination (Fig. 4.1). Clinical observation revealed no changes in food or water consumption, appearance, or general behavior in any animals following RepliVAX WN vaccination. All of the animals` heart rates and respiration rates remained normal, all blood chemistry tests yielded results within normal limits, and no clinical signs of cognitive or neural impairment were observed in any of the vaccinated animals. Taken together, these results indicate that RepliVAX WN vaccination is safe and well-tolerated by rhesus macaques.

A SINGLE DOSE OF REPLIVAX WN INDUCES WNV-SPECIFIC IGM AND IGG RESPONSES

Humoral immunity is believed to play a primary role in clearing flavivirus infections (Kreil et al., 1998a; Beasley et al., 2004b; Shrestha and Diamond, 2004; Shrestha et al., 2008) and provides an excellent predictor of vaccine efficacy (Hoke et al., 1988; Monath et al., 2002b; Barrett, Schober-Bendixen, and Ehrlich, 2003). In order to assess the potency of RepliVAX WN in NHPs, and determine the kinetics of RepliVAX-induced antibody production, WNV-specific IgM and IgG levels were analyzed throughout the study. IgM antibody responses measured by ELISA or HAI were detectable in 3 of 4 vaccinated animals by day 7, and these responses generally increased by day 14 post-vaccination (Fig. 4.2A and Table 4.1). In 3 of the 4 vaccinated animals (26337, 26506, 20075), WNV-specific IgM responses were significantly greater ($p<0.05$) on day 14 than on day 7 (Fig. 4.2A). The presence of WNV-specific IgM was also apparent from the presence of 2-mercaptoethanol (2-ME)–sensitive HAI activity in sera (Table 4.1). By day 14 post-vaccination, low but detectable levels of IgG were observed in 3 of these animals by detection of 2-ME-resistant HAI antibody titers of 1:8 (Table

4.1). At 28 days post-vaccination, neut and HAI titers had increased to 1:32 to 1:64 in all vaccinated animals (Table 4.1). Similar kinetics of antibody response were obtained using an ELISA to detect WNV-specific IgG (Fig. 4.2B). The induction of WNV-specific antibody responses after a single immunization with RepliVAX WN indicates its potential utility as a single-dose vaccine.

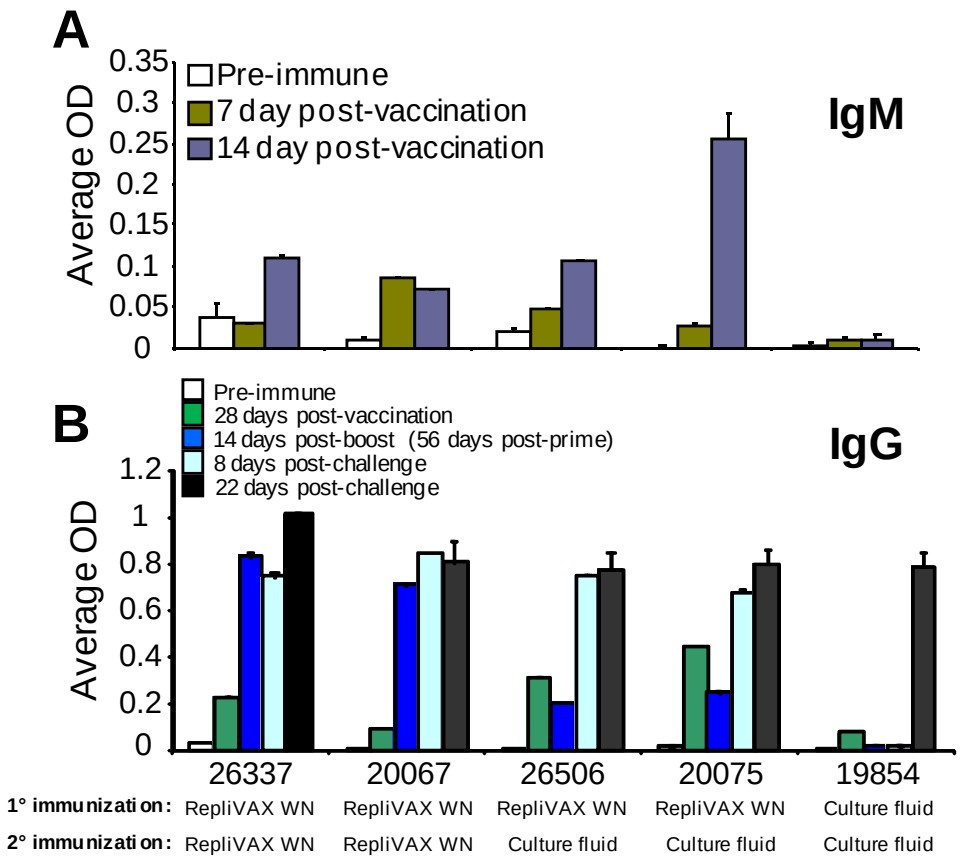

Figure 4.2: WNV-specific antibody levels determined by ELISA. A. IgM levels in the serum of NHPs on the day of vaccination (pre-immune) and days 7 and 14 post-vaccination. **B.** IgG levels in the serum of NHPs on the day of vaccination (pre-immune), day 28 post-vaccination, day 14 post-boost, and days 8 and 22 post-challenge. Bars show average O.D. values and extended bars indicate standard deviation between technical replicates.

ANIMALS BOOSTED WITH A SECOND DOSE OF REPLIVAX WN SHOW INCREASED ANTIBODY TITERS

Two of the four vaccinated animals (26337, 20067) were chosen at random to receive a second SC dose of 10^6 IU of RepliVAX WN 42 days after primary vaccination. The remaining three animals (20075, 26506, 19854) received a mock immunization. As with primary immunization, no adverse clinical responses were observed following the second immunization (data not shown). 14 days after these inoculations, WNV-specific IgG levels were significantly higher (3-4 fold; $p<0.01$) in the boosted animals relative to those primates that received only a single vaccination (Fig. 4.2B), however the effect of boosting was not as pronounced when measured by neut assay (Table 4.1). The neut titers of the 2 boosted animals were 1:32 and 1:128 (compared to 1:32 and 1:64 respectively 28 days after primary vaccination), while the titers of the animals receiving a single dose of RepliVAX WN decreased slightly from 1:32 on day 28 to 1:16 by day 56 (Table 4.1), a trend corroborated by measurement of total WNV-specific IgG levels (Fig. 4.2B). While these results indicate that a second dose of RepliVAX WN induces higher levels of IgG, it is not clear if this correlates to increased neut activity.

ONE OR TWO IMMUNIZATIONS WITH REPLIVAX WN PROTECT RHESUS MACAQUES FROM WNV VIREMIA

Fifty-six days after primary vaccination, and 14 days after a booster was administered to two animals, all NHPs in this study were challenged with a SC inoculation of 10^5 pfu of WNV NY99. Clinical observations were performed daily following challenge, and as expected from previous studies using this model (Pletnev et al., 2003; Arroyo et al., 2004; Ratterree et al., 2004b), no clinical signs of WNV disease were detected in any animals during the 33 day post-challenge observation period (data

Table 4.1 Comparison of RepliVAX WN and WNV-induced antibody titers

		Immune response									
		Pre-immune	Day 7 post-immunization		Day 14 post-immunization		Day 28 post-immunization		Day 14 post-boost		Day 21 post-challenge
ID	Vaccination schedule (immunization/boost)[a]	HAI	HAI	HAI + 2-ME[b]	HAI	HAI + 2-ME[b]	Neut	HAI	Neut	HAI	Neut
26337	RepliVAX WN/ RepliVAX WN	<1:8	1:8	<1:8	1:32	<1:8	1:64	1:64	1:128	1:64	1:512
20067	RepliVAX WN/ RepliVAX WN	<1:8	1:16	<1:8	1:32	1:8	1:32	1:32	1:32	1:32	1:128
20075	RepliVAX WN/ culture fluid	1:8	1:16	<1:8	1:64	1:8	1:32	1:64	1:16	1:16	1:512
26506	RepliVAX WN/ culture fluid	<1:8	1:8	<1:8	1:32	1:8	1:32	1:64	1:16	1:16	1:1024
19854	culture fluid/ culture fluid	1:8	1:8	<1:8	<1:8	<1:8	<1:8	1:8	<1:8	1:8	1:512

[a] All RepliVAX WN inoculations were 10^6 IU; culture fluid, used as a mock inoculum, was recovered from uninfected Vero(VEErep/Pac-Ubi-C*); boost was performed at 42 days post-immunization (see text).
[b] Differences between HAI titer obtained in the presence and absence of 2-ME can be used to infer WNV-specific IgM titers (see text).

not shown). However, data from viremia analyses confirmed the efficacy of RepliVAX WN vaccination. The unvaccinated primate (19854) developed a sustained serum viremia of greater than 100 ffu/ml that persisted for four days (Fig. 4.3), which compared well with data obtained from a cohort of 4 unvaccinated rhesus macaques previously challenged under conditions identical to those used in this study (results not shown). Analysis of WNV viremia revealed that in both animals that received two doses of RepliVAX WN (26337, 20067), and in one receiving a single dose (20075), infectious WNV was undetectable in the serum at any point in time following challenge (Fig. 4.3). Furthermore, the single vaccinated animal that displayed viremia (26506) demonstrated an extremely low level of virus (7.5 ffu/ml) for only one day, immediately following challenge. Thus the viremia detected in this animal appeared to be due to the inoculum, and the overall viral load in this animal was significantly lower ($p<0.02$) than that observed in the unvaccinated animal in this study (19854; Fig. 4.3), and also lower than 4 similarly challenged unvaccinated animals.

Serum IgG levels were analyzed post-challenge to assess the ability of vaccinated animals to mount a recall response upon challenge. All vaccinated animals, regardless of dose schedule, demonstrated robust anti-WNV IgG levels at 8 days post-challenge (Fig 1B). These levels were significantly higher ($p<0.001$) than that observed in the unvaccinated primate, indicating that this was a recall response to RepliVAX WN vaccination. By 22 days post-challenge, however, the unvaccinated primate had developed IgG titers comparable to those observed in vaccinated animals (Fig. 4.2B), and all animals demonstrated similar neut titers (Table 4.1). Interestingly, the lone vaccinated animal that showed a detectable viremia had a post-challenge neut titer of 1:1024, in excess of all other study animals.

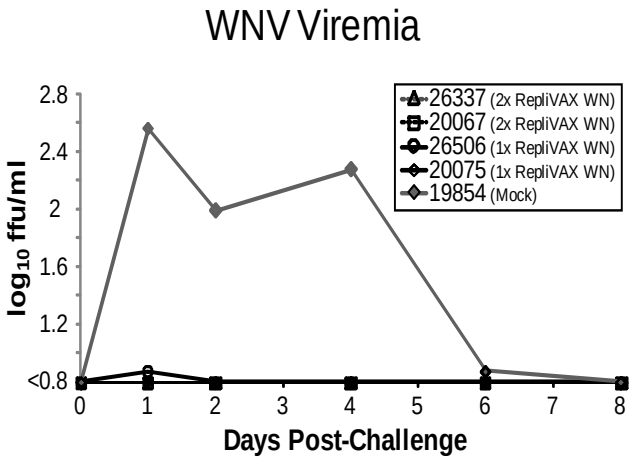

Figure 4.3: Serum viremia levels following WNV challenge. A focus formation assay was used to assess the amount of circulating infectious WNV in the serum of challenged animals at the indicated days following challenge with 10^5 pfu WNV NY99. Animals 26337 and 20067 received two vaccinations with RepliVAX WN prior to challenge, 26506 and 20075 received one dose of RepliVAX WN and 19854 received mock vaccination.

ANALYSES OF CELLULAR RESPONSES TO REPLIVAX WN VACCINATION AND WNV INFECTION

Flow cytometry was performed to identify the phenotype and activation status of T cell and dendritic cell (DC) populations following vaccination with RepliVAX WN and during the acute phase of West Nile virus infection in NHPs. In general, vaccination with RepliVAX WN did not induce detectable variation in the levels of circulating T cell or DC subsets (Fig. 4.4). Challenge with WNV resulted in marked and transient changes in the levels of DCs and T cells, however these changes did not correlate with outcome of infection. Following vaccination, myeloid DCs (MDCs) were present in higher percentages than plasmacytoid DCs (PDCs) in the blood of rhesus macaques, and while both DC subsets increased immediately after challenge with WNV (Fig. 4.4A, left), the increase in circulating levels was of higher amplitude for PDCs. Levels of activation for DCs were evaluated by determining the presence of the co-stimulatory molecule CD86. Both MDCs and PDCs demonstrated a marked increase in the levels of CD86 after challenge with WNV, with PDCs showing a greater fluctuation compared to pre-challenge levels (Fig. 4.4A, right). Both percentages and levels of activation returned to pre-challenge values by one week post-challenge.

The circulating levels of T cell subsets also changed as a result of challenge with WNV, however these fluctuations were not as dramatic as those observed for DCs. Moderate increases in both $CD4^+$ and $CD8^+$ T cells were observed after challenge (Fig. 4.4B, top), whereas changes for NK cells and B cells were minimal (data not shown). Activation of T cells, as measured by expression of CD69, was evident following challenge, particularly in the case of $CD4^+$ T cells (Fig. 4.4B, bottom left). Interestingly, while both the unvaccinated primate (19854) and animals that received a single RepliVAX WN vaccination (26506, 20075) showed similar levels of $CD8^+$ T cell activation following challenge, animals that received two doses of RepliVAX WN (20067, 26337) did not present generalized activation of CD8 T cells (Fig. 4.4B, bottom right).

DISCUSSION

We have previously demonstrated that RepliVAX WN, a novel single-cycle live-attenuated virus vaccine, can be produced using technology compatible to human vaccine production (Widman et al., 2008). In the study presented here we report the initial evaluation of RepliVAX WN produced using these methods in a NHP model of WNV infection. The model selected, parenterally challenged rhesus macaques, displays signs of infection consistent with those that occur in the vast majority of humans infected with WNV. Specifically, WNV infection of rhesus macaques is not associated with symptomatic disease or death. However, this model provides important tests of primate safety and potency, and these animals do display a WNV viremia that persists for multiple days (Ratterree et al., 2004b) permitting this viremia to be used as an endpoint for evaluating vaccine efficacy(Pletnev et al., 2003; Arroyo et al., 2004).

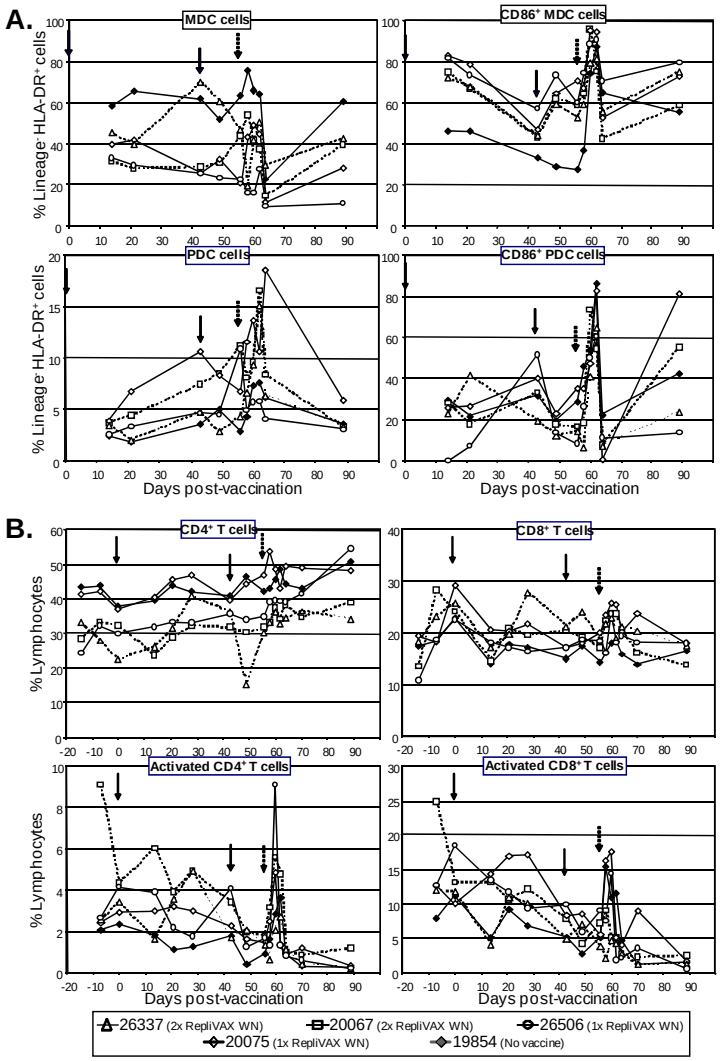

Figure 4.4: Flow cytometry analyses of circulating immune cells in rhesus macaques vaccinated with RepliVAX WN and challenged with WNV. Four rhesus macaques were vaccinated with RepliVAX WN (solid arrows) on day 0; two animals received an additional RepliVAX WN vaccination on day 42 (20067 and 26337, dashed lines), while the other two animals received a mock vaccination on day 42 (20075 and 26506, solid lines). A fifth macaque (19854) was mock vaccinated on days 0 and 42. All animals were challenged with WNV on day 56 (dashed arrow). **A.** Levels of circulating myeloid (top left) and plasmacytoid (bottom left) DCs, as well as activated MDCs (top right) and PDCs (bottom right) were determined using expression of CD86 was used as indicator of DC activation. **B.** Flow cytometry analyses of circulating T lymphocytes in rhesus macaques vaccinated with RepliVAX WN and challenged with WNV. Levels of circulating $CD4^+$ (top left) and $CD8^+$ (top right) T cells, as well as the state of activation for $CD4^+$ (bottom left) and $CD8^+$ (bottom right) T cells were determined by flow cytometry using expression of CD69 was used as indicator of cell activation.

In our studies, vaccination with one or two doses of RepliVAX WN was well-tolerated by all of the animals with no adverse clinical or physiologic signs. These findings were not surprising due to the high level of attenuation of RepliVAX WN (Mason, Shustov, and Frolov, 2006), namely its inability to produce a spreading infection in vaccinees. Moreover, this genetically restricted ability to produce spreading infection makes markers of attenuation used to evaluate traditional live-attenuated viral vaccine candidates such as vaccine viremia and neurovirulence largely unnecessary for RepliVAX WN. Nevertheless, no pathology consistent with encephalitic flavivirus infection was detected in neural tissues examined post-challenge (data not shown), arguing that neither RepliVAX WN nor the challenge virus produced any serious neurological problems.

The potency of RepliVAX WN was evaluated in both single and multi-dose regimens. After a single immunization with 10^6 IU of RepliVAX WN, all vaccinated NHPs developed WNV-specific IgM responses after 7 days that increased sharply in 3 of 4 vaccinated animals by 14 days post-vaccination. The kinetics of IgM appearance suggests a durable presentation of WNV antigens by RepliVAX WN-infected cells, and closely mirrored the kinetics of IgM responses observed in WNV-infected rhesus macaques (Ratterree et al., 2004b). The presence of 2-ME-resistant HAI activity by day 14 post-RepliVAX WN vaccination demonstrated the presence of WNV-specific IgG at this time point post-immunization, and both HAI and neut titers increased by day 28 to levels (1:32 – 1:64) that exceed those believed to correlate with protective immunity against JEV in humans (Hoke et al., 1988; WHO, 1998; Halstead and Tsai, 2004). Although high levels of circulating antibodies were elicited by RepliVAX WN vaccination, global activation and proliferation of DCs and T cells in the blood were not detected at the time points we sampled following primary or secondary vaccination.

Despite the robust immune response elicited by primary RepliVAX WN vaccination, two animals were selected to receive a second identical RepliVAX WN dose in order to assess the usefulness of such a vaccination schedule. ELISA assays for WNV-specific IgG revealed that this boost increased antibody reactivity by 3- to 7-fold over pre-boost values, but neut assays were unable to detect any effect of the boost on neut titers. This could be a result of the induction of a population of non-neutralizing antibodies resulting from administration of a booster dose. While these antibodies may not be detected in virus neutralization in vitro, it is possible that they play a role in virus opsonization in vivo and are thus important in the context of protective immunity. Due to the small scale of this study, however, these results do not strongly support or contradict the usefulness of a second dose of RepliVAX WN, and therefore the optimal administration conditions for RepliVAX WN need further investigation. Interestingly, the lone unvaccinated animal in this study demonstrated a robust neut titer of 1:512 following WNV challenge, significantly higher than the 1:32 – 1:64 neut titers elicited 28 days after a single immunization with RepliVAX WN. This difference in antibody response, along with a relative lack of large-scale DC or T cell activation, can be attributed to the inability of the single-cycle RepliVAX WN to produce viremia that is observed in WNV infection, and highlights a key safety feature of RepliVAX WN over traditional live-attenuated viral vaccines.

Upon challenge with WNV, 3 of 4 vaccinated animals were completely protected from viremia, and the lone vaccinated animal that developed a detectable viremia displayed a titer of only 7.5 ffu/ml of serum at day 1 post-challenge, and WNV was undetectable in this animal on all other days tested. In contrast, WNV was detected in the serum of the single unvaccinated animal at 48 times this level on day 1 post-challenge, remained at elevated levels (≥100 ffu/ml) through day 4, and was still detectable 6 days

post-challenge. The results obtained with this single unvaccinated animal closely mirror those observed previously in a cohort of 4 rhesus macaques challenged with an identical WNV preparation, providing further evidence of the reproducibility of this model, and the effectiveness of RepliVAX WN in preventing WN viremia in NHPs.

All vaccinated animals displayed high levels of WNV-specific IgG levels 8 days after WNV challenge that was not observed in the unvaccinated animal, and based on the sharp rise in IgG levels seen in the singly vaccinated animals, vaccination with RepliVAX WN likely results in formation of a memory B-cell population capable of rapid activation by WNV infection. Flow cytometry was performed on blood samples collected during the post-challenge study period in order to examine the cellular response to acute WNV infection, and what role if any vaccination played in shaping these responses. Challenge with WNV resulted in an increase in the numbers of activated DCs and T cells, in particular PDCs which produce high levels of interferon alpha when exposed to WNV (Silva et al., 2007). Sharp increases in activated $CD4^+$ and $CD8^+$ T cells were also observed in nearly all animals. Interestingly, animals receiving two doses of RepliVAX WN did not demonstrate a rise in activated $CD8^+$ T cells that was seen in all other animals. This is likely a result of high levels of circulating antibody, along with innate immune responses, that retarded the WNV infection enough to prevent large-scale activation of $CD8^+$ T cells. Overall, however, DC and T cell responses in the vaccinated macaques following challenge were similar to those in the unvaccinated animal, despite the lack of detectable viremia, suggesting RepliVAX WN vaccination primed cells for rapid activation in the face of infection.

In conclusion, we have demonstrated that vaccination of rhesus macaques with RepliVAX WN was safe and well-tolerated, and a single dose elicited humoral immune responses within ranges known to correlate with flavivirus vaccine efficacy (WHO,

1998). Administration of a second dose of RepliVAX WN to a subset of NHPs appeared to increase WNV-specific IgG levels over animals receiving a single dose; however the overall benefit of a second vaccination was not clear. Upon challenge with WNV, 3 of 4 vaccinated primates were completely protected from WNV viremia, and singly vaccinated animals demonstrated a strong anamnestic IgG response to WNV challenge. These findings were in sharp contrast to those detected in the challenged unvaccinated animal, which developed a sustained WNV viremia and displayed a delayed IgG response to challenge. Taken together with our previous studies demonstrating safety and efficacy of RepliVAX WN in two rodent models of WNV disease (Widman et al., 2008), the findings presented in this small-scale NHP study support the development of RepliVAX WN as a vaccine for use in man.

MATERIALS AND METHODS

CELL LINES AND VIRUSES

Vero(VEErep/Pac-Ubi-C*) used for RepliVAX WN production and Vero cells used for virus quantification have been described (Widman et al., 2008). The RepliVAX WN used for this study (RepliVAX WN.2 SP) (Widman et al., 2008) was produced in Vero(VEErep/Pac-Ubi-C*) maintained in serum-free medium (SFM; OptiPro; Invitrogen, Carlsbad, CA, supplemented with 10mM HEPES). Briefly, *in vitro* transcribed RepliVAX WN RNA was electroporated into BHK(VEErep/Pac-Ubi-C*) cells (Widman et al., 2008), and this RepliVAX WN harvest was used to infect Vero(VEErep/Pac-Ubi-C*) cells. The clarified RepliVAX WN harvest from Vero(VEErep/Pac-Ubi-C*) cells was enumerated on wild-type Vero cells (Widman et al., 2008), diluted in SFM to 10^6 IU per 500µl, and used for immunization. Challenge studies were performed using WNV

NY99 passaged once in Vero E6 cells. Virus neutralization assays were performed using a snowy owl isolate of WNV NY99 (Xiao et al., 2001).

NON-HUMAN PRIMATE MANIPULATIONS

NHP studies were conducted at the Southwest Foundation for Biomedical Research (SFBR, San Antonio, TX) and were undertaken in compliance with all Federal and Institutional guidelines and protocols. Five male 30-42 month old rhesus macaques (*Macaca mulatta*) of Indian origin were selected from an SPF colony at SFBR based on results of hemagglutination inhibition (HAI) assays that demonstrated a lack of serological responses to WNV, yellow fever virus, Japanese encephalitis virus (JEV), St. Louis encephalitis virus, or any dengue virus serotype. Two weeks prior to vaccination, animals were transferred to an animal biosafety level 2 (ABSL-2) laboratory at SFBR. On day 0 of the study, four animals (26337, 20067, 20075, 26506) were vaccinated with 10^6 IU of RepliVAX WN delivered in 0.5ml subcutaneously (SC) in the upper arm while the fifth animal (19854) received a mock vaccination of clarified SFM from uninfected Vero(VEErep/Pac-Ubi-C*) cells. At predetermined time intervals, animals were sedated and blood samples were collected for hematology, chemistries, and serology. On day 42, two animals (26337, 20067) received a second vaccination with an identical dose of RepliVAX WN while the remaining three animals (20075, 26506, 19854) were administered a mock vaccination. All animals were then transferred to the ABSL-3 facility at SFBR where they were challenged on study day 56 with 10^5 pfu of WNV NY99 delivered in 500ul SC in the upper arm. At predetermined time intervals, animals were sedated and blood samples were collected for hematology, chemistries, serology, and virus recovery. On study day 89 all animals were humanely euthanized and tissues were harvested for histopathologic examination.

IgM and IgG ELISAs

WNV-specific IgM and IgG levels in serum samples were analyzed using the WNV IgM Capture DxSelect and IgG DxSelect ELISA kits (Focus Diagnostics, Cypress, CA) according to manufacturer's protocols. Results are reported as the average optical density observed at 450nm in duplicate wells, and values are corrected for background activity detected from wells that received sample diluent containing no animal sera.

Detection of viremia

Virus in serum samples was quantified using a modification of a focus-formation assay previously described (Bourne et al., 2007). Briefly, Vero cell monolayers in 12-well plates were overlaid with duplicate 1:3 dilutions of serum in culture fluid. After a 1 hr adsorption, cells were overlaid with MEM containing 0.6% tragacanth (MP Biomedicals, Solon, OH), 1% FBS and 10mM HEPES and incubated for 48 hr at 37°C. Following fixation with 50% acetone-50% methanol, the foci were visualized by immunostaining as previously described (Widman et al., 2008).

Neutralization assays

Neutralizing antibody (neut) titers were determined on Vero cells using a focus-reduction assay (Mason, Shustov, and Frolov, 2006). Briefly, serially diluted heat-inactivated sera were incubated with approximately 150 ffu of WNV NY99 for 1hr at 37°C and then inoculated onto Vero cell monolayers in 24-well plates. As with detection of viremia, following a 1 hr absorption, the cells were overlaid with MEM/tragacanth and incubated for 40 hr at 37°C. Following fixation, the foci were visualized by immunostaining as previously described (Widman et al., 2008). Neut titers were reported as the highest serum dilution yielding 50% reduction in focus number compared to those obtained from pre-immune sera of the corresponding animal (Monath et al., 2000b).

HEMAGGLUTININATION INHIBITION ASSAYS

HAI titers were determined by a micro-modification of the Clarke and Casals method (Clarke and Casals, 1958) using gander erythrocytes (Lampire Biological Laboratories, Pipersville, PA). To evaluate the relative contribution of IgM to HAI titers, some tests were run in parallel with a portion of the sera treated with an equal amount of 0.2M 2-mercaptoethanol (2-ME) for 1hr at 37°C to destroy pentameric IgM molecules (Caul, Smyth, and Clarke, 1974).

FLOW CYTOMETRY

Antibodies used for 6-color flow cytometry assays (Appendix A) were specific for human antigens, but have been determined to cross-react with rhesus macaque cell surface proteins (data not shown). Whole blood in sodium EDTA was incubated with a panel of fluorochrome-conjugated monoclonal antibodies for 30 min at room temperature. Erythrocytes were eliminated by a standard whole-blood lysis method (BD PharmLyse Solution, BD-Biosciences, San Jose, CA), and the remaining cells were washed twice with PBS and fixed with 1.6% methanol free-formaldehyde/PBS (Polysciences Inc., Warrington, PA). Cells were kept refrigerated until data was acquired using a CyAn^{TM} ADP instrument (Beckman Coulter Inc, Fullerton, CA) equipped with lasers of 405, 488, and 635nm excitation lines. Electronic compensation and analysis were done using Summit V.4.3 software. For general phenotypes 10,000 events were acquired, but for dendritic cell (DC) analyses up to 100,000 events were recorded. DCs were identified as Lineage (CD2, CD3, CD14, and CD20)$^{-}$ HLA-DR^{+} cells, and further divided into myeloid (CD11c^{+}) or plasmacytoid (CD123^{+}); T cells were identified as CD3^{+} and further divided into CD4^{+} or CD8^{+} cells.

STATISTICAL ANALYSES

Statistical analyses were carried out using Graphpad Prism Analysis Software. Data were analyzed using one-way ANOVA with Bonferroni's post-test (antibodies) or unpaired *t*-test (viremia).

CHAPTER 5 : IN VIVO IMAGING OF LUCIFERASE-EXPRESSING FLAVIVIRUSES DEMONSTRATES THAT THE INTERFERON SYSTEM REGULATES THE PERSISTENCE OF VIRUS-INFECTED CELLS AND AFFECTS THEIR DISTRIBUTION

ABSTRACT

It has been well established that type-I and type-II interferon (IFN) responses play critical roles in controlling flavivirus infection and studies indicate that flaviviruses are capable of blocking IFN production in a number of different ways. We were thus interested in examining the role IFN plays in the development of protective immunity following vaccination with RepliVAX WN, a single-cycle flavivirus (SCFV) vaccine to prevent West Nile virus (WNV)-associated disease. Vaccination with RepliVAX WN induced robust levels of IFNα by 24hr post-inoculation, but was reduced to low levels by 48hr. When AG129 (IFNα/β and IFNγ receptor$^{-/-}$), A129 (IFNα/β receptor$^{-/-}$) and S129 (wild-type) mice were vaccinated, all animals developed similar neutralizing antibody titers, although only vaccinated mice with an intact IFNγ response were protected from WNV challenge. AG129 and S129 mice also displayed similar levels of B cell activation and circulating IgM and IgG levels by 7 days post-vaccination, indicating that IFN responses are not required for the production of antibodies. In vivo imaging of animals inoculated with firefly luciferase (FLUC)-expressing SCFVs revealed the site of inoculation and draining lymph nodes as primary sites of SCFV-infected cell localization. Furthermore, AG129 mice demonstrated markedly higher levels of SCFV gene expression early after inoculation, and although this returned to levels seen in wild type mice by 7 days post-inoculation regardless of genotype, all animals displayed FLUC bioluminescence for nearly 3 weeks. Taken together these results indicate that although

IFN is not required for the development of humoral immune responses, it does play an important role in limiting early viral gene expression.

INTRODUCTION

Recently we have developed a single-cycle flavivirus (SCFV) vaccine candidate, RepliVAX WN, that can be produced in cell lines engineered to express the WNV capsid (C) protein (Widman et al., 2008). The RepliVAX WN genome contains a large internal deletion in the gene coding for C, while retaining the rest of the WNV genome including the prM and E genes. Thus, normal cells infected with RepliVAX WN can initiate a viral infection cycle and produce prM and E-containing sub-viral particles (SVPs) without producing infectious progeny. A single immunization with RepliVAX WN in mice and hamsters provided 100% protection from lethal WNV challenge (Widman et al., 2008) and RepliVAX WN elicited a durable protective immune response in hamsters (Widman et al., 2009).

Interferon (IFN) systems are known to play important roles in innate and adaptive immune responses in animals upon microbial infection (Le Bon and Tough, 2002). Multiple IFNs are classified based on their receptor specificities and gene homologies. Type-I IFNs (IFNα and IFNβ) are induced upon viral infection by recognition of viral components (known as pathogen-associated molecular patterns; PAMPs) such as double-stranded RNA by host factors known as pattern recognition receptors (PRRs) which include the toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), melanoma-differentiation-associated gene 5 (MDA5) and double-stranded RNA-activated protein kinase (PKR). Upon expression, IFNs initiate activation cascades that result in the activation of transcription factors that drive the expression of IFN-stimulated genes

(ISGs) important in the control and elimination of viral infection. Multiple cell types are capable of producing IFNα, whereas fibroblast cells are considered major IFNβ producers (Bach, Aguet, and Schreiber, 1997). Type-II IFN (IFNγ) is produced in large quantities by $CD4^+$ and $CD8^+$ T cells and NK cells. Additionally, studies indicate that antigen presenting cells can also efficiently produce IFNγ (Frucht et al., 2001). When these IFNs bind their receptors on the cell surface, Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) become activated and phosphorylate the signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2) in the cytoplasm. Once phosphorylated, the STATs form a trimeric STAT1-STAT2-IRF9 complex (IFN-stimulated gene factor 3; ISGF3) which activates transcription of IFNα/β and IFNγ-stimulated genes (Garcia-Sastre and Biron, 2006). This class of ISGs includes several antiviral enzymes such as caspase, 2'5'-oligoadenylate synthetase (2'5'-OAS) and PKR, which are involved in replicational and translational arrest and activation of dendritic cells, NK cells and $CD8^+$ memory T cells (Randall and Goodbourn, 2008). Thus, the collective effects of IFN stimulation provide an effective means of controlling viral infection by limiting viral replication and activating systemic immune responses.

The IFN response is a crucial component of the host innate immune response to flavivirus infection. Treatment of animals with IFN has been shown to prevent lethal flavivirus disease (Brooks and Phillpotts, 1999; Leyssen et al., 2003), and animals with defects in IFN signaling are far more susceptible to morbidity and mortality from infection with flaviviruses (Johnson and Roehrig, 1999; Lobigs et al., 2003; Samuel and Diamond, 2005). Studies with mice unable to synthesize the type-I IFN receptor (AG129) have shown IFN plays an important role in limiting the early peripheral replication and dissemination of WNV, and that defects in IFN signaling lead to high viral loads and entry into the CNS (Samuel and Diamond, 2005). Recently, studies have been undertaken

to examine the host factors involved in the recognition of flavivirus infections that lead to the induction of IFN responses. JEV induces IFNβ production in a RIG-I-dependent MDA5-independent mechanism (Kato et al., 2006), while WNV infection results in IFN production that is mediated by both RIG-I (Fredericksen and Gale, 2006) and MDA-5 (Fredericksen et al., 2008). Additionally, PKR has been demonstrated to act as a PRR in WNV infection, since knockdown, deletion or chemical inhibition of PKR results in a reduction of WNV-induced IFN expression (Gilfoy and Mason, 2007). As IFNs play an important role in controlling flavivirus infection, it is not surprising that viruses have evolved countermeasures to disrupt the IFN system. The NS4B protein of dengue virus (Munoz-Jordan et al., 2003), yellow fever virus and WNV (Munoz-Jordan et al., 2005) have all been shown to inhibit IFN signaling by blocking STAT1 phosphorylation, while this function is performed by NS5 of tick-borne flaviviruses (Best et al., 2005). WNV NS2A appears to be capable of inhibiting expression of IFNβ–stimulated genes (Liu et al., 2006), while WNV NS1 inhibits TLR3 signal transduction leading to decreased expression of IFNβ and of NFκB-stimulated gene products (Wilson et al., 2008). Dengue virus NS5 is capable of binding and degrading STAT2 in a ubiquitin and proteasome-dependent manner (Ashour et al., 2009), and JEV NS5 interferes with JAK-STAT signaling (Lin et al., 2006). In addition to type-I IFNs, IFNγ signaling is important in limiting WNV replication in lymphoid tissues, and defects in IFNγ signaling result in increased viral loads that lead to entry of the virus into the CNS (Shrestha et al., 2006). Thus the interplay between flavivirus infection and the IFN response has profound effects on pathogenesis and disease severity in infected hosts.

Since effectiveness of vaccination with SCFVs such as RepliVAX WN is undoubtedly related to the ability of SCFV-infected cells to persist within the vaccinated host, both the innate and adaptive immune responses are likely to control vaccination

efficiency with these products. Previously we have documented that a portion of SCFVs target to immune tissues following inoculation, and that these tissues appear to be the source of high levels of the potent systemic IFNα response detected in these animals (Bourne et al, 2007). Thus the interplay between IFN induced by SCFVs and the known ability of flaviviruses to counteract IFN signaling are of great interest for SCFV vaccine efficacy. Beyond its antiviral effects, IFN has also been shown to be important for the development of an adaptive immune response, and interestingly, recent studies by Diamond and co-workers (Purtha et al., 2008) have reported that in the absence of an intact IFN response, WNV-infected animals show marked deficiencies in B cell activation. To help better understand the role IFN plays in the development of protective immunity to flavivirus infection, we examined murine immune responses to vaccination with RepliVAX WN. Here we demonstrate that RepliVAX WN vaccination elicits high levels of circulating IFNα, and that antibody production is not inhibited in mice unable to respond to IFN, although the repertoire of IgG subclasses was influenced by IFN status. Studies utilizing an SCFV expressing the firefly luciferase (FLUC) gene in place of the prM/E genes encoded by RepliVAX WN (but otherwise structurally identical to RepliVAX) were performed to evaluate the effect of the IFN system on the distribution of infected cells in mice. Footpad (FP) inoculation of SCFVs resulted in extensive FLUC activity at the site of injection, and transient activity throughout the lymphatic system of wt mice. Mice deficient in IFNα/β signaling displayed extremely high levels of viral gene expression, and decreased trafficking of infected cells to lymphoid tissues. IFN status did not affect persistence of SCFV genomes, however, as FLUC activity was detectable in nearly all mice for 3 weeks after inoculation. These results indicate that while IFN is not required for the development of humoral immune responses, it does play a role in vaccine distribution and gene expression.

RESULTS

MICE VACCINATED WITH REPLIVAX WN PRODUCE TYPE-I IFN

A previous study demonstrated that inoculation of mice with SCFVs resulted in the induction of high levels of IFNα (Bourne et al., 2007). These studies utilized SCFVs lacking the prM and E genes (and therefore unable to produce SVPs in infected cells), and thus we were interested in examining the induction of IFN by RepliVAX WN, infection with which results in the production of SVPs. To this end, five AG129 mice lacking type-I and type-II IFN receptors, their parental wt strain S129 and C57BL/6 mice were vaccinated with $3.3x10^7$ IU RepliVAX WN by IP inoculation. As a control, animals from each strain of mouse were injected with UV-inactivated RepliVAX WN at an identical dose. Following vaccination, mice were bled at 8, 24 and 48hr and sera examined by ELISA to measure IFNα and IFNβ levels. At 8hr post-inoculation (hpi), IFNα was not detectable in any of the mouse strains receiving either the replicating or UV-inactivated RepliVAX WN. In S129 and C57BL/6 mice inoculated with RepliVAX WN IFNα was detectable in both strains (average values of 10,000 and 4,000 pg/ml of serum, respectively) at 24 hpi, whereas AG129 mice inoculated with RepliVAX WN as well as all mice inoculated with UV-RepliVAX WN demonstrated low levels of IFNα (approximately 1,000 pg/ml). The RepliVAX WN-induced IFN levels observed in S129 mice were significantly higher than in AG129 mice ($p<0.05$), although differences between BL6 mice and either S129 or AG129 were not significantly different. As expected, from previous studies (Bourne et al., 2007), serum IFN was short-lived, and the IFNα levels in all groups were reduced to less than 1,000 pg/ml by 48hpi. IFNβ was not detected in any groups at any time following vaccination. These results are consistent

with previous studies (Bourne et al., 2007), and indicate that expression of prM and E do not substantially enhance or dampen the type-I IFN response to infection with SCFVs. The lower level of IFN detected in AG129 mice inoculated with replicating RepliVAX WN is intriguing in light of the fact that a number of different IFN-inducing pathways (RIG-I/MDA5, PKR and TLR3) exist that presumably do not require an intact IFN receptor to function. It is possible that the lack of IFN receptors in these AG129 mice altered the behavior of RepliVAX WN following inoculation, perhaps by affecting the ability of infected cells to access lymphatic tissues shown previously to express high levels of IFN mRNA (Bourne et al., 2007). Alternatively, the absence of IFN signaling in the AG129 mice could alter levels of PRRs and other signaling components, blunting the IFN induction by RepliVAX WN infection. Regardless of the mechanism by which IFN signaling is amplified in intact mice, these results demonstrate that RepliVAX WN is capable of inducing IFNα following IP inoculation in mice, and animals unable to respond to IFN produce less IFN in response to vaccination.

IFN RESPONSES ARE NOT REQUIRED FOR THE DEVELOPMENT OF ANTIBODIES, HOWEVER THEY DO PLAY A ROLE IN PROTECTIVE IMMUNITY

Because RepliVAX WN inoculation is capable of inducing IFNα production in mice, we were next interested in assessing the importance of this IFN response in the

Table 5.1: Antibody titers 21 days post-RepliVAX WN vaccination

Group[a]	E titer[b]		NS1 titer[b]		Neut titer[c]
	IgG1	IgG2a	IgG1	IgG2a	
AG129 RepliVAX	54,810	17,438	<100	323	100
A129 RepliVAX	440	21,377	<100	921	123
S129 RepliVAX	3,445	34,457	<100	6,150	125

[a] 5-7 animals of each genotype were vaccinated IP with $2x10^5$ IU RepliVAX WN.
[b] Antibody titers of pooled sera were determined 21 days post-RepliVAX WN vaccination. Values represent the reciprocal dilution of sera at which ELISA O.D. readings are at least 3 standard deviations greater than readings produced from sera obtained from mock vaccinated animals.
[c] Neutralizing antibody titer of pooled sera collected from all animals 21 days post-vaccination (titer shown is the highest dilution of sera giving a 90% reduction of luciferase activity from Vero cells cultured with antibody-treated luciferase-bearing WNVLPs).

development of RepliVAX-induced immunity. To determine if an intact IFN circuit is a requirement for the development of adaptive immune responses to RepliVAX WN vaccination in mice, five to seven AG129, A129 (type-I IFN receptor KO) and S129 mice were vaccinated IP with $2x10^5$ IU of RepliVAX WN. Mice were bled at 21dpi, and the sera were analyzed by ELISA for antibodies against WNV E and NS1 proteins as well as neutralizing antibody titers. AG129 mice developed markedly higher IgG1 responses against E protein (with a reciprocal titer of nearly 55,000) than A129 mice and S129 mice (440 and 3445, respectively), whereas all three strains developed similar IgG2a responses against E (Table 5.1). IgG1 specific for NS1 was undetectable in all mouse strains and S129 mice exhibited markedly higher levels of IgG2a against NS1 (6150) than AG129 (323) or A129 (921) (Table 5.1). Interestingly, all three strains showed similar neutralizing antibody titers, although AG129 showed a slightly lower reciprocal titer (100) than A129 (123) and S129 (125) (Table 5.1), indicating that an intact type-I and type-II IFN system are not required to develop neutralizing antibodies in response to RepliVAX WN vaccination, in apparent contradiction to the work of Purtha et al. (2008).

At 28 dpi, all RepliVAX WN-vaccinated mice (as well as 2 unvaccinated animals of each genotype) were challenged with 1000ffu of WNV. Although all unvaccinated mice died by 9 days post-challenge, the unvaccinated AG129 and A129 mice died by 4 days post-challenge, considerably earlier than S129 mice which did not begin to die until day 8 (results not shown). Interestingly, 86% of vaccinated AG129 mice died by 14 days post-challenge, whereas all vaccinated A129 and S129 mice survived for 14 days. These results are consistent with the reported importance of IFNγ-mediated clearance of infected cells in protection from lethal flavivirus challenge (Johnson and Roehrig, 1999; Lobigs et al., 2003; Shrestha et al., 2006), and add important information to these previous studies by demonstrating that vaccine-induced immunity cannot completely

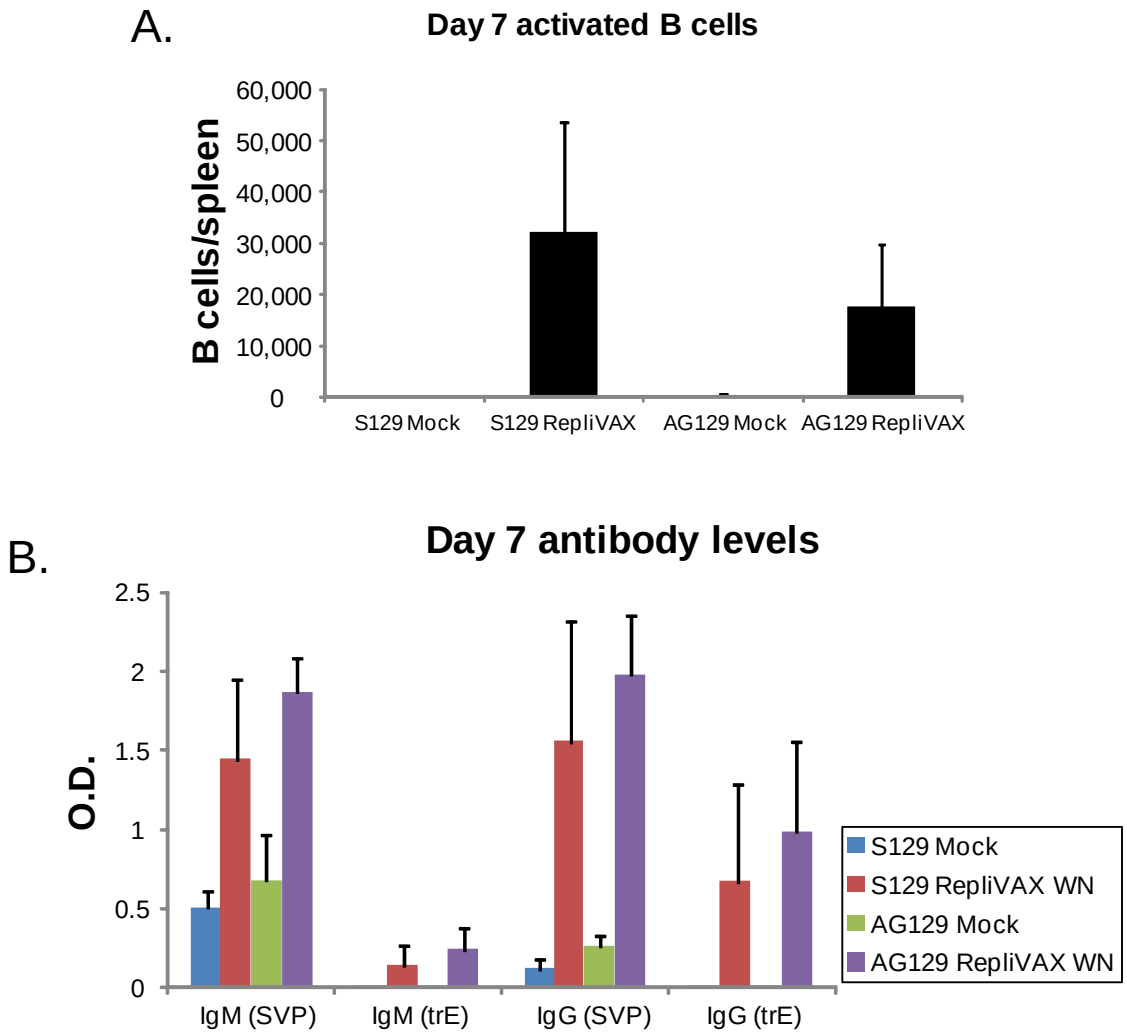

Figure 5.1: WNV-specific IgG-producing B cells and circulating IgG and IgM after vaccination with RepliVAX WN. A. Splenocytes from AG129 and S129 mice were analyzed by ELISPOT for B cells producing IgG capable of recognizing WNV SVPs. Data presented are the mean number of IgG-producing B cells per spleen, and extended bars represent standard deviation. For RepliVAX vaccination, n=5 animals per genotype and n=2 animals per genotype for mock. **B.** ELISA antibody levels measuring total WNV E-specific IgG from AG129 and S129 serum samples. Antibody levels were examined at 1:100 dilutions using purified WNV SVPs or soluble trE as antigen. Extended bars represent standard deviation. n=4 for vaccinated and 2 for mock.

protect animals from lethal challenge in the absence of an IFNγ response (Suzuki, Winkelmann, and Mason, 2009). Taken together, these results suggest that neither IFN system was required for the development of IgM, IgG and neutralizing antibodies following vaccination with RepliVAX WN. However, both neutralizing antibodies and an intact IFNγ signaling pathway are required for protection from lethal WNV challenge.

In order to further examine the role that IFNs play in the development of humoral immunity, we examined early B cell activation following RepliVAX WN administration. Four 10-14 week old female AG129 and S129 mice were inoculated IP with $1x10^6$ IU RepliVAX WN while 2 mice of each genotype received a mock inoculation. 7dpi, animals were euthanized, and spleen and blood samples were collected for analysis of WNV-specific B cell activation by IgG ELISPOT assay and antibody production by ELISA. Using purified SVP antigen, WNV-specific IgG-producing B cells were readily detectable in both S129 and AG129 mice at 7dpi (Fig. 5.1A). Although S129 mice had relative higher average activated B cell counts (32,000 cells/spleen) compared to AG129 (17,600 cells/spleen), this difference was not statistically significant (p=0.22). Interestingly, attempts to detect IgG-producing B cells using a soluble form of the WNV E protein (trE) were unsuccessful, suggesting that antibodies produced by splenic B cells soon after RepliVAX WN administration are specific for distinct conformational epitopes present on WNV particles but not on E monomers. This was further confirmed when circulating IgM and IgG levels were examined 7dpi. Animals vaccinated with RepliVAX WN produced high levels of both SVP-specific IgM and IgG antibodies regardless of their IFN status (Fig. 5.1B). Furthermore, these animals demonstrated much lower levels of trE-specific IgM and IgG, with levels of IgM nearly undetectable (Fig. 5.1B). Taken together, these results show that the ability to respond to IFN does not play a critical role in early B cell activation or antibody production following vaccination, but that early

antibodies demonstrate clear differences in their ability to recognize various forms of WNV antigen.

IN VIVO IMAGING REVEALS FOOTPAD TISSUE AND LYMPH NODES AS PRIMARY SITES OF SCFV REPLICATION

Since we expected that the IFN status of animals would also affect the distribution and persistence of RepliVAX WN-infected cells within vaccinated animals, we decided to examine the behavior and distribution of SCFV-infected cells in vivo. In order to do this, we utilized firefly luciferase (FLUC)-expressing SCFVs (FLUC-SCFV), which are structurally identical to RepliVAX WN particles, but contain genomes encoding FLUC in place of the WNV prM and E genes (Fayzulin et al., 2006; Gilfoy, Fayzulin, and Mason, 2009). C57BL/6 mice were inoculated SC in both rear footpads (FP) with $1x10^8$ IU purified FLUC-SCFV, and imaged at 24hr intervals to examine the distribution of SCFV-infected cells in vivo. As seen in Fig. 5.2, FP tissue at the site of inoculation was a major site of SCFV gene expression as measured by FLUC bioluminescence. Expression in this tissue was consistent and sustained, with FLUC bioluminescence detectable for 7 days after SCFV inoculation (data not shown). In addition to FP tissue, extensive FLUC bioluminescence was observed emanating from a number of draining lymph nodes (LN) including the popliteal, iliac, inguinal, and caudal LN (Fig. 5.2 and data not shown). The FLUC signal observed in the LNs was transient in nature, with activity usually detectable for 24-48hrs in a particular tissue, and only observed in the first 96hrs following inoculation (in contrast to FP signal which persisted for many days). These results suggest RepliVAX infects a class of cells that migrate from the site of inoculation through the lymphatic system where they interact with resident LN immune cells; although it cannot be ruled out that RepliVAX particles themselves migrate to draining lymph nodes where they infect resident cells.

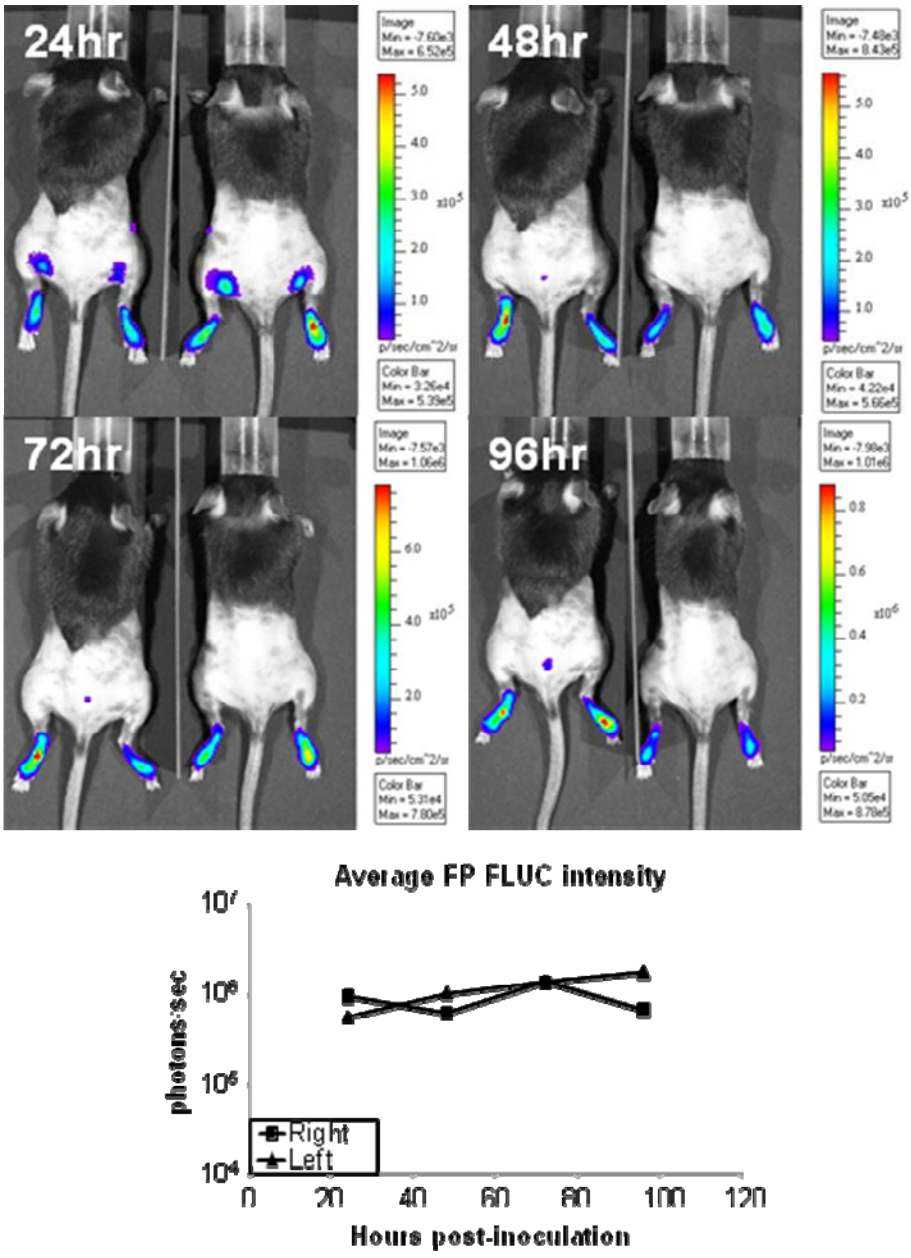

Figure 5.2 : In vivo imaging of C57BL/6 mice inoculated with FLUC-SCFV. C57BL/6 mice were inoculated SC in both rear FP with 1x10^8 IU FLUC-SCFV and imaged at 24hr intervals. At time of imaging mice were administered D-luciferin salt solution IP at a dose of 0.15mg/g and anesthetized 20min later in order to obtain images. Bioluminescence was observed using 1min exposures at medium binning. Footpad (FP) FLUC bioluminescence was analyzed by measuring total flux emanating from each footpad, and is reported as the average over time of both FP from each mouse. Representative images of multiple experiments.

TYPE-I IFN RESPONSES PLAY A ROLE IN LIMITING SCFV GENE EXPRESSION

Although IFN responses did not appear to be required for the development of RepliVAX-induced antibodies, we were interested in characterizing the role IFNs play in the gene expression of FLUC-SCFVs and the behavior of infected cells in vivo. To this end, three AG129 and S129 mice were inoculated SC in both rear FP with $1x10^8$ IU FLUC-SCFV and imaged at 24hr intervals. As with C57BL/6 mice, FLUC bioluminescence was observed extensively in FP tissues in both AG129 and S129 mice, and in the LN of S129 mice transiently over a 72hr period following inoculation. Interestingly, the magnitude of this FLUC bioluminescence in AG129 mice was greater than in the S129 mice, with FP FLUC intensity (measured in photons per second; p/sec) nearly $3\log_{10}$ higher than S129 (Fig. 5.3 bottom). In vivo imaging failed to detect significant FLUC bioluminescence from the LN of AG129 mice (Fig. 5.3 top), and A129 mice also showed markedly less LN bioluminescence than the wt S129 or C57BL/6 mouse strains throughout a number of studies (data not shown). This may indicate that IFN-mediated signaling plays a role in the mechanism by which SCFVs access lymphatic tissues, and may in part explain the lower levels of IFN observed in AG129 mice (see above). These results demonstrate the important role IFNs play in controlling initial infection and early gene expression following inoculation with SCFVs.

SCFV GENE EXPRESSION IS DETECTABLE NEARLY THREE WEEKS AFTER INOCULATION

In a previous study, hamsters vaccinated with RepliVAX WN demonstrated a rise in antibody titers between 4 and 8 weeks post-vaccination (Widman et al., 2009). Thus, we were interested in examining the persistence and magnitude of FLUC activity over a longer period in mice following inoculation with FLUC-SCFVs. AG129, A129, S129,

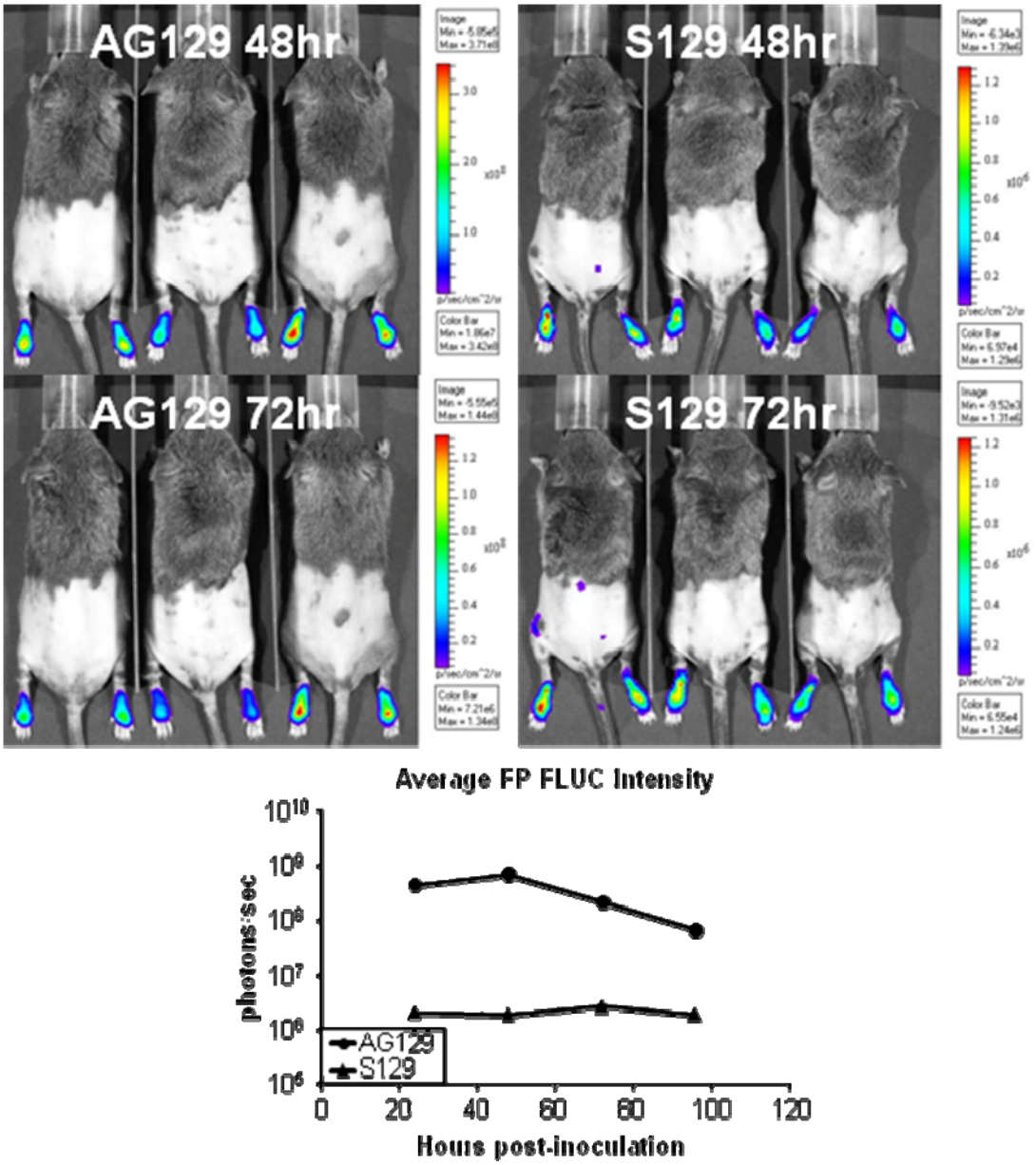

Figure 5.3 : In vivo imaging of AG129 and S129 mice inoculated with FLUC-SCFV. AG129 and S129 mice were inoculated SC in both rear FP with $1x10^8$ IU FLUC-SCFV and imaged at 24hr intervals. At time of imaging mice were administered D-luciferin salt solution IP at a dose of 0.15mg/g and anesthetized 20min later in order to obtain images. Bioluminescence was observed using 1min (S129) or 1-5sec (AG129) exposures at medium binning. FP FLUC bioluminescence was analyzed by measuring total flux emanating from each footpad, and is reported as the average over time of all FP from each mouse genotype. n=3 mice per genotype.

and C57BL/6 mice were again inoculated SC in both rear FP with $1x10^8$ IU FLUC-SCFV and imaged at regular intervals. As with previous studies, FP FLUC bioluminescence remained consistently detectable in all animals over the study period, while FLUC bioluminescence in the LN was observed in most animals over the first 96 hours after inoculation (data not shown). AG129 and A129 mice both demonstrated extremely high FLUC intensity emanating from the FP during the first 96hpi, indicating that the ability to respond to IFNγ does not play a significant role in limiting early gene expression by SCFVs (Fig. 5.4) under these conditions. S129 and C57BL/6 displayed a lower level of FLUC bioluminescence in the FP tissue during the first 4dpi, and by 7dpi all animals

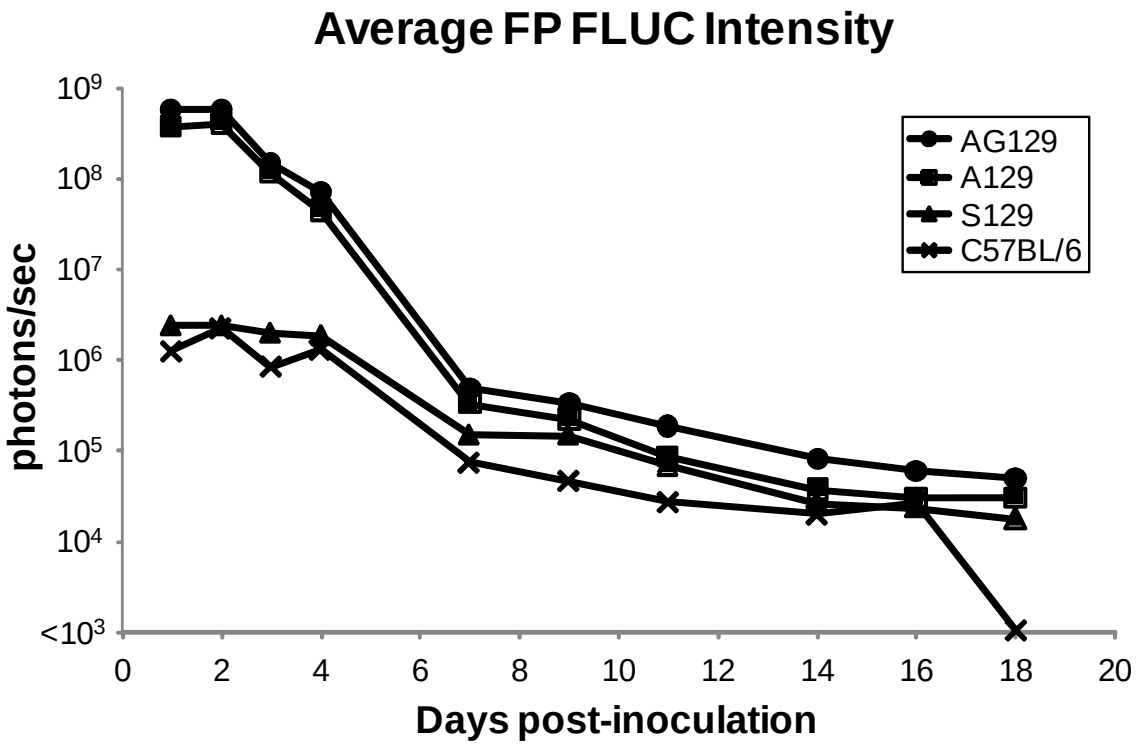

Figure 5.4: Average FP FLUC bioluminescence in mice over 18 days. AG129, A129, S129 and C57BL/6 mice were inoculated SC in both rear FP with $1x10^8$ IU FLUC-SCFV and imaged at 24hr intervals. FP FLUC bioluminescence was analyzed by measuring total flux emanating from each footpad, and is reported as the average over time of all FP from each mouse genotype. N=5 mice per genotype.

demonstrated a similar level of FP FLUC bioluminescence. Interestingly, this FLUC bioluminescence at the site of inoculation remained relatively steady over an 18-day observation period (Fig. 5.4), and nearly all animals (except C57BL/6) had detectable FLUC bioluminescence on day 18. The ability of SCFV gene expression to persist for weeks after inoculation may permit sustained antigen expression in the context of RepliVAX vaccination, the consequences of which may lead to strong immune responses. Thus, RepliVAX WN vaccination would be expected to produce immunogenic SVPs and NS1 proteins from this site for weeks after inoculation, providing a continuous source of antigen to boost the immune response to this vaccine.

DISCUSSION

The interplay between viral infection and host immune response is a critical component in determining the outcome of infection. Although innate immune responses such as IFNs are potent means of limiting viral replication and spread, many viruses including flaviviruses have evolved countermeasures by which they are able to interrupt aspects of the IFN signaling pathway. In particular, multiple flaviviruses have been reported to interfere with STAT1 phosphorylation and nuclear translocation (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Liu et al., 2006), the consequences of which are reduced levels of IFNs and IFN-stimulated genes.

We were interested in examining the IFN response to RepliVAX WN in order to assess the levels to which vaccination could induce IFN production, and to determine if IFN signaling was necessary for the development of protective immunity. We observed that 24hr following administration of RepliVAX WN, high levels of circulating IFNα were detected in wild-type (wt) mice (S129 and C57BL/6), similar to those observed in a previous study (Bourne et al., 2007). IFNα levels in S129 mice were higher than

C57BL/6 mice, possibly due to the reported differences in the number of plasmacytoid dendritic cells in these mouse strains (Asselin-Paturel et al., 2003). Interestingly, the levels of IFNα in C57BL/6 mice induced by RepliVAX WN vaccination were lower than those detected following SCFV infection in a previous study (Bourne et al., 2007). This difference could be due to the fact that these earlier studies used an SCFV with a shorter replicon genome [which replicates to higher levels than RepliVAX genomes (Suzuki, R., unpublished data)], making the previously used SCFV a more potent inducer of IFN. RepliVAX WN induced significantly lower levels of IFNα in AG129 mice than in S129 mice. This is consistent with the inability of the AG129 mice amplify IFN responses as a result of autocrine signaling through the IFN receptor. Nevertheless, they still produced significant levels of IFN, consistent with the expected presence of the intact primary IFN-producing pathways such as RIG-I, MDA5 and PKR that are important for IFN production during WNV infection (Gilfoy and Mason, 2007; Fredericksen et al., 2008) that should function independently of the IFN receptor. Nevertheless, the reduction of IFN production by the IFN receptor-deleted animals is an indication that IFN self-amplification is an important source of systemic IFN in WNV infection.

A recent report indicated that an intact IFN signaling pathway was necessary for B cell activation in the context of WNV infection (Purtha et al., 2008). It has also been reported that the NS2A (Liu et al., 2006), NS4B (Munoz-Jordan et al., 2005) and NS1 (Wilson et al., 2008) proteins of WNV are all capable of antagonizing the IFN response. Thus, we were interested in examining the role IFN signaling plays in the development of a protective humoral immune response to RepliVAX WN vaccination. We observed that AG129 mice develop antibody responses similar to wt S129 mice as early as 7dpi. At this time, similar levels of B cells producing WNV-specific IgG were detected in the spleens of these animals, and levels of circulating IgM and IgG were comparable between mouse

genotypes. At 21dpi, neutralizing antibody titers in AG129, A129 and S129 mice were similar in all animals, although differences in IgG profiles suggested variation in immune responses based on IFN status. These results clearly indicate that IFN signaling is not an absolute requirement for the production of antibodies in response to viral infection. While Diamond and co-workers observed significantly lower levels of B cell activation in the draining LN of AG129 mice in response to WNV challenge (Purtha et al., 2008), it appears that this does not have a profound effect on circulating levels of WNV-specific antibodies. Additionally, because the AG129 model of WN disease is so exquisitely sensitive that time to death is often less than 4 days, distinct B cell subsets in these profoundly ill animals may have been difficult to accurately characterize in these previous studies (Purtha et al., 2008).

It has been well established that relatively high levels of murine IgG1 are indicative of a T_H2-biased immune response while high levels of IgG2a correlate to a T_H1 response (Abbas, Murphy, and Sher, 1996). Generally, natural infections with replicating agents induce T_H1-dominant immune responses (Coutelier et al., 1988). Our WNV E-specific antibody results from S129 and A129 mice demonstrate that RepliVAX WN vaccination can induce T_H1-dominant immune responses similar to those induced by natural infection in mice, even in the absence of type-I IFN signaling. Encouragingly, S129 mice also produced easily detectable levels of IgG1; indicating vaccination produced a balanced immune response in these wt animals. AG129 mice, however, produced extremely high levels of IgG1 from vaccination, far in excess of those observed in other strains, while the IgG2a levels in these mice were slightly lower than other mice. This phenomenon has also been observed with lymphocytic choriomeningitis virus (van den Broek et al., 1995), although these studies were unable to detect IgG2a in AG129 mice and it was thus believed that the inability of AG129 mice to respond to IFNγ

signaling prevented antibody subclass switching (Finkelman et al., 1988; Snapper, Peschel, and Paul, 1988). Interestingly, we observed high levels of WNV E-specific IgG2a in AG129 mice following vaccination, indicating that IFNγ signaling is not a strict requirement for the production of IgG2a and that RepliVAX WN vaccination is capable of eliciting balanced IgG response even in the absence of IFN signaling. It seems more likely that the behavior of SCFV infection itself rather than IFN signaling per se may be responsible for the T_H2-biased immune response observed in AG129 animals, a response similar to those observed following administration of protein subunit vaccines to wt mice (Smucny et al., 1995; Simmons et al., 2001). The extremely high levels of antigen produced soon after infection of AG129 mice with SCFVs may be treated by the host immune system in a similar manner to the large amount of antigen delivered by administration of an inactivated or subunit vaccine, and thus a T_H2-biased response with high levels of IgG1is elicited. These results indicate that IFN signaling plays a role in shaping the antibody response to WNV infection, and that vaccination with RepliVAX WN generates balanced antibody profiles in wt mice similar to those induced by natural replicating infection despite the inability of RepliVAX to produce a spreading infection.

Antibody responses to WNV NS1 were also analyzed and also revealed an interesting trend. NS1-specific IgG2a was detectable in all mice regardless of genotype, although S129 mice produced 6-20x as much as A129 and AG129 mice, respectively. IgG1 specific for NS1 was undetectable in all mice regardless of genotype, indicating that this subclass of IgG may not be produced in large quantities in response to SCFV vaccination. Mouse IgG1 antibodies fix complement poorly if at all, while IgG2a molecules are the most potent complement-fixing immunoglobulin in mice. Antibodies specific for flavivirus NS1 have long been known to fix complement, likely due to presence of NS1 on the surface of infected cells (Schlesinger et al., 1990). Therefore in

the context of the host immunologic response, IgG2a antibodies are functionally advantageous over IgG1 in mediating flavivirus clearance, and therefore may be preferentially produced. Interestingly, despite the accumulation of a large amount of WNV E-specific IgG1 in AG129 mice, no NS1-specific IgG1 was detected in these animals, further indicating that IFN signaling is not required for IgG subclass switching although likely plays a role in many instances.

In addition to affecting the IgG antibody repertoire, IFNγ signaling also had profound effects on mouse survival of WNV challenge following vaccination with RepliVAX WN. When vaccinated AG129, A129 and S129 mice were challenged with WNV, only vaccinated animals with an intact IFNγ response circuit survived. Interestingly, even animals unable to respond to IFNα/β (A129) were protected if IFNγ signaling was intact, indicating that a strong type-I IFN response may not be necessary to confer protection following vaccination. These findings confirm that high levels of neutralizing antibodies are not always sufficient to protect animals from virus challenge, as vaccinated AG129 mice succumbed to WNV infection despite high antibody titers. IFNγ plays an important role in limiting WNV load in lymphoid tissues and delaying viral entry into the CNS (Shrestha et al., 2006), and these mice unable to respond to IFNγ probably developed high peripheral viral load following challenge, overwhelming the neutralizing antibody response present from vaccination. Alternatively, lack of IFNγ-stimulated events may also lead to inefficient memory responses preventing effective anamnestic responses that contribute to protection, although this was not explored in the context of this study. Together these results demonstrate that IFNγ-mediated immune responses play crucial roles in protection from lethal WNV challenge in animals receiving vaccination.

We were also interested in examining the role that IFNs play in the distribution of RepliVAX-infected cells in vivo, and the duration and magnitude of gene expression following inoculation. For this we utilized in vivo imaging of animals inoculated with FLUC-SCFVs, which allows for real-time monitoring of infection. Using this technology, we were able to observe that following FP inoculation, infected cells are most prevalent at the site of inoculation, but that a subset of infected cells localize to the draining LN. While this confirmed a previous finding (Bourne et al., 2007), the ability to follow infection over time revealed that infected cells do not remain at a single LN after inoculation, but are observed transiently and in more distal LN from the site of injection at later time points. This suggests that these infected cells are migratory in nature, and traffic throughout the lymphatic system where they presumably function to stimulate immune responses. Interestingly, we did not observe the same level of FLUC bioluminescence in the LN of mice unable to respond to IFNα/β (AG129 and A129) as in wt strains (S129 and C57BL/6) despite the abilities of all of these mouse strains to produce similar levels of antibodies. This and recent findings that keratinocytes are capable of priming naive T cells with endogenous antigen (Kim et al., 2009), suggest that infected cells at the site of SCFV inoculation may play an important role in immune activation, and that interaction of SCFV-infected cells with resident cells of the draining LN may not be a driving force in the development of a protective immune response. Alternatively, it is also possible that the increased level of gene expression observed by IVIS in the absence of IFNα/β signaling (see below) overcomes deficits present in the adaptive immune responses of mice lacking this signaling, permitting these animals to develop antibody titers at similar levels as wt mice despite the loss of the adjuvanting effects of IFN on the adaptive immune response.

Although the ability to respond to IFNα/β had little effect on antibody production, it did have a dramatic effect on SCFV gene expression, as bioluminescence in the FP of A129 and AG129 mice was nearly 1000x greater than that observed in wt mice. This result was intriguing in light of the fact that numerous studies have reported the ability of WNV proteins to interfere with IFN signaling, primarily by blocking STAT1 phosphorylation. It seems that in the context of infection with a replicating RNA genome in vivo, these mechanisms do not act quickly enough to completely block IFN signaling and are insufficient to counteract the action of IFN once initiated, or that WNV cannot completely block the effects of IFN on initially infected cells. The enhanced viral gene expression observed in mice unable to respond to type-I IFN was short lived, however, and by 7dpi, FLUC bioluminescence in these mice was comparable to that of wt mice. This suggests that intact adaptive immune responses had been activated to control infection, alternative non-IFN-mediated innate responses decreased viral replication, or a large subset of initially infected cells had been killed by viral genome replication. Despite these early differences in viral gene expression, however, SCFV persistence did not appear to be affected by IFN status, as all animals except C57BL/6 demonstrated detectable FLUC bioluminescence at the site of inoculation for nearly 3 weeks. The ability of SCFV genomes to persist for many weeks in the host has important implications in the development of new live-attenuated viral vaccines. Prolonged antigen expression has been linked to development of $CD4^+$ T cells (Obst et al., 2005) and specifically to the formation of memory $CD4^+$ T cells (Jelley-Gibbs et al., 2005). In addition to activating B cells, $CD4^+$ T cells are also crucial in the maintenance of memory $CD8^+$ T cell populations (Sun, Williams, and Bevan, 2004). Thus, the persistence of vaccine genomes and the expression of antigen for an extended period of time will likely

induce long-term protective immune responses and is thus an important consideration in vaccine development.

In conclusion, we have shown that vaccination of mice with RepliVAX WN induces high levels of circulating IFNα, however the ability to respond to signaling from either type-I or type-II IFNs is not required for the development of antibody responses to vaccination. In vivo imaging identified FP and LN tissue as primary sites of infected cell localization, and the ability to respond to IFNα/β had a profound effect on controlling SCFV gene expression during the first 4 days of infection. Overall, SCFV gene expression was detectable for at least 18 days in nearly all mouse strains. Taken together, these results indicate that IFNs play important roles in the early events of WNV infection, but are not required for the development of antibody responses to vaccination.

MATERIALS AND METHODS

VIRUSES AND CELL LINES

Vero cells used for enumeration of RepliVAX WN and FLUC-SCFV particles and for neutralization assays have been previously described (Rossi et al., 2005). RepliVAX WN (previously referred to as RepliVAX WN.2 SP) was produced in BHK(VEErep/Pac-Ubi-C*) cells as previously described (Widman et al., 2008) and diluted in vaccine diluent (L15 containing 10mM HEPES and 0.5% fetal bovine serum). UV-inactivation of RepliVAX WN was performed using a published protocol (Bourne et al., 2007). The FLUC-SCFV particles containing a WNV replicon genome expressing a humanized firefly luciferase gene (FLUC) and the foot-and-mouth disease virus 2A genes in place of the WNV prM and E genes (previously referred to as WNV hFLuc VRP) were propagated in BHK(VEErep/C*-prM-E-Pac) cells as previously described (Gilfoy,

Fayzulin, and Mason, 2009). WNV NY99 passaged twice in Vero cells and used for mouse challenge has also been previously described (Widman et al., 2009).

To obtain the infectious titers (>10^9 IU/ml) needed for in vivo imaging, FLUC-SCFV particles were concentrated and purified prior to inoculation. Briefly, FLUC-SCFV-containing cell culture supernatant was passed through 100,000 kilodalton (kd) cut-off centrifugal filters (Amicon Ultra, Millipore, Billerica, MA) at 1500xg. The resulting concentrate was applied to a 40-10% continuous sucrose gradient and centrifuged at 35,000rpm for 2.5hr at 4°C, at which time the gradient was fractionated and the fractions containing the highest number of infectious particles were pooled. Residual sucrose was eliminated by two 15ml L15 washes of the selected fractions in a centrifugal filter, and the final concentrate was resuspended in L15 containing 10mM HEPES and 0.1% mouse serum albumin.

WNV subviral particles (SVPs) used for ELISPOT and ELISA were produced by infection of Vero cells with RepliVAX WN under conditions analogous to those used to infect BHK(VEErep/Pac-Ubi-C*) (Widman et al., 2008). Clarified cell culture supernatant containing WNV SVPs was concentrated using centrifugal filtration as above, and SVPs were purified on a sucrose gradient prior to use.

ANIMAL IMMUNOGENICITY STUDIES

Five female 10-14 week old AG129 [S129 lineage mice which are unable to produce receptors for either type-I(α/β) or type-II(γ) IFNs (van den Broek et al., 1995)] were bred from founders provided by W. Klimstra (LSUHSC), S129 (129S6/SvEvTac; Taconic, Hudson, NY), and C57BL/6 (Taconic) mice were inoculated IP with $3.3x10^7$ IU RepliVAX WN, while 5 mice of each genotype received UV-inactivated RepliVAX WN at the same concentration. Animals were bled for sera at 8, 24 and 48 hours post-inoculation (hpi) to assess IFN production in response to vaccination.

To assess the role IFNs play in the development of protective immunity, five to seven male AG129, A129 [S129 lineage mice which are unable to produce receptors for type I (α/β) IFNs (Muller et al., 1994)] were provided by L. Soong (UTMB), and S129 mice ranging from 9-13 weeks in age were inoculated IP with $2x10^5$ IU RepliVAX WN, while two animals of each genotype received a vaccination of vaccine diluent. All animals were bled for sera at 7 and 21 days post-inoculation (dpi) to examine antibody levels. At 28dpi, all animals were challenged with 1000ffu (corresponding to approx. $10LD_{50}$ in outbred mice of similar age) of WNV NY99 and monitored for 16 days for morbidity and mortality. Animals scored moribund were humanely euthanized in compliance with UTMB Animal Care and Use requirements and scored as "dead" the following day.

IGM, IGG AND IFN ELISAS

ELISA protocols used to determine WNV-specific IgG levels have been described (Widman et al., 2009). Serial 2-fold dilutions of pooled sera from each mouse genotype and treatment group were applied to ELISA plates coated with recombinant WNV E or NS1 proteins. After incubation and washing, bound IgG was detected using HRP-conjugated antibodies specific for mouse IgG, IgG1, and IgG2a (KPL, Gaithersburg, MD). Normalized optical density (OD) readings were analyzed using nonlinear regression, and titers are reported as the reciprocal of the highest serum dilution producing an OD reading greater than three standard deviations above OD values from sera obtained from mock vaccinated animals.

For ELISPOT experiments, IgM and IgG ELISAs were performed as described previously (Widman et al., 2008). Briefly, serum diluted 1:100 was added to ELISA microtiter plates coated with recombinant soluble WNV E protein (trE) or purified WNV subviral particles (SVPs). After a 1hr incubation, plates were washed and WNV-specific

antibodies were detected by addition of mouse IgG and IgM-specific antibodies (KPL) followed by an HRP-conjugated secondary antibody and tetramethyl-benzidine (TMB) substrate. After quenching, optical density (OD) values were read at 450nm.

IFNα and IFNβ ELISAs (PBL Biomedical Laboratories, Piscataway, NJ) were performed using manufacturer's protocols. Briefly, animal sera were diluted in dilution buffer and added to a microtiter plate. Following 1hr incubation, samples were washed and IFN in samples was detected by addition of mouse IFN-specific antibody followed by an HRP-conjugated secondary antibody and addition of TMB. The reaction was quenched and absorbance was read at 450nm. IFN levels were calculated using the curve generated from side-by-side dilutions of an IFN standard diluted in the presence of normal sera to overcome the effect of serum on the standard curve (Bourne et al., 2007). Statistical differences were determined using t-test.

NEUTRALIZATION ASSAYS

Virus neutralization assays were performed using a previously described protocol (Widman et al., 2008). Briefly, 2-fold serially diluted heat-inactivated sera sampled were incubated with a standardized amount of FLUC-SCFV at 37°C for 1hr before inoculation onto Vero cells prepared in a black-walled 96 well plate. After a 24hr incubation, cell lysis buffer containing 25% Steady-Glo reagent (Promega, Madison, WI) was added in a 1:1(v:v) ratio to culture fluid in the plate, and FLUC activity was read on a microplate luminometer. Neutralizing antibody titers were determined by nonlinear regression analysis of FLUC readings, and are presented as the reciprocal dilution of serum at which 90% of FLUC activity was inhibited as compared to cells infected with FLUC-SCFV incubated with serum diluent alone.

ENZYME-LINKED IMMUNOSPOT ASSAY (ELISPOT)

To examine early B cell activation by RepliVAX WN, four 12 week old AG129 and S129 mice were inoculated IP with $1x10^6$ IU RepliVAX WN while 2 animals of each genotype received an IP inoculation of vaccine diluent as a control. 7dpi, animals were humanely euthanized and blood and spleens collected for analyses by ELISA and ELISPOT. ELISPOT assays were performed by modification of a published protocol (Milligan and Bernstein, 1995). Microtiter filter plates (Millipore) were coated with trE or SVPs in carbonate buffer (pH=8.8) ON at 4°C. Following washing and blocking with 2.5% bovine serum albumin in PBS, single-cell suspensions of splenocytes were plated and incubated at 37°C for 24hr. After washing, WNV-specific IgG was detected by ON incubation at 4°C with a biotinylated mouse IgG-specific antibody followed by a 1hr 37°C incubation with HRP-conjugated streptavadin. Following treatment of the plates with aminoethylcarbazole developing solution, spots were visualized and enumerated using ImmunoSpot 4.0 software (Cellular Technology, Cleveland, OH)

IN VIVO IMAGING

For initial in vivo imaging studies, two 10 week old C57BL/6 mice were utilized. To examine role of IFN in FLUC-SCFV behavior, three 10 week old AG129 and S129 mice were used, and in FLUC-SCFV persistence study, five 10-15 week old AG129, A129, S129, and C57BL/6 mice were used. Real-time in vivo imaging of mice was performed using a Xenogen IVIS 200 (Caliper LS, Hopkinton, MA) and data were analyzed using Living Image 3.0 software (Caliper LS). The posterior half of all animals was shaven 2-3 days prior to subcutaneous (SC) inoculation in the rear footpads (FP) with approximately $1x10^8$ IU FLUC-SCFV. At 24hr intervals following inoculation, animals were given an IP injection of D-luciferin (Caliper LS) in a solution of phosphate-buffered saline corresponding to a dose of 0.15 mg/g body weight. After allowing 20min

for dissemination of D-luciferin, animals were anesthetized by isoflurane inhalation, and imaged in the IVIS 200 at medium binning with exposure times ranging from 1-60sec. Images were analyzed by drawing regions of interest around visible sites of FLUC activity and measuring total flux (photons per second; p/sec). Reported footpad averages are the average total flux from all footpads from all animals in a treatment group.

CHAPTER 6 : CONCLUSIONS AND FUTURE DIRECTIONS

Diseases caused by members of the flavivirus family of viruses are responsible for hundreds of millions of cases of severe illness every year, and currently there is little available to treat or prevent disease. Japanese encephalitis virus, yellow fever virus, dengue virus, and West Nile virus are all responsible for severe human disease. Half of the world's population is estimated to be at risk for dengue infection, and West Nile virus is responsible for the largest outbreak of viral encephalitis in the history of North America. Despite these immense disease threats, no licensed vaccines exist to prevent either of these diseases, although a number of candidates are in development.

Earlier studies reported the development of RepliVAX WN, a single-cycle flavivirus vaccine to prevent WN disease (Mason, Shustov, and Frolov, 2006). The RepliVAX WN genome contains a large deletion in the gene encoding C, and is grown in packaging cell lines engineered to express WNV C in the context of a Venezuelan equine encephalitis replicon (VEErep). In these C-expressing cells, RepliVAX WN can be grown to titers exceeding 10^7 IU/ml in much the same way as traditional live-attenuated viruses. In normal cells, however, RepliVAX is unable to produce infectious progeny and instead produces SVPs and NS1, important WNV antigens that stimulate strong protective immune responses. The ability of the RepliVAX WN genome to replicate in host cells facilitates the development of T cell responses to infection, the determinants of which lie primarily in the non-structural protein region contained in RepliVAX WN (Brien, Uhrlaub, and Nikolich-Zugich, 2007; Brien, Uhrlaub, and Nikolich-Zugich, 2008).

REPLIVAX WN HAS USEFUL CHARACTERISTICS OF BADLY NEEDED VACCINES

An important aspect to vaccine development is the safe and efficient generation of vaccine stocks, and to this end a modified packaging cell line [BHK(VEErep/Pac-Ubi-C*)] was developed in order to propagate RepliVAX WN. These BHK(VEErep/Pac-Ubi-C*) cells were shown to be stable and capable of repeated passage without a decline in packaging efficiency. Attempts to force intergenomic recombination between RepliVAX and VEErep/Pac-Ubi-C* by blind passage did not yield recombinant infectious virus, thus demonstrating a high degree of safety in vaccine production. Furthermore, as a proof of principle, Vero cells acceptable for production of human vaccines were successfully used as packaging cells [Vero(VEErep/Pac-Ubi-C*)] for the propagation of RepliVAX WN under serum-free conditions, indicating that this vaccine is capable of being safely produced in a manner analogous to those used to derive other cell culture-based human vaccines.

In the process of blind passaging RepliVAX in BHK(VEErep/Pac-Ubi-C*) cells to evaluate recombination potential, a better growing RepliVAX WN mutant population was selected for. Two mutations affecting the cleavage efficiencies of sites at either end of the signal sequence for prM of RepliVAX were identified and used to generate a second-generation RepliVAX WN with an enhanced in vitro growth phenotype similar to that of the passaged material. When evaluated in mice and hamsters, a single dose of RepliVAX WN was capable of inducing strong antibody responses and protecting all vaccinated animals from WNV challenge. Interestingly, second-generation RepliVAX WN was able to induce significantly higher levels of antibodies against WNV E and NS1 than the original RepliVAX at the same dose, indicating that the enhanced in vitro growth phenotype of second-generation RepliVAX correlated to enhanced potency in vivo. These results demonstrate the ability to use natural selection as a means by which better

single-cycle vaccines can be produced, and the feasibility and potential utility of RepliVAX WN as a vaccine to prevent WN disease.

Viable vaccine candidates must be able to elicit immune responses that remain protective over long periods of time. We evaluated RepliVAX WN in a hamster model of WNE utilized by others to evaluate the durability of vaccine-induced immune responses. WNV specific antibody levels generally increased for the first 2 months following immunization, and remained steady and at high levels during a 6 month observation period. Importantly, all vaccinated animals were protected from WNV challenge more than 6 months after receiving a single vaccination. These results demonstrate that RepliVAX WN is capable of inducing durable immune responses in hamsters, and may indicate its ability to provide long-term protection from disease.

Before nearly any products, and especially vaccine candidates, are permitted to be tested in man, studies must be performed using non-human primates. We initiated a small-scale study in order to evaluate the safety, immunogenicity, and efficacy of RepliVAX WN in the rhesus macaque model of WN infection. Macaques administered one or two doses of RepliVAX WN displayed no ill effects from vaccination, and a single dose elicited detectable WNV-specific IgG, IgM and neutralizing antibody responses. A second dose of RepliVAX WN enhanced antibody titers, while analysis of DC and T cell subsets failed to detect strong systemic responses to vaccination. Upon challenge, three of four vaccinated animals were completely protected from WNV viremia, including one animal receiving a single dose. This was in sharp contrast to an unvaccinated macaque, which developed a viremia lasting 6 days. These results demonstrate RepliVAX WN is safe and immunogenic in NHPs, and warrants future study as a vaccine to prevent WN disease.

While the results of these studies are encouraging, further characterization of RepliVAX WN-induced immune responses are necessary, particularly in primates. Alternative doses and vaccination schedules need to be carefully evaluated in a large number of primates in order to determine ideal conditions to administer RepliVAX WN, and to gain a better understanding of the immune responses elicited from vaccination. More stringent efficacy studies are also needed in order to confirm the limited data presented here. NHPs do not develop clinically detectable WN disease unless the challenge inoculum is administered intracranially, a route that bypasses many early events in WNV infection, and thus vaccine evaluation in these animals can be difficult to interpret. As others have used IC inoculation to evaluate WNV vaccine candidates (Pletnev et al., 2003; Arroyo et al., 2004) in macaques, a study of this type may be useful for RepliVAX WN evaluation in lieu of a more useful primate model.

IFN IS NOT REQUIRED FOR ANTIBODY PRODUCTION, BUT IT DOES PLAY IMPORTANT ROLES IN VACCINE VIRUS DISTRIBUTION AND GENE EXPRESSION

IFNs play critical roles in the control and clearance of flavivirus infections, and in turn flaviviruses have developed strategies to block the host IFN response in order to persist. Thus is was of great interest to us to examine the role IFNs play in the development of RepliVAX WN-induced protective immunity. We hypothesized that robust induction of IFNs by RepliVAX WN, perhaps facilitated by localization of vaccine-infected cells to areas of immunologic importance, was in part responsible for the potency of RepliVAX WN. Although RepliVAX WN induced high levels of circulating IFNα in mice, IFN responses were not required for the development of neutralizing antibodies, although the ability to switch IgG subclasses in response to IFNγ stimulation did play a role in shaping the repertoire of IgG produced. Furthermore, the ability to respond to IFN played no role in the activation of WNV-specific B cells or the production of IgG and IgM following vaccination. IFNγ responses were important in the

development of protective immunity, as only mice with an intact type-II IFN system survived WNV challenge following vaccination. Interestingly, all vaccinated A129 animals survived challenge, indicating that immunization can induce protective immune response in the absence of IFNα/β signaling. These results demonstrate that IFN signaling is not a strict requirement for the production of antibodies, and that RepliVAX WN vaccination induces balanced humoral immune responses in animals with or without IFN signaling.

Although we did not observe significant differences in endpoint antibody titers between vaccinated AG129, A129 and S129, we were interested in examining the behavior of RepliVAX WN in vivo. Using real-time in vivo imaging and FLUC-SCFVs similar to RepliVAX, we observed that following SC FP inoculation, SCFV-infected cells localize primarily at the site of inoculation although a subset of infected cells localize to the draining LN. Bioluminescence observed in the LN was transient and progressed to more distal LN over time, suggesting that SCFVs infect a class of migratory cells that traffic to LN where they presumably stimulate resident lymphocytes. AG129 and A129 mice were observed to have nearly 1,000-times more FP FLUC bioluminescence than S129 mice, demonstrating the importance of IFNα/β signaling in limiting early viral gene expression in vivo. This inability to control gene expression is likely the reason naive AG129 and A129 mice succumb to WNV challenge so rapidly. Interestingly. nearly all strains of mice displayed signs of SCFV gene expression 18 days post-inoculation, suggesting that RepliVAX WN may be able to persist for many weeks in a vaccinated animal, despite its ability to induce high levels of IFNα. The dramatic difference in viral gene expression between mice able and unable to respond to IFN stimulation is enigmatic in light of the increasing evidence that WNV proteins are capable of blocking STAT1 phosphorylation and IFN signaling, and may indicate that

WNV is not capable of blocking IFN signaling rapidly enough to prevent an antiviral response. These results suggest the ability to induce a balanced humoral immune response, access lymphoid tissues early after infection and persist in the host may be responsible for RepliVAX WN potency and efficacy.

From the results of these studies, many questions still remain. The role of T cell responses in the protective immunity observed following RepliVAX WN vaccination is not well understood. Studies to identify responding T cell populations, and the kinetics of this response, may allow for RepliVAX WN to be improved. Furthermore they will shed light on the early immunological events of WNV infection and add to the understanding of T cell responses to WNV infection. The ability to visualize and image clearance of viral infection using in vivo imaging provides a powerful tool by which functions of the innate and adaptive immune response will continue to be examined in the context of WNV infection.

The results presented herein have served to advance the field of flavivirus immunology, and set into motion the development of a promising vaccine candidate for WN disease. A better understanding of the role of innate immune responses play in the development of adaptive immunity has been gained, and future studies will continue to advance our knowledge of these elements.

APPENDIX

APPENDIX A: ANTIBODIES USED FOR FLOW CYTOMETRY

Antigen	Clone Name	Fluorochrome	Supplier
CD14	322A-1	FITC	BD-Biosciences, San Jose, CA
CD20	H299	FITC	BD-Biosciences, San Jose, CA
CD2	T11	FITC	BD-Biosciences, San Jose, CA
CD159a	Z199	PE	Beckman Coulter, Fullerton, CA
CD4	L200	PerCP-Cy5.5	BD-Biosciences, San Jose, CA
CD20	2H7	APC	BD-Biosciences, San Jose, CA
CD8	SK1	PerCP-Cy5.5 or APC-H7	BD-Biosciences, San Jose, CA
CD3	SP34-2	Pacific Blue	BD-Biosciences, San Jose, CA
HLA-DR	L243(G46-6)	APC-H7	BD-Biosciences, San Jose, CA
CD123	7G3	APC	BD-Biosciences, San Jose, CA
CD11c	S-HCL-3	PE	BD-Biosciences, San Jose, CA
CD3e	SP34-2	FITC	BD-Biosciences, San Jose, CA
CD95	DX2	Alexa Fluor 488	BD-Biosciences, San Jose, CA
CD86	IT2.2	Pacific Blue	Biolegend, San Diego, CA
CD40	5C3	PE-Cy5.5	e-Bioscience, San Diego, CA
CD16	3G8	APC	Invitrogen Corporation, Carlsbad, CA
CD69	FN50	FITC	DAKO, Carpinteria, CA

Antibodies used to perform flow cytometric analyses of NHP plasma (See Chapter 4).

REFERENCES

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature.* **383**(6603)**,** 787-793.

Aberle, J. H., Aberle, S. W., Kofler, R. M., and Mandl, C. W. (2005). Humoral and cellular immune response to RNA immunization with flavivirus replicons derived from tick-borne encephalitis virus. *J Virol.* **79**(24)**,** 15107-15113.

Ackermann, M., and Padmanabhan, R. (2001). De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. *J Biol Chem.* **276**(43)**,** 39926-39937.

Aihara, H., Takasaki, T., Toyosaki-Maeda, T., Suzuki, R., Okuno, Y., and Kurane, I. (2000). T-cell activation and induction of antibodies and memory T cells by immunization with inactivated Japanese encephalitis vaccine. *Viral Immunol.* **13**(2)**,** 179-186.

Allison, S. L., Stadler, K., Mandl, C. W., Kunz, C., and Heinz, F. X. (1995). Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. *J Virol.* **69**(9)**,** 5816-5820.

Alter, M. J. (2007). Epidemiology of hepatitis C virus infection. *World J Gastroenterol.* **13**(17)**,** 2436-2441.

Amberg, S. M., and Rice, C. M. (1999). Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. *J Virol.* **73**(10)**,** 8083-8094.

Anderson, J. F., Andreadis, T. G., Vossbrinck, C. R., Tirrell, S., Wakem, E. M., French, R. A., Garmendia, A. E., and Van, K. H. J. (1999). Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. *Science.* **286**(5448)**,** 2331-2333.

Anderson, J. F., and Main, A. J. (2006). Importance of vertical and horizontal transmission of West Nile virus by Culex pipiens in the Northeastern United States. *J Infect Dis.* **194**(11)**,** 1577-1579.

Anonymous. (2006). Acambis announces encouraging data from Phase 2 trial of West Nile virus vaccine. Acambis. Cambridge, UK, Cambridge, MA.

Anonymous. (2007). Alphavax announces results from Phase I influenza vaccine clinical trial. Alphavax. Research Triangle Park, NC.

Arroyo, J., Miller, C., Catalan, J., Myers, G. A., Ratterree, M. S., Trent, D. W., and Monath, T. P. (2004). ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy. *J Virol.* **78**(22)**,** 12497-12507.

Artsob, H., Gubler, D. J., Enria, D. A., Morales, M. A., Pupo, M., Bunning, M. L., and Dudley, J. P. (2009). West Nile Virus in the New World: Trends in the Spread and Proliferation of West Nile Virus in the Western Hemisphere. *Zoonoses Public Health.*

Ashour, J., Laurent-Rolle, M., Shi, P. Y., and Garcia-Sastre, A. (2009). NS5 of dengue virus mediates STAT2 binding and degradation. *J Virol.* **83**(11)**,** 5408-5418.

Asnis, D. S., Conetta, R., Teixeira, A. A., Waldman, G., and Sampson, B. A. (2000). The West Nile Virus outbreak of 1999 in New York: The Flushing Hospital experience. *Clinical Infectious Diseases.* . **30**(3)**,** 413-418.

Asselin-Paturel, C., Brizard, G., Pin, J. J., Briere, F., and Trinchieri, G. (2003). Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol.* **171**(12)**,** 6466-6477.

Bach, E. A., Aguet, M., and Schreiber, R. D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol.* **15,** 563-591.

Baqar, S., Hayes, C. G., Murphy, J. R., and Watts, D. M. (1993). Vertical transmission of West Nile virus by Culex and Aedes species mosquitoes. *Am J Trop Med Hyg.* **48**(6)**,** 757-762.

Barrett, P. N., Schober-Bendixen, S., and Ehrlich, H. J. (2003). History of TBE vaccines. *Vaccine.* **21 Suppl 1,** S41-49.

Bazan, J. F., and Fletterick, R. J. (1989). Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. *Virology.* **171**(2)**,** 637-639.

Beasley, D. W., Davis, C. T., Estrada-Franco, J., Navarro-Lopez, R., Campomanes-Cortes, A., Tesh, R. B., Weaver, S. C., and Barrett, A. D. (2004a). Genome sequence and attenuating mutations in West Nile virus isolate from Mexico. *Emerg Infect Dis.* **10**(12)**,** 2221-2224.

Beasley, D. W., Li, L., Suderman, M. T., Guirakhoo, F., Trent, D. W., Monath, T. P., Shope, R. E., and Barrett, A. D. (2004b). Protection against Japanese encephalitis virus strains representing four genotypes by passive transfer of sera raised against ChimeriVax-JE experimental vaccine. *Vaccine.* **22**(27-28)**,** 3722-3726.

Beasley, D. W., Whiteman, M. C., Zhang, S., Huang, C. Y., Schneider, B. S., Smith, D. R., Gromowski, G. D., Higgs, S., Kinney, R. M., and Barrett, A. D. (2005). Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J Virol.* **79**(13)**,** 8339-8347.

Best, S. M., Morris, K. L., Shannon, J. G., Robertson, S. J., Mitzel, D. N., Park, G. S., Boer, E., Wolfinbarger, J. B., and Bloom, M. E. (2005). Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. *J Virol.* **79**(20)**,** 12828-12839.

Bin, H., Grossman, Z., Pokamunski, S., Malkinson, M., Weiss, L., Duvdevani, P., Banet, C., Weisman, Y., Annis, E., Gandaku, D., Yahalom, V., Hindyieh, M., Shulman, L., and Mendelson, E. (2001). West Nile fever in Israel 1999-2000: from geese to humans. *Ann N Y Acad Sci.* **951,** 127-142.

Blackwell, J. L., and Brinton, M. A. (1997). Translation elongation factor-1 alpha interacts with the 3' stem-loop region of West Nile virus genomic RNA. *J Virol.* **71**(9)**,** 6433-6444.

Blitvich, B. J. (2008). Transmission dynamics and changing epidemiology of West Nile virus. *Anim Health Res Rev.* **9**(1)**,** 71-86.

Blitvich, B. J., Fernandez-Salas, I., Contreras-Cordero, J. F., Marlenee, N. L., Gonzalez-Rojas, J. I., Komar, N., Gubler, D. J., Calisher, C. H., and Beaty, B. J. (2003). Serologic evidence of West Nile virus infection in horses, Coahuila State, Mexico. *Emerg Infect Dis.* **9**(7)**,** 853-856.

Bourne, N., Scholle, F., Silva, M. C., Rossi, S. L., Dewsbury, N., Judy, B., De Aguiar, J. B., Leon, M. A., Estes, D. M., Fayzulin, R., and Mason, P. W. (2007). Early

production of type i interferon during West Nile virus infection: role for lymphoid tissues in IRF3-independent interferon production. *J Virol.* **81**(17)**,** 9100-9108.

Brandt, W. E., Cardiff, R. D., and Russell, P. K. (1970). Dengue virions and antigens in brain and serum of infected mice. *J Virol.* **6**(4)**,** 500-506.

Brandt, W. E., Chiewslip, D., Harris, D. L., and Russell, P. K. (1970). Partial purification and characterization of a dengue virus soluble complement-fixing antigen. *J Immunol.* **105**(6)**,** 1565-1568.

Brien, J. D., Uhrlaub, J. L., and Nikolich-Zugich, J. (2007). Protective capacity and epitope specificity of CD8(+) T cells responding to lethal West Nile virus infection. *Eur J Immunol.* **37**(7)**,** 1855-1863.

Brien, J. D., Uhrlaub, J. L., and Nikolich-Zugich, J. (2008). West Nile virus-specific CD4 T cells exhibit direct antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. *J Immunol.* **181**(12)**,** 8568-8575.

Brinton, M. A., and Dispoto, J. H. (1988). Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. *Virology.* **162**(2)**,** 290-299.

Brock, K. V. (2003). The persistence of bovine viral diarrhea virus. *Biologicals.* **31**(2)**,** 133-135.

Brooks, T. J., and Phillpotts, R. J. (1999). Interferon-alpha protects mice against lethal infection with St Louis encephalitis virus delivered by the aerosol and subcutaneous routes. *Antiviral Res.* **41**(1)**,** 57-64.

Cannon, G., and Weissman, D. (2002). RNA based vaccines. *DNA Cell Biol.* **21**(12)**,** 953-961.

Cardiff, R. D., and Lund, J. K. (1976). Distribution of dengue-2 antigens by electron immunocytochemistry. *Infect Immun.* **13**(6)**,** 1699-1709.

Castillo-Olivares, J., and Wood, J. (2004). West Nile virus infection of horses. *Vet Res.* **35**(4)**,** 467-483.

Caul, E. O., Smyth, G. W., and Clarke, S. K. (1974). A simplified method for the detection of rubella-specific IgM employing sucrose density fractionation and 2-mercaptoethanol. *J Hyg (Lond).* **73**(3)**,** 329-340.

CDC. West Nile Virus - Statistics, Surveillance, and Control - Case Count 2008. http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount08_detailed.htm. Accessed: May 20, 2009.

Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., Bazan, J. F., Fletterick, R. J., and Rice, C. M. (1990). Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proc Natl Acad Sci U S A.* **87**(22)**,** 8898-8902.

Chen, C. J., Kuo, M. D., Chien, L. J., Hsu, S. L., Wang, Y. M., and Lin, J. H. (1997a). RNA-protein interactions: involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA. *J Virol.* **71**(5)**,** 3466-3473.

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M. (1997b). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med.* **3**(8)**,** 866-871.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science.* **244**(4902)**,** 359-362.

Chu, J. J., and Ng, M. L. (2004a). Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J Virol.* **78**(19)**,** 10543-10555.

Chu, J. J., and Ng, M. L. (2004b). Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. *J Biol Chem.* **279**(52)**,** 54533-54541.

Chung, K. M., Thompson, B. S., Fremont, D. H., and Diamond, M. S. (2007). Antibody recognition of cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance of West Nile Virus-infected cells. *J Virol.* **81**(17)**,** 9551-9555.

Clarke, D. H., and Casals, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg.* **7**(5)**,** 561-573.

Cleaves, G. R., and Dubin, D. T. (1979). Methylation status of intracellular dengue type 2 40 S RNA. *Virology.* **96**(1)**,** 159-165.

Clum, S., Ebner, K. E., and Padmanabhan, R. (1997). Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient in vitro processing and is mediated through the hydrophobic regions of NS2B. *J Biol Chem.* **272**(49)**,** 30715-30723.

Clyde, K., and Harris, E. (2006). RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. *J Virol.* **80**(5)**,** 2170-2182.

Courageot, M. P., Frenkiel, M. P., Dos, S. C. D., Deubel, V., and Despres, P. (2000). alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. *J Virol.* **74**(1)**,** 564-572.

Coutelier, J. P., van der Logt, J. T., Heessen, F. W., Vink, A., and van Snick, J. (1988). Virally induced modulation of murine IgG antibody subclasses. *J Exp Med.* **168**(6)**,** 2373-2378.

Cui, T., Sugrue, R. J., Xu, Q., Lee, A. K., Chan, Y. C., and Fu, J. (1998). Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. *Virology.* **246**(2)**,** 409-417.

Davis, B. S., Chang, G. J. J., Cropp, B., Roehrig, J. T., Martin, D. A., Mitchell, C. J., Bowen, R., and Bunning, M. L. (2001). West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. *J Virol.* **75**(9)**,** 4040-4047.

Davis, C. T., Beasley, D. W., Guzman, H., Siirin, M., Parsons, R. E., Tesh, R. B., and Barrett, A. D. (2004). Emergence of attenuated West Nile virus variants in Texas, 2003. *Virology.* **330**(1)**,** 342-350.

Davis, C. W., Nguyen, H. Y., Hanna, S. L., Sanchez, M. D., Doms, R. W., and Pierson, T. C. (2006). West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. *J Virol.* **80**(3)**,** 1290-1301.

Davis, N. L., Caley, I. J., Brown, K. W., Betts, M. R., Irlbeck, D. M., McGrath, K. M., Connell, M. J., Montefiori, D. C., Frelinger, J. A., Swanstrom, R., Johnson, P. R., and Johnston, R. E. (2000). Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J Virol.* **74**(1)**,** 371-378.

De Nova-Ocampo, M., Villegas-Sepulveda, N., and del Angel, R. M. (2002). Translation elongation factor-1alpha, La, and PTB interact with the 3' untranslated region of dengue 4 virus RNA. *Virology.* **295**(2)**,** 337-347.

Delenda, C., Frenkiel, M. P., and Deubel, V. (1994). Protective efficacy in mice of a secreted form of recombinant dengue-2 virus envelope protein produced in baculovirus infected insect cells. *Arch Virol.* **139**(1-2)**,** 197-207.

Della-Porta, A. J., and Westaway, E. G. (1977). Immune response in rabbits to virion and nonvirion antigens of the Flavivirus kunjin. *Infect Immun.* **15**(3)**,** 874-882.

Despres, P., Combredet, C., Frenkiel, M. P., Lorin, C., Brahic, M., and Tangy, F. (2005). Live measles vaccine expressing the secreted form of the West Nile virus envelope glycoprotein protects against West Nile virus encephalitis. *J Infect Dis.* **191**(2)**,** 207-214.

Diamond, M. S., Shrestha, B., Marri, A., Mahan, D., and Engle, M. (2003). B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J Virol.* **77**(4)**,** 2578-2586.

Dudek, T., and Knipe, D. M. (2006). Replication-defective viruses as vaccines and vaccine vectors. *Virology.* **344**(1)**,** 230-239.

Dupuis, A. P., 2nd, Marra, P. P., and Kramer, L. D. (2003). Serologic evidence of West Nile virus transmission, Jamaica, West Indies. *Emerg Infect Dis.* **9**(7)**,** 860-863.

Durbin, A. P., McArthur, J., Marron, J. A., Blaney, J. E., Jr., Thumar, B., Wanionek, K., Murphy, B. R., and Whitehead, S. S. (2006). The live attenuated dengue serotype 1 vaccine rDEN1Delta30 is safe and highly immunogenic in healthy adult volunteers. *Hum Vaccin.* **2**(4)**,** 167-173.

Eidson, M., Komar, N., Sorhage, F., Nelson, R., Talbot, T., Mostashari, F., and McLean, R. (2001). Crow deaths as a sentinel surveillance system for West Nile virus in the Northeastern United States, 1999. *Emerg Infect Dis.* **7**(4)**,** 615-620.

Elliott, M. B., Chen, T., Terio, N. B., Chong, S. Y., Abdullah, R., Luckay, A., Egan, M. A., Boutilier, L. A., Melville, K., Lerch, R. A., Long, D., Eldridge, J. H., Parks, C. L., Udem, S. A., and Hancock, G. E. (2007). Alphavirus replicon particles encoding the fusion or attachment glycoproteins of respiratory syncytial virus elicit protective immune responses in BALB/c mice and functional serum antibodies in rhesus macaques. *Vaccine.* **25**(41)**,** 7132-7144.

Elshuber, S., Allison, S. L., Heinz, F. X., and Mandl, C. W. (2003). Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus. *J Gen Virol.* **84**(Pt 1)**,** 183-191.

Falgout, B., Miller, R. H., and Lai, C. J. (1993). Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B-NS3 protease activity. *J Virol.* **67**(4)**,** 2034-2042.

Falgout, B., Pethel, M., Zhang, Y. M., and Lai, C. J. (1991). Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol.* **65**(5)**,** 2467-2475.

Falkler, W. A., Jr., Diwan, A. R., and Halstead, S. B. (1973). Human antibody to dengue soluble complement-fixing (SCF) antigens. *J Immunol.* **111**(6)**,** 1804-1809.

Farrell, H. E., McLean, C. S., Harley, C., Efstathiou, S., Inglis, S., and Minson, A. C. (1994). Vaccine potential of a herpes simplex virus type 1 mutant with an essential glycoprotein deleted. *J Virol.* **68**(2)**,** 927-932.

Fayzulin, R., Scholle, F., Petrakova, O., Frolov, I., and Mason, P. W. (2006). Evaluation of replicative capacity and genetic stability of West Nile virus replicons using highly efficient packaging cell lines. *Virology.* **351**(1)**,** 196-209.

Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J., and Holland, P. V. (1975). Transfusion-associated hepatitis not due to viral hepatitis type A or B. *N Engl J Med.* **292**(15)**,** 767-770.

Finkelman, F. D., Katona, I. M., Mosmann, T. R., and Coffman, R. L. (1988). IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J Immunol.* **140**(4)**,** 1022-1027.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J Virol.* **66**(1)**,** 341-348.

Fredericksen, B. L., and Gale, M., Jr. (2006). West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *J Virol.* **80**(6)**,** 2913-2923.

Fredericksen, B. L., Keller, B. C., Fornek, J., Katze, M. G., and Gale, M., Jr. (2008). Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J Virol.* **82**(2)**,** 609-616.

Frucht, D. M., Fukao, T., Bogdan, C., Schindler, H., O'Shea, J. J., and Koyasu, S. (2001). IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol.* **22**(10)**,** 556-560.

Garcia-Sastre, A., and Biron, C. A. (2006). Type 1 interferons and the virus-host relationship: a lesson in detente. *Science.* **312**(5775)**,** 879-882.

Garcia-Tapia, D., Hassett, D. E., Mitchell, W. J., Jr., Johnson, G. C., and Kleiboeker, S. B. (2007). West Nile virus encephalitis: sequential histopathological and immunological events in a murine model of infection. *J Neurovirol.* **13**(2)**,** 130-138.

Garmendia, A. E., Van, K. H. J., French, R. A., Anderson, J. F., Andreadis, T. G., Kumar, A., and West, A. B. (2000). Recovery and identification of West Nile virus from a hawk in winter. *J Clin Microbiol.* **38**(8)**,** 3110-3111.

Gehrke, R., Ecker, M., Aberle, S. W., Allison, S. L., Heinz, F. X., and Mandl, C. W. (2003). Incorporation of tick-borne encephalitis virus replicons into virus-like particles by a packaging cell line. *J Virol.* **77**(16)**,** 8924-8933.

Germi, R., Crance, J. M., Garin, D., Guimet, J., Lortat, J. H., Ruigrok, R. W. H., Zarski, J. P., and Drouet, E. (2002). Heparan sulfate-mediated binding of infectious dengue virus type 2 and Yellow fever virus. *Virology* **292**(1)**,** 162-168.

Gilfoy, F., Fayzulin, R., and Mason, P. W. (2009). West Nile virus genome amplification requires the functional activities of the proteasome. *Virology.* **385**(1)**,** 74-84.

Gilfoy, F. D., and Mason, P. W. (2007). West Nile virus-induced interferon production is mediated by the double-stranded RNA-dependent protein kinase PKR. *J Virol.* **81**(20)**,** 11148-11158.

Girard, Y. A., Klingler, K. A., and Higgs, S. (2004). West Nile virus dissemination and tissue tropisms in orally infected Culex pipiens quinquefasciatus. *Vector Borne Zoonotic Dis.* **4**(2)**,** 109-122.

Girard, Y. A., Popov, V., Wen, J., Han, V., and Higgs, S. (2005). Ultrastructural study of West Nile virus pathogenesis in Culex pipiens quinquefasciatus (Diptera: Culicidae). *J Med Entomol.* **42**(3)**,** 429-444.

Goddard, L. B., Roth, A. E., Reisen, W. K., and Scott, T. W. (2002). Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis.* **8**(12)**,** 1385-1391.

Godsey, M. S., Jr., Blackmore, M. S., Panella, N. A., Burkhalter, K., Gottfried, K., Halsey, L. A., Rutledge, R., Langevin, S. A., Gates, R., Lamonte, K. M., Lambert, A., Lanciotti, R. S., Blackmore, C. G., Loyless, T., Stark, L., Oliveri, R., Conti, L., and Komar, N. (2005). West Nile virus epizootiology in the southeastern United States, 2001. *Vector Borne Zoonotic Dis.* **5**(1)**,** 82-89.

Gollins, S. W., and Porterfield, J. S. (1985). Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. *J Gen Virol.* **66**(9)**,** 1969-1982.

Gollins, S. W., and Porterfield, J. S. (1986). The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. *J Gen Virol.* **67**(9)**,** 1941-1950.

Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V., and Blinov, V. M. (1989a). N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res.* **17**(10)**,** 3889-3897.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989b). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* **17**(12)**,** 4713-4730.

Gubler, D. J. (2007). The continuing spread of West Nile virus in the western hemisphere. *Clin Infect Dis.* **45**(8)**,** 1039-1046.

Gubler, D. J., Kuno, G., and Markoff, L. (2007). Flaviviruses. 5th ed. *In* "Fields Virology" (B. N. Fields, D. M. Knipe and P. M. Howley, eds.) Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, PA.

Guirakhoo, F., Bolin, R. A., and Roehrig, J. T. (1992). The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. *Virology.* **191**(2)**,** 921-931.

Guirakhoo, F., Weltzin, R., Chambers, T. J., Zhang, Z. X., Soike, K., Ratterree, M., Arroyo, J., Georgakopoulos, K., Catalan, J., and Monath, T. P. (2000). Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. *J Virol.* **74**(12)**,** 5477-5485.

Guyatt, K. J., Westaway, E. G., and Khromykh, A. A. (2001). Expression and purification of enzymatically active recombinant RNA-dependent RNA polymerase (NS5) of the flavivirus Kunjin. *J Virol Methods.* **92**(1)**,** 37-44.

Hall, R. A., Khromykh, A. A., Mackenzie, J. M., Scherret, J. H., Khromykh, T. I., and Mackenzie, J. S. (1999). Loss of dimerisation of the nonstructural protein NS1 of kunjin virus delays viral replication and reduces virulence in mice, but still allows secretion of NS1. *Virology . Nov.* **264**(1)**,** 66-75.

Halstead, S. B. (2003). Neutralization and antibody-dependent enhancement of dengue viruses. *Adv Virus Res.* **60,** 421-467.

Halstead, S. B., and Tsai, T. F. (2004). Japanese encephalitis vaccine. 5th ed. *In* "Vaccine" (S. Plotkin and W. A. Orenstein, eds.), pp. 919-957. W.B. Saunders Company, Philadelphia, PA.

Hanna, S. L., Pierson, T. C., Sanchez, M. D., Ahmed, A. A., Murtadha, M. M., and Doms, R. W. (2005). N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. *J Virol.* **79**(21)**,** 13262-13274.

Hayes, C. G. (2001a). West Nile virus: Uganda, 1937, to New York City, 1999. *Ann N Y Acad Sci.* **951,** 25-37.

Hayes, C. G. (2001b). West Nile virus: Uganda, 1937, to New York City, 1999.

Hayes, E. B., Komar, N., Nasci, R. S., Montgomery, S. P., O'Leary, D. R., and Campbell, G. L. (2005a). Epidemiology and transmission dynamics of West Nile virus disease. *Emerg Infect Dis.* **11**(8)**,** 1167-1173.

Hayes, E. B., Sejvar, J. J., Zaki, S. R., Lanciotti, R. S., Bode, A. V., and Campbell, G. L. (2005b). Virology, pathology, and clinical manifestations of West Nile virus disease. *Emerg Infect Dis.* **11**(8)**,** 1174-1179.

Heinz, F. X., Stiasny, K., Puschner-Auer, G., Holzmann, H., Allison, S. L., Mandl, C. W., and Kunz, C. (1994). Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. *Virology.* **198**(1)**,** 109-117.

Hevey, M., Negley, D., Pushko, P., Smith, J., and Schmaljohn, A. (1998). Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology.* **251**(1)**,** 28-37.

Hoke, C. H., Nisalak, A., Sangawhipa, N., Jatanasen, S., Laorakapongse, T., Innis, B. L., Kotchasenee, S., Gingrich, J. B., Latendresse, J., Fukai, K., and et al. (1988). Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med.* **319**(10)**,** 608-614.

Houe, H. (1999). Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet Microbiol.* **64**(2-3)**,** 89-107.

Huang, C. Y., Silengo, S. J., Whiteman, M. C., and Kinney, R. M. (2005). Chimeric dengue 2 PDK-53/West Nile NY99 viruses retain the phenotypic attenuation markers of the candidate PDK-53 vaccine virus and protect mice against lethal challenge with West Nile virus. *J Virol.* **79**(12)**,** 7300-7310.

Ishikawa, T., Takasaki, T., Kurane, I., Nukuzuma, S., Kondo, T., and Konishi, E. (2007). Co-immunization with West Nile DNA and inactivated vaccines provides synergistic increases in their immunogenicities in mice. *Microbes Infect.* **9**(9)**,** 1089-1095.

Ishikawa, T., Widman, D. G., Bourne, N., Konishi, E., and Mason, P. W. (2008). Construction and evaluation of a chimeric pseudoinfectious virus vaccine to prevent Japanese encephalitis. *Vaccine.* **26**(22)**,** 2772-2781.

Jan, L. R., Yang, C. S., Henchal, L. S., Sumiyoshi, H., Summers, P. L., Dubois, D. R., and Lai, C. J. (1993). Increased immunogenicity and protective efficacy in outbred and inbred mice by strategic carboxyl-terminal truncation of Japanese encephalitis virus envelope glycoprotein. *Am J Trop Med Hyg.* **48**(3)**,** 412-423.

Jelley-Gibbs, D. M., Brown, D. M., Dibble, J. P., Haynes, L., Eaton, S. M., and Swain, S. L. (2005). Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med.* **202**(5)**,** 697-706.

Johansson, M., Brooks, A. J., Jans, D. A., and Vasudevan, S. G. (2001). A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. *J Gen Virol.* **82**(4)**,** 735-745.

Johnson, A. J., and Roehrig, J. T. (1999). New mouse model for dengue virus vaccine testing. *J Virol.* **73**(1)**,** 783-786.

Jones, C. T., Patkar, C. G., and Kuhn, R. J. (2005). Construction and applications of yellow fever virus replicons. *Virology.* **331**(2)**,** 247-259.

Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K. E., and Padmanabhan, R. (1995). Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. *J Biol Chem.* **270**(32)**,** 19100-19106.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Reis e Sousa, C., Matsuura, Y., Fujita, T., and Akira, S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature.* **441**(7089)**,** 101-105.

Khromykh, A. A., Kenney, M. T., and Westaway, E. G. (1998). trans-Complementation of flavivirus RNA polymerase gene NS5 by using Kunjin virus replicon-expressing BHK cells. *J Virol.* **72**(9)**,** 7270-7279.

Khromykh, A. A., Meka, H., Guyatt, K. J., and Westaway, E. G. (2001a). Essential role of cyclization sequences in Flavivirus RNA replication. *J Virol.* **75**(14)**,** 6719-6728.

Khromykh, A. A., Varnavski, A. N., Sedlak, P. L., and Westaway, E. G. (2001b). Coupling between replication and packaging of flavivirus RNA: Evidence derived from the use of DNA-based full-length cDNA clones of Kunjin virus. *J Virol.* **75**(10)**,** 4633-4640.

Khromykh, A. A., Varnavski, A. N., and Westaway, E. G. (1998). Encapsidation of the flavivirus kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins in trans. *J Virol.* **72**(7)**,** 5967-5977.

Kiermayr, S., Kofler, R. M., Mandl, C. W., Messner, P., and Heinz, F. X. (2004). Isolation of capsid protein dimers from the tick-borne encephalitis flavivirus and in vitro assembly of capsid-like particles. *J Virol.* **78**(15)**,** 8078-8084.

Kim, B. S., Miyagawa, F., Cho, Y. H., Bennett, C. L., Clausen, B. E., and Katz, S. I. (2009). Keratinocytes Function as Accessory Cells for Presentation of Endogenous Antigen Expressed in the Epidermis. *J Invest Dermatol.*

Klenk, K., Snow, J., Morgan, K., Bowen, R., Stephens, M., Foster, F., Gordy, P., Beckett, S., Komar, N., Gubler, D., and Bunning, M. (2004). Alligators as West Nile virus amplifiers. *Emerg Infect Dis.* **10**(12)**,** 2150-2155.

Kofler, R. M., Aberle, J. H., Aberle, S. W., Allison, S. L., Heinz, F. X., and Mandl, C. W. (2004). Mimicking live flavivirus immunization with a noninfectious RNA vaccine. *Proc Natl Acad Sci U S A.* **101**(7)**,** 1951-1956.

Kofler, R. M., Heinz, F. X., and Mandl, C. W. (2002). Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. *J Virol.* **76**(7)**,** 3534-3543.

Komar, N. (2003). West Nile virus: epidemiology and ecology in North America. *Adv Virus Res.* **61,** 185-234.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R., and Bunning, M. (2003a). Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis.* **9**(3)**,** 311-322.

Komar, N., Panella, N. A., Burns, J. E., Dusza, S. W., Mascarenhas, T. M., and Talbot, T. O. (2001). Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. *Emerg Infect Dis.* **7**(4)**,** 621-625.

Komar, O., Robbins, M. B., Klenk, K., Blitvich, B. J., Marlenee, N. L., Burkhalter, K. L., Gubler, D. J., Gonzalvez, G., Pena, C. J., Peterson, A. T., and Komar, N. (2003b). West Nile virus transmission in resident birds, Dominican Republic. *Emerg Infect Dis.* **9**(10)**,** 1299-1302.

Konishi, E., and Mason, P. W. (1993). Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J Virol.* **67**(3)**,** 1672-1675.

Konishi, E., Pincus, S., Fonseca, B. A., Shope, R. E., Paoletti, E., and Mason, P. W. (1991). Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology.* **185**(1)**,** 401-410.

Konishi, E., Pincus, S., Paoletti, E., Shope, R. E., Burrage, T., and Mason, P. W. (1992). Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology.* **188**(2)**,** 714-720.

Konishi, E., Win, K. S., Kurane, I., Mason, P. W., Shope, R. E., and Ennis, F. A. (1997). Particulate vaccine candidate for Japanese encephalitis induces long- lasting virus-specific memory T lymphocytes in mice. *Vaccine.* **15**(3)**,** 281-286.

Kramer, L. D., and Bernard, K. A. (2001). West Nile virus infection in birds and mammals. *Ann N Y Acad Sci.* **951,** 84-93.

Kreil, T. R., Maier, E., Fraiss, S., and Eibl, M. M. (1998a). Neutralizing antibodies protect against lethal flavivirus challenge but allow for the development of active humoral immunity to a nonstructural virus protein. *J Virol.* **72**(4)**,** 3076-3081.

Kreil, T. R., Maier, E., Fraiss, S., and Eibl, M. M. (1998b). Neutralizing antibodies protect against lethal flavivirus challenge but allow for the development of active humoral immunity to a nonstructural virus protein. *J Virol.* **72**(4)**,** 3076-3081.

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S., and Strauss, J. H. (2002). Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. *Cell.* **108**(5)**,** 717-725.

Kummerer, B. M., and Rice, C. M. (2002). Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J Virol.* **76**(10)**,** 4773-4784.

Kurane, I., and Takasaki, T. (2000). Immunogenicity and protective efficacy of the current inactivated Japanese encephalitis vaccine against different Japanese encephalitis virus strains. *Vaccine.* **18 Suppl 2,** 33-35.

La Rocca, S. A., Herbert, R. J., Crooke, H., Drew, T. W., Wileman, T. E., and Powell, P. P. (2005). Loss of interferon regulatory factor 3 in cells infected with classical swine fever virus involves the N-terminal protease, Npro. *J Virol.* **79**(11)**,** 7239-7247.

Lanciotti, R. S., Ebel, G. D., Deubel, V., Kerst, A. J., Murri, S., Meyer, R., Bowen, M., McKinney, N., Morrill, W. E., Crabtree, M. B., Kramer, L. D., and Roehrig, J. T. (2002). Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. *Virology.* **298**(1)**,** 96-105.

Lanciotti, R. S., Roehrig, J. T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K. E., Crabtree, M. B., Scherret, J. H., Hall, R. A., MacKenzie, J. S., Cropp, C. B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H. M., Stone, W., McNamara, T., and Gubler, D. J. (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science.* **286**(5448)**,** 2333-2337.

Langevin, S. A., Bunning, M., Davis, B., and Komar, N. (2001). Experimental infection of chickens as candidate sentinels for West Nile virus. *Emerg Infect Dis.* **7**(4)**,** 726-729.

Le Bon, A., and Tough, D. F. (2002). Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol.* **14**(4)**,** 432-436.

Lee, E., and Lobigs, M. (2000). Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. *J Virol.* **74**(19)**,** 8867-8875.

Lee, E., Stocks, C. E., Amberg, S. M., Rice, C. M., and Lobigs, M. (2000). Mutagenesis of the signal sequence of yellow fever virus prM protein: Enhancement of signalase cleavage in vitro is lethal for virus production. *Journal of Virology.* **74**(1)**,** 24-32.

Lee, J. S., Groebner, J. L., Hadjipanayis, A. G., Negley, D. L., Schmaljohn, A. L., Welkos, S. L., Smith, L. A., and Smith, J. F. (2006). Multiagent vaccines vectored by Venezuelan equine encephalitis virus replicon elicits immune responses to Marburg virus and protection against anthrax and botulinum neurotoxin in mice. *Vaccine.* **24**(47-48)**,** 6886-6892.

Lemon, S. M., Walker, C., Alter, M. J., and Yi, M. (2007). Hepatitis C Virus. 5th ed. *In* "Fields Virology" (B. N. Fields, D. M. Knipe and P. M. Howley, eds.) Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, PA.

Leyssen, P., Drosten, C., Paning, M., Charlier, N., Paeshuyse, J., De Clercq, E., and Neyts, J. (2003). Interferons, interferon inducers, and interferon-ribavirin in treatment of flavivirus-induced encephalitis in mice. *Antimicrob Agents Chemother.* **47**(2)**,** 777-782.

Li, W., Li, Y., Kedersha, N., Anderson, P., Emara, M., Swiderek, K. M., Moreno, G. T., and Brinton, M. A. (2002). Cell proteins TIA-1 and TIAR interact with the 3' stem-loop of the West Nile virus complementary minus-strand RNA and facilitate virus replication. *J Virol.* **76**(23)**,** 11989-12000.

Lieberman, M. M., Clements, D. E., Ogata, S., Wang, G., Corpuz, G., Wong, T., Martyak, T., Gilson, L., Coller, B. A., Leung, J., Watts, D. M., Tesh, R. B., Siirin, M., Travassos da Rosa, A., Humphreys, T., and Weeks-Levy, C. (2007).

Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. *Vaccine.* **25**(3)**,** 414-423.

Lin, C., Amberg, S. M., Chambers, T. J., and Rice, C. M. (1993). Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. *J Virol.* **67**(4)**,** 2327-2335.

Lin, R. J., Chang, B. L., Yu, H. P., Liao, C. L., and Lin, Y. L. (2006). Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. *J Virol.* **80**(12)**,** 5908-5918.

Lin, Y. L., Chen, L. K., Liao, C. L., Yeh, C. T., Ma, S. H., Chen, J. L., Huang, Y. L., Chen, S. S., and Chiang, H. Y. (1998). DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. *J Virol.* **72**(1)**,** 191-200.

Lindenbach, B. D., and Rice, C. M. (1997). trans-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. *J Virol.* **71**(12)**,** 9608-9617.

Lindenbach, B. D., and Rice, C. M. (1999). Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J Virol.* **73**(6)**,** 4611-4621.

Lindenbach, B. D., Thiel, H. J., and Rice, C. M. (2007). Flaviviridae: The viruses and their replication. 5th ed. *In* "Fields Virology" (B. N. Fields, D. M. Knipe and P. M. Howley, eds.) Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, PA.

Liu, W. J., Chen, H. B., and Khromykh, A. A. (2003). Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. *J Virol.* **77**(14)**,** 7804-7813.

Liu, W. J., Wang, X. J., Clark, D. C., Lobigs, M., Hall, R. A., and Khromykh, A. A. (2006). A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *J Virol.* **80**(5)**,** 2396-2404.

Liu, W. J., Wang, X. J., Mokhonov, V. V., Shi, P. Y., Randall, R., and Khromykh, A. A. (2005). Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *J Virol.* **79**(3)**,** 1934-1942.

Lo, M. K., Tilgner, M., Bernard, K. A., and Shi, P. Y. (2003). Functional analysis of mosquito-borne flavivirus conserved sequence elements within 3' untranslated region of West Nile virus by use of a reporting replicon that differentiates between viral translation and RNA replication. *J Virol.* **77**(18)**,** 10004-10014.

Lobigs, M., and Lee, E. (2004). Inefficient signalase cleavage promotes efficient nucleocapsid incorporation into budding flavivirus membranes. *J Virol.* **78**(1)**,** 178-186.

Lobigs, M., Mullbacher, A., Wang, Y., Pavy, M., and Lee, E. (2003). Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus. *J Gen Virol.* **84**(Pt 3)**,** 567-572.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science.* **285**(5424)**,** 110-113.

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., Brahic, M., and Tangy, F. (2004). A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. *J Virol.* **78**(1)**,** 146-157.

Lozach, P. Y., Burleigh, L., Staropoli, I., Navarro-Sanchez, E., Harriague, J., Virelizier, J. L., Rey, F. A., Despres, P., Arenzana-Seisdedos, F., and Amara, A. (2005). Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. *J Biol Chem.* **280**(25)**,** 23698-23708.

Ma, L., Jones, C. T., Groesch, T. D., Kuhn, R. J., and Post, C. B. (2004). Solution structure of dengue virus capsid protein reveals another fold. *Proc Natl Acad Sci U S A.* **101**(10)**,** 3414-3419.

Mackenzie, J. M., Khromykh, A. A., Jones, M. K., and Westaway, E. G. (1998). Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology.* **245**(2)**,** 203-215.

Mackenzie, J. S., Gubler, D. J., and Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med.* **10**(12 Suppl)**,** S98-109.

Manz, R. A., Thiel, A., and Radbruch, A. (1997). Lifetime of plasma cells in the bone marrow. *Nature.* **388**(6638)**,** 133-134.

Martin, J. E., Pierson, T. C., Hubka, S., Rucker, S., Gordon, I. J., Enama, M. E., Andrews, C. A., Xu, Q., Davis, B. S., Nason, M., Fay, M., Koup, R. A., Roederer, M., Bailer, R. T., Gomez, P. L., Mascola, J. R., Chang, G. J., Nabel, G. J., and Graham, B. S. (2007). A West Nile virus DNA vaccine induces neutralizing antibody in healthy adults during a phase 1 clinical trial. *J Infect Dis.* **196**(12)**,** 1732-1740.

Mason, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology.* **169**(2)**,** 354-364.

Mason, P. W., Pincus, S., Fournier, M. J., Mason, T. L., Shope, R. E., and Paoletti, E. (1991a). Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology.* **180**(1)**,** 294-305.

Mason, P. W., Pincus, S., Fournier, M. J., Mason, T. L., Shope, R. E., and Paoletti, E. (1991b). Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology.* **180**(1)**,** 294-305.

Mason, P. W., Shustov, A. V., and Frolov, I. (2006). Production and characterization of vaccines based on flaviviruses defective in replication. *Virology.* **351**(2)**,** 432-443.

Mattar, S., Edwards, E., Laguado, J., Gonzalez, M., Alvarez, J., and Komar, N. (2005). West Nile virus antibodies in Colombian horses. *Emerg Infect Dis.* **11**(9)**,** 1497-1498.

Matusan, A. E., Pryor, M. J., Davidson, A. D., and Wright, P. J. (2001). Mutagenesis of the Dengue virus type 2 NS3 protein within and outside helicase motifs: Effects on enzyme activity and virus replication. *J Virol.* **75**(20)**,** 9633-9643.

McLean, R. G., Ubico, S. R., Bourne, D., and Komar, N. (2002). West Nile virus in livestock and wildlife. *Curr Top Microbiol Immunol.* **267,** 271-308.

Men, R. H., Bray, M., and Lai, C. J. (1991). Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J Virol.* **65**(3)**,** 1400-1407.

Miller, B. R., Nasci, R. S., Godsey, M. S., Savage, H. M., Lutwama, J. J., Lanciotti, R. S., and Peters, C. J. (2000). First field evidence for natural vertical transmission of West Nile virus in Culex univittatus complex mosquitoes from Rift Valley Province, Kenya. *Am J Trop Med Hyg.* **62**(2)**,** 240-246.

Milligan, G. N., and Bernstein, D. I. (1995). Generation of humoral immune responses against herpes simplex virus type 2 in the murine female genital tract. *Virology.* **206**(1)**,** 234-241.

MMWR. (2002a). Possible West Nile virus transmission to an infant through breast-feeding--Michigan, 2002. *Morb Mortal Wkly Rep.* **51**(39)**,** 877-878.

MMWR. (2002b). Update: Investigations of West Nile virus infections in recipients of organ transplantation and blood transfusion--Michigan, 2002. *Morb Mortal Wkly Rep.* **51**(39)**,** 879.

Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature.* **427**(6972)**,** 313-319.

Mok, H., Lee, S., Utley, T. J., Shepherd, B. E., Polosukhin, V. V., Collier, M. L., Davis, N. L., Johnston, R. E., and Crowe, J. E., Jr. (2007). Venezuelan equine encephalitis virus replicon particles encoding respiratory syncytial virus surface glycoproteins induce protective mucosal responses in mice and cotton rats. *J Virol.* **81**(24)**,** 13710-13722.

Monath, T. P. (2001). Prospects for development of a vaccine against the West Nile virus. *Ann N Y Acad Sci.* **951,** 1-12.

Monath, T. P., Levenbook, I., Soike, K., Zhang, Z. X., Ratterree, M., Draper, K., Barrett, A. D., Nichols, R., Weltzin, R., Arroyo, J., and Guirakhoo, F. (2000a). Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: dose-response effectiveness and extended safety testing in rhesus monkeys. *J Virol.* **74**(4)**,** 1742-1751.

Monath, T. P., Levenbook, I., Soike, K., Zhang, Z. X., Ratterree, M., Draper, K., Barrett, A. D. T., Nichols, R., Weltzin, R., Arroyo, J., and Guirakhoo, F. (2000b). Chimeric yellow fever virus 17D-Japanese encephalitis virus vaccine: Dose-response effectiveness and extended safety testing in rhesus monkeys. *J Virol.* **74**(4)**,** 1742-1751.

Monath, T. P., Liu, J., Kanesa-Thasan, N., Myers, G. A., Nichols, R., Deary, A., McCarthy, K., Johnson, C., Ermak, T., Shin, S., Arroyo, J., Guirakhoo, F., Kennedy, J. S., Ennis, F. A., Green, S., and Bedford, P. (2006). A live, attenuated recombinant West Nile virus vaccine. *Proc Natl Acad Sci U S A.* **103**(17)**,** 6694-6699.

Monath, T. P., McCarthy, K., Bedford, P., Johnson, C. T., Nichols, R., Yoksan, S., Marchesani, R., Knauber, M., Wells, K. H., Arroyo, J., and Guirakhoo, F. (2002a). Clinical proof of principle for ChimeriVax: recombinant live, attenuated vaccines against flavivirus infections. *Vaccine.* **20**(7-8)**,** 1004-1018.

Monath, T. P., Nichols, R., Archambault, W. T., Moore, L., Marchesani, R., Tian, J., Shope, R. E., Thomas, N., Schrader, R., Furby, D., and Bedford, P. (2002b). Comparative safety and immunogenicity of two yellow fever 17D vaccines (ARILVAX and YF-VAX) in a phase III multicenter, double-blind clinical trial. *Am J Trop Med Hyg.* **66**(5)**,** 533-541.

Morrison, L. A., and Knipe, D. M. (1994). Immunization with replication-defective mutants of herpes simplex virus type 1: sites of immune intervention in pathogenesis of challenge virus infection. *J Virol.* **68**(2)**,** 689-696.

Mostashari, F., Bunning, M. L., Kitsutani, P. T., Singer, D. A., Nash, D., Cooper, M. J., Katz, N., Liljebjelke, K. A., Biggerstaff, B. J., Fine, A. D., Layton, M. C., Mullin, S. M., Johnson, A. J., Martin, D. A., Hayes, E. B., and Campbell, G. L. (2001). Epidemic West Nile encephalitis, New York, 1999: Results of a household-based seroepidemiological survey. *Lancet North American Edition.* **358**(9278)**,** 261-264.

Mukhopadhyay, S., Kim, B. S., Chipman, P. R., Rossmann, M. G., and Kuhn, R. J. (2003). Structure of west nile virus. *Science.* **302**(5643)**,** 248.

Mukhopadhyay, S., Kuhn, R. J., and Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol.* **3**(1)**,** 13-22.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. *Science.* **264**(5167)**,** 1918-1921.

Munoz-Jordan, J. L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W. I., and Garcia-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J Virol.* **79**(13)**,** 8004-8013.

Munoz-Jordan, J. L., Sanchez-Burgos, G. G., Laurent-Rolle, M., and Garcia-Sastre, A. (2003). Inhibition of interferon signaling by dengue virus. *Proc Natl Acad Sci U S A.* **100**(24)**,** 14333-14338.

Murray, K., Baraniuk, S., Resnick, M., Arafat, R., Kilborn, C., Cain, K., Shallenberger, R., York, T. L., Martinez, D., Hellums, J. S., Hellums, D., Malkoff, M., Elgawley, N., McNeely, W., Khuwaja, S. A., and Tesh, R. B. (2006). Risk factors for encephalitis and death from West Nile virus infection. *Epidemiol Infect.* **134**(6)**,** 1325-1332.

Muylaert, I. R., Chambers, T. J., Galler, R., and Rice, C. M. (1996). Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse neurovirulence. *Virology.* **222**(1)**,** 159-168.

Nasci, R. S., Savage, H. M., White, D. J., Miller, J. R., Cropp, B. C., Godsey, M. S., Kerst, A. J., Bennett, P., Gottfried, K., and Lanciotti, R. S. (2001). West Nile virus in overwintering Culex mosquitoes, New York City, 2000. *Emerg Infect Dis.* **7**(4)**,** 742-744.

Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D., Murray, K., Huang, A., Rosenberg, A., Greenberg, A., Sherman, M., Wong, S., and Layton, M. (2001). The outbreak of West Nile virus infection in the New York City area in 1999. *N Engl J Med.* **344**(24)**,** 1807-1814.

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J. L., Arenzana-Seisdedos, F., and Despres, P. (2003). Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Rep.* **4**(7 Suppl)**,** 1-6.

Nguyen, L. H., Knipe, D. M., and Finberg, R. W. (1992). Replication-defective mutants of herpes simplex virus (HSV) induce cellular immunity and protect against lethal HSV infection. *J Virol.* **66**(12)**,** 7067-7072.

Obst, R., van Santen, H. M., Mathis, D., and Benoist, C. (2005). Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J Exp Med.* **201**(10)**,** 1555-1565.

Odelola, H. A., and Oduye, O. O. (1977). West Nile virus infection of adult mice by oral route. *Arch Virol.* **54**(3)**,** 251-253.

Peiris, J. S., and Porterfield, J. S. (1979). Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature.* **282**(5738)**,** 509-511.

Petrakova, O., Volkova, E., Gorchakov, R., Paessler, S., Kinney, R. M., and Frolov, I. (2005). Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in Mammalian cells. *J Virol.* **79**(12)**,** 7597-7608.

Platonov, A. E., Shipulin, G. A., Shipulina, O. Y., Tyutyunnik, E. N., Frolochkina, T. I., Lanciotti, R. S., Yazyshina, S., Platonova, O. V., Obukhov, I. L., Zhukov, A. N., Vengerov, Y. Y., and Pokrovskii, V. I. (2001). Outbreak of West Nile virus infection, Volgograd Region, Russia, 1999. *Emerg Infect Dis.* **7**(1)**,** 128-132.

Platt, K. B., Tucker, B. J., Halbur, P. G., Tiawsirisup, S., Blitvich, B. J., Fabiosa, F. G., Bartholomay, L. C., and Rowley, W. A. (2007). West Nile virus viremia in eastern chipmunks (Tamias striatus) sufficient for infecting different mosquitoes. *Emerg Infect Dis.* **13**(6)**,** 831-837.

Pletnev, A. G., Claire, M. S., Elkins, R., Speicher, J., Murphy, B. R., and Chanock, R. M. (2003). Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus. *Virology.* **314**(1)**,** 190-195.

Pletnev, A. G., and Men, R. (1998). Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. *Proc Natl Acad Sci U S A.* **95**(4)**,** 1746-1751.

Pletnev, A. G., Putnak, R., Speicher, J., Wagar, E. J., and Vaughn, D. W. (2002). West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy. *Proc Natl Acad Sci U S A.* **99**(5)**,** 3036-3041.

Pletnev, A. G., Swayne, D. E., Speicher, J., Rumyantsev, A. A., and Murphy, B. R. (2006). Chimeric West Nile/dengue virus vaccine candidate: preclinical evaluation in mice, geese and monkeys for safety and immunogenicity. *Vaccine.* **24**(40-41)**,** 6392-6404.

Preugschat, F., and Strauss, J. H. (1991). Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus in vitro and in vivo. *Virology.* **185**(2)**,** 689-697.

Prikhod'ko, G. G., Prikhod'ko, E. A., Pletnev, A. G., and Cohen, J. I. (2002). Langat flavivirus protease NS3 binds caspase-8 and induces apoptosis. *J Virol.* **76**(11)**,** 5701-5710.

Purtha, W. E., Chachu, K. A., Virgin, H. W. t., and Diamond, M. S. (2008). Early B-cell activation after West Nile virus infection requires alpha/beta interferon but not antigen receptor signaling. *J Virol.* **82**(22)**,** 10964-10974.

Pushko, P., Parker, M., Ludwig, G. V., Davis, N. L., Johnston, R. E., and Smith, J. F. (1997). Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology.* **239**(2)**,** 389-401.

Qiao, M., Ashok, M., Bernard, K. A., Palacios, G., Zhou, Z. H., Lipkin, W. I., and Liang, T. J. (2004). Induction of sterilizing immunity against West Nile Virus (WNV), by immunization with WNV-like particles produced in insect cells. *J Infect Dis.* **190**(12)**,** 2104-2108.

Ramanathan, M. P., Chambers, J. A., Pankhong, P., Chattergoon, M., Attatippaholkun, W., Dang, K., Shah, N., and Weiner, D. B. (2006). Host cell killing by the West Nile Virus NS2B-NS3 proteolytic complex: NS3 alone is sufficient to recruit caspase-8-based apoptotic pathway. *Virology.* **345**(1)**,** 56-72.

Randall, R. E., and Goodbourn, S. (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol.* **89**(Pt 1)**,** 1-47.

Ratterree, M. S., da Rosa, A. P., Bohm, R. P., Jr., Cogswell, F. B., Phillippi, K. M., Caillouet, K., Schwanberger, S., Shope, R. E., and Tesh, R. B. (2003). West Nile virus infection in nonhuman primate breeding colony, concurrent with human epidemic, southern Louisiana. *Emerg Infect Dis.* **9**(11)**,** 1388-1394.

Ratterree, M. S., Gutierrez, R. A., Travassos da Rosa, A. P., Dille, B. J., Beasley, D. W., Bohm, R. P., Desai, S. M., Didier, P. J., Bikenmeyer, L. G., Dawson, G. J., Leary, T. P., Schochetman, G., Phillippi-Falkenstein, K., Arroyo, J., Barrett, A. D., and Tesh, R. B. (2004a). Experimental infection of rhesus macaques with West Nile virus: level and duration of viremia and kinetics of the antibody response after infection. *J Infect Dis.* **189**(4)**,** 669-676.

Ratterree, M. S., Gutierrez, R. A., Travassos da Rosa, A. P., Dille, B. J., Beasley, D. W., Bohm, R. P., Desai, S. M., Didier, P. J., Bikenmeyer, L. G., Dawson, G. J., Leary, T. P., Schochetman, G., Phillippi-Falkenstein, K., Arroyo, J., Barrett, A. D., and Tesh, R. B. (2004b). Experimental infection of rhesus macaques with West Nile virus: level and duration of viremia and kinetics of the antibody response after infection. *J Infect Dis.* **189**(4)**,** 669-676.

Ren, J., Ding, T., Zhang, W., Song, J., and Ma, W. (2007). Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? *Virol J.* **4,** 83.

Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., and Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature.* **375**(6529)**,** 291-298.

Reyes-Del Valle, J., Chavez-Salinas, S., Medina, F., and Del Angel, R. M. (2005). Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *J Virol.* **79**(8)**,** 4557-4567.

Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H. (1985). Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science.* **229**(4715)**,** 726-733.

Roosendaal, J., Westaway, E. G., Khromykh, A., and Mackenzie, J. M. (2006). Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in

rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. *J Virol.* **80**(9)**,** 4623-4632.

Root, J. J., Oesterle, P. T., Nemeth, N. M., Klenk, K., Gould, D. H., McLean, R. G., Clark, L., and Hall, J. S. (2006). Experimental infection of fox squirrels (Sciurus niger) with West Nile virus. *Am J Trop Med Hyg.* **75**(4)**,** 697-701.

Rossi, S. L., Zhao, Q., O'Donnell, V. K., and Mason, P. W. (2005). Adaptation of West Nile virus replicons to cells in culture and use of replicon-bearing cells to probe antiviral action. *Virology.* **331**(2)**,** 457-470.

Ruggli, N., Bird, B. H., Liu, L., Bauhofer, O., Tratschin, J. D., and Hofmann, M. A. (2005). N(pro) of classical swine fever virus is an antagonist of double-stranded RNA-mediated apoptosis and IFN-alpha/beta induction. *Virology.* **340**(2)**,** 265-276.

Ruggli, N., Tratschin, J. D., Schweizer, M., McCullough, K. C., Hofmann, M. A., and Summerfield, A. (2003). Classical swine fever virus interferes with cellular antiviral defense: evidence for a novel function of N(pro). *J Virol.* **77**(13)**,** 7645-7654.

Samuel, M. A., and Diamond, M. S. (2005). Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *J Virol.* **79**(21)**,** 13350-13361.

Samuel, M. A., Morrey, J. D., and Diamond, M. S. (2007). Caspase 3-dependent cell death of neurons contributes to the pathogenesis of West Nile virus encephalitis. *J Virol.* **81**(6)**,** 2614-2623.

Sardelis, M. R., Turell, M. J., O'Guinn, M. L., Andre, R. G., and Roberts, D. R. (2002). Vector competence of three North American strains of Aedes albopictus for West Nile virus. *J Am Mosq Control Assoc.* **18**(4)**,** 284-289.

Savage, H. M., Ceianu, C., Nicolescu, G., Karabatsos, N., Lanciotti, R., Vladimirescu, A., Laiv, L., Ungureanu, A., Romanca, C., and Tsai, T. F. (1999). Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *Am J Trop Med Hyg.* **61**(4)**,** 600-611.

Schepp-Berglind, J., Luo, M., Wang, D., Wicker, J. A., Raja, N. U., Hoel, B. D., Holman, D. H., Barrett, A. D., and Dong, J. Y. (2007). Complex adenovirus-mediated expression of West Nile virus C, PreM, E, and NS1 proteins induces both humoral and cellular immune responses. *Clin Vaccine Immunol.* **14**(9)**,** 1117-1126.

Schlesinger, J. J., Brandriss, M. W., Cropp, C. B., and Monath, T. P. (1986). Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. *J Virol.* **60**(3)**,** 1153-1155.

Schlesinger, J. J., Brandriss, M. W., Putnak, J. R., and Walsh, E. E. (1990). Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. *J Gen Virol.* **71**(3)**,** 593-599.

Schmidt, J. R., and Said, M. I. (1964). Isolation of West Nile Virus from the African Bird Argasid, Argas Reflexus Hermanni, in Egypt. *J Med Entomol.* **1**(1)**,** 83-86.

Schneider, B. S., and Higgs, S. (2008). The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. *Trans R Soc Trop Med Hyg.* **102**(5)**,** 400-408.

Schneider, B. S., Soong, L., Girard, Y. A., Campbell, G., Mason, P., and Higgs, S. (2006). Potentiation of West Nile encephalitis by mosquito feeding. *Viral Immunol.* **19**(1)**,** 74-82.

Scholle, F., Girard, Y. A., Zhao, Q., Higgs, S., and Mason, P. W. (2004). trans-Packaged West Nile virus-like particles: infectious properties in vitro and in infected mosquito vectors. *J Virol.* **78**(21)**,** 11605-11614.

Sejvar, J. J. (2007). The long-term outcomes of human West Nile virus infection. *Clin Infect Dis.* **44**(12)**,** 1617-1624.

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

Shafee, N., and AbuBakar, S. (2003). Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis. *J Gen Virol.* **84**(8)**,** 2191-2195.

Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H., and Takashima, I. (2004). Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *J Gen Virol.* **85**(12)**,** 3637-3645.

Shiryaev, S. A., Kozlov, I. A., Ratnikov, B. I., Smith, J. W., Lebl, M., and Strongin, A. Y. (2007). Cleavage preference distinguishes the two-component NS2B-NS3 serine proteinases of Dengue and West Nile viruses. *Biochem J.* **401**(3)**,** 743-752.

Shrestha, B., and Diamond, M. S. (2004). Role of CD8+ T cells in control of West Nile virus infection. *J Virol.* **78**(15)**,** 8312-8321.

Shrestha, B., Gottlieb, D., and Diamond, M. S. (2003). Infection and injury of neurons by West Nile encephalitis virus. *J Virol.* **77**(24)**,** 13203-13213.

Shrestha, B., Ng, T., Chu, H. J., Noll, M., and Diamond, M. S. (2008). The relative contribution of antibody and CD8+ T cells to vaccine immunity against West Nile encephalitis virus. *Vaccine.* **26**(16)**,** 2020-2033.

Shrestha, B., Wang, T., Samuel, M. A., Whitby, K., Craft, J., Fikrig, E., and Diamond, M. S. (2006). Gamma interferon plays a crucial early antiviral role in protection against West Nile virus infection. *J Virol.* **80**(11)**,** 5338-5348.

Silva, M. C., Guerrero-Plata, A., Gilfoy, F. D., Garofalo, R. P., and Mason, P. W. (2007). Differential activation of human monocyte-derived and plasmacytoid dendritic cells by West Nile virus generated in different host cells. *J Virol.* **81**(24)**,** 13640-13648.

Simmons, M., Murphy, G. S., Kochel, T., Raviprakash, K., and Hayes, C. G. (2001). Characterization of antibody responses to combinations of a dengue-2 DNA and dengue-2 recombinant subunit vaccine. *Am J Trop Med Hyg.* **65**(5)**,** 420-426.

Slifka, M. K., Matloubian, M., and Ahmed, R. (1995). Bone marrow is a major site of long-term antibody production after acute viral infection. *J Virol.* **69**(3)**,** 1895-1902.

Smith, T. J., Brandt, W. E., Swanson, J. L., McCown, J. M., and Buescher, E. L. (1970). Physical and biological properties of dengue-2 virus and associated antigens. *J Virol.* **5**(4)**,** 524-532.

Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H. (1940). A neurotropic virus isolated from the blood of a native of Uganda. *Am J Trop Med Hyg.* **20,** 471-492.

Smucny, J. J., Kelly, E. P., Macarthy, P. O., and King, A. D. (1995). Murine immunoglobulin G subclass responses following immunization with live dengue virus or a recombinant dengue envelope protein. *Am J Trop Med Hyg.* **53**(4)**,** 432-437.

Snapper, C. M., Peschel, C., and Paul, W. E. (1988). IFN-gamma stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J Immunol.* **140**(7)**,** 2121-2127.

Steele, K. E., Linn, M. J., Schoepp, R. J., Komar, N., Geisbert, T. W., Manduca, R. M., Calle, P. P., Raphael, B. L., Clippinger, T. L., Larsen, T., Smith, J., Lanciotti, R. S., Panella, N. A., and McNamara, T. S. (2000). Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. *Vet Pathol.* **37**(3)**,** 208-224.

Stevens, T. M., and Schlesinger, R. W. (1965). Studies on the nature of dengue viruses. I. Correlation of particle density, infectivity, and RNA content of type 2 virus. *Virology.* **27**(1)**,** 103-112.

Stocks, C. E., and Lobigs, M. (1998). Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. *J Virol.* **72**(3)**,** 2141-2149.

Stohlman, S. A., Wisseman, C. L., Jr., Eylar, O. R., and Silverman, D. J. (1975). Dengue virus-induced modifications of host cell membranes. *J Virol.* **16**(4)**,** 1017-1026.

Styer, L. M., Bernard, K. A., and Kramer, L. D. (2006). Enhanced early West Nile virus infection in young chickens infected by mosquito bite: effect of viral dose. *Am J Trop Med Hyg.* **75**(2)**,** 337-345.

Styer, L. M., Kent, K. A., Albright, R. G., Bennett, C. J., Kramer, L. D., and Bernard, K. A. (2007). Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. *PLoS Pathog.* **3**(9)**,** 1262-1270.

Su, C. M., Liao, C. L., Lee, Y. L., and Lin, Y. L. (2001). Highly sulfated forms of heparin sulfate are involved in Japanese encephalitis virus infection. *Virology* **286**(1)**,** 206-215.

Sun, J. C., Williams, M. A., and Bevan, M. J. (2004). CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol.* **5**(9)**,** 927-933.

Suzuki, R., Winkelmann, E. R., and Mason, P. W. (2009). Construction and characterization of a single-cycle chimeric flavivirus vaccine candidate that protects mice against lethal challenge with dengue virus type 2. *J Virol.* **83**(4)**,** 1870-1880.

Swayne, D. E., Beck, J. R., Smith, C. S., Shieh, W. J., and Zaki, S. R. (2001). Fatal encephalitis and myocarditis in young domestic geese (Anser anser domesticus) caused by West Nile virus. *Emerg Infect Dis.* **7**(4)**,** 751-753.

Tan, B. H., Fu, J., Sugrue, R. J., Yap, E. H., Chan, Y. C., and Tan, Y. H. (1996). Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity. *Virology.* **216**(2)**,** 317-325.

Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Birx, D. L., Steinman, R. M., Schlesinger, S., and Marovich, M. A. (2003). DC-SIGN

(CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med.* **197**(7)**,** 823-829.

Tesh, R. B., Arroyo, J., Travassos Da Rosa, A. P., Guzman, H., Xiao, S. Y., and Monath, T. P. (2002). Efficacy of killed virus vaccine, live attenuated chimeric virus vaccine, and passive immunization for prevention of West Nile virus encephalitis in hamster model. *Emerg Infect Dis.* **8**(12)**,** 1392-1397.

Tesh, R. B., Siirin, M., Guzman, H., Travassos da Rosa, A. P., Wu, X., Duan, T., Lei, H., Nunes, M. R., and Xiao, S. Y. (2005). Persistent West Nile virus infection in the golden hamster: studies on its mechanism and possible implications for other flavivirus infections. *J Infect Dis.* **192**(2)**,** 287-295.

Thurner, C., Witwer, C., Hofacker, I. L., and Stadler, P. F. (2004). Conserved RNA secondary structures in Flaviviridae genomes. *J Gen Virol.* **85**(5)**,** 1113-1124.

Tonry, J. H., Xiao, S. Y., Siirin, M., Chen, H., da Rosa, A. P., and Tesh, R. B. (2005). Persistent shedding of West Nile virus in urine of experimentally infected hamsters. *Am J Trop Med Hyg.* **72**(3)**,** 320-324.

Tsai, T. F., Popovici, F., Cernescu, C., Campbell, G. L., and Nedelcu, N. I. (1998). West Nile encephalitis epidemic in southeastern Romania. *Lancet.* **352**(9130)**,** 767-771.

Turell, M. J., Dohm, D. J., Sardelis, M. R., Oguinn, M. L., Andreadis, T. G., and Blow, J. A. (2005). An update on the potential of north American mosquitoes (Diptera: Culicidae) to transmit West Nile Virus. *J Med Entomol.* **42**(1)**,** 57-62.

United States General Accounting Office (2000). "West Nile virus outbreak : lessons for public health preparedness : report to congressional requesters." The Office, Washington, D.C. (P.O. Box 37050, Washington, DC 20013).

USDA. (2005). USDA Issues License for West Nile Virus DNA Vaccine for Horses. USDA Animal and Plant Health Inspection Service. Washington, DC.

van den Broek, M. F., Muller, U., Huang, S., Aguet, M., and Zinkernagel, R. M. (1995). Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. *J Virol.* **69**(8)**,** 4792-4796.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med.* **11**(7)**,** 791-796.

Warrener, P., Tamura, J. K., and Collett, M. S. (1993). RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria. *J Virol.* **67**(2)**,** 989-996.

Watts, D. M., Tesh, R. B., Siirin, M., Rosa, A. T., Newman, P. C., Clements, D. E., Ogata, S., Coller, B. A., Weeks-Levy, C., and Lieberman, M. M. (2007). Efficacy and durability of a recombinant subunit West Nile vaccine candidate in protecting hamsters from West Nile encephalitis. *Vaccine.* **25**(15)**,** 2913-2918.

Weir, E. (2000). West Nile fever heads north. *Can Med Assoc J.* **163**(7)**,** 878.

Wengler, G. (1993). The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology.* **197**(1)**,** 265-273.

Wengler, G., and Gross, H. J. (1978). Studies on virus-specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses. *Virology.* **89**(2)**,** 423-437.

Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K., and Khromykh, A. A. (1997). Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *J Virol.* **71**(9)**,** 6650-6661.

White, L. J., Parsons, M. M., Whitmore, A. C., Williams, B. M., de Silva, A., and Johnston, R. E. (2007). An immunogenic and protective alphavirus replicon particle-based dengue vaccine overcomes maternal antibody interference in weanling mice. *J Virol.* **81**(19)**,** 10329-10339.

WHO. (1998). Japanese encephalitis vaccines. *Wkly Epidemiol Rec.* **73**(44)**,** 337-344.

Widman, D. G., Frolov, I., and Mason, P. W. (2008). Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. *Adv Virus Res.* **72,** 77-126.

Widman, D. G., Ishikawa, T., Fayzulin, R., Bourne, N., and Mason, P. W. (2008). Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. *Vaccine.* **26**(22)**,** 2762-2771.

Widman, D. G., Ishikawa, T., Winkelmann, E. R., Infante, E., Bourne, N., and Mason, P. W. (2009). RepliVAX WN, a single-cycle flavivirus vaccine to prevent West Nile disease, elicits durable protective immunity in hamsters. *Vaccine.* **27**(41)**,** 5550-5553.

Wilson, J. R., de Sessions, P. F., Leon, M. A., and Scholle, F. (2008). West Nile virus nonstructural protein 1 inhibits TLR3 signal transduction. *J Virol.* **82**(17)**,** 8262-8271.

Winkler, G., Maxwell, S. E., Ruemmler, C., and Stollar, V. (1989). Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. *Virology.* **171**(1)**,** 302-305.

Winkler, G., Randolph, V. B., Cleaves, G. R., Ryan, T. E., and Stollar, V. (1988). Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology.* **162**(1)**,** 187-196.

Work, T. H., Hurlbut, H. S., and Taylor, R. M. (1953). Isolation of West Nile virus from hooded crow and rock pigeon in the Nile delta. *Proc Soc Exp Biol Med.* **84**(3)**,** 719-722.

Work, T. H., Hurlbut, H. S., and Taylor, R. M. (1955). Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. *Am J Trop Med Hyg.* **4**(5)**,** 872-888.

Xiao, S. Y., Guzman, H., Zhang, H., Travassos da Rosa, A. P., and Tesh, R. B. (2001). West Nile virus infection in the golden hamster (Mesocricetus auratus): a model for West Nile encephalitis. *Emerg Infect Dis.* **7**(4)**,** 714-721.

Yamshchikov, V. F., and Compans, R. W. (1993). Regulation of the late events in flavivirus protein processing and maturation. *Virology.* **192**(1)**,** 38-51.

Yocupicio-Monroy, R. M., Medina, F., Reyes-del Valle, J., and del Angel, R. M. (2003). Cellular proteins from human monocytes bind to dengue 4 virus minus-strand 3' untranslated region RNA. *J Virol.* **77**(5)**,** 3067-3076.

Yon, C., Teramoto, T., Mueller, N., Phelan, J., Ganesh, V. K., Murthy, K. H., and Padmanabhan, R. (2005). Modulation of the nucleoside triphosphatase/RNA

helicase and 5'-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. *J Biol Chem.* **280**(29)**,** 27412-27419.

Yoshii, K., Goto, A., Kawakami, K., Kariwa, H., and Takashima, I. (2008). Construction and application of chimeric virus-like particles of tick-borne encephalitis virus and mosquito-borne Japanese encephalitis virus. *J Gen Virol.* **89**(Pt 1)**,** 200-211.

Zhang, W., Chipman, P. R., Corver, J., Johnson, P. R., Zhang, Y., Mukhopadhyay, S., Baker, T. S., Strauss, J. H., Rossmann, M. G., and Kuhn, R. J. (2003a). Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nat Struct Biol.* **10**(11)**,** 907-912.

Zhang, Y., Corver, J., Chipman, P. R., Zhang, W., Pletnev, S. V., Sedlak, D., Baker, T. S., Strauss, J. H., Kuhn, R. J., and Rossmann, M. G. (2003b). Structures of immature flavivirus particles. *Embo J.* **22**(11)**,** 2604-2613.

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D.G. Widman, I. Frolov, and P.W. Mason. 2008. Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. *Advances in Virus Research.* **72**:77-126.

D.G. Widman, T. Ishikawa, R. Fayzulin, N. Bourne, and P.W. Mason. 2008. Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. *Vaccine.* **26:**2762–2771.

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Abstracts

D.G. Widman, T. Ishikawa, N. Bourne, and P.W. Mason. Characterization of the in vivo behavior of single-cycle flavivirus particles. Selected for oral presentation at the 28th Annual Meeting of the American Society for Virology. Vancouver, BC. July 11-15, 2009.

D.G. Widman, T. Ishikawa, R. Carrion Jr, N. Bourne, and P.W. Mason. RepliVAX WN, a single-cycle flavivirus vaccine, is safe and efficacious in a rhesus macaque model of West Nile

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D.G. Widman, T. Ishikawa, R. Carrion Jr, N. Bourne, and P.W. Mason. Evaluation of RepliVAX WN, a single-cycle flavivirus vaccine, in a rhesus macaque model of West Nile disease. Selected for presentation at the 3rd Annual NIH National Graduate Student Research Festival. Bethesda, MD. September 11-12, 2008.

D.G. Widman, T. Ishikawa, R. Fayzulin, N. Bourne, and P.W. Mason. Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. Selected for oral presentation at the 27th Annual Meeting of the American Society for Virology. Ithaca, NY. July 12-16, 2008.

D.G. Widman, R.Z. Fayzulin, E. Infante, T. Ishikawa, and P.W. Mason. Developing a second-generation pseudo-infectious flavivirus vaccine candidate for West Nile (WN) encephalitis. Presented at the 8th International Symposium on Positive-Strand RNA Viruses. Washington, D.C. May 26-30, 2007.

D.G. Widman, N. Dewsbury, R.Z. Fayzulin, I. Frolov, and P.W. Mason. Improving yield and testing safety of a novel pseudo-infectious virus vaccine candidate for West Nile (WN) encephalitis. Presented at the Sealy Center for Vaccine Development Changing Landscape of Vaccine Development- Vaccines for Global Health. Galveston, Texas. November 8-10, 2006.

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BIBLIOGRAPHY:

PUBLISHED:

1. D.G. Widman, T. Ishikawa, E.R. Winkelmann, E. Infante, N. Bourne, and P.W. Mason. RepliVAX WN, a single-cycle flavivirus vaccine to prevent West Nile disease, elicits durable protective immunity in hamsters. *Vaccine*. **26:**2762–2771. 2009.

2. D.G. Widman, I. Frolov, and P.W. Mason. Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. *Advances in Virus Research.* 72:77-126. 2008.

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4. T. Ishikawa, D.G. Widman, N. Bourne, E. Konishi, and P.W. Mason. Construction and evaluation of a chimeric pseudoinfectious virus vaccine to prevent Japanese encephalitis. *Vaccine.*26:2772-2781. 2008.

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