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Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system

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Summary Safer vaccines are needed to prevent flavivirus diseases. To help develop these products we have produced a pseudoinfectious 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.

Introduction

West Nile virus (WNV) is a positive-sense, single-stranded RNA virus belonging to the *Flavivirus* genus of the *Flaviviridae* family. Medically important members of this genus include dengue virus, yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV) [1]. Since its introduction into the US, WNV has spread throughout the hemisphere and become endemic

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in North America. Although WNV infection is often asymptomatic, it can produce human disease ranging from febrile illness to fatal encephalitis. To date there have been over 25,000 human cases reported in the United States [2].

The WNV genome consists of a single open reading frame encoding a polyprotein that is cleaved into three structural and seven non-structural proteins. The structural proteins C, M (produced in cells as a precursor, prM), and E make up the viral particle, while the non-structural (NS) proteins are required for genome replication and polyprotein processing [1]. WNV virions consist of a C-containing nucleocapsid surrounded by a host-derived membrane containing M and E. During flavivirus infection subviral particles (SVPs) composed of M and E but lacking nucleocapsid are released with mature virions. Expression of prM and E in the absence of C produces SVPs [3,4], and several vaccine candidates, including subunit [5—9], DNA [10—12], RNA [13] and live-vectors [3,14—18] have been developed based on SVPs. Animal studies have demonstrated that these candidates elicit protective immune responses that include both humoral and cell-mediated activities [7,15]. Among these candidates, DNA vaccines suffer from poor immunogenicity [10—12], subunit vaccines are difficult to produce in large quantities [5,7,9], and live-vectored vaccines have proven ineffective in the face of pre-existing vector immunity [18].

Currently there are few vaccines available to prevent flavivirus diseases. The YFV-17D live-attenuated vaccine (LAV), although extremely effective, has recently been associated with a number of severe adverse events including the development of viscerotropic YF disease [19—22]. Moreover, this vaccine is not recommended for use in infants, pregnant women, or immunocompromised individuals. A promising line of research into new vaccines has focused on genetically derived chimeras of YFV-17D (ChimeriVax) encoding prM and E of other flaviviruses [23—25]. Other vaccine candidates have been produced by using a similar chimerization strategy to insert prM and E genes into an attenuated dengue type 4 backbone [26—28]. Despite the fact that ChimeriVax-WN has been successfully evaluated in non-human primates [29], the 17D backbone in ChimeriVax presents the same potential hazards associated with the YFV vaccine, and it is unclear if chimeric viruses will display unwanted pathogenic characteristics. Licensed inactivated viral vaccines (INV) exist to prevent JE and TBE, but these INVs are expensive and require multiple vaccinations [30]. Recently, the JEV INV was removed from the universal vaccination campaign in Japan due to concerns over adverse reactions [31,32].

A promising line of research has focused on development of attenuated flaviviruses containing large in-frame deletions within the C gene. Specifically, C-deleted genomes of TBEV have been shown to be replicationally competent, capable of producing SVPs, and unable to spread between cells, making them a useful RNA vaccine [13,33]. Utilizing a similar strategy we have developed a trans-encapsidation system to package C-deleted genomes, producing a novel WN vaccine candidate, RepliVAX WN [34]. Unlike the RNA vaccines described above, RepliVAX WN is a special type of LAV that can be produced in cell lines expressing the missing C [34,35]. When RepliVAX WN infects normal cells, it cannot produce infectious progeny due to the lack of the C protein, however these cells produce SVPs that have demonstrated ability to protect animals from flavivirus infection (see above) and RepliVAX WN efficiently protects mice against West Nile encephalitis (WNE) [34].

Here we describe an improved system for growth of RepliVAX, and development of an improved second-generation RepliVAX WN (RepliVAX WN.2). Our new propagation system consists of cell lines based on either baby hamster kidney (BHK) cells or vaccine-certified Vero cells expressing a form of the WNV C protein engineered for long-term stability and an inability to recombine with the RepliVAX genome. RepliVAX WN.2 produced in these cells was more potent than our original RepliVAX WN and efficiently protected two different animals from WNE.

Materials and methods

Cell cultures and viruses

The baby hamster kidney cells used for all studies and Vero cells used for titration and blind passaging studies have been previously described [34]. Vaccine-substrate Vero cells (S. Whitehead, NIH, Bethesda, MD) were maintained in OptiPro serum-free medium (SFM) (Gibco/Invitrogen, Carlsbad, CA). Packaging cell lines were produced by puromycin (10 μg/ml) selection of cell lines harboring Venezuelan equine encephalitis virus replicons (VEErep) encoding the desired flavivirus genes (see below).

The snowy owl isolate of WNV NY99 [36] (R.B. Tesh, UTMB) was used for all animal studies. Mice were challenged with 1000 ffu of virus (corresponding to 10 LD_{50} in 8-week-old mice), and hamsters were challenged with 1×10^6 ffu.

Plasmid constructs, RNA transcription and transfections

DNA plasmid constructions were performed using standard recombinant DNA techniques. The RepliVAX WN genome (Fig. 1) containing a 70-codon deletion in C [34] was introduced into a bacterial artificial chromosome (BAC) to increase stability. Second-generation RepliVAX WN genomes (RepliVAX WN.2 SP, RepliVAX WN.2 NS, and RepliVAX WN.2 SP—NS) containing cleavage-site mutations were incorporated into the BAC-propagated cDNAs by PCR-based mutagenesis. Sequences of plasmids and RepliVAX recovered from transfected cells were determined by standard techniques (see below). WNV infectious clone work was conducted using a BAC cDNA encoding our previously described full-length genome of a 2002 WNV isolate [37].

The VEErep encoding the mutated WNV C gene (C*; Fig. 1) was generated using previously described methods [38]. An expression cassette engineered to contain the WNV C gene downstream of a *puromycin N-acetyl transferase* (*Pac*)/*ubiquitin* fusion was inserted into a plasmid harboring a non-cytopathic VEErep, and *in vitro* transcription reactions obtained from this plasmid were used to produce replicon-bearing cells [38].

WNV ELISA antigens were obtained from cell lines expressing WNV NS1 or a soluble truncated E generated from non-cytopathic VEE replicons [39] by using similar methods. VEErep/Pac-Ubi-WNNS1NS2A was engineered to encode the *Pac*/*ubiquitin* fusion, 31 codons of the N terminus of C, 30 codons of the C terminus of E (the signal sequence of NS1),

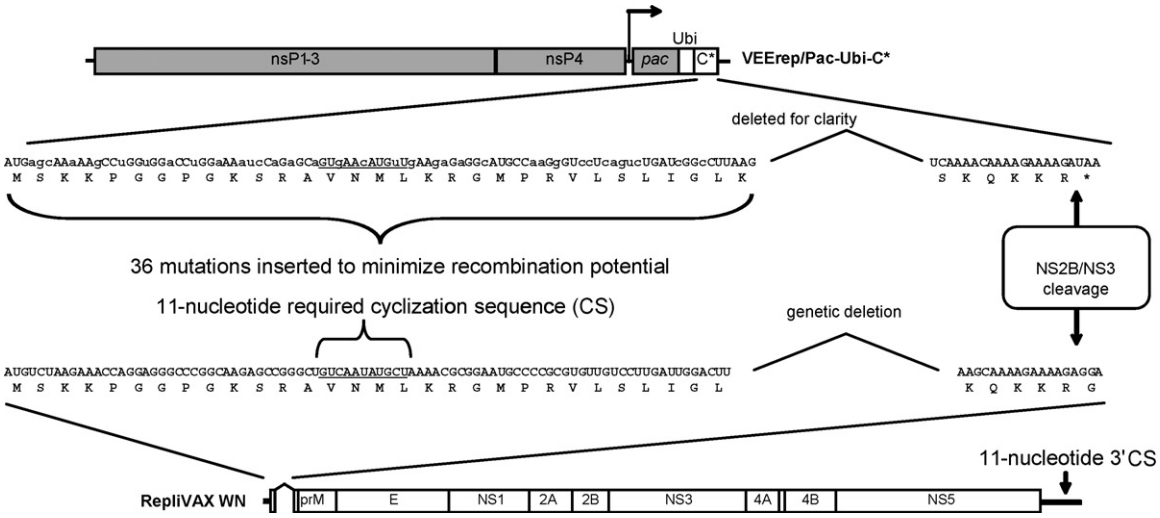

Figure 1 Schematic representation of the RepliVAX WN genome and the VEE replicon used to supply the C protein in packaging cells. Top: non-cytopathic VEE replicon (VEErep/Pac-Ubi-C*) encoding the complete C of WNV along with the *Pac* selectable marker expressed as a fusion protein with ubiquitin. This 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.

and the NS1-NS2A genes. VEErep/WNprMtrE-Pac was engineered to encode prM followed by the first 421 codons of E (80% of full-length E) fused to a 6× histidine tag at its C terminus.

Passaging of RepliVAX WN

RepliVAX WN particles obtained from electroporation of synthetic RepliVAX WN RNA into BHK(VEErep/Pac-Ubi-C*) cells were used for subsequent infections. RepliVAX WN passaging was performed at a multiplicity of infection (MOI) of 0.01 in MEM containing 1% FBS, 10 mM HEPES, and antibiotics (maintenance-media). Medium was replaced at 24 h intervals post-infection, and the 72 h sample was used for subsequent infections.

Analyses of RepliVAX WN

Sequence analyses of RepliVAX WN were performed by standard methods [37]. Vero cell monolayers were infected with undiluted RepliVAX harvests, and 24 h later total RNA was isolated, converted to cDNA, and specific oligonucleotide primers were used to amplify cDNA fragments that were gel-purified and sequenced by standard techniques (Protein Chemistry Core Laboratory, UTMB).

The techniques used to characterize and enumerate RepliVAX WN have been described previously [34,37]. Infectious titers (infectious units [IU]/ml) were determined by infecting Vero cell monolayers with serial dilutions of virus, fixing the cells after 28 h, and immunohistochemically detecting infected cells with a polyclonal mouse hyperimmune ascites fluid (MHIAF) specific for WNV. To assess focus-forming ability, BHK(VEErep/Pac-Ubi-C*) were infected with serial dilutions of RepliVAX WN, overlaid with a 0.6% tragacanth solution, incubated for 72 h, fixed, and stained as described above. Growth curves were performed by analogous methods.

RepliVAX WN.2 animal studies

Groups of fifteen 5-week-old female Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, IN) were immunized intraperitoneally (i.p.) or subcutaneously (s.c.) with RepliVAX WN, RepliVAX WN.2, or diluent alone (mock). All inocula were delivered in 100 μl of L-15 medium containing 10 mM HEPES and 0.5% FBS. Animals were monitored for vaccine-induced side effects including lethargy and hind-limb paralysis. At 21 days post-vaccination serum was collected from all of the animals by retro-orbital bleed. Seven days later animals were challenged i.p. with 10 LD_{50} of WNV NY99 and monitored for changes in weight and health 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.

Groups of ten 4-week-old female Syrian hamsters (Harlan Sprague Dawley) were immunized i.p. with RepliVAX WN or diluent (mock) in a volume of 100 μl of maintenance-media. Animals were monitored for 3 weeks for vaccine-induced side effects, and at day 21 post-vaccination sera were obtained from all animals. Seven days later, hamsters were challenged i.p. with 1×10^6 ffu of WNV NY99 diluted in 100 μl PBS + 10% FBS, and weight and health were monitored for 3 weeks. Animals scored moribund were humanely euthanized in compliance with UTMB Animal Care and Use requirements and scored as ''dead'' the following day.

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 with NS1 or E protein harvested from VEErep-bearing cell lines (see above), and then incubated with individual sera (diluted 1:100) for 1 h. Goat anti-mouse IgG HRP-conjugated antibody (KPL, Gaithersburg, MD) was added to the plates for 1 h, and the bound HRP was detected by incubation with TMB (Sigma), prior to quenching with 1 M HCl. The reaction product was quantitated spectrophotometrically at 450 nm, and values were corrected for background activity detected from wells that received diluent in place of sera.

Neutralizing antibody (neut) titers were determined by measuring the serum dilution that produced a 90% reduction of luciferase activity from Vero cells infected with a firefly luciferase-encoding WNV VLP (WNVLP), an assay that has been shown to be comparable to classical focus reduction assays [40]. Serial twofold dilutions of heat-inactivated pooled serum samples were incubated for 1 h at 37 °C with a fixed amount of luciferase-encoding WNVLPs [38]. These VLP/serum mixtures were used to infect Vero cell monolayers in black 96-well plates (Greiner Bio-One, Monroe, NC) for 2 h at which time the infection medium was replaced with maintenance-media and allowed to incubate for 24 h. A solution containing cell lysis buffer 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 read on a luminometer (Applied Biosystems, Foster City, CA) and light output was expressed as the percent activity obtained from lysates prepared from monolayers infected with WNVLPs incubated at 37 °C in diluent only.

Statistical analyses

Serology data and 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.

Results

Production of RepliVAX WN in C-expressing cell lines

We have previously demonstrated that C-deleted WNV genomes (RepliVAX WN) could be packaged into infectious particles by trans-complementation with C produced in packaging cell lines, and demonstrated that these particles are unable to spread in cells that do not express C [34]. However, the packaging cell lines used in these studies encoded all three WNV structural proteins [34], a strategy that could facilitate intergenomic recombination producing a fully infectious WNV genome. Furthermore, we noted that some VEErep genomes can undergo intragenomic recombination, eliminating foreign genes [38] which could compromise long-term propagation of packaging cells. We thus set out to produce a genetically stable cell line with reduced possibility of intergenomic recombination.

To generate a new VEErep construct with enhanced genetic stability and increased safety, plasmid DNA encoding a non-cytopathic VEE replicon was engineered so that its subgenomic promoter drove expression of a *Pac*/*ubiquitin* fusion protein followed by the WNV C gene (VEErep/Pac-Ubi-C*) (Fig. 1). In order to reduce sequence homology and intergenomic recombination potential with the truncated C (trC) gene in RepliVAX WN, 36 silent mutations [38] were incorporated into the 5′ region of the C gene (C*) contained in VEErep/Pac-Ubi-C* (Fig. 1). These changes also disrupted the 5′ cyclization sequence (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. Although previous studies indicated that addition of prM coding regions to C-expressing VEEreps enhanced RepliVAX packaging [34], VEErep/Pac-Ubi-C* contains a stop codon upstream of the prM signal sequence (Fig. 1) to further reduce the possibility of intergenomic recombination. 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 focus formation assays. RepliVAX WN infections of this cell line routinely produced titers of $>10^7$ IU/ml, while three other clones produced 0.5—1 log lower levels (results not shown). As expected from fusion of *Pac* to the C gene through *ubiquitin*, this cell line was extraordinarily stable. 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).

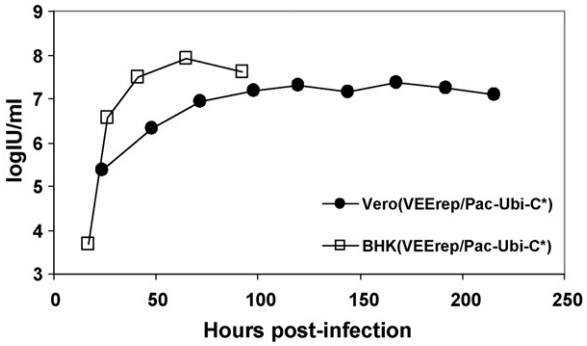

Figure 2 Growth curve analyses showing yield of RepliVAX WN.2 SP from clonally derived cultures of either BHK(VEErep/Pac-Ubi-C*) cells (open squares) or Vero(VEErep/Pac-Ubi-C*) (closed circles) infected at an MOI of 0.05.

To demonstrate that RepliVAX WN could be produced in a cell line acceptable for human vaccine production, a vaccine-compatible Vero cell line was electroporated with *in vitro*-transcribed VEErep/Pac-Ubi-C* RNA and replicon-expressing cells were selected by addition of Pur to the culture medium. RepliVAX WN.2 infectious titers from clonally derived Vero(VEErep/Pac-Ubi-C*) cells did not reach levels equivalent to those of BHK(VEErep/Pac-Ubi-C*) cells (Fig. 2), but RepliVAX WN-producing Vero cell monolayers remained viable for over 200 h post-infection.

Repeated passaging of RepliVAX WN through packaging cells was unable to force productive recombination

In order to examine the unlikely possibility that the RepliVAX WN 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—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 three successive passages on WT Vero cells after which no immunopositive cells were detected in the Vero monolayer, indicating that no viable progeny capable of causing a spreading infection 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 and PCR-amplified for sequence analyses. At each passage level, the size of the amplified trC region was identical to that of the parental original genome, and sequence analyses failed to detect any evidence of recombination or other changes in the trC gene.

Serial passaging of RepliVAX WN in packaging cells selects for genomes with mutations in proteolytic cleavage sites

From blind passage studies we noted that passage 10 (p10) RepliVAX WN produced larger and more heterogeneous foci than passage 0 (p0) RepliVAX WN (Fig. 3A). Side-by-side growth analyses revealed that the p10 RepliVAX WN achieved titers up to fivefold higher than p0 (results not shown). Sequencing of the entire genome of this p10 population revealed two non-synonymous changes, one of which was an A to U substitution in the codon at the +3 position to the NS2B/NS3 protease cleavage site that separated trC from the prM signal peptide. This change, which was only observed in a portion of the population, resulted in a K to M substitution (QKKR|GGKT → QKKR|GGmT) (Fig. 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 signalase cleavage site preceding prM resulting in an S to 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 context of RepliVAX WN it appears that only prM is essential for particle formation. No other non-synonymous changes were detected in the structural gene region (trC through codon 145 of E) in p20 or p30 RepliVAX WN.

Individual members of the RepliVAX WN p10 population were isolated using standard limiting dilution purification. The foci of infection produced by 10 of 12 independent isolates were of comparable size to the large foci produced by RepliVAX WN p10. The structural genes of four of these isolates (Fig. 3B) were sequenced, and three of these were found to contain only the S → C substitution while the remaining isolate contained the K → M substitution (Fig. 3B). The abundance of the signalase cleavage site mutant in the p10 population suggests that it was more fit than the other mutation under these blind passage conditions.

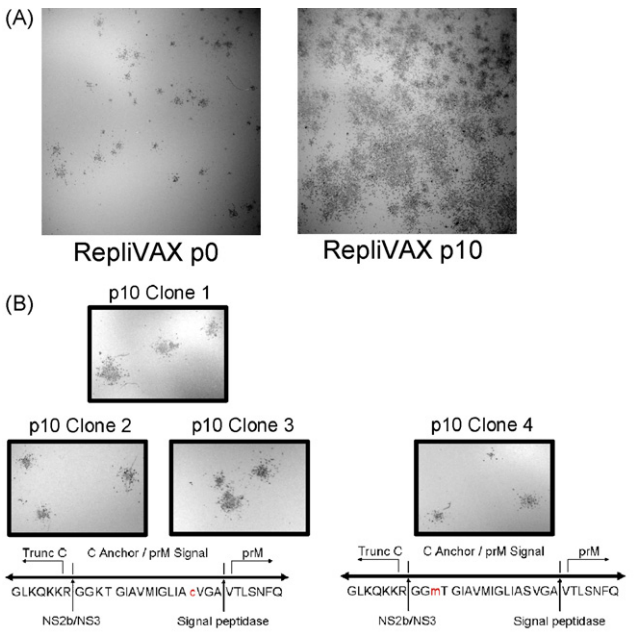

Figure 3 Characteristics of RepliVAX WN p10. (A) Immunostained foci of infection produced by infection of BHK(VEErep/Pac-Ubi-C*) with p0 and p10 RepliVAX WN, showing that RepliVAX WN p10 produces larger and more heterogeneous foci than the p0 RepliVAX WN. (B) Foci formed by four isolated clones from 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.

Incorporation of proteolytic cleavage site mutations into the RepliVAX WN genome

To demonstrate that the mutations described above were responsible for the enhanced growth properties of p10 RepliVAX WN, RepliVAX WN genomes containing the mutations were engineered. RepliVAX WN.2 plasmids were constructed by inserting the signalase cleavage site mutation (RepliVAX WN.2 SP), NS2B/NS3 cleavage site mutation (RepliVAX WN.2 NS), or both (RepliVAX WN.2 SP—NS) into BAC plasmids encoding the RepliVAX WN genome, and high titers (10^7 to 10^8 IU/ml) of RepliVAX WN.2 particles were obtained from electroporation of BHK(VEErep/Pac-Ubi-C*) cells with synthetic RNAs.

RepliVAX WN.2 demonstrate enhanced growth kinetics when propagated in C-expressing cells

To determine the role of each mutation in growth, RepliVAX WN.2 particles 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 original RepliVAX WN (Fig. 4A). When all three RepliVAX WN.2 were compared to the parental RepliVAX WN in one-step growth experiments, enhanced infectious yield was observed for a brief time early after infection (Fig. 4B). All of these RepliVAX WN.2 infections produced significantly higher yields ($p < 0.05$) at 18 and 26 h post-infection than original RepliVAX WN; however by 49 h production reached comparable levels in all four infections.

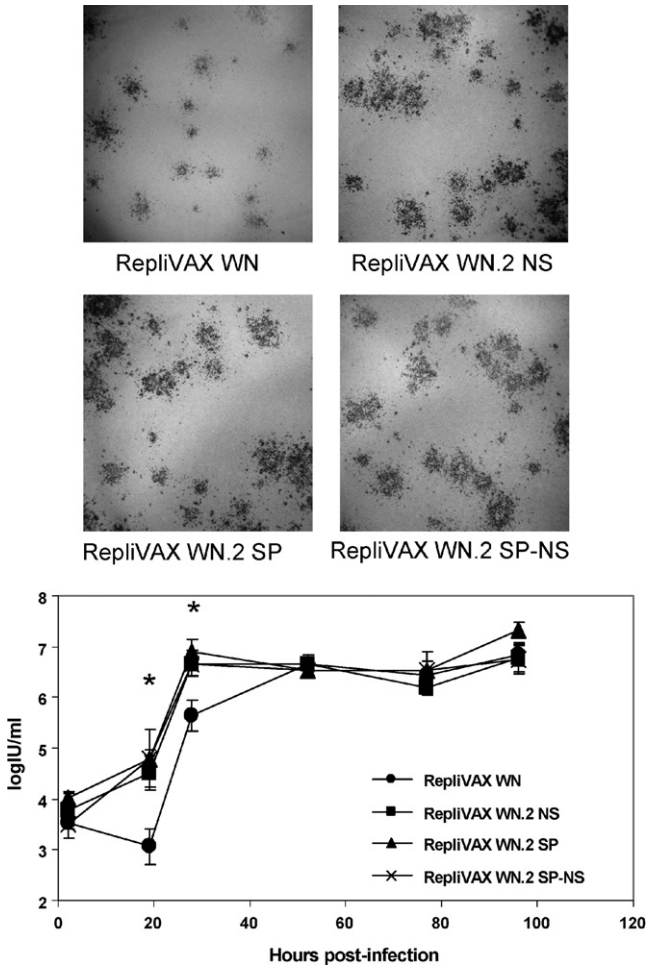

Figure 4 Characterization of RepliVAX WN.2. (A) Immunostained foci produced on BHK(VEErep/Pac-Ubi-C*) cells infected with RepliVAX WN, RepliVAX WN.2 NS, RepliVAX WN.2 SP, and RepliVAX WN.2 SP—NS. (B) One-step growth curves showing yield of RepliVAX WN.2 NS (closed square), RepliVAX WN.2 SP (closed triangle), 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 ''*'' denoted significance.

Mutations selected for in the RepliVAX WN genome did not enhance the growth characteristics of WNV containing a functional C gene

To test the effect of the RepliVAX WN cleavage site mutations on live virus growth, these mutations were introduced into live WNV. When the three WNV mutants were compared side-by-side with their parental WNV, the mutants produced infectious foci indistinguishable from wt virus (Supplemental Fig. 1A). Furthermore, the growth profiles of all four viruses were indistinguishable when assayed at MOIs of 1 or 0.05 (Supplemental Fig. 1B), indicating that the growth-enhancing phenotype observed in RepliVAX WN could not be detected in the context of live WNV.

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. Swiss Webster mice were immunized with escalating doses of RepliVAX WN.2 SP (referred to from this point on as RepliVAX WN.2 for simplicity), original RepliVAX WN, or diluent (mock). All inocula were examined using i.p. vaccination previously shown to be potent and efficacious [34]. In addition, since live-attenuated flavivirus vaccines have been tested in non-human primates [24—26,28,29,41] and man [29,42,43] using a s.c. route, two groups of mice were vaccinated s.c. with 1×10^6 or 4×10^4 IU of RepliVAX WN.2. None of the animals immunized by any route showed adverse effects. Three weeks after vaccination ELISA IgG titrations for WNV E and NS1 demonstrated that all vaccinated animals seroconverted. Among i.p.-immunized animals these analyses revealed a RepliVAX WN.2 dose-dependent response to both proteins (Fig. 5). RepliVAX WN.2 administered i.p. at a dose of 1×10^6 IU elicited a significantly greater IgG response ($p < 0.02$) to WNV E than i.p. doses of 2×10^5 and 4×10^4 IU

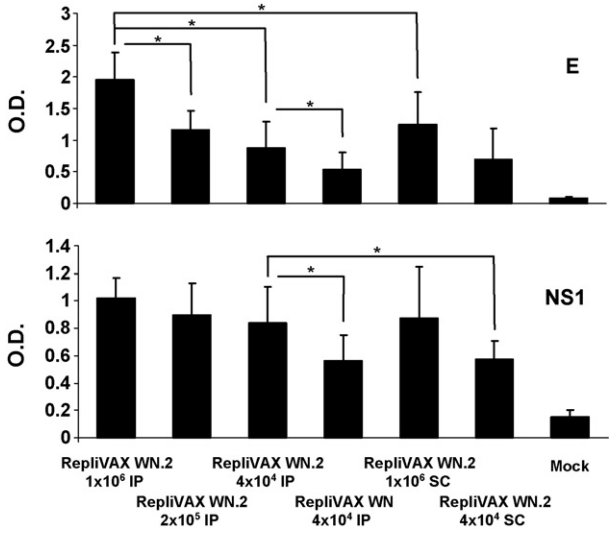

Figure 5 ELISA antibody levels specific for WNV E and NS1 proteins in sera from vaccinated mice. Bars show average OD values for the indicated antigens from pre-challenge sera obtained from animals vaccinated as indicated. Extended bars indicate standard deviation. ''*'' indicates significant difference.

Table 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	1×10^6 i.p.	1:160	100 (15/15)	100 (15/15)
RepliVAX WN.2	2×10^5 i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN.2	4×10^4 i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN	4×10^4 i.p.	1:80	100 (15/15)	100 (15/15)
RepliVAX WN.2	1×10^6 s.c.	1:160	100 (15/15)	100 (15/15)[d]
RepliVAX WN.2	4×10^4 s.c.	1:80	100 (15/15)	100 (15/15)
Diluent	0	<1:20	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 10 LD_{50} WNV NY99.
[c] Protection from illness (defined by a greater than 10% weight loss during the 21-day post-challenge observation period).
[d] One animal in this group demonstrated a weight loss of 10% on days 3—5 post-challenge and 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.

of RepliVAX WN.2 (Fig. 5). Interestingly, at the lowest dose tested, i.p. RepliVAX WN.2 vaccination induced a significantly greater antibody titer against E ($p < 0.02$) and NS1 ($p < 0.02$) than RepliVAX WN administered by the same route (Fig. 5). This suggests that the enhanced growth characteristics observed for RepliVAX WN.2 *in vitro* may correlate to enhanced immunogenicity. S.c. immunization with RepliVAX WN.2 induced readily detectable antibody responses to E and NS1, but these responses were lower than those elicited by i.p. administration (Fig. 5). In the case of the 1×10^6 IU dose, these differences were statistically significant ($p < 0.05$) for E but not NS1, however the difference observed at the lowest dose tested (4×10^4 IU) was statistically significant ($p < 0.05$) for NS1 but not E. Ninety percent neut titers ranging from 1:80 to 1:160 were observed in pooled sera from each vaccination group (Table 1). Interestingly, despite slightly lower ELISA titers detected in animals among some groups (see above, Fig. 5), neut titers from pooled sera were indistinguishable (Table 1).

Following this single vaccination, mice were challenged with 10 LD_{50} of WNV NY99 and monitored for signs of illness. After challenge, 6/15 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 1). The unexpectedly low WNV-induced mortality in the control animals is enigmatic, since an earlier challenge study using the same-aged outbred mice and 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). All mice in the vaccine groups were protected from weight loss and other symptoms indicative of WNV-induced illness (>10% loss from weight at day of challenge) except for one animal in the 1×10^6 IU RepliVAX WN.2 s.c. group (Table 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).

Following immunization of 5-week-old female hamsters i.p. with 1×10^6 or 2×10^5 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), and a dose-dependent IgG response to E and NS1 (Fig. 6). The hamsters were challenged i.p. with 1×10^6 ffu of WNV NY99 and monitored for signs of illness. 1×10^6 ffu was chosen for challenge even though a lower dose (1×10^4 IU) has been used by others for hamster vaccine [44,45] and pathogenicity [36] studies because this higher dose, which is approximately 10,000 times higher than the minimal infectious dose [36], causes clinically apparent disease in most animals ([36] and R.B. Tesh, personal communication). At this challenge dose, 60% of 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, no RepliVAX WN.2-vaccinated animals died or displayed weight loss during 3 weeks following challenge. Collectively these results

Table 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	1×10^6 i.p.	1:160	100 (10/10)	100 (10/10)
RepliVAX WN.2	2×10^5 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 1×10^6 ffu WNV NY99.
[c] Protection from illness (defined by a greater than 10% weight loss during 21-day post-challenge observation period).

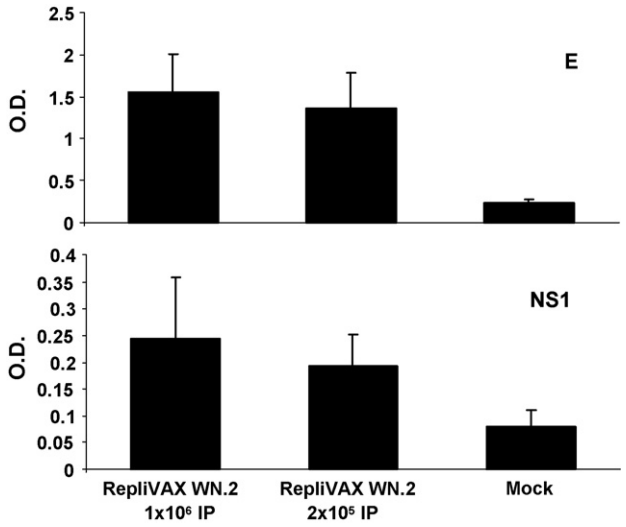

Figure 6 ELISA antibody levels specific for WNV E and NS1 proteins in sera from vaccinated hamsters. Bars show average OD values for the indicated antigens from pre-challenge sera obtained from animals vaccinated as indicated. Extended bars indicate standard deviation.

demonstrate the safety and efficacy of RepliVAX WN.2 as a vaccine against WNE in two rodent model systems.

Discussion

There is a great need for effective and safe vaccines to prevent flavivirus diseases. 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 and the RepliVAX WN genome. To document that these cell lines would not recombine with RepliVAX WN, an aggressive blind-passage procedure was performed in BHK(VEErep/Pac-Ubi-C*) cells. These studies demonstrated that after 30 serial passes there was no genetic (RT-PCR) or phenotypic (recovery of live virus) evidence of recombination. We also developed a stable line of cells expressing VEErep/Pac-Ubi-C* from Vero cells certified by the WHO for human vaccine manufacture that were capable of producing RepliVAX WN.2 at high titers and unlike BHK cells, repeated harvests could be obtained from these Vero cells.

Our studies also showed that natural selection of better-growing variants could be used to improve RepliVAX WN. Serial passaging of RepliVAX WN particles in BHK(VEErep/Pac-Ubi-C*) cells resulted in selection of genomes containing mutations affecting the signal sequence of prM in the RepliVAX WN genome. Isolation and propagation of individual members of this population and reverse genetics studies demonstrated that either mutation conferred an enhanced growth phenotype. Based on our inability to distinguish between these mutants and the slightly higher frequency with which the signalase cleavage site mutation was observed in the naturally selected population, RepliVAX WN.2 SP was chosen for vaccination studies.

The position of these growth-enhancing mutations was not unexpected since it has been shown that sequences of the NS2B/NS3 and signalase cleavage sites between C and prM are important determinants for SVP and infectious particle formation [46,47]. Studies have documented the importance of the NS2B/NS3 cleavage site in the production of infectious YFV progeny [48] and other studies using infectious YFV revealed that the natural signalase cleavage site at the start of prM is suboptimal and mutations that increased the efficiency of processing at this site blocked production of infectious virus [49]. Based on the idea that this signalase site was suboptimal, Kofler et al. rationally altered their C-deleted TBEV RNA vaccine to enhance signalase cleavage at this site, and achieved an increase in SVP production *in vitro* and immunogenicity *in vivo* [13,33]. Although the results of the YFV studies are consistent with cleavage at the NS2B/NS3 site prior to signalase cleavage, work by Lobigs and Lee provided additional data to indicate that coordination of these cleavage events is essential for efficient encapsidation of nucleocapsid [50]. Information on the importance of cleavage site utilization in virus viability from studies to produce chimeric flaviviruses designed to express foreign prM/E cassettes demonstrated that alteration of the +3 position of the NS2B/NS3 cleavage site was particularly beneficial for growth of a WNV/DENV4 chimera [27].

Taken together, this suggests that the selective pressure we applied to RepliVAX WN (as well as a chimeric RepliVAX WN expressing the JEV prM/E gene cassette in place of the corresponding WNV genes (RepliVAX JE)) [40] could have selected mutations at the +3 position of the NS2B/NS3 cleavage site as in RepliVAX WN.2 NS (or RepliVAX JE.2) [40] that enhanced NS2B/NS3 cleavage or selected a mutation resulting in replacement of the β-turn-promoting serine at the signalase cleavage site (RepliVAX WN.2 SP) reducing cleavage by signalase. Alternatively, the mutations could have altered the hydropathic properties of the signal peptide allowing it to shift within the lipid bilayer thereby altering cleavage activity of these proteases at either face of the ER membrane. Interestingly, we 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-containing 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 WN efficacy data to a hamster model. Murine studies demonstrated that incorporation of the single amino acid change at the signalase cleavage site of RepliVAX WN.2 produced a detectable increase in potency when delivered i.p., and demonstrated that at all i.p. doses tested, RepliVAX WN.2 and the parental RepliVAX WN were 100% efficacious. RepliVAX WN.2 was also efficacious when delivered by the s.c. route.

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 detect a difference in SVP production from cells infected *in vitro* with RepliVAX WN.2 or the parental RepliVAX WN (results not shown). How-

ever, work from our group on RepliVAX JE demonstrated a large increase in both infectious titer and SVP production following introduction of a similar mutation at the +3 position of its NS2B/NS3 cleavage site [40]. Despite the lack of demonstrable enhancement of the RepliVAX WN.2 mutation on intracellular replication (results not shown) or production of SVPs *in vitro*, RepliVAX WN.2 displayed a statistically significant enhancement of *in vivo* IgG responses to WNV E and NS1 relative to the parental RepliVAX WN. This could be an indication that by enhancing the release of SVPs, that this mutation also facilitated the enhanced release of NS1 *in vivo*.

These results provide additional support for the utility of RepliVAX WN as a vaccine for WNE. We demonstrated that RepliVAX WN can be safely passaged without recombination, we produced a stable propagation system in cells suitable for human vaccine manufacture, and we produced a better growing/more potent RepliVAX WN derivative. Our studies also confirmed previous efficacy and potency results in mice, provided potency and efficacy data for a vaccination route compatible with human use, and 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 $\geq$1:80, in excess of the 1:10 titers reported as sufficient to afford protection in humans against JE [51–53]. These findings, along with those by Ishikawa et al. [40], demonstrate the utility of RepliVAX as safe, economical, and efficacious vaccines to prevent flavivirus diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2008.03.009.

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