West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy

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A candidate live attenuated vaccine strain was constructed for West Nile virus (WN), a neurotropic flavivirus that has recently emerged in the U.S. Considerable attenuation for mice was achieved by chimerization with dengue virus type 4 (DEN4). The genes for the structural premembrane and envelope proteins of DEN4 present in an infectious cDNA clone were replaced by the corresponding genes of WN strain NY99. Two of 18 cDNA clones of a WN/DEN4 chimera yielded full-length RNA transcripts that were infectious when transfected into susceptible cells. The two infectious clones shared a motif in the transmembrane signal domain located immediately downstream of the NS2B-NS3 protease cleavage site that separates the DEN4 capsid protein and the WN premembrane protein of the chimera. This motif, Asp and Thr at a position 3 and 6 amino acids downstream of the cleavage site, respectively, was not present in the 16 noninfectious cDNA clones. The WN/DEN4 chimera was highly attenuated in mice compared with its WN parent; the chimera was at least 28,500 times less neurovirulent in suckling mice inoculated intracerebrally and at least 10,000 times less virulent in adult mice inoculated intraperitoneally. Nonetheless, the WN/DEN4 chimera and a deletion mutant derived from it were immunogenic and provided complete protection against lethal WN challenge. These observations provide the basis for pursuing the development of a live attenuated WN vaccine.

protective immunity | dengue virus | viral chimera

West Nile virus (WN) belongs to the family *Flaviviridae* that comprises more than 60 viruses, many of which are important human pathogens. WN is a member of the Japanese encephalitis virus (JE) serocomplex of mosquito-borne flaviviruses that includes St. Louis encephalitis, JE, and Murray Valley encephalitis viruses (1, 2). Like other members of the JE antigenic complex, WN is maintained in a natural cycle that involves mosquito vectors and birds, whereas humans are usually an incidental host. For many years, WN has been recognized as one of the most widely distributed flaviviruses with a geographic range including Africa, Australia, Europe, the Middle East, and West Asia (2, 3). During 1999, WN first established itself in the U.S. in the northeast and mid-Atlantic states, and more recently, this virus extended its range to include the southeastern states (4, 5). In endemic regions, most human WN infections are asymptomatic or cause mild illness with symptoms of low-grade fever, headache, body aches, rash, myalgia, and polyarthropathy. However, human epidemics with severe disease have been reported in Israel, France, Romania, and Russia. In acute severe illness, the virus can cause hepatitis, meningitis, and encephalitis, leading to paralysis and coma, resulting in death. The neuropathologic lesions are similar to those of JE, with diffuse central nervous system inflammation and neuronal degeneration. The virus is also found in the spleen, liver, lymph nodes, and lungs of infected individuals. During the 1999 outbreak of WN in the U.S., more than 60 people became ill, and seven died. Because of the recent and unexpected spread of WN from the northeast to the southeast of the U.S., this virus is considered a significant emerging disease threat that has embedded itself over a considerable region of the country. Currently, a licensed human vaccine is not available for prevention of WN disease. Mosquito control is the only practical strategy to combat the spread of disease, but effective spraying is difficult to perform in urban areas. Clearly, an effective vaccine is needed to protect at-risk populations.

Recent advances in recombinant DNA technology have allowed us to develop an approach for constructing live attenuated flavivirus vaccines (6–9). Our approach was made possible by the conservation among flaviviruses of genome organization, number of viral proteins, replicative strategy, gene expression, virion structure, and morphogenesis (10). All flaviviruses have a positive-sense nonsegmented RNA genome that encodes a single long polyprotein processed to yield capsid (C), premembrane (preM), and envelope glycoprotein (E) structural proteins, followed by nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, in that order. These shared properties suggested that viable chimeric viruses could be produced by replacing the genes for the viral structural proteins in a full-length infectious cDNA clone of a flavivirus with the corresponding viral genes (in cDNA form) of another flavivirus. When tested, this strategy was successful for chimeras that contained the sequence for viral structural proteins preM and E of tick-borne encephalitis virus (TBEV) or tick-borne Langat virus (LGT), whereas all other sequences were derived from the full-length infectious cDNA of mosquito-borne dengue type 4 virus (DEN4). This indicated that viral structural proteins of a disparate flavivirus, TBEV or LGT, could function in the context of cis-acting 5′ and 3′ sequences and nonstructural proteins of DEN4. Significantly, both chimeras proved to be highly attenuated in mice with respect to peripheral virulence, the ability of a virus to spread to the central nervous system (CNS) from a peripheral site of inoculation and cause encephalitis. Nonetheless, the chimeras proved to be immunogenic and able to induce resistance in mice against challenge with TBEV or LGT (6–8). It appeared that a favorable balance between reduction in virus replication *in vivo* (attenuation) and induction

Abbreviations: WN, West Nile virus; JE, Japanese encephalitis virus; TBEV, tick-borne encephalitis virus; LGT, Langat virus; DEN4, dengue type 4 virus; FFU/ml, focus-forming units per milliliter; IC, intracerebral.

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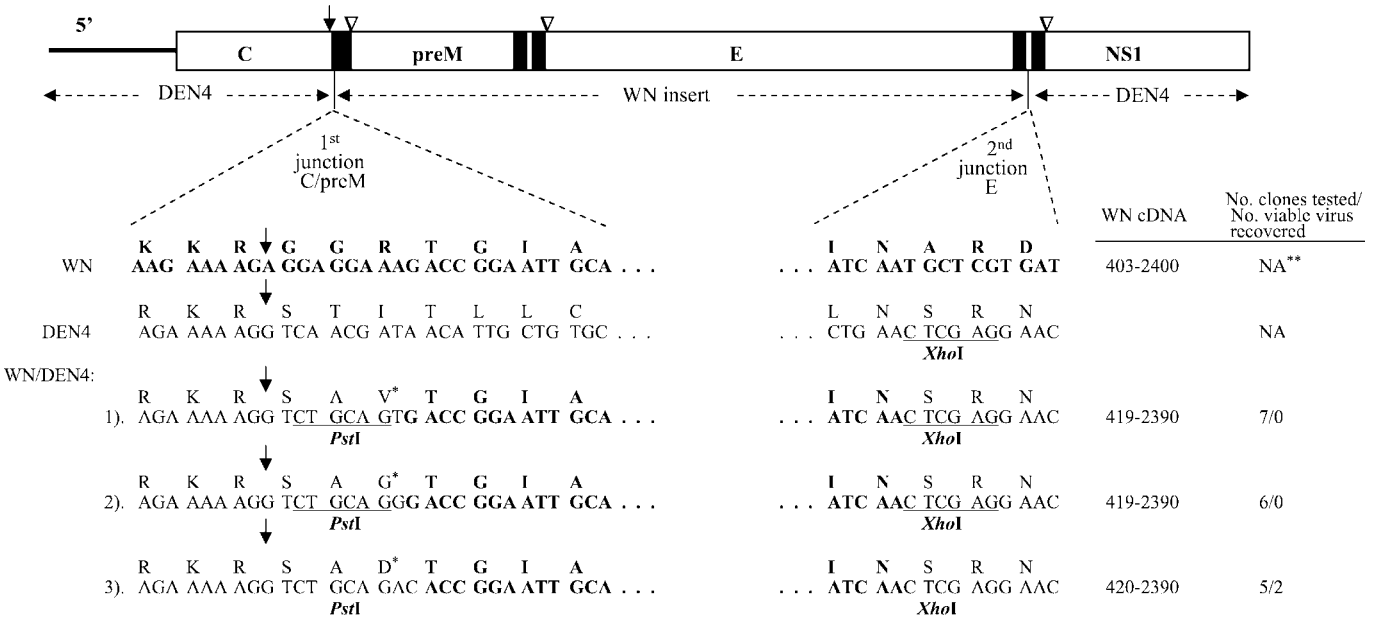

Fig. 1. Structure of chimeric WN/DEN4 cDNAs. The top bar depicts the chimeric virus cDNA genome from the 5′ terminus of the genome to the 3′ terminus of the NS1 gene. The solid black boxes represent hydrophobic domains in the polyprotein. The vertical solid arrow indicates position of a potential NS2B-NS3 protease cleavage site in the polyprotein between the C and preM proteins. Cleavage sites for cellular signalase are indicated by open triangle (▽). Restriction enzyme-cleaved WN cDNA fragment-bearing sequence for WN premembrane (preM) and envelope glycoprotein (E) structural protein genes were inserted into DEN4 cDNA at *Pst*I and *Xho*I sites, which are underlined. The second junction is located in the COOH terminus of the WN E protein between the two hydrophobic domains. The amino acid and nucleotide sequences of WN are presented in bold letters, and the nucleotide numbering system is from GenBank accession no. AF196835. Infectivity of RNA transcripts from full-length cDNA constructs was tested by transfecting simian or mosquito cells and by evaluating cell cultures for evidence of infection by immunofluorescence assay. * indicates amino acids in chimeric constructs that vary at the 3+ position downstream of protease cleavage site. **, not applicable.

of protective immunity had been achieved. We interpret this to mean that tick-borne flavivirus preM and E can interact in the context of DEN4 nonstructural proteins and cis-acting 5′ and 3′ sequences at a level sufficient for infectivity and induction of immunity but not sufficient for full expression of virulence that requires a high level of replication *in vivo* and ability to spread into the CNS.

As a logical extension of this strategy, we constructed viable WN/DEN4 chimeras in which the structural preM and E protein genes of the distantly related mosquito-borne WN were substituted for the corresponding genes of DEN4. We also generated a WN/DEN4 chimera with a 30-nucleotide deletion in the 3′ noncoding region that had previously been shown to render DEN4 safe but still immunogenic in adult volunteers (11). Studies in mice were then performed to evaluate neurovirulence, peripheral virulence, immunogenicity, and protective efficacy of the newly constructed WN/DEN4 chimeric viruses.

Materials and Methods

Cells and Viruses. Simian *Vero* cells (World Health Organization seed passage 143) and mosquito C6/36 cells were obtained from L. Potash (Novavax, Rockville, MD). The *Vero* cells are qualified for use in production of candidate human vaccines. Simian $LLCMK_2$ cells were purchased from the American Type Culture Collection (Manassas, VA). The WN wild-type strain NY99–35262 used in this study was kindly provided by R. Lanciotti (Centers for Disease Control and Prevention, Fort Collins, CO); it was originally isolated from a Chilean flamingo at the Bronx Zoo (New York) in 1999 (5). A virus suspension prepared in *Vero* cells had a titer of 2.6×10^7 focus-forming units per milliliter (FFU/ml), as determined with *Vero* cells by using an immunostaining focus-forming assay (12) and WN-specific mouse antibodies. A *Vero* cell preparation of wild-type DEN4 Caribbean strain 814669 with a titer of 1.1×10^8 FFU/ml was kindly provided by S. Whitehead (National Institute of Allergy and Infectious Diseases).

Chimeric WN/DEN4 cDNA and Recovery of Infectious Virus. Plasmid p2A(*Xho*I) (13) containing the DEN4 full-length infectious cDNA, previously used for recovery of chimeric TBEV/DEN4 and LGT/DEN4 viruses (6, 7), was used for construction of WN/DEN4 cDNA. This was achieved by substituting cDNA of the WN preM and E protein genes for those of the corresponding DEN4 genes (Fig. 1). The source of WN cDNA was a PCR product that included nucleotides 233 to 2758 of the WN strain NY99 genome. This was also kindly provided by R. Lanciotti. The nucleotide sequence of the structural protein genes in this PCR fragment was determined and compared with the published sequence of WN NY99 (GenBank accession no. AF196835). Three nucleotide differences ($C_{1893} \rightarrow$ U, $C_{2370} \rightarrow$ U and $C_{2385} \rightarrow$ A) were identified in the E protein sequence, none of which resulted in an amino acid substitution.

Prior experience with construction and analysis of tick-borne/DEN4 chimeras indicated that we could not predict *a priori* the sequence of the DEN4 C protein/tick-borne flavivirus preM protein junction required for viability (6, 7). For this reason, we adopted an empirical approach and tested several different C/preM junction sequences (Fig. 1). This was not necessary for the downstream junction, because it was located within the COOH-terminal region of WN E. Initially, three sets of C/preM junctions were tested, but only one yielded a viable WN/DEN4 chimera (Fig. 1). The primers used for construction of the chimeras by PCR used oligonucleotide 5′-TCAAAACAAAAGAAAAGATCTGCAGTGACCGGAATTGCAGTCATGATTGGC-3′ or 5′-TCAAAACAAAAGAAAAGATCTGCAGGGACCGGAATTGCAGTCATGATTGGC-3′ or 5′-TCAAAACAAAAGAAAAGATCTGCAGACACCGGAATTGCAGTCATGATTGGC-3′ as a forward primer and oli-

Table 1. Mutations that were identified in genome of the WN/DEN4 or WN/DEN4-3′ Δ30 chimera during cloning and rescue of chimera from cDNA in simian *Vero* cells

Virus	Region of genome	Nucleotide (position)*	WN/DEN4			WN/DEN4-3′ Δ30			Amino acid change
				Recombinant virus‡			Recombinant virus‡		
			pDNA†	Clone 55	Clone 18	pDNA	Clone 1	Clone 78	
WN	preM	U_{428}	C	C	C	C	C	C	$Ile_6 \rightarrow Thr$
		A_{847}	G	G	G	G	G	G	$Ile_{146} \rightarrow Val$
	E	A_{1566}	G	G	G	G	G	G	silent
		A_{1810}	G	G	G	G	G	G	$Thr_{282} \rightarrow Ala$
DEN4	NS3	A_{4799}	A	C/a	A	A	A	A	$Glu_{92} \rightarrow Asp$
	NS4B	C_{7141}	C	C	C	C	C	U	$Thr_{105} \rightarrow Ile$
		U_{7162}	U	C/u	C	U	C	U	$Leu_{112} \rightarrow Ser$

*Numbering of nucleotide sequence of structural protein genes derived from the sequence of WN NY99 genome (GenBank accession no. AR196835) and numbering of nucleotide sequence of nonstructural protein genes derived from the sequence of DEN4 genome (GenBank accession no. AR326825).
†Plasmid DNA.
‡Clone rescued in *Vero* cells.

gonucleotide 5′-CCGCAAGAAACGTCATAGCAATTGAC-CTGTCACTCGAGTTGATTCCCATCCACAACAGAAGA-GC-3′ as a reverse primer. Stable full-length WN/DEN4 cDNA clones were identified after transformation of *Escherichia coli* BD 1528 with a ligation mixture that contained the PCR product and the vector, both of which were digested by *Pst*I and *Xho*I (Fig. 1). Sequences at the junctions between WN and DEN4 genes in each chimeric plasmid were verified.

Plasmid DNA containing full-length WN/DEN4 cDNA was linearized with Asp_{718}. *In vitro* RNA synthesis and transfection of cells with the RNA transcripts were performed as described previously (12). Briefly, RNA transcripts of full-length WN/DEN4 constructs listed in Fig. 1 were used to transfect simian $LLCMK_2$, simian *Vero* cells, or mosquito C6/36 cells in the presence of LipofectAmine 2000 reagent (GIBCO/BRL) in a BSL-3 laboratory generously provided by L. Markoff (Center for Biologics Evaluation and Research, Food and Drug Administration). Transfected cells were examined by immunofluorescence assay for the presence of WN or DEN4 proteins by using a WN- or DEN4-specific hyperimmune mouse ascitic fluid. Two infectious chimeric viruses containing group 3 junctions (Fig. 1), namely, WN/DEN4 clones 18 and 55, were isolated. The recovered chimeras were amplified once in simian *Vero* or mosquito C6/36 cells, and viral RNA was isolated and then reverse transcribed into cDNA that was used for sequence analysis (Table 1). In a similar manner, the sequence of the *Vero* cell-derived WN/DEN4 clone 18 was determined after an additional purification by two rounds of terminal end-point dilution and amplification in *Vero* cells. The resulting virus suspension had a titer of 1.7×10^6 FFU/ml.

To introduce a deletion into the 3′ noncoding region (NCR) of WN/DEN4 genome, the DNA fragment between the *Xho*I site (nucleotide 2345 of DEN4 genome; GenBank accession no. AF326827) and the Asp_{718} site at the 3′ end of plasmid WN/DEN4–18 DNA was replaced by the corresponding *Xho*I-Asp_{718}-fragment derived from full-length cDNA of a DEN4 mutant, clone p4Δ30 (11). This mutant had 30 nucleotides deleted from the 3′ NCR of the genome between nucleotides 10478 and 10507. Full-length RNA generated by SP6 polymerase from 10 different plasmids was tested for infectivity by transfection of simian *Vero* cells. Two individual WN/DEN4–3′Δ30 cDNA clones were infectious. The rescued deletion mutants, WN/DEN4–3′Δ30 clones 1 and 78, were purified twice by terminal end-point dilution and amplified in *Vero* cells to a titer of 1.4×10^5 and 6×10^4 FFU/ml, respectively. Viral RNA was isolated, and the complete sequence of the 3′ deletion mutant genome was determined (Table 1).

Evaluation of Parental and Chimeric Viruses in Mice. Neurovirulence of *Vero* cell culture-propagated parental WN (strain NY99), parental DEN4 (strain 814669), chimeric WN/DEN4 (clone 18), and its deletion mutant (clone 1) was evaluated in a BSL-3 facility at the Walter Reed Army Institute of Research, an American Association of Laboratory Animal Care-accredited facility, under Institutional Animal Care and Use Committee-approved protocol number I05–01. Three-day-old Swiss–Webster mice (Taconic Farms) in groups of 9–12 were inoculated by the intracerebral (IC) route with decimal dilutions ranging from 0.1 to 10^5 FFU of virus in 0.03 ml of MEM/0.25% human serum albumin. Mice were observed for 21 days for development of fatal encephalitis. The LD_{50} of each virus was determined by the method of Reed and Muench (14). Parental and chimeric viruses were also analyzed for peripheral virulence by i.p. inoculation of 3-week-old Swiss female mice in groups of 10. Mice were inoculated with decimal dilutions of virus ranging from 0.1 to 10^5 FFU and were observed for 28 days for fatal encephalitis. Moribund mice were humanely euthanized.

Mice that survived i.p. inoculation were bled on day 28 to evaluate the WN-specific neutralizing antibody response. Serum from mice in each group was pooled, and the WN-neutralizing antibody titer of the serum pool was determined by FFU reduction assay on *Vero* cells as described previously (9, 12). Briefly, a 1:10 dilution of pooled sera was prepared in MEM containing 2% FBS and then heat inactivated for 30 min at 56°C. Serial 2-fold dilutions of inactivated pooled sera were mixed with an equal volume of a virus suspension containing ≈50 FFU of WN. The mixture was incubated for 30 min at 37°C, and 0.4 ml was then added to duplicate wells of *Vero* cells in a six-well plate. After 1 h of absorption at 37°C, the inoculum was removed, and cells were overlaid with MEM containing 2% FBS, 50 μg/ml of gentamicin, 0.25 μg/ml of fungizone, and 1% tragacanth gum. Antibody titer was determined after 2 days of incubation by an immunostaining focus-forming assay (12) that used WN-specific hyperimmune mouse ascitic fluid. Neutralizing antibody titer was the highest dilution of pooled sera that reduced focus formation 50% compared with sera collected from nonimmunized mice.

The surviving mice were challenged i.p. on day 29 with 100 i.p. LD_{50} (10^3 FFU) of parental WN virus and observed for fatal encephalitis for a period of 21 days. Moribund mice were humanely euthanized.

Results

Construction and Recovery of Chimeric WN/DEN4 Viruses. We constructed 18 plasmids that contained full-length chimeric

Table 2. Neurovirulence and peripheral virulence of parental WN or DEN4 and their chimeric WN/DEN4 virus or its 3′ deletion mutant WN/DEN4-3′ Δ30 in Swiss mice, as assayed by IC or i.p. inoculation

Virus	Neurovirulence		Peripheral virulence	
	LD_{50} (FFU) after IC inoculation of 3-day-old Swiss mice	Reduction from WN parent	LD_{50} (FFU) after i.p. inoculation of 3-week-old Swiss mice	Reduction from WN parent
DEN4	407	—	>100,000*	—
WN	0.35	—	10	—
WN/DEN4 chimera (clone 18)	>10,000*	28,571×	>100,000*	>10,000×
WN/DEN4-3′ Δ30 chimera (clone 1)	>1,000*	2,857×	>10,000*	>1,000×

Each decimal dilution was tested in 9–12 mice in the group.
*Highest concentration tested.

WN/DEN4 cDNA, which included the structural preM and E protein genes of the WN strain NY99 with all other sequences derived from DEN4. Full-length RNA generated by SP6 RNA polymerase from only two of the 18 chimeric cDNAs was infectious when transfected into mosquito C6/36 or simian *Vero* cells. Evidence for virus infectivity was detected by immunofluorescence assay (IFA). In the case of the two viable chimeric viruses, 80–100% of transfected cells were infected by day 5, as indicated by IFA by using WN-specific hyperimmune mouse ascitic fluid. The two viable chimeric viruses (WN/DEN4 clones 18 and 55) had the C/preM intergenic junction sequence of group 3 shown in Fig. 1. The presence of this junction was confirmed by sequence analysis of the recovered chimeras. Also, the complete genomic sequence of the two chimeras rescued from cDNA in *Vero* cells was determined and compared with the consensus sequence of their parental WN NY99 and DEN4 viruses as well as the nucleotide sequence of the WN/DEN4 viral chimera insert in the plasmid DNA from which infectious RNA transcripts were derived (Table 1). Analysis of plasmid DNAs revealed four differences in nucleotide sequence from the consensus WN sequence determined by reverse transcription–PCR of a high-titered suspension of WN strain NY99. Three of these differences produced amino acid substitutions in preM ($Ile_6 \rightarrow$ Thr and $Ile_{146} \rightarrow$ Val) and E ($Thr_{282} \rightarrow$ Ala). In addition, variability between (*i*) Glu_{92} and Asp and (*ii*) Leu_{112} and Ser was identified in the DEN4 NS3 and NS4B nonstructural proteins of the WN/DEN4 clone 55. Also, sequence of the *Vero* cell-grown WN/DEN4 clone 18 differed from its progenitor plasmid cDNA sequence in the DEN4 NS4B gene. A change $U_{7162} \rightarrow$ C that caused the substitution $Leu_{112} \rightarrow$ Ser was identified. Interestingly, a different substitution at this locus, $Leu_{112} \rightarrow$ Phe, was previously observed by Blaney *et al.* (15) on passage of wild-type DEN4 in *Vero* cells.

After our success in constructing full-length infectious WN/DEN4 cDNAs, we constructed chimeric virus mutants with a 30-nucleotide deletion in their 3′ noncoding region. Two mutants, WN/DEN4–3′Δ30 clone 1 and clone 78, were recovered from transfected *Vero* cells. The complete sequence of both these clones was analyzed (Table 1). The sequence of clone 78 differed from the sequence of plasmid cDNA from which its infectious RNA transcripts were derived. A change of $C_{7141} \rightarrow$ U produced an amino acid substitution $Thr_{105} \rightarrow$ Ile in the NS4B protein. The WN/DEN4–3′Δ30 clone 1 also exhibited only one nucleotide difference from the plasmid cDNA sequence. This resulted in the same NS4B amino acid change ($Leu_{112} \rightarrow$ Ser) that was observed in WN/DEN4 clone 18.

The WN/DEN4 chimera replicated more efficiently in *Vero* cells than did WN/DEN4–3′Δ30. The unmodified WN/DEN4 chimera reached a titer of 10^6 FFU/ml on day 6 in cells infected with a multiplicity of infection of 0.01; this was ≈10-fold higher than the titer attained by the deletion mutant by day 6. The titer of the unmodified chimera was nearly the same as that attained by parental DEN4 under the same conditions (data not shown).

Mouse Neurovirulence. Before evaluating chimeric viruses for virulence in mice, the *Vero* cell-rescued chimeric WN/DEN4 virus and its 3′ deletion mutant were cloned biologically twice by terminal end-point dilution and then amplified in qualified *Vero* cells. The titer attained by the *Vero* cell-adapted WN/DEN4 clone 18 and WN/DEN4–3′Δ30 clone 1 was 1.7×10^6 FFU/ml and 1.4×10^5 FFU/ml, respectively.

Both chimeric WN/DEN4 virus and the deletion mutant WN/DEN4–3′Δ30 as well as parental WN strain NY99 and DEN4 strain 814669 were evaluated in 3-day-old Swiss mice for neurovirulence by direct IC inoculation (Table 2). Wild-type WN NY99 grown in *Vero* cells was highly neurovirulent with an intracerebral LD_{50} of 0.35 FFU in suckling Swiss mice. Wild-type DEN4 also grown in *Vero* cells was less neurovirulent with an IC LD_{50} of 407 FFU. Both WN/DEN4 and WN/DEN4–3′Δ30 chimeric viruses exhibited a significant reduction in neurovirulence compared with their WN and DEN4 parents. All of the mice inoculated IC with 10^3 FFU of WN/DEN4 or its 3′ deletion mutant survived during a 21-day observation period. At a higher dose of 10^4 FFU, only 4 of 11 mice inoculated with WN/DEN4 died. Thus, in suckling mice the WN/DEN4 chimera was more than 28,571 times less neurovirulent than its WN parent. The chimera with the 30-nucleotide deletion was also significantly less neurovirulent than its WN parent. These observations are consistent with earlier observations that chimerization of TBEV or LGT with DEN4 significantly reduced their neurovirulence for mice (6, 7, 16).

Peripheral Virulence in Mice. Subsequently, we evaluated the chimeric viruses for peripheral virulence, i.e., the ability of virus inoculated by the i.p. route to spread from a peripheral site to the central nervous system and cause encephalitis. Both chimeras were highly attenuated compared with their WN parent (Tables 2 and 3). Notably, i.p. inoculation of 10^4 FFU of the deletion mutant chimera or 10^5 FFU of the unmodified chimera did not induce fatal encephalitis in 3-week-old Swiss mice, whereas the i.p. LD_{50} for the WN parent was 10 FFU.

Immunogenicity and Protective Efficacy of Chimeric Viruses in Mice. The two chimeras were immunogenic; a single i.p. inoculation of 10^2 FFU of the WN/DEN4 chimera induced a moderate level of serum WN neutralizing antibodies (1:93), whereas a 10-fold higher concentration (10^3 FFU) induced a very high titer of WN neutralizing antibodies (1:1,189). Also, 10^3 FFU of the chimeric WN/DEN4–3′Δ30 deletion mutant stimulated a high level of such antibodies (1:292). i.p. challenge of the immunized mice on day 29 with 100 i.p. LD_{50} (10^3 FFU) of parental WN indicated that the chimeras provided 90–100% protection against this high-dose WN challenge (Table 3). There was a good correlation between the titer of serum WN neutralizing antibodies that developed in response to immunization and the degree of resistance induced. All unvaccinated control mice developed signs of CNS disease 7–13 days after challenge with 100 i.p. LD_{50}

Table 3. Peripheral virulence, antibody response, and protective efficacy of parental (WN or DEN4) viruses and chimeric WN/DEN4 virus or its 3′ deletion mutant WN/DEN4-3′ Δ30 in 3-week-old Swiss mice

Mice inoculated i.p. with	Dose inoculated, FFU	Mortality after i.p. inoculation	Mean titer of WN neutralizing antibody in pooled sera on day 28	Mortality after survivors inoculated i.p. with 100 i.p. LD_{50} of WN on day 29
WN	0.1	0/10	<1:10	10/10 (100%)
	1	0/10	1:24	10/10 (100%)
	10	5/10	1:40	4/5 (80%)
	100	10/10		
	1,000	9/10		
	10,000	10/10		
WN/DEN4 chimera (clone 18)	1	0/10	1:26	10/10 (100%)
	10	0/10	1:21	9/10 (90%)
	100	0/10	1:93	7/10 (70%)
	1,000	0/10	1:1,189	0/10 (0%)
	10,000	0/10	1:585	0/9* (0%)
	100,000	0/10	1:924	0/10 (0%)
WN/DEN4-3′ Δ30 chimera (clone 1)	1	0/10	1:28	9/10 (90%)
	10	0/10	<1:10	9/10 (90%)
	100	0/10	1:14	8/10 (80%)
	1,000	0/10	1:292	1/10 (10%)
	10,000	0/10	1:269	0/10 (0%)
DEN4	1,000	0/10	<1:10	10/10 (100%)
	10,000	0/10	1:13	8/10 (80%)
	100,000	0/10	1:22	10/10 (100%)
Control			<1:10	10/10 (100%)

*One of the 10 mice inoculated died as a result of trauma. WN virus was not detected in the brain by tissue culture (*Vero* cell) assay.

of WN, and these animals died shortly thereafter. To determine whether there was an age-related resistance of mice to WN, another group of 7-week-old mice also served as controls; they were the same age as immunized mice at time of challenge. This group of older control mice was challenged with one i.p. LD_{50}, determined in 3-week-old mice. Seven of eight mice died during the 21-day observation period, indicating that age-dependent resistance of mice to WN was not a factor in the observed protective effect of immunization.

Discussion

Initially (6, 7), we demonstrated that, although preM and E proteins of distantly related tick-borne and mosquito-borne flaviviruses are highly divergent, these proteins could be interchanged without loss of virus viability. This approach has been used to create new chimeric flaviviruses (17–22).

Previously, we succeeded in constructing and recovering viable tick-borne/DEN4 chimeras (6, 7, 16). In these instances, the tick-borne flavivirus parent was TBEV, a highly virulent virus or Langat virus, a naturally attenuated tick-borne virus. Thus, the two components of these chimeras had disparate vector hosts, namely ticks and, in the case of DEN4, mosquitoes. Decreased efficiency of gene product interactions was thought to be the basis for the marked attenuation exhibited by these chimeras. Nonetheless, although highly attenuated in mice, the TBEV/DEN4 and LGT/DEN4 chimeras were immunogenic and provided considerable protection against their parental tick-borne flavivirus. In the present study, both virus parents of the WN/DEN4 chimeras are transmitted by mosquitoes. However, vector preference differs, *Aedes* for DEN4 and *Culex* for WN (2, 3).

In the present study, we constructed viable WN/DEN4 chimeras that contained a DEN4 genome whose genes for structural preM and E proteins were replaced by the corresponding genes of WN strain NY99. Among flaviviruses, the hydrophobic domain between C and preM ("transmembrane signal domain") varies in sequence and also in length, from 14 to 20 amino acids (23). It acts as a signal sequence for translocation of preM protein into the endoplasmic reticulum lumen where posttranslation maturation of this protein occurs (10). This signal peptide is flanked at its NH_2-terminal region by the viral protease NS2B-NS3 cleavage site and at its COOH-terminal region by a cellular signalase cleavage site. Three different junctions at the protease cleavage site between DEN4 C and WN preM protein were engineered in chimeric constructs (Fig. 1). The C/preM fusion sequence at the viral protease cleavage site (KR$\downarrow$S) in the chimeras was constructed to be similar to that of the DEN4 parent, which provides its NS2B-NS3 protease for the processing of the chimeric polyprotein. However, each of the three chimeric constructs contain a unique substitution in the transmembrane signal sequence at the third amino acid position downstream of the protease cleavage site (Fig. 1). Thus, the transmembrane signal of the constructs is similar in length and differs in sequence only at the third amino acid position downstream of the protease cleavage site. Viable WN/DEN4 virus was recovered only when construct number 3 (Fig. 1) was used to prepare RNA transcripts for transfection. Infectious virus was recovered from two of five separate clones that encoded Asp in the 3+ amino acid position. And only these two clones contained a second-site mutation at the 6+ amino acid position downstream of the protease cleavage site that substituted Thr for Ile (Table 1). In contrast, none of the 13 clones that encoded Gly or Val at the 3+ amino acid position produced infectious virus after transfection, suggesting that the transmembrane signal sequence between C and preM is a determinant of viability in the context of a WN/DEN4 chimera. This is consistent with an earlier observation made with yellow fever virus that the transmembrane signal sequence between C and preM protein plays a role in viability and neurovirulence (24).

The WN strain NY99 exhibited considerable virulence in Swiss mice; its IC LD_{50} was ≈0.4 FFU for suckling mice and its i.p. LD_{50} was 10 FFU for 3-week-old Swiss mice (Table 2). Nearly the same level of neurovirulence was observed for a wild-type strain of WN isolated in Israel that was evaluated in CD-1 (ICR)

mice: IC LD_{50} and i.p. LD_{50} were estimated to be 1.1 and 4.3 plaque-forming units, respectively (25). In addition, a high degree of genomic similarity (>99.8%) between the WN NY99 and the WN Israel-1998 was recently confirmed by sequence analysis (5), indicating that both highly pathogenic strains of WN, representing North American and Middle Eastern viruses, are closely related. Wild-type DEN4 Caribbean strain 814669 was moderately neurovirulent for suckling mice with an IC LD_{50} of 407 FFU, and it was ≈20 times more virulent than its cDNA cloned virus (7). In contrast, the WN/DEN4 chimera and its 3′ deletion mutant were significantly less neurovirulent than their wild-type DEN4 or WN parent. Only at a high dose of 10^4 FFU did a minority of mice die that were inoculated IC with WN/DEN4 chimera. Also, the WN/DEN4 chimera inoculated IC at this dose caused death of suckling mice later than did parental WN virus: 4–5 days after infection for wild-type WN compared with 9–13 days after infection for the chimera.

Despite the high peripheral virulence of wild-type WN strain NY99 (i.p. LD_{50} of 10 FFU), chimerization of WN with DEN4 completely ablated this property of its WN parent. Thus, 3-week-old Swiss mice survived i.p. inoculation of 10^4 or 10^5 FFU of chimeric virus. Our observations are consistent with earlier findings that a similar large reduction of peripheral neurovirulence of TBEV or LGT occurs after chimerization with DEN4 (6, 7, 16).

Although highly attenuated, the WN/DEN4 chimeras stimulated a moderate-to-high level of serum neutralizing antibodies against WN NY99 (Table 3). There was a strong correlation between the level of neutralizing antibodies to WN induced by immunization and resistance to subsequent lethal WN challenge. The immune response of mice inoculated with the chimeras was dose-dependent and indicated that the unmodified WN/DEN4 chimera was slightly more immunogenic than the corresponding 3′ deletion mutant. However, 90–100% protection against WN challenge was achieved when a single 10^3 FFU dose of WN/DEN4 chimera or its 3′ deletion mutant was used for immunization. A higher dose (10^4 FFU) of either chimera provided complete protection to WN challenge. Thus, the WN preM and E proteins of the chimeric viruses represent effective antigens able to induce complete protection to challenge with highly virulent WN. Our observations concerning safety, immunogenicity, and protective efficacy of the chimeric WN/DEN4 vaccine candidates in mice provide a basis for extending our evaluation of the vaccine candidates to nonhuman primates and domestic animals, such as horses, which are at high risk.

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