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Biological properties of chimeric West Nile viruses

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Abstract

Recently, we have described a lineage 2 attenuated WN virus suitable for the development of a live WN vaccine. To design vaccine candidates with an improved immunogenicity, we assembled an infectious clone of the NY99 strain and created several chimeric constructs with reciprocal exchanges of structural protein genes between attenuated W956 and virulent NY99 and investigated their biological properties. Our data indicated that, while the growth rates of NY99 and chimeric viruses in tissue culture are determined primarily by properties of the structural proteins, determinants responsible for a highly cytopathic phenotype of NY99 or lack thereof for W956 are located within the nonstructural protein region of the WN genome. The high virulence of NY99 and the attenuated phenotype of W956 were found to be associated with determinants in the nonstructural region. Chimeric viruses carrying the NY99 structural proteins were attenuated in neuroinvasiveness and demonstrated an immunogenicity superior to W956.

Keywords: West Nile; NY99 strain; Infectious clone; Chimeric virus; Vaccine; Antibody; Neutralization; Pathogenicity; Mouse

Introduction

West Nile (WN) virus isolated over 60 years ago (the prototype B956 strain; Smithburn et al., 1940) has been known in Europe and the Middle East for decades as a causative agent of self-limiting epidemics and epizootics (Murgue et al., 2001; Savage et al., 1999). Since its sudden emergence in the U.S. (Lanciotti et al., 1999), hundreds of human mortality cases and thousands of confirmed illness cases have been reported to the CDC (2005). Although highly related to certain strains circulating in the Middle East, the NY99 strain is perhaps the most pathogenic and virulent WN strain known to date (Monath, 2001), especially for mice that succumb to encephalitis after peripheral inoculation of very small doses (Beasley et al., 2002).

In humans, WN infection is often inapparent or occurs as a mild febrile disease (Monath and Heinz, 1996). However, this virus was also associated with severe neurological symptoms (Flatau et al., 1981; Smithburn et al., 1940); recent outbreaks of WN infection were characterized by an increased CNS involvement (Roehrig et al., 2002; Solomon and Vaughn, 2002). In the mouse model, in which flaviviruses are inherently neurovirulent, both neurovirulence and neuroinvasiveness have been positively associated with determinants in the envelope proteins (Cecilia and Gould, 1991; Chambers et al., 1999; Gualano et al., 1998; Hasegawa et al., 1992; Holzmann et al., 1990, 1997; Jiang et al., 1993; McMinn, 1997; Pletnev et al., 1992; Pletnev et al., 1993). Other evidence indicates that mutations in other parts of the flavivirus genome can also contribute to loss or acquisition of neurovirulence (Butrapet et al., 2000; Duarte dos Santos et al., 2000; Dunster et al., 1999; Muylaert et al., 1996; Ni et al., 1995; Xie et al., 1998). Based on serological data and genetic characterization, West Nile viruses have been grouped into at least two distinct lineages (Berthet et al., 1997; Price and O'Leary, 1967). Representatives with moderate and high virulence have been found in both WN virus groups (Beasley et al., 2002). The NY99 strain belongs to lineage 1, which also includes other WN strains that have been associated with human and equine outbreaks (Roehrig et al., 2002). The envelope protein (E) of many flaviviruses is glycosylated, and WN virus is not an exception to this rule, although a few non-glycosylated strains have been identified (Beasley et al., 2001; Berthet et al., 1997; Wengler et al., 1985). The importance of E glycosylation for expression of the virulent phenotype of lineage 1 WN viruses has been demonstrated experimentally (Beasley et al., 2005; Shirato et al., 2004).

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However, evidence documenting negative effects of E glycosylation on the WN virulence in mice or on its infectivity in cell culture has been reported as well (Chambers et al., 1998; Hanna et al., 2005).

A number of subunit or recombinant WN vaccines for veterinary and human use are currently under development (Kahler, 2003; Lai and Monath, 2003; Ng et al., 2003; Nusbaum et al., 2003; Pletnev et al., 2002; Tesh et al., 2002). In contrast to subunit or inactivated vaccines, a live WN vaccine may be expected to elicit a long lasting balanced humoral- and cell-mediated immune response normally seen after YF17D vaccination (Yamshchikov et al., 2005). However, the high virulence and pathogenicity of the NY99 strain (Beasley et al., 2002; Roehrig et al., 2002) make it questionable for use in development of a live-attenuated WN vaccine. Recently, we have reported characterization of a lineage 2 WN isolate, W956 (Yamshchikov et al., 2004), which appears sufficiently attenuated, immunogenic and cross-protective to be considered as a candidate for further development of live WN vaccine. One may expect that increasing the antigenic match to the strain circulating in the U.S. could improve the immunogenicity of a candidate for vaccine development. As a first step in evaluating this approach, we have assembled an infectious clone of the NY99 strain and created several chimeric viruses carrying reciprocal exchanges of the W956 and NY99 structural protein genes. In this study, we compared biological properties of the parent and chimeric viruses in tissue culture and in the mouse model and evaluated their immunogenicity in mice.

Results

Design of NY99 and other infectious DNA constructs

Earlier we reported the assembly of an infectious clone of WN lineage 2 virus (pSP6**W956**; Yamshchikov et al., 2001b). To simplify handling, it was converted to the infectious DNA (iDNA) format by engineering the CMV promoter transcription start to the beginning of WN genome cDNA. The antisense strand hepatitis δ ribozyme followed by the bovine growth hormone transcription termination signal (BG) was engineered to the end of WN cDNA for an increased fidelity of 3′-end formation, giving rise to the final construct pCMV**W956**δBG (further referred to as pCMV**W956**; Fig. 1).

A NY99 infectious clone was assembled using genetic material of isolate 385–99 (Xiao et al., 2001) according to the strategy we developed earlier (Yamshchikov et al., 2001a) with artificial introns inserted into viral genome cDNA for stabilization of the plasmid in *E. coli*. Upon transfection of eukaryotic cells, introns are spliced out resulting in precise restoration of viral ORF and initiation of the viral infectious cycle. Two such introns were inserted at positions 2383 and 3472 of the NY99 genome. The infectivity of the final construct CMV**NY99**(i2383i3472)δBG (further referred to as CMV**NY99**) is demonstrated in Fig. 2. Except for the two introns and an introduced *Mfe*I site at the end of E gene, NY99 genome cDNA in CMV**NY99** is authentic to the 385–99 genome (GenBank #DQ211652). The presence of both i2384 and i3472 was found to be crucial for the stability of the plasmid.

The pCMV**W956** and pCMV**NY99** constructs were used to create plasmids carrying reciprocal exchanges of the structural protein genes of two viruses (Fig. 1). The pCMV**[CprME$_{NY99}$]W956** chimera carries genes of all NY99 structural proteins instead of those of W956. In the pCMV**[prME$_{NY99}$]W956** chimera, only the prM-E region of NY99 (not including its prM signal sequence) from position 466 to position 2405 was used to replace the corresponding region in the pCMV**W956** construct. The E protein of NY99 is glycosylated at the NYS site ($E_{154–156}$). A variant of pCMV**[prME$_{NY99}$]W956** was prepared that encodes NY99 E in which this glycosylation site was mutated (NYS → SYS). The last chimeric construct pCMV**[CprME$_{W956}$]NY99** was created by transferring the *Bgl*II–*Mfe*I fragment coding for CprME of W956 into pCMV**NY99**.

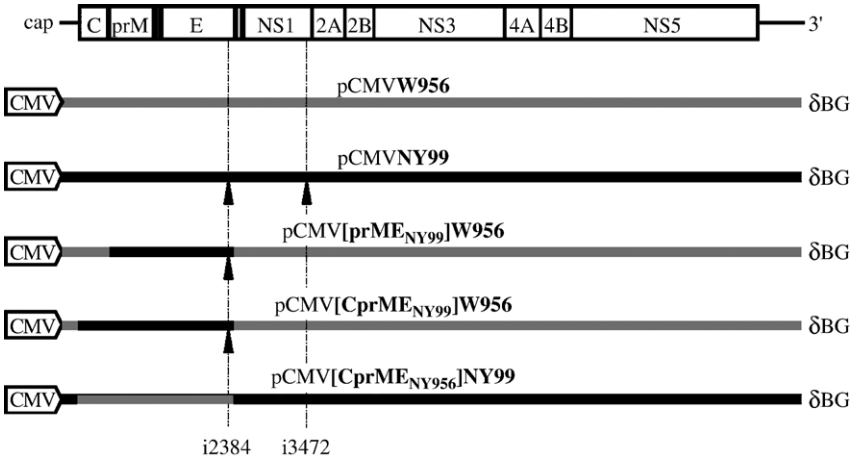

Fig. 1. Parent and chimeric infectious DNA constructs used in this study. A schematic representation of the WN virus genome with genes of recognized virus-specific proteins (Lindenbach and Rice, 2001) is shown on the top. Approximate locations of the intron present in particular constructs are marked by a filled arrowhead, genome positions of intron insertions are shown below. CMV—human cytomegalovirus promoter/enhancer, δ—antisense hepatitis δ ribozyme, BG—bovine growth hormone transcription termination and polyadenylation sequence. For clarity, the schematic is not drawn exactly to the scale.

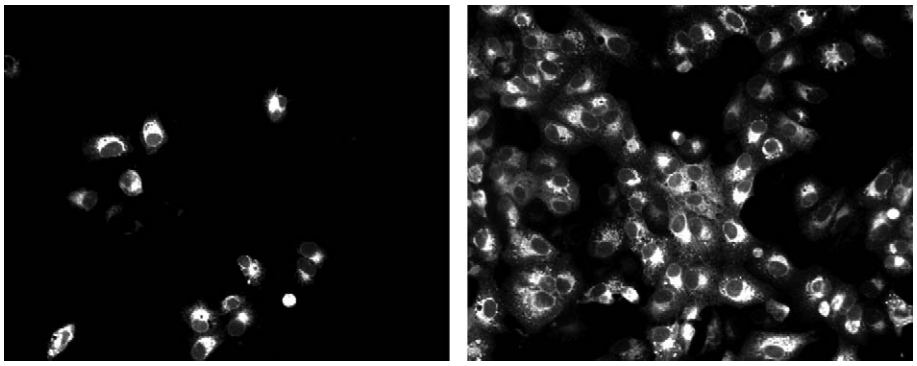

Fig. 2. Virus recovery from the WN infectious DNA. WN antigens in transfected cells were visualized by indirect immunofluorescence with WN-specific HMAF and anti-mouse IgG-fluorescein conjugate. Vero cells 24 h (left) and 40 h (right) after transfection with pCMVNY99 and Lipofectamine 2000 (Invitrogen).

Characterization of recovered viruses in tissue culture

All constructs described above were infectious in tissue culture, that is, lipid-mediated direct transfection of Vero, BHK or mosquito C6/36 cells with either of these plasmids resulted in the establishment of a productive infection. Foci of virus multiplication were easily detectable by indirect immunofluorescence 24 h after transfection, and by 40 h after transfection, the entire monolayer was infected (Fig. 2 for pCMV**NY99**). The specific infectivity of iDNA was in the range 3–8 × 10^6 pfu/μg DNA in Vero cells. For brevity, viruses recovered from the iDNA constructs (shown in parentheses) were designated W956 (pSP6**W956** or pCMV**W956**), $NY99_{REC}$ (pCMV**NY99**), $NY99_{CME}$ (pCMV**[CprME$_{NY99}$]W956**), $NY99_{ME}$ (pCMV**[prME$_{NY99}$]W956**), $NY99_{ME}$(SYS) (pCMV**[prME$_{NY99}$]W956** with the mutated glycosylation site in E) and $W956_{CME}$ (pCMV**[CprME$_{W956}$]NY99**). Genomes of all virus isolates were completely sequenced at Vero passage 2 and demonstrated the expected nucleotide sequences constructed on the basis of W956 (GenBank #M12294) and 385–99 (GenBank #DQ211652) genome sequences. In addition, the E gene of $NY99_{ME}$(SYS) was sequenced at Vero passage 3 and no signs of reversion to the wild type sequence were found.

The glycosylation status of glycoproteins specified by the parent and chimeric viruses was verified by immunoprecipitation, deglycosylation with PNGase F and SDS-PAGE. The prM and NS1 proteins of both NY99 and W956 each carry glycosylation sites, and all are glycosylated as shown in Fig. 3. While the E protein of NY99 is also glycosylated at the NYST site (position 154–157 in the E protein), the E protein of W956 (as well as its parent 956D117B3 and its ancestor B956) carries a deletion of the entire 4 aa site. Accordingly, the E protein of W956 is not glycosylated (Fig. 3). As expected, $NY99_{ME}$(SYS) also produced non-glycosylated E (data not shown). In contrast, cells infected with either NY99 or both chimeric $NY99_{CME}$ and $NY99_{ME}$ produce glycosylated E (Fig. 3). WN HMAF used for immunoprecipitation was prepared against W956 (Yamshchikov et al., 1997, 2001b). It appears less efficient in immunoprecipitation of secreted NS1 of NY99, which is represented by a diffuse band due to the heterogeneity of the oligosaccharide moiety (Fig. 3, left) and is better revealed after deglycosylation (Fig. 3, right). Both chimeric viruses specify NS1 of W956, which is efficiently recovered as for W956 (Fig. 3).

The growth characteristics of viruses at passage 2 were compared in Vero cells. As shown in Fig. 4A, $NY99_{CME}$ and $NY99_{ME}$ accumulated at the rate essentially similar to NY99. To the opposite, the growth curve of the $W956_{CME}$ chimera that carries the structural protein genes of W956 and the rest of the genome from NY99 was found to be very similar to W956 (Fig. 4B). For comparison, Fig. 4B shows a single step growth curve for $NY99_{REC}$ that was recovered from the parent infectious DNA construct pCMV**NY99**. The growth rate of $NY99_{REC}$ is very similar to parent NY99 (Fig. 4A). We did not measure specifically the growth rate of $NY99_{ME}$(SYS). Based on the titer of the passage 2 progeny (1.3 × 10^7 pfu/ml) attained by 4 days

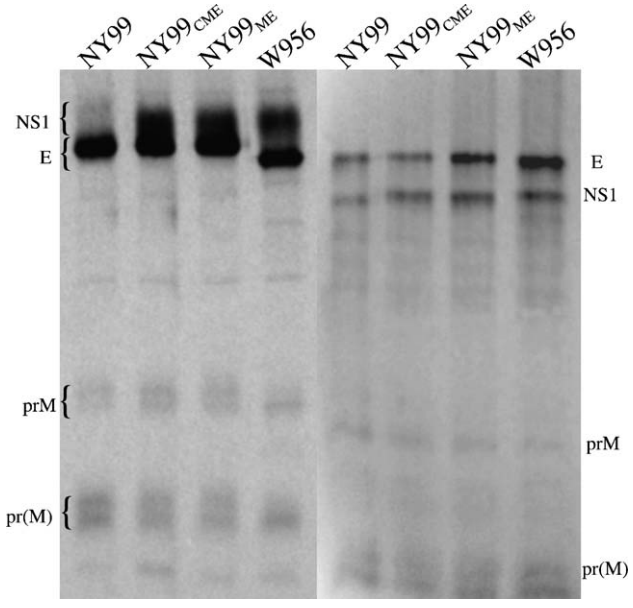

Fig. 3. PAGE analysis of proteins specified by parent and chimeric viruses. [^{35}S]-labeled WN-specific proteins were recovered by immunoprecipitation (left panel) from media 24 h after infection of Vero with specified viruses used at MOI = 10. The same samples after deglycosylation with PNGase F are shown on the right panel. Virus designations are the same as in text. WN glycoproteins are identified on the left and right; pr(M) is the glycosylated amino terminal part of prM that is cleaved during virus maturation and is secreted from infected cells.

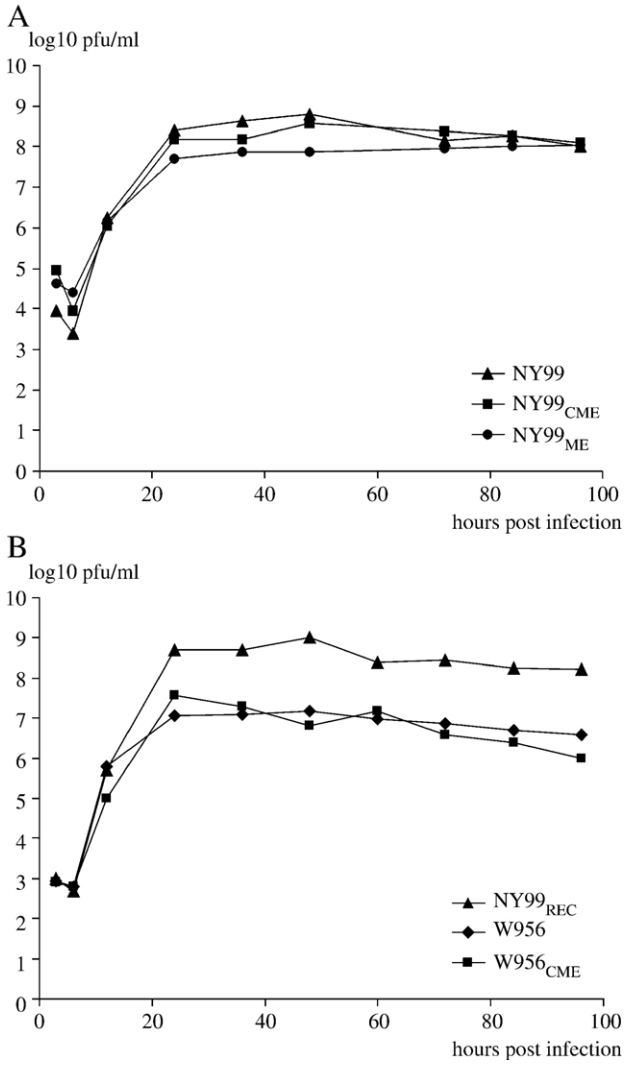

Fig. 4. Growth characteristics of parent and chimeric viruses. Vero cells were infected at MOI = 1, viral progeny was harvested at specified time intervals and titers were determined in Vero. $NY99_{CME}$—virus recovered from pCMV[$CprME_{NY99}$]W956, $NY99_{ME}$—virus recovered from pCMV [$prME_{NY99}$]W956, $NY99_{REC}$—virus recovered from pCMVNY99, W956—virus recovered from pCMVW956, and $W956_{CME}$—virus recovered from (pCMV[$CprME_{W956}$]NY99.

post-infection, the growth rate of $NY99_{ME}$(SYS) appears to resemble W956 and $W956_{CME}$.

The prominent phenotypic difference between parent NY99 and W956 is the high cytopathic effect in Vero cells that is characteristic for the former and is absent for the latter (Fig. 5). Surprisingly, both $NY99_{CME}$ and $NY99_{ME}$ carrying the structural protein genes of NY99 have retained the phenotype of W956, which does not form distinctive plaques in Vero cells (Fig. 5, the upper panel). Foci of virus multiplication are barely visible on fixed and stained monolayers as pale areas without discernible cell destruction. Immunohistochemical staining of fixed cells shows that $NY99_{CME}$ and $NY99_{ME}$ actually form larger foci, which is indicative of a higher efficiency of viral spread, resembling NY99 more than W956 (Fig. 5, the lower panel). This phenotypic marker agrees with the higher accumulation rates of both $NY99_{CME}$ and $NY99_{ME}$ demonstrated above, which is characteristic for NY99. In contrast, $W956_{CME}$ carrying the structural protein genes of W956 in the NY99 genome displays an essentially reversed phenotype. It demonstrates a clearly cytopathic phenotype of NY99 (Fig. 5, the upper panel), yet by its growth rate (Fig. 4B) and by the plaque/foci size (Fig. 5, the lower panel), it resembles W956.

Virulence and pathogenicity of recovered viruses

Peripheral virulence of the parent and chimeric viruses was assessed in 4-week-old outbred Swiss Webster mice, which were infected i.m. with serial 10-fold dilutions of viral stocks. LD_{50} values were calculated by the Reed&Muench method (Burleson et al., 1992) based on survival 2 weeks after inoculation. As shown in Table 1, the attenuated phenotype of W956 (i.m. $LD_{50} = 0.9 \times 10^6$ pfu) is contrasted by the high virulence of NY99 (i.m. $LD_{50} = 17$ pfu). Infection with NY99 uniformly resulted in rapidly progressing encephalitis accompanied by multiple paralysis and resolved by death often in less than 12 h after onset of neurological symptoms. This is summarized by a short average survival time (AST) with a rather little dependence on the virus dose and insignificant deviation among animals, as shown in Table 1. Permanent sequelae, such as hind limb paralysis, were often observed

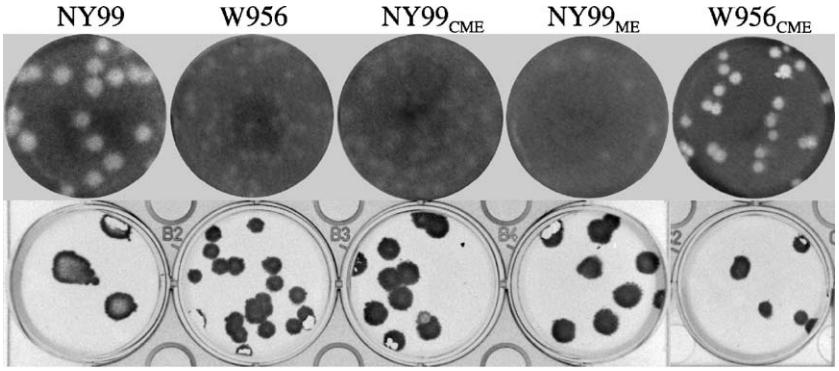

Fig. 5. Cytopathic properties and plaque morphology of parent and recovered viruses. Confluent monolayers of Vero cells were infected with serial 10-fold dilutions of specified viruses and incubated under 1% methyl cellulose for 5 days. Selected wells were either fixed in 10% formalin and stained with methyl violet (upper panel) or fixed in methanol-acetic acid at −20 °C and immunostained with WN-specific HMAF followed by a peroxidase conjugate and the DAB substrate (lower panel). Visualized foci were photographed in visible light.

Table 1
Virulence of the parent viruses in mice

W956[a]	D/T[b]	%[b]	AST	NY99[a]	D/T[b]	%[b]	AST
2.6×10^6	3/6	50	10.7 ± 2.5	–	–	–	–
2.6×10^5	2/6	33	9.5 ± 2.1	8.8×10^4	6/6	100	7.5 ± 0.8
2.6×10^4	1/6	17	13.0 ± 0.0	8.8×10^3	6/6	100	8.0 ± 1.3
2.6×10^3	0/6	0	N/A	8.8×10^2	5/6	83	8.0 ± 0.8
2.6×10^2	0/6	0	N/A	8.8×10^1	5/6	83	8.0 ± 1.4
2.6×10^1	0/6	0	N/A	8.8×10^0	3/6	50	9.0 ± 1.0
$LD_{50} = 0.9 \times 10^6$ pfu				$LD_{50} = 1.7 \times 10^1$ pfu			

[a] Intramuscular injection, pfu/mouse.
[b] Mortality at 2 weeks after inoculation.

among survivors. In contrast, infection with lethal doses of W956 resolved in 2–3 days and was characterized by immobility, anorexia and a substantial weight loss, but paralytic symptoms were uncommon. Infection with lethal doses of W956 was characterized by overall longer AST, which also did not show a clear dependence on the virus dose but was characterized by a substantial deviation between animals (Table 1). Virus was isolated from brains of all succumbed mice indicating that W956 is neuroinvasive when administered in high doses. Mice, which became symptomatic, apparently could not contain the infection and usually did not survive; those that have survived even high doses of W956 usually did not demonstrate visibly identifiable illness.

Transfer of the NY99 structural protein genes into the genome of attenuated W956 did not result in transfer of the NY99 highly virulent phenotype, although an about 100-fold increase in virulence was observed based on values of LD_{50} shown in Tables 1 and 2. Mice inoculated with either $NY99_{CME}$ and $NY99_{ME}$ chimeric viruses demonstrated scattered deaths with a lack of clear dependence on the virus dose, which did not allow to calculate reliable LD_{50} values. For either chimeric virus, however, AST was more dependent on the virus dose. As shown in Table 2, in the upper dose range, AST was closer to that after NY99 infection, but it gradually increased to resemble W956 in the lower dose range of chimeric viruses. Elimination of the glycosylation site in $NY99_{ME}$ by (NYS → SYS) mutation completely eliminated the observed ~100-fold increase in virulence (Table 2). The resulting $NY99_{ME}$(SYS) virus appeared as attenuated as W956 despite the presence of NY99 prM-E. We could not calculate LD_{50} of this virus because mortality did not exceed 50% at the maximal dose we could administer. Both chimeric viruses carrying glycosylated NY99 E produced symptomatic patterns resembling either NY99 (paralytic encephalitis) or W956 (no paralytic symptoms). We could not identify a statistically significant correlation of the particular symptomatic pattern and the dose. The only mouse that succumbed to the highest dose of $NY99_{ME}$(SYS) demonstrated a symptomatic pattern undistinguishable from that caused by W956, although this single case is insufficient to draw conclusions. The survival time was similar to W956 as well.

Surprisingly, transfer of the structural protein genes from attenuated W956 into the genome of virulent NY99 produced a chimeric virus that demonstrated essentially the same virulence in mice as parent NY99 (Table 2). Infection with $W956_{CME}$ uniformly produced a rapidly developing paralytic encephalitis. Similarly to infection with NY99, a sudden onset of symptoms was followed by terminal resolution in less than 12 h. Opposite to the two other chimeric viruses, the AST dependence on the dose of $W956_{CME}$ was very similar to that of NY99 (compare AST in Tables 1 and 2).

Immune response to chimeric viruses

Mice that survived infection with chimeric viruses $NY99_{CME}$, $NY99_{ME}$ and $NY99_{ME}$(SYS) were bled at 4 weeks after inoculation, and immune sera were combined in each virus dose group to obtain averaged results. The presence of virus-specific antibodies was evaluated by ELISA with the NY99 viral antigen and in plaque reduction-neutralization titer (PRNT) assays with NY99 virus. For comparison, we also evaluated the humoral immune response in mice that survived infection with W956. Similar comparison with NY99 could not be done due to the low survival rate of infected mice; some of the few animals that survived the lowest NY99 doses did not demonstrate any virus-specific immunity and likely did not experience infection. Although animals demonstrated high titers of WN-specific IgG 4 weeks after inoculation with all tested

Table 2
Virulence of the chimeric viruses in mice

$NY99_{CME}$[a]	D/T[b]	%[b]	AST[c]	$NY99_{ME}$[a]	D/T[b]	%[b]	AST[c]	$NY99_{ME}$(SYS)[a]	D/T[b]	%[b]	AST[c]	$W956_{CME}$[a]	D/T[b]	%[b]	AST[c]
2.2×10^6	5/6	83	7.3 ± 2.9	5.75×10^6	4/6	67	7.8 ± 2.2	1.3×10^6	1/4	25	10.0 ± 0.0	3.1×10^6	5/6	83	6.6 ± 0.9
2.2×10^5	3/6	50	7.7 ± 0.6	5.75×10^5	1/6	17	8.0 ± 0.0	1.3×10^5	0/4	0	–	3.1×10^5	6/6	100	6.8 ± 0.8
2.2×10^4	3/6	50	9.3 ± 2.1	5.75×10^4	6/6	100	9.7 ± 1.5	1.3×10^4	0/4	0	–	3.1×10^4	6/6	100	8.0 ± 0.9
2.2×10^3	3/6	50	10.0 ± 0.0	5.75×10^3	2/6	33	9.5 ± 0.7	1.3×10^3	0/4	0	–	3.1×10^3	6/6	100	8.5 ± 0.5
2.2×10^2	0/6	0	–	5.75×10^2	2/6	33	8.5 ± 0.7	1.3×10^2	0/4	0	–	3.1×10^2	6/6	100	8.2 ± 0.4
2.2×10^1	3/6	50	10.3 ± 0.6	5.75×10^1	4/6	67	11.5 ± 2.4	1.3×10^1	0/4	0	–	3.1×10^1	6/9	66	9.2 ± 1.7
–	–	–	–	–	–	–	–	–	–	–	–	3.1×10^0	0/3	0	N/A
–	–	–	–	–	–	–	–	–	–	–	–	3.1×10^{-1}	0/3	0	N/A
$LD_{50} = 1.0 \times 10^4$ pfu[d]				$LD_{50} = 1.2 \times 10^4$ pfu[d]				$LD_{50} > 1.3 \times 10^6$ pfu[e]				$LD_{50} = 2.1 \times 10^1$ pfu			

[a] Intramuscular injection, pfu/mouse.
[b] Mortality at 2 weeks after inoculation.
[c] Average survival time.
[d] Reliable values cannot be calculated.
[e] LD_{50} cannot be calculated because 50% endpoint could not be reached.

viruses (Table 3), mice inoculated with the chimeric viruses $NY99_{CME}$, $NY99_{ME}$ and $NY99_{ME}$(SYS) developed, in general, a 4- to 10-fold stronger NY99-specific humoral response. Comparison of NY99 neutralizing titers shown in Table 3 demonstrates that all chimeric viruses have induced titers of NY99 neutralizing antibodies superior to those caused by W956 immunization. The difference is especially visible in the lower dose range. While at the lowest W956 dose used animals did not demonstrate any detectable immunity indicating lack of infection, all chimeric viruses at comparable ($NY99_{CME}$ and $NY99_{ME}$) and even lower doses ($NY99_{ME}$(SYS)) induced strong virus-specific and neutralizing immune response. This suggests that virions containing NY99 structural proteins possess a higher infectivity resulting in a lower effective infectious dose. Accordingly, all animals that demonstrated the presence of neutralizing antibodies have survived subsequent challenge with $100LD_{50}$ of NY99. There were no survivors in the lowest W956 dose group as well as among control unimmunized mice.

Discussion

Earlier, we described a highly attenuated lineage 2 isolate WN1415 that is highly immunogenic and cross-protective against NY99. We reasoned that the suitability of this isolate (referred here as W956 to indicate its relation to the prototype B956 strain) for WN vaccine development can be substantially increased by combining its attenuated nature with the antigenic identity of NY99. To test that, we have created chimeric lineage 1 and lineage 2 West Nile viruses and investigated their biological properties in tissue culture as well as virulence and immunogenicity in mice. Chimeric iDNA carrying reciprocal exchanges of the structural protein genes of two viruses was assembled using corresponding lineage 1 and lineage 2 infectious DNA constructs pCMV**NY99** and pCMV**W956**.

Growth properties of the parent (NY99 and W956) and the recovered ($NY99_{REC}$, $NY99_{CME}$, $NY99_{ME}$ and $W956_{CME}$) viruses were examined in Vero cells. As expected, NY99, $NY99_{REC}$, $NY99_{CME}$ and $NY99_{ME}$ that carry the prM-E genes of NY99 demonstrated the essentially similar growth characteristics. In contrast, the growth rate of $W956_{CME}$ was very similar to W956 with peak titers lower than observed for $NY99_{REC}$ by 1–2 logs. The differences in the growth rate correlate with the plaque size or the size of virus multiplication foci visualized by immunohistochemical staining. The combined evidence indicates that the higher growth rate characteristic for NY99 is determined by the properties of its structural proteins. In contrast, determinants that are responsible for the cytopathic effect of NY99 (or lack thereof for W956) are associated with the nonstructural proteins (NSps). This conclusion is strongly supported by the essentially reversed phenotype of $W956_{CME}$ that carries the structural protein genes of W956 in the NY99 genome. Determinants or mechanisms of flavivirus cytopathicity are not clearly defined. For WN viruses, either necrosis or apoptosis has been observed depending on the virus dose (Chu and Ng, 2003). A number of evidence implicated NS3 in induction of apoptosis by dengue and WN viruses (Duarte dos Santos et al., 2000; Ramanathan et al., 2006). Although 3 and 26 aa differences distinguish NS2B and NS3 of W956 and NY99, all these are shared by noncytopathic W956 and its cytopathic ancestor B956 (Yamshchikov et al., 2004), indicating that neither the NS2B–NS3 protease or NS3 alone is not responsible for the observed differences in cytopathicity.

Both neuroinvasiveness and neurovirulence of flaviviruses in the mouse model have been associated with determinants in the envelope protein (Cecilia and Gould, 1991; Chambers et al., 1999; Gualano et al., 1998; Hasegawa et al., 1992; Holzmann et al., 1990, 1997; Jiang et al., 1993; McMinn, 1997; Pletnev et al., 1992, 1993). Thus, one could anticipate that transfer of the NY99 structural protein genes will result in transfer of the NY99 highly virulent phenotype. However, this was not found to be the case, although an about 100-fold increase in virulence was observed based on LD_{50} values of attenuated W956 and both chimeric viruses. It needs to be underscored, however, that LD_{50} values for $NY99_{CME}$ and $NY99_{ME}$ could not be reliably determined by the Reed&Muench method due to scattered mortality without clear dependence on the virus dose, and calculated values should be considered as approximate. Both chimeric viruses demonstrated so-called attenuated neuroinvasive phenotype with characteristic lack of clear dose-dependent response (Arroyo et al., 2001; Beasley et al., 2005; Shirato et al., 2004). It has been shown that neuroinvasiveness of NY99 and other lineage 1 strains at least in part is determined by the glycosylation status of the E protein (Beasley et al., 2005;

Table 3
Development of NY99-specific humoral immunity[a]

WN1415[b]			$NY99_{CME}$[b]			$NY99_{ME}$[b]			$NY99_{ME}$(SYS)[b]		
Dose	ELISA[c]	PRNT[d]	Dose	ELISA[c]	PRNT[d]	Dose	ELISA[c]	PRNT[d]	Dose	ELISA[c]	PRNT[d]
2.6×10^6	80,000	320	2.2×10^6	10,000	80	5.8×10^6	>128,000	1280	1.3×10^6	>128,000	3200
2.6×10^5	32,000	120	2.2×10^5	>128,000	2133	5.8×10^5	>128,000	1920	1.3×10^5	>128,000	1280
2.6×10^4	32,000	60	2.2×10^4	>128,000	1707	5.8×10^4	ND	ND	1.3×10^4	>128,000	480
2.6×10^3	32,000	240	2.2×10^3	128,000	640	5.8×10^3	128,000	3840	1.3×10^3	128,000	640
2.6×10^2	32,000	60	2.2×10^2	80,000	640	5.8×10^2	32,000	1920	1.3×10^2	64,000	240
2.6×10^1	0	0	2.2×10^1	80,000	320	5.8×10^1	32,000	960	1.3×10^1	128,000	480

[a] Serum samples at 4 weeks post-inoculation combined for each virus dose group and assayed in triplicate.
[b] Immunizing virus administered i.m. at the specified doses as determined by back-titration.
[c] Endpoint dilution titers of combined for each dose group serum samples assayed in triplicate.
[d] Endpoint dilution plaque reduction-neutralization titers of combined for each dose group serum samples assayed in triplicate.

Shirato et al., 2004). On the other hand, glycosylation of E was associated with a reduced infectivity of WNV of either lineage in tissue culture (Hanna et al., 2005) and with attenuation of the mouse virulence for lineage 1 viruses (Chambers et al., 1998). Molecularly cloned W956, parent 956D117B3 and their ancestor B956, which displayed various levels of attenuation (Yamshchikov et al., 2004), carry a deletion of four amino acids that compose the $E_{154–157}$ glycosylation locus (NYST in the majority of lineage 1 viruses). Replacement of the authentic E gene in W956 with the gene of glycosylated E from NY99 produced $NY99_{CME}$ and $NY99_{ME}$ with the increased virulence. When the glycosylation site in $NY99_{ME}$ was eliminated by NYS → SYS mutagenesis, this increase in virulence was completely eliminated since the resulting $NY99_{ME}$(SYS) appeared as attenuated as WN956 despite the presence of NY99 prM-E. This does not contradict the existing evidence supporting the importance of E glycosylation for expression of the virulent phenotype. However, transfer of the gene coding for non-glycosylated E from W956 into the NY99 genome produced the $W956_{CME}$ chimeric virus with essentially the same neuroinvasiveness and virulence in mice as NY99 despite its somewhat slower growth in tissue culture. Thus, the effect of E glycosylation on virulence and/or neuroinvasiveness is most likely virus-specific.

The combined evidence indicates that determinants of NY99 high virulence, as well as the genetic basis of W956 attenuation, are found in the nonstructural protein region. Recently reported evidence indicates that NY99 interferes with IFN signaling pathways in infected cells (Guo et al., 2005; Liu et al., 2005; Munoz-Jordan et al., 2005; Pantelic et al., 2005; Scholle and Mason, 2005). While reports contradict each other in regard to possible mechanisms and roles of individual proteins, there is a general agreement on the crucial role of NY99 NSps in such interference. It remains to be seen, however, whether such interference play an important role in NY99 pathogenesis in vivo. Another phenotypic marker shared between highly virulent NY99 and $W956_{CME}$ is the cytopathic nature of both viruses. Recent demonstration of the specific role that toll-like receptor 3 (Tlr3) plays in mediating NY99 neuroinvasion in mice (Wang et al., 2004) leads to a tempting suggestion. Tlr3 recognizes dsRNA (Schroder and Bowie, 2005) that is produced as an intermediate during replication of many RNA viruses, including WN virus. The high cytopathicity of NY99 may contribute to its neuroinvasive phenotype by increasing the release of dsRNA from infected cells. When compounded by interference with IFN signaling, which may have broad consequences from an inefficient early control of viremia due to dampened innate immune response to a cytokine-induced compromise of the blood brain barrier (Veldhuis et al., 2003), the high cytopathicity of NY99 may constitute a significant factor of its virulence. Although this seems a feasible explanation for the apparent association of virulence and cytopathicity that we have observed in this study, it remains to be experimentally supported.

The above conclusion implies that attenuation of highly virulent NY99 by mutagenesis of the E protein may not be adequate to yield a sufficiently safe vaccine. Even if a very high level of attenuation can be achieved by mutations introduced in E at multiple loci, the function of the E protein is directly connected to the virus infectivity and is under a high selective pressure for reversion to the maximal functionality. With virulence determinants being in the NSp region, rapid selection of viruses with a higher infectivity may result in rapid reversion to a highly virulent phenotype. Such consideration corroborates the suitability of the chimeric flavivirus approach (Lai and Monath, 2003) for development of a WN vaccine, in which WN virulence determinants located in its NSp genome region are eliminated by replacement with NSp genome regions of attenuated vaccine vectors. We suggest that utilization of an attenuated WN virus as a viral vector may offer additional advantages of the highly homologous nonstructural proteins that carry major flavivirus T-cell epitopes (Co et al., 2002; Lobigs et al., 1994; Mathew et al., 1996; Okamoto et al., 1998; Parrish et al., 1991; Spaulding et al., 1999). Comparison of humoral responses in mice demonstrated the superior immunogenicity of the chimeric viruses. Notably, some increase in virulence resulted from chimerization of attenuated W956 with NY99 prM-E completely eliminated by removal of the E glycosylation site without affecting the virus growth rate and its superior immunogenicity. Although rigorous testing of the genetic stability of the introduced NYS → SYS mutation is warranted, the high immunogenicity, good growth characteristics and the highly attenuated nature makes W956 and $NY99_{ME}$(SYS) suitable for development of WN live vaccines, with the latter to be more attractive than the former due to closer antigenic similarity to NY99. Targeted mutagenesis of the E protein may be utilized to eliminate the residual virulence, for example, by introducing attenuating mutations found in the Japanese encephalitis SA14-14-2 vaccine as suggested earlier (Monath, 2001). We are currently in the process of testing these alternatives.

Materials and methods

DNA manipulation, cloning and characterization of recombinant constructs

DNA manipulations were done using the standard procedures (Sambrook and Russell, 2001) with commercially available enzymes in conditions recommended by manufacturers. Total RNA from infected cells was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). PCR and RT-PCR amplification was done using high fidelity KOD polymerase (Novagen, Madison, WI). A set of primers for amplification and sequencing of 385–99 cDNA fragments was designed on the basis of the 382–99 sequence (GenBank #AF196835). Sequencing of cDNA fragments and plasmids was done using ABI310 Genetic Analyzer (Perkin-Elmer) with the manufacturer's kits and protocols. Throughout the study, NIH Guidelines for Research involving Recombinant DNA Molecules were followed. *E. coli* strain HB101 was used for cloning and maintenance of recombinant constructs.

Assembly of parent and chimeric infectious DNA constructs

The infectious clone of lineage 2 WN 956D117B3 strain, here referred to as pSP6**W956**, has been described earlier (Yamshchikov et al., 2001b). A 739-bp fragment containing the CMV promoter/enhancer including its transcription start was amplified from the pCIneo plasmid (Promega, Madison, WI). The CMV transcription start was engineered by PCR to the beginning of the WN genome cDNA, and resulting joint CMV-5′UTR was used as *Cla*I–*Bgl*II fragment to replace the corresponding SP6-5′UTR fragment in pSP6**W956**. A 350-bp fragment containing the hepatitis δ ribozyme followed by the bovine growth hormone transcription termination and polyadenylation signal (the δBG cassette, Fig. 1) was amplified from JE infectious DNA reported earlier (Yamshchikov et al., 2001a) and was inserted at the end of the WN genome cDNA, with the cleavage site of the ribozyme precisely engineered to the 3′-end of the WN genome. The resulting pCMV**W956** was characterized by complete sequencing of the WN genome cDNA insert.

A similar *Cla*I–*Bgl*II CMV-5′UTR joint fragment was created using 385–99 cDNA, which was used to assemble a CMV-driven subgenomic replicon construct lacking genes C, prM and E (positions 162–2352). The δBG cassette was engineered at the 3′end of the 385–99 genome as above. The C-prM-E structural protein gene cassette of 385–99, which was modified by PCR-mediated insertion of a 132 bp intron at position 2384, and by an introduced *Mfe*I site (position 2405), was assembled separately. The 132-bp artificial intron carrying stop codons in three frames was amplified from pCIneo. The modified structural gene cassettes were inserted as *Bgl*II–*Bsp*EI fragments into the 385–99 replicon plasmid replacing the corresponding fragment carrying the deletion. The final stabilized pCMV**NY99** infectious DNA construct was created by PCR-mediated insertion of a second copy of the 132 bp intron at position 3472 into the construct carrying i2384. After complete sequencing of the 385–99 genome cDNA insert in the final construct, all 12 revealed mutations have been eliminated by replacing fragments carrying mutations with the corresponding fragments amplified from de novo synthesized cDNA.

Reciprocal exchanges of the C-prM-E genes between pCMV**W956** and pCMV**NY99** were done by exchange of the corresponding *Bgl*II–*Mfe*I fragments (from position 89, 8 nt upstream from the beginning of NY99 and W956 ORF, to position 2393 in W956 and 2405 in NY99 at the amino terminus of the NS1 signal sequence). A fragment including only the prM-E genes of 385–99 was created by PCR-mediated engineering of the carboxy terminus of the internal prM signal peptide of W956 (including the signalase cleavage site) to the amino terminus of mature prM of 385–99 with subsequent transfer of the chimeric C-prM-E cassette as the *Bgl*II–*Mfe*I fragment.

Mutagenesis of the glycosylation site was done by PCR using a pair of mutagenizing primers that introduce the N → S mutation (…AAC… → …TCC… in the sense strand) in the NYS glycosylation site in the NY99 E protein. These primers and primers flanking unique *Eco*NI and *Mfe*I sites were used to amplify a mutagenized fragment from the pCMV**NY99** template. Resulting PCR fragment was digested with *Eco*NI and *Mfe*I, a 1283-bp fragment was purified on agarose gel and used to replace the corresponding *Eco*NI–*Mfe*I fragment in pCMV**[prME$_{NY99}$]W956**). Sequences of all primers used in this section are available on request.

Cells and viruses

Vero (ATCC CRL-1586) were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT) and 1× antibiotic–antimycotic mixture (Invitrogen, Carlsbad, CA). The molecularly cloned isolate W956 (earlier designated W1415) has been described (Yamshchikov et al., 2001b, 2004). The 385–99 isolate of the NY99 strain at Vero passage 1 was kindly provided by R. Tesh (Galveston, TX), and the working stock (8.8×10^7 pfu/ml) was prepared by additional passage in Vero. Total RNA isolated from infected cells after harvesting the passage 2 virus was used to prepare a set of cDNA fragments covering the entire 385–99 genome.

Recovery of chimeric viruses, determination of virus titers and characterization of viruses in tissue culture

Transfection of Vero cells with infectious DNA was done using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.8 μg of DNA and 3 μl of the reagent in 100 μl of Opti-MEM (Invitrogen) was added to a 70–80% confluent Vero monolayer in a 12-well plate cluster. After 6–8 h incubation under the normal growth conditions, the transfection medium was replaced with the fresh growth medium and incubation was continued for additional 2–3 days to allow sufficient accumulation of the passage 1 progeny. Working stocks of passage 2 viruses were prepared by infecting Vero with the passage 1 progeny at MOI = 0.1 and harvesting the progeny from the medium 4–5 days post-infection. Titers of viral stocks at passage 2 prepared in Vero cells were 1.4×10^8 (NY99$_{CME}$), 5.7×10^7 (NY99$_{ME}$), 1.3×10^7 (NY99$_{ME}$(SYS)) and 3.1×10^7 (W956$_{CME}$) pfu/ml. Total RNA isolated from the infected cells was used for RT-PCR amplification of overlapping cDNA fragments, which were gel-purified and sequenced as above.

For indirect immunofluorescence, cells were seeded on glass coverslips in 24-well plate clusters and half of the DNA and transfection reagent was used under the same conditions. Cells fixed with acidic methanol were processed for WN-specific indirect immunofluorescence as described (Yamshchikov et al., 2001b).

Viral proteins were labeled with [^{35}S]Met 24 h after infection of Vero with specified viruses at MOI = 10; labeling, immunoprecipitation, deglycosylation with PNGase F and PAGE analysis were done as described earlier (Yamshchikov and Compans, 1993, 1995; Yamshchikov et al., 1997). Virus

titers were determined by a 96-well microassay (Yamshchikov et al., 2004).

The specific infectivities of iDNA constructs, virus cytopathic properties and plaque morphology were analyzed as described (Yamshchikov et al., 2001a, 2001b, 2004). Briefly, transfected or infected Vero monolayers were incubated in the growth medium containing 1% methyl cellulose, washed, fixed and either stained with methyl violet or immunostained with WN-specific hyperimmune ascites fluid (HMAF) and the peroxidase–DAB procedure.

Virus infection and challenge

Three to four-week-old female Swiss Webster outbred mice purchased from Charles River (Wilmington, MA) were maintained in a BL3 facility according to the NIH guidelines and used in IACUC-approved protocols. Mice were infected into the *Tibialis anterior* muscle (i.m.) with virus dilutions prepared in PBS plus 0.2% normal mouse serum and observed for 3 weeks. Mice that developed the encephalitic syndrome such as paralysis were euthanized and counted as lethal cases. LD_{50} (50% lethal dose) was calculated by the Reed&Muench method (Burleson et al., 1992). Mice that survived the primary infection were challenged at 4 weeks with 100 i.m. LD_{50} of NY99 virus and were observed for additional 3 weeks.

Analysis of NY99-specific humoral immune response

Endpoint dilution titers of NY99-specific IgG were determined in triplicate by standard antibody-capture ELISA using the NY99 viral coating antigen produced from solubilized pelleted virions as described earlier (Yamshchikov et al., 2004). Readings that differ by two standard deviations from pre-immune sera at the same dilution were considered positive. Titers of neutralizing antibodies were determined using NY99 virus and plaque reduction-neutralization titer (PRNT) microassay (constant virus-variable serum) as described (Yamshchikov et al., 2004, 2005). Briefly, confluent Vero cells in 96-well microplates were infected in duplicate with 50 pfu of NY99 in the presence of serially diluted immune sera, incubated for 24 h, fixed and stained using WN-specific HMAF and ABC peroxidase–DAB procedure.

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