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Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus

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Abstract

Two molecularly engineered, live-attenuated West Nile virus (WN) vaccine candidates were highly attenuated and protective in rhesus monkeys. The vaccine candidates are chimeric viruses (designated WN/DEN4) bearing the membrane precursor and envelope protein genes of WN on a backbone of dengue 4 virus (DEN4) with or without a deletion of 30 nucleotides (Δ30) in the 3′ noncoding region of DEN4. Viremia in WN/DEN4- infected monkeys was reduced 100-fold compared to that in WN- or DEN4-infected monkeys. WN/DEN4-3′Δ30 did not cause detectable viremia, indicating that it is even more attenuated for monkeys. These findings indicate that chimerization itself and the presence of the Δ30 mutation independently contribute to the attenuation phenotype for nonhuman primates. Despite their high level of attenuation in monkeys, the chimeras induced a moderate-to-high titer of neutralizing antibodies and prevented viremia in monkeys challenged with WN. The more attenuated vaccine candidate, WN/DEN4-3′Δ30, will be evaluated first in our initial clinical studies.
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Introduction

West Nile virus (WN) is a member of the family Flaviviridae, and it belongs to the Japanese encephalitis virus (JE) serocomplex that includes such important human pathogens as Murray Valley encephalitis, Kunjin, Japanese encephalitis, and St. Louis encephalitis viruses (Burke and Monath, 2001; Hayes, 1989). Similar to other members of this serocomplex, WN is maintained in nature in a mosquito–bird–mosquito cycle, with humans, horses, and other mammals serving as incidental hosts. WN is widely distributed in Africa and Europe, where it has usually been associated with mild illness that includes symptoms of low-grade fever, headache, rash, myalgia, and polyarthropathy. In 1999, WN was detected for the first time in the Northeastern part of the USA, and during the next 3 years, the virus extended its range to include the southern and western states and southern Canada (Campbell et al., 2002; CDC Report,

2002). Severe illness has been seen in the USA and is most common in the elderly, in whom this virus can cause hepatitis, meningitis, and encephalitis, leading to paralysis, coma, and death. Specifically, during the 2002 outbreak of WN in the USA, there were 3389 WN illnesses reported that included 201 deaths (CDC Report, 2002). Consequently, WN illness is considered an emerging disease in the USA and presents a very significant public health threat. Currently, a licensed vaccine is not available for the prevention of WN disease in humans. Mosquito control is the only strategy available to control WN, but spraying is ineffective in urban areas, and despite intense efforts, the virus has spread rapidly within North America and is likely to spread throughout the hemisphere. Clearly, an effective human vaccine is urgently needed to protect at-risk populations, especially since the magnitude of the epidemic continues to increase (Campbell et al., 2002; CDC Report, 2002).

Advaces in recombinant DNA technology have allowed us to develop a novel strategy for constructing live-attenuated flavivirus vaccines that we have been pursuing for the past decade (Pletnev et al., 1992, 2000, 2001; Pletnev and Men, 1998). Our approach was made possible by the con-

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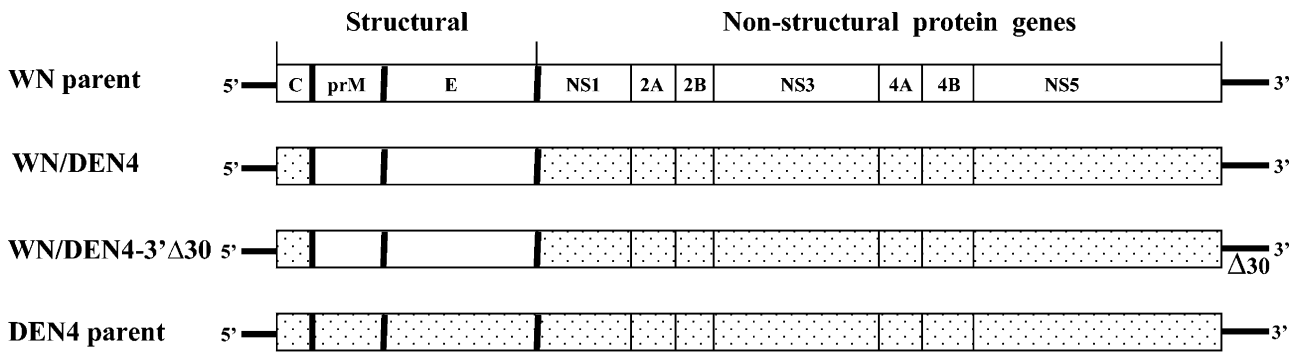*E-mail address:* apletnev@niaid.nih.gov (A.G. Pletnev).

Fig. 1. Structure of full-length genome of parental WN or DEN4 and their chimeric viruses. The 5'- and 3'-termini as well as the shaded regions are from DEN4; the unshaded regions are from WN. The vertical solid lines represent hydrophobic domains in the polyprotein. Δ30 indicates the position of a 30-nucleotide deletion in the 3' noncoding region of the DEN4 genome between nucleotides 10478 and 10507 (Durbin et al., 2001). Chimeric viruses were recovered after transfection of Vero cells with RNA transcripts derived from full-length cDNA as described previously (Pletnev et al., 2002). The prM protein is cleaved by a host cell protease to yield the mature M protein present in virions.

servation among flaviviruses of genome organization, number of viral proteins, replicative strategy, gene order and expression, virion structure, and morphogenesis (Lindenbach and Rice, 2001). All flaviviruses have a positive-sense, nonsegmented RNA genome that encodes a single long polyprotein that is processed by viral or cellular proteases to yield capsid (C), membrane (M), and envelope glycoprotein (E) structural proteins followed by nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 1). 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 of another distantly related flavivirus. Since the E protein induces neutralizing protective antibodies, an attenuated chimeric virus can be used as vaccine against the donor of the E protein (Burke and Monath, 2001; Pletnev et al., 1992, 2000, 2001; Pletnev and Men, 1998). Vaccine candidates for tick-borne encephalitis virus (TBE) were created by replacing the membrane precursor (prM) and E structural protein genes of the mosquito-borne wild-type dengue virus type 4 (DEN4) with the corresponding genes from TBE or the partially attenuated tick-borne Langat virus (LGT), and the resulting chimeric viruses were attenuated with respect to neurovirulence and highly attenuated with respect to neuroinvasiveness in mice (Pletnev et al., 1992, 2000, 2001; Pletnev and Men, 1998). Each chimera proved to be immunogenic and able to induce resistance in mice against challenge with TBE or LGT. These findings indicated that a favorable balance between reduction in virus replication in vivo (attenuation) and induction of protective immunity could be achieved through chimerization of flaviviruses.

As a logical extension of this strategy, we previously constructed a viable WN/DEN4 chimeric virus (Fig. 1) in which the structural prM and E protein genes of WN were substituted for the corresponding genes of the distantly related DEN4 that differed in amino acid sequence by 61.5% for prM and 55.9% for E (Pletnev et al., 2002). The DEN4 is a desirable backbone for these chimeras for two reasons: (i) primary infection of humans with DEN4 rarely results in the severe liver or central nervous system (CNS) disease seen with other flaviviruses such as yellow fever or Japanese encephalitis virus (Burke and Monath, 2001), and (ii) a large menu of mutations, including genetically stable deletion mutations, are available that can attenuate DEN4 for CNS or hepatic tissue as well as for mosquitoes (Blaney et al, 2001, 2002; Hanley et al., 2002; Durbin et al., 2001; Troyer et al., 2001). Previous studies in mice indicated that WN/DEN4 vaccine candidate was highly attenuated when tested by either peripheral or direct intracerebral inoculation, whereas its WN parent virus was highly virulent by both routes (Pletnev et al., 2002). Although the WEN/DEN4 virus was highly attenuated when tested by direct intracerebral inoculation in mice, it retained some residual neurovirulence at 10^4 focus-forming units (FFU), the highest dose tested. However, it was not clear whether chimerization itself specified the observed attenuation since the DEN4 virus that constitutes the backbone of the chimera itself is highly attenuated in mice for neurovirulence and especially neuroinvasiveness. Use of an animal model in which both parental viruses replicate efficiently was needed to address this question, and rhesus monkeys represent such an animal model (Pletnev et al., 2001; Durbin et al., 2001; Whitehead et al., 2003).

To generate a further attenuated derivative of WN/DEN4 (Fig. 1), we selected from the menu of DEN4 attenuating mutations (Blaney et al., 2001, 2002; Hanley et al., 2002; Durbin et al., 2001; Troyer et al., 2001) a 30-nucleotide deletion mutation in the 3' noncoding region of DEN4 (referred to here as the Δ30 mutation). The Δ30 deletion mutation was chosen for inclusion in the WN/DEN4 virus because it is a genetically stable mutation that attenuates dengue virus for mice, monkeys, mosquitoes, and humans (Blaney et al., 2001, 2002; Hanley et al., 2002; Durbin et al., 2001; Troyer et al., 2001; Whitehead et al., 2003). Immunization with either the WN/DEN4 or the WN/DEN4-3'Δ30 chimeric virus completely protected mice against lethal challenge with WN (Pletnev et al., 2002). The present studies in rhesus monkeys were

Table 1
WN/DEN4 chimeric viruses are attenuated and protective in rhesus monkeys

Immunizing virus (dose, FFU)	Monkey number	Viremia ($\log_{10}$FFU/ml) on day postinoculation[a]							Serum NT antibody titer on postimmunization day 42		Viremia ($\log_{10}$FFU/ml) on day postchallenge with WN					Serum NT antibody titer on postchallenge day 42
		1	2	3	4	5	6	7	WN	DEN4	1	2	3	4	5	WN
1. WN/DEN4 (10^5)	1	—	—	—	—	—	0.7	0.7	416	<20	—	—	—	—	—	981
	2	—	0.7	—	—	—	—	0.7	1126	<20	—	—	—	—	—	2082
	3	—	—	—	—	—	—	—	751	<20	—	—	—	—	—	1210
	4	—	—	—	0.7	—	1.0	—	541	<20	—	—	—	—	—	1456
									GMT: 661							GMT: 1377
2. WN/DEN4 (10^6)	5	—	—	—	—	—	—	—	675	<20	—	—	—	—	—	970
	6	—	0.7	—	—	—	—	—	467	<20	—	—	—	—	—	1006
	7	—	—	—	—	—	—	—	270	<20	—	—	—	—	—	1039
	8	—	—	—	—	0.7	—	—	727	<20	—	—	—	—	—	2334
									GMT: 501							GMT: 1240
3. WN/DEN4-3′ Δ30 (10^5)	9	—	—	—	—	—	—	—	109	<20	—	—	—	—	—	1437
	10	—	—	—	—	—	—	—	254	<20	—	—	—	—	—	462
	11	—	—	—	—	—	—	—	170	<20	—	—	—	—	—	629
	12	—	—	—	—	—	—	—	247	<20	—	—	—	—	—	817
									GMT: 186							GMT: 764
4. WN (10^5)	13	—	2.4	2.2	2.6	2.6	0.7	—	1305	<20	—	—	—	—	—	2136
	14	1.0	2.2	2.6	2.1	2.3	1.0	—	1324	<20	—	—	—	—	—	2524
									GMT: 1318							GMT: 2322
5. WN (10^6)	15	0.7	2.2	2.7	2.6	2.3	1.0	—	599	<20	—	—	—	—	—	1424
	16	1.9	2.6	2.9	2.6	2.0	—	—	842	<20	—	—	—	—	—	1837
									GMT: 708							GMT: 1617
6. DEN4[b] (10^6)	17	2.2	2.0	—	—	—	—	—	<20	754	—	1.7	2.2	2.0	0.7	635
	18	2.2	1.9	1.5	1.4	—	—	—	<20	559	—	1.7	2.2	2.0	0.7	600
	19	2.2	2.1	0.7	—	—	—	—	<20	674	—	0.7	1.4	2.4	0.7	448
	20	2.3	2.6	1.5	—	—	—	—	<20	565	1.0	1.8	2.0	0.7	—	277
									GMT: <20	633						GMT: 467

Note. GMT, geometric mean titer; NT, neutralizing.

[a] Viremia was not seen in any monkey after day 7 postinoculation. The limit of detectable viremia in serum was 0.7 $\log_{10}$FFU/ml.

[b] The level of replication of the DEN4 wild-type virus used in this study was comparable to that of the recombinant DEN4 virus that served as backbone for the WN/DEN4 (Durbin et al., 2001).

initiated to determine whether the addition of the genetically stable Δ30 deletion mutation to WN/DEN4 further attenuated it for a nonhuman primate and to determine whether the WN/DEN4 and WN/DEN4-3′Δ30 chimeric viruses exhibited a satisfactory balance between attenuation and immunogenicity in a species that is more relevant than mice for the evaluation of a vaccine that is destined for use in humans. WN/DEN4-3′Δ30 virus was found to be more attenuated for rhesus monkeys than WN/DEN4, yet it retained sufficient immunogenicity to completely prevent viremia following WN virus challenge. WN/DEN4-3′Δ30, therefore, represents a promising WN vaccine candidate. Furthermore, these data strongly suggest that both chimerization and the Δ30 mutation independently attenuate this chimeric virus for rhesus monkeys.

Results

Evaluation of WN/DEN4 chimeras in nonhuman primates

Attenuation, immunogenicity, and protective efficacy of the WN/DEN4 and WN/DEN4-3′Δ30 chimeric viruses were studied in a total of 20 rhesus monkeys at the dose of virus indicated in Table 1. Since peripheral inoculation of WN virus causes an asymptomatic infection in rhesus monkeys, the level of attenuation of chimeric viruses was evaluated by comparing the duration and magnitude of viremia in monkeys infected with a chimeric virus with that of monkeys infected with the wild-type parental WN virus, strain NY99, originally isolated from a Chilean flamingo at the Bronx Zoo in 1999 (Lanciotti et al., 1999) or the DEN4 parental virus, Caribbean strain 814669 (Durbin et al., 2001). Both wild-type parental viruses were isolated on multiple days following infection and achieved a virus titer in serum between $10^{2.2}$ and $10^{2.9}$ FFU/ml, indicating that they were suitable controls for comparison with the chimeric vaccine candidates. The level of viremia of WN wild-type virus was similar at both doses (10^5 and 10^6 FFU).

In contrast to wild-type WN, WN/DEN4 induced only a brief viremia in five of eight monkeys inoculated with 10^5 or 10^6 FFU (Table 1). Viremia lasted 1 or 2 days and attained a peak titer approximately 100-fold lower than that

observed in WN or DEN4 virus-infected monkeys. The level of virus replication of WN/DEN4 remained flat throughout the entire period of viremia. Notably, each of the four monkeys inoculated with 10^5 FFU of the WN/DEN4-3′ $\Delta 30$ mutant failed to develop a detectable viremia ($<10^{0.7}$ FFU/ml), and this degree of additional attenuation from that of WN/DEN4 was statistically significant (Mann–Whitney U test; $df = 7$, $P < 0.05$). These observations demonstrate (i) that WN/DEN4 replicated in a nonhuman primate less well than either WN or DEN4 parental virus, indicating that chimerization of WN and DEN4 was responsible for the attenuation of the chimeric virus observed during infection of a nonhuman primate and (ii) that the WN/DEN4-3′$\Delta 30$ virus was even more attenuated for monkeys than the unmodified WN/DEN4 chimera, indicating that the $\Delta 30$ mutation independently attenuates WN/DEN4 for monkeys.

Although the WN/DEN4 chimera and its deletion mutant were significantly attenuated for rhesus monkeys, these chimeric viruses induced a moderate-to-high level of serum WN neutralizing antibodies (range of 1:270 to 1:1126) in each immunized animal. The serum neutralizing antibody titer of monkeys that received WN/DEN-3′$\Delta 30$ was 3.5-fold lower than that of monkeys infected with a comparable dose of WN/DEN4 (groups 1 and 3, Table 1; Mann–Whitney U test: $df = 7$, $P < 0.05$), a finding consistent with the further attenuation caused by the $\Delta 30$ deletion mutation. Nonetheless, both chimeric viruses induced complete resistance to challenge with 10^5 FFU of wild-type WN virus administered subcutaneously on day 42 postimmunization. WN challenge virus was not detected in the blood of any of the 12 monkeys immunized with WN/DEN4 or its further attenuated deletion mutant. The WN challenge virus replicated efficiently only in monkeys previously immunized with DEN4 virus. Each monkey of this group developed WN viremia that lasted 4 days and attained a peak titer of $10^{2.0}$ to $10^{2.4}$ FFU/ml. This indicated that the observed resistance induced by the chimeras was not mediated by their DEN4 components.

The titer of serum neutralizing antibodies against WN was measured 42 days following challenge with this virus. Only 1 of 16 monkeys (monkey 9) immunized with the WN/DEN4 or WN/DEN4-3′$\Delta 30$ chimera or their WN parent developed a significant (four-fold or greater) increase in serum neutralizing antibody titer after WN challenge. The total lack of viremia of immunized monkeys following WN challenge and the failure of 11 of 12 monkeys immunized with a vaccine candidate to develop a significant antibody response to WN challenge suggests that WN infection of immunized monkeys had been largely prevented.

Discussion

Live-attenuated WN chimeric vaccine candidates are also being generated in which the WN wild-type virus prM and E protein genes are substituted for those in the yellow fever virus (YF), strain 17D, a widely used, licensed vaccine (Arroyo et al., 2001; Monath, 2001). This virus appears to be attenuated both by chimerization and by the presence of point mutations in the YF 17D backbone. In the present study, two molecularly engineered, live-attenuated WN virus vaccine candidates exhibited different levels of attenuation and were protective in rhesus monkeys and, therefore, have promise for use as a vaccine to prevent WN disease in humans. The first vaccine candidate, WN/DEN4, exhibited greatly reduced viremia during infection of monkeys compared to its WN or DEN4 parental virus, demonstrating that chimerization itself resulted in attenuation for a nonhuman primate. This is not surprising since a number of chimeric flaviviruses have been generated between two wild-type flaviviruses, and many of these chimeric viruses have proven to be attenuated in vivo (Pletnev et al., 1992, 1993, 2000, 2001; Bray et al., 1996; Pletnev and Men, 1998). Importantly, this observation extends previous findings that demonstrated that the Langat/DEN4 chimera was more attenuated than either parent in rhesus monkeys (Pletnev et al., 2001). This indicates that chimerization of two mosquito-borne flaviviruses as well as chimerization between a tick-borne and a mosquito-borne flavivirus results in attenuation of replication in rhesus monkeys. The decreased efficiency of interactions of gene products derived from disparate virus parents is likely the basis for the marked attenuation exhibited by these chimeric viruses in mice and monkeys. Importantly, the WN/DEN4 vaccine candidate achieved a satisfactory balance between attenuation and immunogenicity in both mice (Pletnev et al., 2002) and monkeys (present study) in that a high titer of neutralizing antibodies was induced, and immunized animals were resistant to infection with WN challenge virus. These findings are consistent with recent studies in mice that showed that B cells and antibodies play critical roles in prevention of WN infection (Diamond et al., 2003). Although the genetic basis for the attenuation phenotype of WN/DEN4 is not completely understood, it is reasonable to propose that this phenotype would be stable following replication in vivo since there is significant sequence divergence between the WN prM and E and the corresponding DEN4 proteins that are replaced in the chimeric viruses. This sequence divergence suggests that many nucleotide changes in the WN/DEN4 virus genome would have to occur following replication in vivo to restore virulence. If the attenuation phenotype were unstable, one would expect the level of replication of the vaccine virus to increase during the period of replication in vivo (Tolpin et al., 1981), but this was not observed. Instead, the pattern of viremia observed in each of the eight monkeys infected with the WN/DEN4 chimeric virus remained flat throughout the entire period of detectable infection as expected for virus with a stable attenuation phenotype.

Failure to detect viremia in monkeys infected with WN/DEN4-3′$\Delta 30$ chimera and the induction of a lower level of serum WN antibodies indicated that this virus was more

attenuated for a nonhuman primate than WN/DEN4 and that the Δ30 mutation independently attenuated the chimera for monkeys. Thus, WN/DEN4-3′ Δ30 chimeric virus is highly attenuated by two genetically stable attenuating mutations, namely, chimerization and the Δ30 mutation. Despite its high level of attenuation in monkeys, WN/DEN4-3′Δ30 chimera induced a moderate titer of serum neutralizing antibodies and completely prevented viremia when monkeys were challenged with WN, indicating that it had achieved a satisfactory balance between attenuation and immunogenicity. These findings identify the WN/DEN4-3′Δ30 chimera as a promising live-attenuated virus vaccine for humans, and we plan to initiate clinical trials this year with this candidate.

Materials and methods

Cells and viruses

Simian Vero cells (WHO seed passage 143) were obtained from FDA/WHO via the ATCC. The Vero cells had been qualified for use in production of candidate human vaccines and were used to prepare WN and DEN4 parent viruses and their two chimeric derivatives. The WN wild-type strain NY99 used in this study was kindly provided by Dr. R. Lanciotti (CDC, Fort Collins, CO); it was originally isolated from a Chilean flamingo at the Bronx Zoo (New York) in 1999 (Lanciotti et al., 1999). Wild-type DEN4 Caribbean strain 814669 prepared in Vero cells was kindly provided by Dr. S. Whitehead (NIAID). Chimeric WN/DEN4 and WN/DEN4-3′Δ30 viruses recovered from full-length infectious cDNA in Vero cells were the same as described previously (Pletnev et al., 2002).

Evaluation of parental and chimeric viruses in monkeys

Twenty rhesus monkeys (Macaca mulatta), weighing 3 to 5 kg, were screened for neutralizing antibody before the study was initiated and were found to be seronegative for WN and DEN4. Groups of four monkeys were inoculated subcutaneously (sc) with 10^5 or 10^6 FFU of chimeric WN/DEN4 (clone 18), 10^5 FFU of WN/DEN4-3′ Δ30 deletion mutant (clone 1), or 10^6 FFU of wild-type DEN4 strain 814669. Two monkeys were inoculated with 10^5 FFU of wild-type WN strain NY99, and two monkeys received 10^6 FFU of this virus. A 0.5 ml inoculum was administered at two sites, one on each upper shoulder. Monkeys were bled daily for 12 days for detection of viremia and on day 42 for measurement of neutralizing antibody titer. On day 42 postimmunization each of the immunized monkeys was challenged sc with 10^5 FFU of wild-type WN. Monkeys were bled daily for 12 days to test for viremia as well as on day 84 for measurement of neutralizing antibody. The quantity of virus in monkey serum was determined by direct titration on Vero cells using WN-specific or DEN4-specific

mouse antibodies to immunostain foci of virus infection as described previously (Pletnev et al., 2001, 2002). Serum WN or DEN4 neutralizing antibody titer was determined by FFU reduction assay for individual serum samples using wild-type WN or DEN4, respectively (Pletnev et al., 2002).

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