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Research Paper

Infectivity of West Nile/Dengue Chimeric Viruses for West Nile and Dengue Mosquito Vectors

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ABSTRACT

West Nile virus (WN), an agent of significant human and veterinary disease, is endemic in the Old World and rapidly spreading throughout the Americas. Vaccines are needed to halt the geographic expansion of this virus and prevent disease where it is established. However, to preclude introduction of a vaccine virus into the environment, a live attenuated WN vaccine should have low potential for transmission by mosquitoes. A chimeric WN vaccine candidate was previously generated by replacing the membrane and envelope structural protein genes of recombinant dengue type 4 virus (rDEN4) with those of WN; a derivative of this virus, WN/DEN4-3′Δ30, contains a 30-nucleotide deletion in the 3′ untranslated region. To assess the potential for transmission by mosquitoes of these vaccine candidates, the ability of each chimeric virus to infect the mosquito midgut, disseminate to the head, and pass into the saliva was compared to that of their wild-type parental WN and DEN4 viruses in three vector species. The WN/DEN4 chimeric viruses were significantly attenuated in both *Culex tarsalis*, a vector able to transmit WN but not dengue, and in *Ae. aegypti*, a vector able to transmit dengue but not WN. However, the chimeric viruses were as infectious as either wild-type virus for *Ae. albopictus*, a vector able to transmit both dengue and WN. These results indicate that chimerization caused a contraction in vector host range rather than universal attenuation for mosquitoes *per se*. This restriction in potential vectors renders it less likely that WN/DEN4 and WN/DEN4-3′Δ30 would be transmitted from vaccinees to mosquitoes. Key Words: Dengue virus—West Nile virus—Mosquito. Vector-Borne Zoonotic Dis. 5, 1–10.

INTRODUCTION

West Nile virus (WN) is a member of the mosquito-borne lineage of the family *Flaviviridae*, which includes such important human pathogens as dengue virus serotypes 1–4 (DEN1–4), yellow fever virus (YF), and Japanese encephalitis virus (JE) (Hayes 1989, Leyssen et al. 2002). WN is maintained in an enzootic cycle between mosquitoes and birds, with humans, horses, and other domestic and wild animals as incidental hosts (Hayes 1989). In the past 5 years, the geographic range of WN has expanded from the Old World into the Americas, resulting in disease outbreaks in humans, domestic animals, and birds (2003). The continuing spread of WN highlights the inability of mosquito control to halt the spread of this virus and the need for effective vaccines for human and veterinary use.

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To generate a live attenuated WN vaccine, our laboratory has capitalized on the striking conservation of genome organization among flaviviruses, all of which possess a single-stranded, positive-sense RNA genome that codes for a single polypeptide and is organized as follows: 5′ UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ UTR (UTR: untranslated region; C: capsid protein; prM: membrane precursor protein; E: envelope protein; NS: nonstructural protein) (Brinton 2002, Rice 1996). It is possible to create viable chimeric viruses in which the prM and E structural genes of one flavivirus are replaced with those from a second virus against which the vaccine is targeted. Attenuation can result either from chimerization itself, from attenuating mutations incorporated into the non-structural genes or UTR's, or from an interaction between these two factors. A structural gene chimeric WN vaccine candidate in which the WN prM-E genes replaced those of a wild-type recombinant dengue type 4 virus (rDEN4) (Pletnev et al. 2003, Pletnev et al. 2002) showed a 100-fold decrease in viremia in rhesus monkeys relative to its parental wild-type viruses (Pletnev et al. 2003). A derivative of WN/DEN4, created by substituting the WN prM-E genes into DEN4 vaccine candidate rDEN4Δ30 (Durbin et al. 2001), which carries an attenuating 30-nucleotide deletion in the 3′ UTR, infected each of four rhesus monkeys but did not cause a detectable viremia. Despite substantial attenuation in replication, both WN/DEN4 and WN/DEN4-3′Δ30 induced a neutralizing antibody response and protected monkeys from challenge with wild type WN (Pletnev et al. 2003). In ongoing clinical trials of WN/DEN4 and WN/DEN-3′Δ30 in horses, both viruses replicated to very low titers (<1.5 $\log_{10}$ PFU/mL) but generated a high level of neutralizing antibody (F. Fuller, A. Pletnev, et al., unpublished data). Thus both attenuated chimeric viruses are being considered as potential vaccines for both veterinary and human use, but prior to the initiation of large-scale clinical trials it was necessary to evaluate the ability of each vaccine candidate to be transmitted by mosquitoes. To preclude introduction of a vaccine virus into the environment, an acceptable live attenuated WN vaccine should have low potential for transmission.

When a mosquito feeds on a viremic host, the process of transmission may be interrupted at four different points (Hardy et al. 1983): (i) the virus may replicate to insufficient titer in the vertebrate host to infect the mosquito; (ii) the virus may fail to infect the mosquito midgut, due to either vector specificity or attenuation of the virus; (iii) the virus may fail to disseminate from the midgut to the salivary glands because of the presence of a midgut escape barrier; and (iv) a disseminated infection may fail to pass into the mosquito saliva, due either to a salivary gland infection barrier or a salivary gland escape barrier. Previous studies, discussed above, suggest that low titers in vaccinees will act as one barrier against transmission. However multiple safeguards against transmission are desirable. Therefore, in the current study, we investigated the role of the latter three barriers in reducing the potential for mosquito transmission of WN/DEN4 and WN/DEN4-3′Δ30.

In nature WN is primarily transmitted by various species of *Culex* mosquitoes, although the virus has been isolated from 29 mosquito species belonging to 10 genera in the United States alone (Campbell et al. 2002). In previous studies, we have shown that chimerization between DEN2 and DEN4 can decrease the ability of DEN2 to infect *Aedes aegypti* midguts, thereby reducing the potential for transmission (Whitehead et al. 2003). In addition, we have shown that the Δ30 mutation specifically restricts the ability of rDEN4 to disseminate from the mosquito midgut to the head, which is an indication of whether or not the mosquito is infectious, providing a further safeguard against potential transmission (Hanley et al. 2003).

In the current study, we compared the infectivity of the vaccine candidates WN/DEN4 and WN/DEN4-3′Δ30 to their WN and DEN4 wild-type parents for three species of potential vector mosquitoes in North America: (i) *Culex tarsalis*, a vector species competent for WN (Goddard et al. 2002, Turell et al. 2002) but not DEN (Rodhain et al. 1997), (ii) *Aedes aegypti*, which is the primary vector of DEN (Rodhain et al. 1997) but is not competent for WN (Turell et al. 2001), and (iii) *Aedes albopictus*, which is a competent vector of both WN (2000, Kutz et al. 2003, Sardelis et al. 2002, Turell et al. 2001a,b)

and DEN (Rodhain et al. 1997). Herein, a vector is defined as competent if it permits infection, replication and transmission of a virus (Goddard et al. 2002). Thus, this study investigates the effect of both chimerization and the Δ30 mutation on attenuation and host range of the WN/DEN4 chimeric viruses for potential mosquito vectors that differ in their susceptibility to virus infection and relative roles in WN transmission in North America.

METHODS AND MATERIALS

Cells and virus

Simian Vero cells were maintained at 37°C in an atmosphere of 5% CO_2 in MEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.05 mg/mL gentamicin (Invitrogen). The WN wild-type strain NY99 was kindly provided to us by Dr. R. Lanciotti (CDC, Ft. Collins, CO); it was originally isolated from a Chilean flamingo in the Bronx Zoo (New York) and passaged in Vero cells as previously described (Pletnev et al. 2002). The DEN4 wild-type Caribbean strain 814669 (Durbin et al. 2001) was kindly provided by Dr. Stephen Whitehead (NIAID). Chimeric virus WN/DEN4 was recovered from a full-length infectious cDNA clone (Pletnev et al. 2002) and passaged three times in Vero cells maintained in serum-free media. To generate a WN/DEN4-3′Δ30 virus as a vaccine candidate for human use and for use in this study, the chimeric virus was rescued from a full-length cDNA clone and amplified in qualified simian Vero cells (WHO Seed, passage 145) as described previously for the experimental lot of WN/DEN4-3′Δ30 (Pletnev et al. 2002). Subsequently, virus was biologically cloned by two successive passages at terminal dilution and finally amplified by three serum-free passages in qualified Vero cells. Sequence analysis of the newly derived WN/DEN4-3′Δ30 (vaccine lot) revealed that during its recovery and amplification it accumulated four coding mutations ($Gly_{314} \rightarrow Arg$ in E; $Gln_2 \rightarrow Pro$ in NS2A; $Thr_{103} \rightarrow Ala$ and $Val_{216} \rightarrow Ala$ in NS4B) that differed from the sequence of the previously described experimental WN/DEN4-3′Δ30 virus recovered from the same cDNA clone in unqualified Vero cells (Pletnev et al. 2002). Also, a mutation in NS4B, $Leu_{112} \rightarrow Ser$, that occurred in the experimental WN/DEN4-3′Δ30 virus was not found in the newly generated vaccine lot of WN/DEN4-3′Δ30. Despite the accumulation of different suites of incidental mutations, the vaccine lot of WN/DEN4-3′Δ30 showed a similar phenotype to the experimental lot in rhesus monkeys (A. Pletnev, unpublished data)

Mosquito maintenance, infection, and experimental transmission

Mosquitoes were maintained at 28°C with a 16:8 light/dark photoperiod and provided a 10% sucrose solution in cotton wicks. *Ae. aegypti* and *Ae. albopictus* were derived from previously described colonies maintained at Walter Reed Army Institute of Research (Troyer et al. 2001). *Culex tarsalis* were derived from a colony maintained at the University of California, Davis (Bellamy et al. 1958).

Mosquitoes were infected orally as previously described (Goddard et al. 2002) by feeding on hanging droplets comprised of two parts defibrinated rabbit blood (Microbiological Media, San Ramon, CA) containing 2.5% sucrose and one part virus. Because the titer of the wild-type DEN4 stock used in this experiment was approximately 10-fold higher than that of the other three viruses, two different bloodmeals were used for DEN4: one made with undiluted virus and one made with virus diluted tenfold. Infectious blood was diluted in bovine serum albumin-PBS and stored at −80°C until the titer was determined as described below. The titer of each of the bloodmeals used in this experiment fell within 0.5 $\log_{10}$pfu/mL, and usually within 0.1 $\log_{10}$PFU/mL, of expected values, indicating that titer remained steady during dilution in the bloodmeal (data not shown). Engorged mosquitoes were incubated at the conditions specified above for 14 days for each virus and then processed as described below. In addition, a subset of mosquitoes that had fed on the chimeric viruses was incubated for an additional 7 days (21 days total) to determine whether chimeric viruses might show delayed replication.

Mosquito saliva was collected as previously described (Goddard et al. 2002). Briefly, starved mosquitoes were immobilized by exposure to triethylamine and their proboscises were inserted into a capillary tube containing a 1:1 FBS and 10% sucrose solution for 10 min. Saliva was expelled into 250 μL of diluent (20% FBS, 1% mycostatin. 1% gentamicin, 4% penicillin, and 4% streptomycin in phosphate-buffered saline, PBS) and frozen at −80°C until the titer was determined. Additionally, mosquito bodies were frozen at −80°C until dissection. Mosquito head and midgut preparations were made on glass slides as previously described (Troyer et al. 2001). Immunofluorescence assay (IFA) was conducted as described previously (Troyer et al. 2001). Because previous studies had shown that immunoperoxidase assays using DEN serotype-specific monoclonal antibodies sometimes failed to stain DEN interserotypic chimeras carrying the prM-E genes from the serotype for which the antibodies were specific (K. Hanley unpublished data), a 1:1 mixture of a WN-specific antibody in hyperimmune mouse ascites fluid (HMAF) and a flavivirus-specific mouse monoclonal antibody (HB-112), each diluted 1:200 in PBS, were used as the primary antibody.

To determine WN titer in bloodmeal aliquots and in saliva, confluent Vero cell monolayers on 12-well plates were inoculated in duplicate with serial tenfold dilutions of virus, incubated for 2 days at 37°C in an atmosphere of 5% CO_2 and immunostained as previously described (Pletnev et al. 2002) using a WN-specific antibody in HMAF at a concentration of 1:1000 as the primary antibody. Because DEN4 and the WN/DEN4 chimeric viruses produce smaller plaques than WN, the procedure for immunostaining these viruses was slightly modified in that 24-well plates were inoculated with virus, incubated for 5 days, and immunostained using either a DEN4-specific HMAF at a concentration of 1:1000 (for DEN4) or a 1:1 mixture of WN and DEN4 HMAF at a final concentration of 1:1000 (for the chimeric viruses).

Statistical analyses

In this study, each mosquito was assayed for virus infection of the midgut, dissemination to the head, and presence of virus in the saliva. Each chimeric virus was analyzed on day 14 and day 21 post-infection in each species, and there was no difference between the two days in the percent infection of midguts or heads detected (Fisher's exact test, $p > 0.05$ for all comparisons), consequently day 14 and day 21 data for each virus in each mosquito species were combined in subsequent analyses. Unless otherwise specified, statistical comparisons were made using a Fisher's exact test.

RESULTS

Culex tarsalis: infection, dissemination, and presence of virus in saliva

Data for infection and dissemination in all three mosquito species are shown in Table 1. *Cx. tarsalis* has previously been shown to be a competent vector for WN (Goddard et al. 2002), and in the current study, as expected, wild-type WN produced a relatively high percentage of both midgut and disseminated infections in this species. *Culex* mosquitoes are not generally susceptible to DEN (Rodhain et al. 1997). In the current study, wild-type DEN4, even at a dose 10-fold higher than that of WN, produced a significantly lower percentage of midgut infections in *Cx. tarsalis* than wild type WN and failed to disseminate (for the justification of the comparisons made and results of statistical comparisons, see Table 2). The two chimeric viruses, WN/DEN4 and WN/DEN4-3′Δ30, behaved like their DEN4 parent, producing a significantly lower percentage of midgut infections than wild-type WN and failing to disseminate. The two chimeric viruses did not differ in their ability to infect the midgut (Table 2).

To further analyze the lack of dissemination by chimeric viruses in *Cx. tarsalis*, the observed number of disseminated infections generated by the chimeric viruses was compared to the expected number of disseminated infections. Data for the WN/DEN4 and WN/DEN4-3′Δ30 were combined for this analysis. To generate the expected number of disseminated infections, the percentage of midgut infections that disseminated to the head for the infectious wild-type WN parent virus was multiplied

TABLE 1. INFECTIVITY OF THE CHIMERIC WN/DEN4 AND WN/DEN40-3′Δ30 VACCINE CANDIDATES AND THEIR WILD-TYPE PARENTAL VIRUSES, WN AND DEN4, IN THREE SPECIES OF VECTOR MOSQUITOES

					Percentage infected	
Mosquito	*Virus*	*Day*[a]	*Bloodmeal titer (PFU)*[b]	*No.*	*Midgut*[c]	*Head*[d]
Culex tarsalis	DEN4	14	2.6×10^7	40	5	0
	WN	14	3.3×10^6	40	46	33
	WN/DEN4	14	4.0×10^6	15	7	0
	WN/DEN4	21	4.0×10^6	28	21	0
	WN/DEN4-3′Δ30	14	1.5×10^6	15	13	0
	WN/DEN4-3′Δ30	21	1.5×10^6	35	9	0
Aedes aegypti	DEN4	14	2.6×10^7	40	55	25
	DEN4	14	2.6×10^6	40	23	8
	WN	14	3.3×10^6	40	5	0
	WN/DEN4	14	4.0×10^6	40	3	0
	WN/DEN4	21	4.0×10^6	40	5	0
	WN/DEN4-3′Δ30	14	1.5×10^6	35	14	0
	WN/DEN4-3′Δ30	21	1.5×10^6	20	10	0
Aedes albopictus	DEN4	14	2.6×10^7	40	78	67
	DEN4	14	2.6×10^6	40	23	18
	WN	14	3.3×10^6	40	8	0
	WN/DEN4	14	4.0×10^6	40	10	5
	WN/DEN4	21	4.0×10^6	40	15	8
	WN/DEN4-3′Δ30	14	1.5×10^6	20	10	5
	WN/DEN4-3′Δ30	21	1.5×10^6	40	8	5

[a]Number of days that mosquitoes were incubated after feeding on an infectious bloodmeal.
[b]Virus titer in bloodmeal.
[c]Percentage of mosquitoes with detectable WN and/or DEN4 virus antigen in midgut tissue; virus antigen was identified by IFA.
[d]Percentage of mosquitoes with detectable virus antigen in head tissue.

by the number of midgut infections by the chimeric viruses. In *Cx. tarsalis*, 18 mosquitoes had a detectable WN midgut infection, of these 13 also had a detectable head infection, resulting in a rate of dissemination of midgut infections of 72%. If the rate of dissemination among the chimeric viruses had been similar, we would have expected to see 9 disseminated in-

TABLE 2. SAMPLE SIZES (N) AND p-VALUES FOR COMPARISONS OF THE PERCENTAGE OF MIDGUTS AND HEADS INFECTED BY THE SPECIFIED VIRUSES IN THE SPECIFIED MOSQUITO SPECIES USING FISHER'S EXACT TEST

Comparison		*Cx. tarsalis*			*Ae. aegypti*			*Ae. albopictus*		
Virus 1	*Virus 2*	*N*	*Midgut*[a]	*Head*[a]	*N*	*Midgut*	*Head*	*N*	*Midgut*	*Head*
WN	DEN4	80[b]	<0.0001	<0.0001	80	ns	ns	80	ns	ns
WN	WN/DEN4	83	<0.01	<0.001						
WN	WN/DEN4-3′Δ30	90	<0.002	<0.001						
DEN	WN/DEN4				120	0.002	ns	120	ns	ns
DEN	WN/DEN4-3′Δ30				95	ns	ns	100	ns	ns
WN/DEN4	WN/DEN4-3′Δ30	93	ns	ns	135	ns	ns	140	ns	ns

[a]P-value for the comparison of percentage midguts or heads infected by the two specified viruses. Two strategies were employed to limit the number of statistical comparisons and reduce the likelihood of Type I errors. First, comparisons in each mosquito species were made only between the two wild-type viruses, between the chimeric viruses and the more infectious wild-type virus, and between the two chimeric viruses. Second, a conservative alpha value of 0.01 was used to judge the significance of each comparison, thereby decreasing the likelihood of detecting a spurious significant difference. ns, a non-significant p-value (e.g., $p > 0.01$). Empty cells indicate that a statistical comparison was not made.
[b]For this comparison, data from the higher dose (2.6×10^7 PFU) of DEN4 was used; for all other comparisons, data for the lower dose of DEN4 (2.6×10^6 PFU) was used.

fections (72% of 12 midgut infections). However, the chimeric viruses produced no disseminated infections, indicating that not only were they less likely to infect the midgut, but when they did infect the midgut, they were also significantly less likely to disseminate than their WN parent virus (one-group chi-squared test, $n = 50$, $p < 0.05$).

Saliva samples were taken from each mosquito prior to dissection. In *Cx. tarsalis*, virus was not detected in the saliva of any of the mosquitoes that fed on DEN, WN/DEN4 or WN/DEN4-3′Δ30. Of the 40 mosquitoes that fed on WN, 18 (46%) had midgut infections and 13 (33%) had disseminated infections, but only one (3%) had detectable virus in the saliva. The one positive sample had a titer of 400 PFU per sample. Given the high prevalence of disseminated infections, the observation that only 3% had detectable virus present in the saliva was surprising. Colony maintenance of mosquitoes can affect susceptibility to arboviruses (Vazeille et al. 2003); to determine whether the low percentage of WN virus in saliva was a result of loss of susceptibility in the colony mosquitoes, we repeated the infections outlined in Table 1 in wild-caught *Cx. tarsalis* from Yolo County, CA. Similar to colony-reared *Cx. tarsalis*, we detected virus only in wild-caught *Cx. tarsalis* exposed to WN; of these 26 mosquitoes only one (5%) had detectable virus in the saliva, with a titer of 26 PFU per sample. Midgut and head infections were not analyzed in wild-caught mosquitoes. To determine whether another cell type might be more sensitive for detecting virus in mosquito saliva, a subset of samples were re-tested in LLC-MK_2 and C6/36 cell monolayers. Use of different cell cultures did not result in detection of an increased number of mosquitoes with virus in their saliva (data not shown).

Aedes aegypti: infection, dissemination, and presence of virus in saliva

Ae. aegypti is a competent vector of DEN (Rodhain et al. 1997) but not WN (Turell et al. 2001). In the current study, wild-type DEN4 generated a high percentage of midgut and disseminated infections in this species, whereas WN, at an equivalent dose, generated a similar percentage of midgut infections but no disseminated infections (Tables 1 and 2). The two chimeric viruses behaved like their WN parent; WN/DEN4 produced a significantly lower percentage of midgut infections than wild-type DEN4 and failed to generate a disseminated infection while WN/DEN4-3′Δ30 produced a similar percentage of midgut infections but failed to generate any disseminated infections (Tables 1 and 2). Because the overall number of disseminated infections was low in this species, comparisons among viruses were not significant. The two chimeric viruses did not differ from each other in their ability to infect the midgut (Tables 1 and 2). Because the chimeric viruses produced few midgut infections, the expected number of disseminated infections in *Ae. aegypti*, based on the number of disseminated infections generated by wild-type DEN4 was low, precluding a meaningful statistical analysis.

In *Ae. aegypti*, virus was not detected in the saliva any of the mosquitoes that fed on WN, WN/DEN4 or WN/DEN4-3′Δ30. Of the 40 mosquitoes that fed on a bloodmeal containing 2.6×10^7 PFU/mL of DEN4, 22 (55%) had midgut infections, 10 (25%) had disseminated infections, and three (8%) had detectable virus in the saliva. Two of these positive saliva samples had a titer of 1 PFU per sample, and one had a titer of 2 PFU/sample.

Aedes albopictus: infection, dissemination, and presence of virus in saliva

Ae. albopictus is a competent vector of both DEN (Rodhain et al. 1997) and WN (Sardelis et al. 2002, Turell et al. 2001a,b). The percentage of midgut infections generated by DEN4 and WN in this species was not significantly different, but WN produced no disseminated infections (Tables 1 and 2). Notably, the two chimeric viruses generated a similar percentage of midgut infections as wild-type DEN4 and both chimeras produced a similar proportion of disseminated infections to DEN4. The two chimeric viruses did not differ from each other in their ability to either infect or disseminate in *Ae. albopictus*, indicating that the Δ30 mutation had no effect on rates of dissemination in this mosquito (Tables 1 and 2). The per-

centage of disseminated infections generated by the chimeric viruses did not differ from the expected value based on the rate of dissemination by wild-type DEN4 (one-sample chi-squared test, $n = 17$, $p = 0.6$).

In *Ae. albopictus*, virus was not detected in the saliva any of the mosquitoes that fed on WN, WN/DEN4 or WN/DEN4-3′Δ30. Of the 40 mosquitoes that fed on a bloodmeal containing 2.6×10^7 PFU/mL of DEN, 31 (78%) had midgut infections, 27 (48%) had disseminated infections, and one (3%) had detectable virus in the saliva. That sample had a titer of 5 PFU per sample.

DISCUSSION

Several barriers to mosquito transmission of vaccine candidates WN/DEN and WN/DEN-3′Δ30 have been identified. In previous studies, chimeric WN/DEN4 virus induced a brief, low-level viremia in rhesus monkeys that was at least a hundred-fold reduced compared to wild-type WN or DEN4, and WN/DEN4-3′Δ30 did not generate a detectable viremia (Pletnev et al. 2003). These titers are several orders of magnitude lower than those in horses experimentally infected with wild-type WN ($\leq 10^3$ PFU/mL), which were insufficient to infect >600 *Ae. albopictus* fed on these horses (Bunning et al. 2002). Moreover, wild-type WN titers in human blood ($<10^2$ PFU/mL, as estimated from one blood donation [Iwamoto et al. 2003]), are even lower than those detected in horses. Thus, the chimeric viruses are unlikely to reach sufficient titers in vaccinees to become infectious for mosquitoes.

The WN/DEN chimeric vaccine candidates were also unable to generate disseminated infections in two mosquito vectors that play a significant role of the maintenance of WN and DEN, respectively. In this study, *Cx. tarsalis* and *Ae. aegypti* manifested the expected patterns of vector competence, showing higher levels of infections by WN and DEN4, respectively. In these two vector species, the chimeric viruses behaved like their less infectious wild-type parent, infecting only a low percentage of mosquitoes and failing to disseminate. In *Ae. albopictus*, in contrast, WN and DEN4 showed, with a similar level of infection, and both chimeric viruses produced similar percentage of both midgut and disseminated infections to the more infectious wild-type parent. Thus, chimerization led to a constriction of the vector range of the chimeric viruses and loss of ability to infect vectors competent for only one of the two parent viruses. However, the chimeric viruses were not attenuated in a vector permissive for both parents, indicating that chimerization *per se* did not cause attenuation. In contrast to previous studies (Hanley et al. 2003, Troyer et al. 2001), the Δ30 mutation did not confer an identifiable decrease in dissemination in *Ae. albopictus*, the permissive vector species. This result emphasizes the importance of testing the phenotypic effects of mutations in each new virus into which they are inserted.

Passage of the chimeric viruses into the saliva of mosquitoes was not detected in any of the three vector species used in this study. However, this data must be interpreted with caution because of the unexpectedly low transmission rate (<8%) of wild-type viruses in competent vectors. In a previous study using the same method for infecting mosquitoes and collecting saliva (Goddard et al. 2002), *Cx. tarsalis* that fed on bloodmeals containing approximately 10^7 PFU/mL of wild-type WN showed similar infection rates to those of the current study, but significantly more of these infections (>80%) were detected in saliva. In another study, 33% of *Ae. aegypti* that were orally infected with DEN4 transmitted virus to a hanging blood drop (Jirakanjanakit et al. 1999). Moreover, wild-type WN was not detected in the saliva of *Ae. albopictus*, a species previously identified as a competent vector for WN. Four factors may contribute to the relatively low percentage of saliva samples positive for wild-type virus in the current study. The first is strain differences affecting the susceptibility of the vectors to virus infection (Rosen et al. 1985). Although wild-caught *Cx. tarsalis* from Yolo county were used by both Goddard et al. (2002) and the current study, it is possible that changes in the susceptibility of the population to WN occurred in the interval between the two studies (Huber et al. 2002, Paupy et al. 2003). The second factor is differences in mutations present in the wild-type viruses that arise as they are passaged in

tissue culture. Vero-cell growth promoting mutations occur regularly in dengue viruses passaged in these cells (Blaney et al. 2003) and have been shown to affect the infectivity of dengue virus for *Ae. aegypti* (Hanley et al. 2003). Although the wild-type WN viruses used in Goddard et al. (2002) and the current study were derived from the same progenitor and each was passaged twice in Vero cells, they do not necessarily possess the same Vero cell adaptation mutations. A wide variety of adaptation mutations have been detected in independently passaged dengue viruses (Blaney et al. 2003), which vary in their effects on attenuation for mosquitoes (Hanley et al., unpublished data). If the wild-type WN and DEN4 viruses indeed acquired mutations that restricted access of virus to mosquito saliva, this would serve to further decrease the likelihood that an infected mosquito would transmit the virus to a new host. The third factor is the titer of the bloodmeals used in this study, which are high relative to a wild-type WN infection in mammals but low relative to a wild-type WN infection in some species of birds (Komar et al. 2003). Finally, technical differences between this study and Goddard et al. (2002) may contribute to the relatively low number of virus-positive saliva samples detected in this study. In this study, virus titer was quantified through immunoperoxidase staining of infected Vero cell monolayers, whereas plaque assays were used by Goddard et al. (2002).

Thus patterns of infectivity of WN/DEN4 and WN/DEN4-3′Δ30 for mosquitoes fed artificial bloodmeals support the safety of local communities during clinical trials with these vaccine candidates. However, artificial bloodmeals may be several orders of magnitude less infectious than natural bloodmeals (Weaver et al. 1993). Thus further investigation of the infectivity of these chimeric viruses for mosquito vectors fed on human vaccinees is warranted; such studies have been conducted previously for rDEN4Δ30, and transmission from vaccinees to *Ae. albopictus* was not detected (Troyer et al. 2001).

Patterns of infectivity of these chimeric viruses also provide intriguing insights into the determinants of vector specificity. Previous studies had implicated the structural genes as the primary determinants of such specificity (Beaty et al. 1981, 1982, Brault et al. 2002, Olson et al. 2000, Sundin et al. 1987, Woodward et al. 1991). For example, chimeras between enzootic and epidemic strains of Venezuelan equine encephalitis virus (VEEV) have been used to locate the determinants of vector specificity for this virus (Brault et al. 2002). In the VEEV system, enzootic strains are transmitted by *Culex* species but fail to infect *Ae. taeniorhynchus*, whereas epidemic strains are transmitted primarily by *Ae. taeniorhynchus*. Chimeric viruses carrying the PE2 gene (the glycoprotein precursor) of the epidemic strain in the background of the enzootic strain are capable of infecting *Ae. taeniorhynchus*, whereas chimeric viruses carrying the PE2 gene of the enzootic strain in the background of the epidemic strain were poorly infectious for *Ae. taeniorhynchus.* Thus, the determinants of vector specificity are located in the viral envelope for this pair of viruses (Brault et al. 2002).

In contrast to VEEV, the determinants of vector specificity identified in the current study appear to be located in both the structural genes and in the genes encoding the non-structural proteins or in the untranslated regions of the genome, since chimeric viruses carrying the WN prM and E genes in a DEN4 background failed to infect vectors specific for either WN or DEN4. A further exploration of the determinants of vector specificity in this system will require the construction of a complementary DEN4/WN virus in which the DEN4 prM-E structural genes replace those of wild-type WN.

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