GROWTH CHARACTERISTICS OF CHIMERIVAX™-DEN2 VACCINE VIRUS IN Aedes aegypti AND Aedes albopictus Mosquitoes

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Abstract. The chimeric yellow fever (YF) 17D-dengue type 2 (ChimeriVax™-DEN2) vaccine virus developed by Acambis, Inc. (Cambridge, MA) contains the prM and E genes of wild-type (wt) dengue 2 (DEN-2) (strain PUO-218) virus in the YF vaccine virus (strain 17D) backbone. The potential of ChimeriVax™-DEN2 virus to infect and be transmitted by Aedes aegypti, the principal DEN and YF virus mosquito vector, and Aedes albopictus, a species that occurs in areas of active transmission of YF and DEN viruses, was evaluated. Mosquitoes were intrathoracically (IT) inoculated with virus or were fed a virus-laden blood meal, and the replication kinetics of ChimeriVax™-DEN2 were compared with the wt DEN-2 and YF 17D vaccine viruses. Replication of YF 17D virus is attenuated in cultured Ae. albopictus C6/36 mosquito cells and in Ae. aegypti and Ae. albopictus mosquitoes. Growth of ChimeriVax™-DEN2 virus similarly was restricted in C6/36 cells and in mosquitoes. ChimeriVax™-DEN2 replicated in 56% of IT inoculated Ae. aegypti, and virus disseminated to head tissue in 36%, with a mean viral titer of 1.8 log10 PFU/mosquito. Of mosquitoes, 16% of Ae. aegypti and 24% of Ae. albopictus were infected 14 days after a blood meal containing ChimeriVax™-DEN2, but virus did not disseminate to head tissue. In contrast, DEN-2 replicated in all IT inoculated and orally infected Ae. aegypti (mean titer 5.5 log10 PFU/mosquito), and virus disseminated to head tissue in 95%. Of Ae. albopictus, 84% were infected after a blood meal containing DEN-2 virus; dissemination occurred in 36%. Replication of ChimeriVax™-DEN2 virus in mosquitoes corresponded to that of YF 17D vaccine virus, which is restricted in its ability to infect and replicate in mosquitoes. Therefore, transmission of ChimeriVax™-DEN2 virus by vector mosquitoes is unlikely.

INTRODUCTION

Dengue (DEN) viruses are important arthropod-borne pathogens, which cause dengue fever (DF) and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) in humans.1 The virus is transmitted through the bite of infected female Aedes (Sabethes) mosquitoes, primarily Aedes aegypti, and, depending on the geographic area, Aedes albopictus and Aedes polynesiensis.2 The mosquito vectors and the DEN viruses are found throughout the tropics and sub-tropics; thus, approximately 2.3 billion people are at risk of contracting DEN infection. Currently, there are 100 million DF cases and hundreds of thousands of DHF/DSS cases per year.3

DEN viruses are positive-stranded RNA flaviruses comprised of 4 closely related but antigenically distinct serotypes, designated DEN-1, DEN-2, DEN-3, and DEN-4. Infection by one DEN serotype does not protect against infection with other serotypes because antibodies raised against one serotype cross-react with virus antigen of a second serotype but are not cross-protective.1 Previous infection by one serotype may enhance infection of the second DEN serotype by opsonization. Antibody-dependent enhancement of the second DEN serotype during an infection is hypothesized to cause DHF/DSS, which has a 20% fatality rate if left untreated.4 All 4 serotypes are circulating throughout the tropics; consequently an individual may be exposed to all 4 DEN serotypes in their lifetime.1 Serologic analysis has shown that patients can be infected concurrently with more than one DEN serotype.5,6 Therefore, a tetravalent vaccine against all 4 DEN serotypes is essential. There is currently no effective vaccine against DEN viruses, and efforts to develop a vaccine have been complicated by the need to administer vaccines against all 4 serotypes simultaneously.

Yellow fever (YF) 17D is a live, attenuated virus that has been a safe and effective vaccine in use for > 50 years.7 A chimeric, recombinant vaccine virus, ChimeriVax™, has been developed by Acambis Inc. (Cambridge, MA) that uses the yellow fever (YF) 17D virus capsid and nonstructural genes and the noncoding regions as a vector. The premembrane, membrane (prM) and envelope (E) genes are replaced with homologous genes of a flavivirus of interest.8 ChimeriVax™ virus has been developed for Japanese encephalitis (JE).9,10 DEN serotypes 1–4,11,12 and West Nile13 viruses. Previous studies showed that growth of ChimeriVax™-JE, Acambis, Inc., Cambridge, MA and ChimeriVax™-DEN2 viruses is attenuated in vaccinated nonhuman primates, and yet the immunogenic response provides robust immune responses and protection against virus infection.11,14 The potential of ChimeriVax™-JE virus to infect and be transmitted by mosquitoes also was evaluated.15 ChimeriVax™-JE did not infect the JE vector mosquito Culex tritaeniorhynchus, and replication of ChimeriVax™-JE in the YF vectors Ae. aegypti and Ae. albopictus was limited and similar to that of YF 17D virus. We report here on vector competence studies with ChimeriVax™-DEN2 vaccine virus.

ChimeriVax™-DEN2 virus contains the prM and E genes of wild-type (wt) DEN-2 (strain PUO-218) virus in YF 17D virus (Figure 1).11 ChimeriVax™-DEN2 was evaluated successfully in preclinical tests and currently is being produced under good manufacturing practice (GMP) for a Phase I study.12 Before a candidate vaccine can be used, the possibility that a vector mosquito species could be infected with the vaccine virus from the blood of a vaccinated individual and transmit it must be evaluated. We tested the potential for ChimeriVax™-DEN2 virus to transmit to Aedes aegypti, the primary vector of YF and DEN viruses, and Ae. albopictus, a species of possible importance. Mosquitoes either were inoculated with virus or were infected orally to compare the replication kinetics of ChimeriVax™-DEN2 virus with YF17D vaccine and wt DEN-2 viruses. Inoculation was included to preclude the potential infection barriers in the midgut associated with oral feeding. Oral infection and dissemination of ChimeriVax™-DEN2 virus were tested in 2
populations of laboratory-reared *Ae. aegypti*; one field-collected population of *Ae. aegypti* from the Pacific island of Vanuatu, where dengue epidemiology have occurred; and a laboratory strain of *Ae. albopictus*.

**MATERIALS AND METHODS**

**Mosquitoes.** *Aedes aegypti* from the laboratory colonies DQ (Dequincy, LA) and PR (Puerto Rico) and *Ae. albopictus* LC (Lake Charles, LA) were provided by Roger Nasci (Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, Fort Collins, CO). *Aedes aegypti* collected in the Republic of Vanuatu (F2), an island in the Pacific Ocean, were provided by Thomas Burkot (consultant to the Secretariat of the Pacific Community). F2 and F3 generations from these mosquitoes were used in this study. Larvae were reared in aluminum pans, approximately 200 per pan, on a diet of 0.4% liver powder and rabbit chow (3 pellets/pan). Pupae were transferred to screened cages. Adult mosquitoes were maintained on a 16-hour light, 8-hour dark cycle at 27°C and 85% humidity on a diet of 5% sugar water.

**Virus preparation.** Yellow fever 17D vaccine virus (Aventis-Pasteur, Swiftwater, PA, lot VA293AA) was used unpasaged. ChimeriVax-DEN2 virus was obtained from Acambis Inc. at passage 2 (Vero Green monkey kidney) and was passaged once in Vero cells, harvested at 4 days, and stored in 15% fetal bovine serum (FBS) at −70°C. DEN-2 PUO-218 virus was provided by Acambis Inc (C6/36-2, Vero-1), passed once in C6/36 cells, harvested at 6 days, and stored in 15% FBS at −70°C. Stock virus titers were as follows: YF 17D, 6 log<sub>10</sub> plaque-forming units (PFU)/ml; ChimeriVax-DEN2, 7.3 log<sub>10</sub> PFU/ml; and DEN-2, 7.1 log<sub>10</sub> PFU/ml.

**Virus titration.** Virus titer was assessed by plaque titration. Individual mosquitoes were triturated by pestle in 1.7-ml tubes (Kontes Glass Co, Vineland, NJ) in 1 ml of BA-1 diluent (1X M-199 medium with Hank’s salts, 0.05M Tris, pH 7.6, 1% bovine albumin, 0.35 g/L of sodium bicarbonate, 100 of U/ml penicillin, 100 μg/mL of streptomycin, 1 μL/ml of fungizone). Mosquito suspensions were clarified by centrifugation at 10,000 rpm for 5 minutes. Virus titer was determined by double-overlay plaque assay in Vero cells as previously described. Second overlays were applied after a 4-day incubation period for YF 17D and ChimeriVax<sup>TM</sup>-DEN2 viruses and after 6 days for wt DEN-2 virus.

**Immunohistochemistry.** Viral dissemination in the mosquito was determined by direct immunofluorescent antibody staining of head tissue as described previously. Fluorescein isothiocyanate (FITC)-conjugated anti-YF virus mouse IgG ascitic fluid (Jackson Laboratories, West Grove, PA, lot 12185; diluted 1:15) was used to detect YF 17D virus. FITC-conjugated antiflavivirus human sera (DFA; CDC, diluted 1:80) was used to detect DEN-2 and ChimeriVax-DEN2 viruses.

**Growth curves.** Ten-day growth curves were conducted for each of the ChimeriVax<sup>TM</sup>-DEN2, DEN-2, and YF 17D viruses in Vero and C6/36 cells. Cell monolayers were infected at a multiplicity of infection of 0.1 PFU per cell. After a 1-hour adsorption, monolayers were washed, and cells were maintained in Dulbecco’s modified minimal essential medium containing gentamicin and 5% FBS. Samples were removed every other day for 10 days, and titers were determined by plaque titration as described previously.

**Intrathoracic inoculation of mosquitoes.** Mosquitoes that had enclosed 1–2 days earlier were cold-anesthetized and inoculated intrathoracically (IT) using a micropipette needle that had been pulled to a point with a Narishige (Tokyo) needle puller. Approximately 0.34 μl of virus standardized to 6.0 log<sub>10</sub> PFU/ml was injected into each mosquito (2.5 log<sub>10</sub> PFU/mosquito). Inoculated mosquitoes were maintained in cartons with 5% sugar water at 27°C and 85% humidity. Three mosquitoes were collected at 24-hour intervals for 8 days; 26 were collected at 14 days postinoculation and were dissected immediately or frozen at −70°C until assayed.

**Oral infection of mosquitoes.** Fresh virus was prepared in Vero cells as described previously. Virus supernatant was collected 4–6 days after infection (70% cytopathic effect, (CPE)) and clarified by centrifugation at 10,000 rpm, 4°C for 20 minutes. Calf’s blood in Alsever’s solution (Colorado Serum Co, Denver, CO) was washed with ice-cold PBS 3 times. Mosquitoes 7–10 days old that had been starved for 24–48 hours were fed an artificial-virus blood meal consisting of equal parts washed calf’s blood and freshly harvested virus, heated quickly to 37°C, and dropped onto the mesh tops of mosquito cartons. Mosquitoes were allowed to feed for 20 minutes, then were cold-anesthetized and sorted. Fully engorged mosquitoes were collected and incubated at 27°C and 85% humidity on sugar water as described previously. Mosquitoes were removed at 14 days and were stored at −70°C until they were assayed for infection.

**Sequencing of virus isolated from mosquitoes.** Genomic sequences of the DEN-2 prME region in ChimeriVax-DEN2 were obtained from 2 mosquitoes that were positive for virus infection at 14 days after IT inoculation. Mosquito tissues were triturated in 1 ml of BA-1 diluent and clarified by centrifugation. Mosquito suspension, 100 μl, was used to inoculate a T-25 cm<sup>2</sup> flask of Vero cells as described previously. Virus supernatant was collected at 5 days, ~70% CPE. Viral RNA was isolated using the QIAamp viral RNA kit (Qiagen, Valencia, CA). The DEN-2 prM and E genes and surrounding YF 17D gene region were amplified using the Titan One Tube RT-PCR Kit (Roche Diagnostics, Mannheim, Germany) and primers designed from the ChimeriVax<sup>TM</sup>-DEN2 virus sequence (forward position 463: 5′-GCTGTGTATGACGGGTGGA-3′; reverse position 2557: 5′-CTTGTTCAGCCAGTCATGAGTC-3′). The resultants 2,094-bp DNA product was purified by gel electrophoresis followed by extraction from the gel using the Qiaquick gel extraction kit (Qiagen, Valencia, CA) and se-
sequenced using an ABI Prism Dye Terminator Cycle sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (PE: Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on an ABI Prism 377 automated DNA Sequencer (PE: Applied Biosystems).

RESULTS

Virus replication in vitro. All viruses replicated in C6/36 and Vero cells (Figure 2). DEN-2 virus reached a titer of 7.1 log_{10} PFU/ml in C6/36 cells on day 4, whereas ChimeriVax™-DEN2 virus was more restricted in replication in these cells, reaching a peak titer of 5.5 log_{10} PFU/ml on day 10. The peak titer of YF 17D in C6/36 cells was 6.5 log_{10} PFU/ml. DEN-2, ChimeriVax™-DEN2, and YF 17D viruses replicated equally well in Vero cells (Figure 2B), with peak titers of approximately 7.5 log_{10} PFU/ml at days 6–8.

Virus replication and dissemination in intrathoracically inoculated mosquitoes. Growth of YF 17D and ChimeriVax™-DEN2 viruses in IT inoculated *Ae. aegypti* and *Ae. albopictus* was limited compared with wt DEN-2 virus (Figure 3). The peak titer in *Ae. aegypti* inoculated with ChimeriVax™-DEN2 virus was 3.5 log_{10} PFU/mosquito at 4 days postinoculation, which was 1 log lower than the peak titer of 4.5 log_{10} PFU/mosquito for YF 17D and > 2 logs lower than the peak titer of 5.9 log_{10} PFU/mosquito in wt DEN-2–infected mosquitoes (Figure 3A). The peak titer of ChimeriVax™-DEN2 virus IT inoculated into *Ae. albopictus* was 3.1 log_{10} PFU/mosquito, which was 1 and 2.5 logs lower than the peak viral titers of YF 17D and wt DEN-2 viruses (Figure 3B).

In a separate experiment, virus replication and dissemination was assayed in IT inoculated *Ae. aegypti* after an extrinsic incubation period of 14 days (Table 1). Of *Ae. aegypti* IT inoculated with 6 log_{10} PFU/ml ChimeriVax™-DEN2 virus (2.5 log_{10} PFU/mosquito), 56% (14 of 25) remained infected at 14 days after inoculation (mean titer of positives, 1.8 log_{10} PFU/ml), and virus was disseminated to the head in 36% (9 of 25) (mean titer of positives 1.4 log_{10} PFU/mosquito). Of mosquitoes IT inoculated with YF17D virus, 92% (24 of 26) were infected at 14 days (mean titer ± SD of positives, 2.1 ± 1.1 log_{10} PFU/mosquito); in 18 of 26 mosquitoes, virus titer did not increase above the inoculum dose of 2.5 log_{10} PFU/mosquito (data not shown). Virus disseminated to head tissue in 69% (18 of 26; mean titer of positives, 1.8 log_{10} PFU/mosquito) of mosquitoes IT inoculated with YF17D. Of mosquitoes IT inoculated with DEN-2 virus became infected (mean titer, 5.0 log_{10} PFU/mosquito) and developed a disseminated infection (mean titer in head tissue, 4.8 log_{10} PFU/mosquito).

Oral infection of mosquitoes. Oral infection and dissemination rates of ChimeriVax™-DEN2 virus were tested in 2 populations of laboratory-reared *Ae. aegypti*, one population of *Ae. aegypti* from the Pacific island of Vanuatu, and a laboratory strain of *Ae. albopictus* (Table 2). After the blood meal, mosquitoes were maintained for an extrinsic incubation period of 2 weeks. Overall, 17% (11 of 66) of *Ae. aegypti* ingesting an artificial blood meal containing 6.3–7.5 log_{10} PFU/ml of ChimeriVax™-DEN2 virus were infected at 14...
days, but virus did not disseminate to head tissue in any of these mosquitoes (0 of 66). In *Ae. albopictus*, 24% (6 of 25) were infected 14 days after a blood meal, but virus did not disseminate to the head. Replication was similar in mosquitoes that ingested a blood meal consisting of 6.5–6.9 log10 PFU/ml of YF 17D virus; 17% (4 of 24) of *Ae. aegypti* and 24% (6 of 25) of *Ae. albopictus* became infected, but no dissemination occurred. Virus titer in mosquitoes infected with ChimeriVax™-DEN2 or YF 17D viruses was low (mean titer of positives, 1 log10 PFU/mosquito) (Table 2). In contrast, 100% (31 of 31) of *Ae. aegypti* ingesting a blood meal consisting of 5.9–6.4 log10 PFU/ml of DEN-2 virus were infected at 14 days, and 84% (21 of 25) of *Ae. albopictus* were infected with DEN-2 virus at 14 days. DEN-2 virus disseminated to the head in 95% (29 of 31) of *Ae. aegypti* and 36% (9 of 25) of *Ae. albopictus*. DEN-2 virus titer was 3–6 log10 PFU/mosquito.

**DISCUSSION**

The YF 17D vaccine has been in use for > 60 years, with > 400 million doses given, and is one of the most successful and safe vaccines developed to date. In early vector competence studies of YF 17D vaccine virus, researchers showed that *Ae. aegypti* did not become infected by feeding on a viremic vaccinee; replication of YF 17D virus in artificially infected mosquitoes was restricted to low titer, and none of these mosquitoes were capable of transmitting the virus. More recently, growth of a chimeric vaccine virus developed against JE, ChimeriVax™-JE, has been shown to be similarly restricted in *Ae. aegypti* and *Ae. albopictus* mosquitoes. In these experiments, the replication kinetics of ChimeriVax™-DEN2 virus were shown to be similar to YF 17D and ChimeriVax™-JE viruses in *Ae. albopictus* C6/36 cells and in *Ae. aegypti* and *Ae. albopictus* mosquitoes.

The mosquito midgut is an important factor in susceptibility to virus infection and vector competence. The ability of a virus to infect the midgut epithelial cells has a genetic basis in the vector mosquito and in the virus strain. It has been shown that a virus can replicate in mosquito species that are not the natural vectors and that are not naturally susceptible to infection with the virus when the virus is introduced by inoculation. To assess the growth of virus in mosquito tissue, without the midgut infection barrier as a factor, mosquitoes were IT inoculated with virus. Replication of ChimeriVax™-DEN2 and YF 17D viruses IT inoculated into mosquitoes was reduced 3 logs compared with wt DEN-2 virus; the titer of ChimeriVax™-DEN2 virus was approximately 100 PFU/mosquito, compared with a mean titer of 10^7 PFU/mosquito in mosquitoes IT inoculated with wt DEN-2 virus. In a competent mosquito vector, virus amplifies throughout the mosquito during the extrinsic incubation period. The mean viral titer in mosquitoes IT inoculated with ChimeriVax™-DEN2 or YF 17D viruses remained below the inoculum dose of approximately 2.5 log10 PFU/mosquito at 14 days. In contrast, in *Ae. aegypti* and *Ae. albopictus* mosquitoes, both competent vectors of DEN virus, DEN-2 virus titer...
increased 2.5 logs to 5 log_{10} PFU/mosquito during the 14-day extrinsic incubation period.

Different geographic populations of *Ae. aegypti* vary in susceptibility to infection by DEN and YF viruses.\(^{20,21}\) In laboratory studies using artificial blood meals similar to those described here, infection rates ranged from 15%–100% between mosquito populations. The Puerto Rican mosquito population is considered to be susceptible to DEN-2 virus infection, whereas the DQ strain is less susceptible.\(^{29}\) Maintenance of mosquito populations in an insectary over many generations results in selection for certain traits and may change the susceptibility for a virus by the mosquitoes.\(^{23}\) To minimize any effect that the use of a laboratory-adapted strain might have on the outcome of these experiments, the vector competence of field-collected *Ae. aegypti* from the Republic of Vanuatu for ChimeriVax™-DEN2 virus also was assessed. All 4 DEN virus serotypes have been recorded in the Republic of Vanuatu, and *Ae. aegypti* is considered to be the primary mosquito vector.\(^{22,23}\)

In our studies, all 3 *Ae. aegypti* populations were 100% susceptible to oral infection by DEN-2 virus. Infection rates of ChimeriVax™-DEN2 virus ranged from 6% in PR mosquitoes to 32% of DQ mosquitoes. Similarly, YF 17D infected from 0% of the PR *Ae. aegypti* to 22% of DQ mosquitoes. All 3 mosquito populations tested were susceptible to infection by wt DEN-2 virus but were poorly infected by ChimeriVax™-DEN2 and YF 17D viruses.

Previous experiments showed that dissemination of virus to head tissue corresponds to salivary gland infection.\(^{24}\) Because the virus is introduced to the vertebrate host through the bite via saliva, dissemination also can be correlated with virus transmission.\(^{24}\) The presence of virus in mosquito head tissue was used as an assay for transmission potential. In our experiments, disseminated infection occurred in 84% of *Ae. aegypti* orally ingesting a blood meal containing DEN-2 virus, which correlates to the competence of *Ae. aegypti* as a vector for DEN-2 virus. Conversely, virus did not disseminate to head tissue in any of the mosquitoes fed an artificial blood meal of either ChimeriVax™-DEN2 or YF 17D virus. Thus, it is unlikely that ChimeriVax™-DEN2 virus would be transmitted by mosquitoes.

To ascertain the stability of the *prM* and *E* nucleotide sequence in ChimeriVax™-DEN2 after replication in mosquitoes, virus was isolated from 2 infected *Ae. aegypti*, and the *prM* and *E* regions were sequenced and compared with ChimeriVax™-DEN2 seed virus sequence. In one of the virus isolates, a 1-nucleotide change resulted in an aspartate-to-glycine amino acid change at amino acid 29 in the preenvelope region of the *M* protein. The premembrane is post-translationally cleaved from the *M* protein during virion maturation.\(^{25}\) The *E* protein is involved in virus-cell attachment and determines tissue tropism. Presumably the protein structure in this gene region is optimized for entry into mosquito and human cells in wt virus, and an amino acid change would not be expected to increase virulence in humans or increase replication efficiency in mosquito tissue. Single amino acid changes can cause attenuation. The amino acid change observed in our study apparently did not affect the replication kinetics of the chimera in mosquitoes because the titer of ChimeriVax™-DEN2 virus in this individual mosquito was 2 log_{10} PFU, and the mean titer of the mosquitoes IT inoculated with ChimeriVax™-DEN2 virus at 14 days was 1.8 log_{10} PFU/mosquito (Table 1).

In nonhuman primates infected with ChimeriVax™-DEN2 virus, restricted replication occurs, resulting in a low level of viremia (peak titer, 1.6 log_{10} PFU/ml).\(^{31,32}\) In these experiments, a low percentage of mosquitoes feeding on an infectious blood meal of 5.9–7.5 log_{10} PFU/ml were infected with ChimeriVax™-DEN2, and the virus did not disseminate to head tissue in any of the mosquitoes. Low host viremia, combined with poor oral infectivity of ChimeriVax™-DEN2 in vector mosquitoes, makes infection of mosquitoes through the bite of a vaccinated host unlikely. In addition, ChimeriVax™-DEN2 virus is not able to disseminate to salivary gland or head tissue in orally infected mosquitoes, and there is little potential for transmission of the vaccine virus by vector mosquitoes.

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