Dynamics of Susceptibility and Transmissibility of the Live, Attenuated, Candidate Vaccines Dengue-1 PDK13, Dengue-3 PGMK30F3, and Dengue-4 PDK48 After Oral Infection in Aedes aegypti

Nuananong Jirakanjanakit, Mi Mi Khin, Sutee Yoksan, and Natth Bhamarapratwi
Center for Vaccine Development, Institute of Science and Technology for Development, Mahidol University at Salaya, Nakornpathom, Thailand

Abstract. Dengue-1 virus PDK13 and isolates from vaccinees (dengue-1 Ib1 and dengue-1 Ib10), dengue-3 PGMK30F3, and dengue-4 PDK48 were studied for their abilities to infect, disseminate, and replicate in Aedes aegypti mosquitoes by the oral route. In general, infection and dissemination rates were poorer for the vaccine compared with the parent viruses. The transmissibility was also lower for dengue-1 PDK13 than the parent virus, whereas it was not detected for isolates from vaccinees and dengue-3 PGMK30F3. Transmissibility of dengue-4 PDK48 was not determined because no dissemination occurred. Replication rates of vaccine strains were also found to be less efficient than the parent viruses. These imply that vaccination with the candidate vaccine is safe. Moreover, vector attenuation of vaccine viruses was consistent with its phenotypic markers of attenuation, which remained stable after a mosquito passage or after human and mosquito passage.

Dengue (DEN) is a global disease of the tropics and one of the most important emerging diseases, with half of the world population at risk.1 Since the 1940s, an approach of disease prevention has been directed towards a search for a vaccine.2,3 Live, attenuated, candidate DEN-1 PDK13, DEN-2, PDK53, DEN-3 PGMK30F3, and DEN-4 PDK48 vaccine viruses were developed by the Center for Vaccine Development at Mahidol University (Nakornpathom, Thailand).4-7 The DEN-1 PDK13, DEN-2 PDK53, and DEN-4 PDK48 candidate vaccine viruses were derived from serial passages of the parent viruses DEN-1 16007, DEN-2 16681 and DEN-4 1036 in primary dog kidney (PDK) cell cultures (National Institute of Public Health and Environment Protection, Bilthoyen, The Netherlands).4,8 The DEN-3 PGMK30F3 vaccine was derived from serial passages of DEN-3 16562 virus in primary green monkey kidney (PGMK) cells for 30 consecutive passages and an additional 3 passages in certified fetal rhescus liver (FRhl) cells (American Type Culture Collection, Rockville, MD). These DEN virus candidate vaccines have met the appropriate requirements of biologic attenuation.4 However, during phase 1 and phase 2 clinical evaluations of DEN candidate vaccine viruses, some volunteers experienced a transient period of viremia, during which dengue viruses were recovered.5 The possibility of the vector Aedes aegypti feeding on a viremic individual exists, especially when immunization programs are conducted. This concern has provoked an investigation of the stability of the biologic characteristics of DEN candidate vaccines after oral infection into Ae. aegypti. It is expected that the vaccine should not infect mosquito by oral route. However, if it does, the vaccine virus should infect the mosquito poorly and be inefficiently transmitted. Moreover, the vaccine virus should not revert to a virulent form, so that immunization with the vaccine will not disseminate pathogenic dengue virus in nature. Therefore, vector attenuation is an important consideration for a dengue candidate vaccine.

Our previous study on DEN-2 PDK53 candidate vaccine after oral infection into Ae. aegypti showed that the capacity for oral infection and dissemination of DEN-2 PDK53 were significantly lower than that of the parent virus, and that transmission by mosquitoes orally infected with the vaccine virus was not demonstrated.9 Moreover, the stability of biologic characteristics of DEN-2 PDK53 has confirmed that immunization with this candidate vaccine was safe. Therefore, similar studies were carried with DEN-1 PDK13, DEN-3 PGMK30F3, and DEN-4 PDK48 vaccines.

Materials and Methods

Viruses. Dengue-1 16007 virus was isolated from the serum of a patient with dengue hemorrhagic fever (DHF) in Bangkok in 1964, DEN-3 16562 virus was isolated from a patient with DHF in The Philippines in 1964, and DEN-4 1036 virus was isolated from a patient with dengue fever (DF) in Indonesia in 1978. All of these viruses were provided by Dr. D. J. Gubler (Centers for Disease Control and Prevention, Fort Collins, CO). The DEN-1 PDK13, DEN-3 PGMK30F3, and DEN-4 PDK48 candidate vaccines were selected based on changes in biologic characteristics indicative of viral attenuation.4 The DEN-1 Ib1 and DEN-1 Ib10 viruses were recovered on day 5 and day 12 post-immunization, respectively, in LLC-MK2 cell cultures from two vaccinees receiving the monovalent DEN-1 PDK13 vaccine.

Each serotype of the viruses used in the present study was passaged 3 times in LLC-MK2 cell cultures. A direct fluorescent antibody test (DFAT)10 was used to check for the presence of viruses. A confirmation of type specificity was done by an indirect fluorescent antibody test (IDFAT)11 using monoclonal antibody against each dengue serotype. Harvested virus suspension with 20% heat-inactivated calf serum was aliquoted into 1-dram vials and stored at −80°C before use.

Mosquitoes. Aedes aegypti (collected from Bangkok, Thailand) reared in the insectary of the Center for Vaccine Development at Mahidol University since 1987 was used. Five-day-old female mosquitoes were deprived of a sugar meal for 48 hr before being tested.

Oral Infection. The experiments were conducted in the same way as the study on DEN-2 PDK53. Briefly, infectious blood meals were prepared from equal volumes of virus suspension, 10% sugar solution, and washed guinea pig erythrocytes. Drops of an infectious blood meal were placed
on nylon mesh covering cardboard cartons holding mosquitoes. After 1 hr, fully engorged mosquitoes were separated and incubated for 14 days at 32°C at a relative humidity of 80%. Mosquitoes were then assayed for infection by the DFAT on a body squash and for dissemination on a head squash of the same mosquito. In vitro transmission was determined from orally infected mosquitoes that transmitted virus when feeding on a drop of blood suspension after 28 days of incubation.

Replication rates were determined by virus assay of infected mosquitoes from various incubation periods. Geometric mean titers from 5 mosquitoes were used. Biologic attributes, plaque size morphology, cytopathic effect in LLC-MK2 cells, temperature sensitivity, and mouse neurovirulence, were checked in infected mosquitoes after incubation for 28 days as previously described.

**Virus assay.** Virus assays for titration of pre-feeding and post-feeding meals and replication rates in infected mosquitoes were done by intrathoracic inoculation using 3-5-day-old *Toxorhynchites splendens*. Viral antigens were detected by the DFAT, and the virus titers were calculated by the method of Reed and Meunch.

**Statistical analysis.** Differences of each parameter between candidate vaccines and the parent viruses were evaluated by the chi-square test. Significant differences were determined at a 95% confidence level.

**RESULTS**

Infectious blood meals were assayed for the amount of viruses from both prefeeding and post-feeding meals. The post-feeding blood meal, collected after 1 hr of mosquito feeding at room temperature (26°C), showed no significant decrease in titer (0.1–0.4 log10 50% mosquito infectious dose [MID50/ml]) compared with the pre-feeding blood meal (Table 1).

Infection, dissemination, and transmission rates of the parent and candidate vaccine viruses were obtained from orally infected *Ae. aegypti* with infectious blood meal titers of 7.3–8.1 log10 MID50/ml (Table 1). Infection rates of DEN-1 PDK13, DEN-1 Ib1, and DEN-1 Ib10 were lower than those of the parent virus DEN-1 16007. However, statistical analysis showed no significant differences from the parent virus, except for DEN-1 Ib10 (χ² = 7.43, P < 0.01). Dissemination rates of DEN-1 PDK13, DEN-1 Ib1, and DEN-1 Ib10 were about half of those of DEN-1 16007. These differences were statistically significant (χ² = 19.55, 13.70, and 31.13, respectively, P < 0.001). In vitro transmission of DEN-1 PDK13 (13%) was only about one-third that of DEN-1 16007 (36%). However, it was not significantly different at a 95% confidence level because only a few mosquitoes were tested (P = 0.1416, by Fisher’s exact test). Moreover, we could not detect any transmission in the mosquitoes infected with the vaccine isolates from vaccinees, since few mosquitoes could be tested for the transmissibility due to low infection rates.

The infection rate for DEN-3 PGMK30F3 was about one-third as high as its parent virus (DEN-3 16562), while the dissemination rate was only one-sixth as high. These differences were statistically significant (χ² = 36.95 and 45.29, P < 0.001). In vitro transmission of DEN-3 PGMK30F3 was not detected; it was 38% for DEN-3 16562. Similar results were obtained from the studies on DEN-4 PDK48, which showed a much lower infection rate compared with its parent virus (DEN-4 1036) (0.8% versus 22%). The DEN-4 PDK48 virus was not disseminated in the mosquitoes; therefore, in vitro transmission was not studied. It was also observed that mosquitoes orally infected with vaccine viruses and isolates from vaccinees showed only a small amount of antigen in head and body squash preparations, 10–20% of the tissue, as detected by the DFAT.

Replication rates of parent viruses in orally infected mosquitoes on various days postinfection were generally found to be approximately 100 times higher than in the vaccine viruses (Table 2). However, the peak titers of most viruses in orally infected mosquitoes were observed by day 21. Replication of DEN-4 PDK48 virus was not demonstrated since the infection rate was very low (0.8%).

Concern for the capability of DEN vaccine viruses to be transmitted from vaccinees prompted us to study the vector...
competence of *Ae. aegypti*. The experiments were carried out to evaluate the result of infecting the mosquito through the parenteral route (intrathoracic inoculation). Successful infection and dissemination rates (100%) were obtained for both DEN-3 PGMK30F3 and DEN-3 16562, while *in vitro* transmission was 57% (26 of 46) for the virus. This was significantly lower ($\chi^2 = 6.8, P < 0.01$) than the 82% (46 of 56) for its parent virus (Table 1). Replication rates of DEN-3 virus after parenteral infection showed higher titers for both DEN-3 PGMK30F3 and DEN-3 16562 compared with the result of oral infection (Table 2). However, the peak titer of DEN-3 16562 after parenteral inoculation was obtained only by day 14, while for DEN-3 PGMK30F3 it was obtained by day 21 and showed a lower titer than the parent virus. It was also observed that the intensity of fluorescence staining became stronger if the vaccine-infected mosquitoes were incubated for a longer period.

Markers originally used as indicators of attenuation were retained in DEN-1 PDK13, DEN-3 PGMK30F3 and DEN-4 PDK48 viruses after passage in mosquitoes and in isolates from vaccinees (DEN-1 Ib1 and DEN-1 Ib10) after passage in humans and mosquitoes (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Viruses*</th>
<th>Infecting titer log$<em>{10}$MID$</em>{50}$/ml$^\dagger$</th>
<th>Geometric mean titer log$<em>{10}$MID$</em>{50}$/ml for varying incubation period (day)$^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN-1 16007 (OR)</td>
<td>8.5</td>
<td>7.2</td>
</tr>
<tr>
<td>DEN-1 PDK13 (OR)</td>
<td>7.6</td>
<td>5.3</td>
</tr>
<tr>
<td>DEN-1 Ib1 (OR)</td>
<td>7.3</td>
<td>5.0</td>
</tr>
<tr>
<td>DEN-1 Ib10 (OR)</td>
<td>6.9</td>
<td>4.9</td>
</tr>
<tr>
<td>DEN-3 16562 (OR)</td>
<td>7.8</td>
<td>5.3</td>
</tr>
<tr>
<td>DEN-3 PGMK30F3 (OR)</td>
<td>8.0</td>
<td>4.7</td>
</tr>
<tr>
<td>DEN-3 16562 (PR)</td>
<td>7.7</td>
<td>7.1</td>
</tr>
<tr>
<td>DEN-3 PGMK30F3 (PR)</td>
<td>7.9</td>
<td>6.3</td>
</tr>
<tr>
<td>DEN-4 1036 (OR)</td>
<td>7.8</td>
<td>4.8</td>
</tr>
<tr>
<td>DEN-4 PDK48 (OR)</td>
<td>8.1</td>
<td>NR</td>
</tr>
</tbody>
</table>

* Parent = DEN-1 16007, DEN-3 16562, and DEN-4 1036; Vaccine = DEN-1 PDK13, DEN-3 PGMK30F3, and DEN-4 PDK48; Isolates from vaccines = DEN-1 Ib1 and DEN-1 Ib10. OR = oral infection; PR = parenteral infection.

$^\dagger$ Mean titer log$_{10}$MID$_{50}$/ml.

$^\ddagger$ Orally infected DEN-2 PDK53 and DEN-4 PDK-TD3FRHLP3 vaccines. A possible explanation is that the virus vaccine had been modified during serial passages in unnatural hosts (PDK, PGMK, or FrHL cells), which affected the ability to infect mosquitoes. It should be noted that DEN-3 PGMK30F3 and DEN-4 PDK48 had more passages in cell culture than DEN-1 PDK13, which could represent a higher level of attenuation. Therefore, it appeared that infection and dissemination capacity correlate well with level of attenuation. Alternatively, the DEN vaccine viruses might have lost their capability to replicate and disseminate into other tissues of orally infected mosquitoes. Miller and others found that viral antigen was present in large amounts only in the mesenteronal tissue of *Ae. aegypti* orally infected with DEN-2 PR159/S1 vaccine virus.15 They proposed that

### Table 3

<table>
<thead>
<tr>
<th>Viruses*</th>
<th>CPE in LLC-MK$_2$ cells$^\ddagger$</th>
<th>Plaque size$^\ddagger$</th>
<th>Temperature sensitivity (°C)</th>
<th>Mouse survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN-1 16007§</td>
<td>+</td>
<td>M + S</td>
<td>40</td>
<td>13.9</td>
</tr>
<tr>
<td>DEN-1 PDK13¶</td>
<td>None</td>
<td>PP</td>
<td>39</td>
<td>19.9</td>
</tr>
<tr>
<td>DEN-1 Ib1§</td>
<td>None</td>
<td>PP</td>
<td>39</td>
<td>20.2</td>
</tr>
<tr>
<td>DEN-1 Ib10§</td>
<td>None</td>
<td>PP</td>
<td>39</td>
<td>19.6</td>
</tr>
<tr>
<td>DEN-3 16562§</td>
<td>+</td>
<td>L</td>
<td>40.5</td>
<td>18.8</td>
</tr>
<tr>
<td>DEN-3 PGMK30F3¶</td>
<td>None</td>
<td>S + PP</td>
<td>38.0</td>
<td>20.8</td>
</tr>
<tr>
<td>DEN-3 PGMK30F3§</td>
<td>None</td>
<td>S + PP</td>
<td>38.0</td>
<td>20.6</td>
</tr>
<tr>
<td>DEN-4 1036§</td>
<td>None</td>
<td>M + S</td>
<td>40.0</td>
<td>8.4</td>
</tr>
<tr>
<td>DEN-4 PDK48¶</td>
<td>None</td>
<td>PP</td>
<td>38.0</td>
<td>11.0</td>
</tr>
<tr>
<td>DEN-4 PDK48§</td>
<td>None</td>
<td>PP</td>
<td>38.0</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* Parent = DEN-1 16007, DEN-3 16562, and DEN-4 1036; Vaccine = DEN-1 PDK13, DEN-3 PGMK30F3, and DEN-4 PDK48; Isolates from vaccines = DEN-1 Ib1 and DEN-1 Ib10.

$^\ddagger$ CPE = cytopathic effect.

$^\ddagger$ M = medium (2–4 mm); S = small (1 mm); PP = pinpoint (<1 mm); L = large (>5 mm).

$^\ddagger$ Before mosquito passage.
virus replicated in the midgut of the mosquito but was unable to mature and escape into the hemocoel, or to attach and replicate in secondary organ systems. Kuberski suggested that dissemination of viruses could occur via the hemolymph. Therefore, failure of virus to infect the salivary gland and neutral tissue might be due to the low level of infectious particles in the hemolymph, which could be below the threshold required to infect these tissues. Our study did not indicate whether viral particles in body squash preparations were from the mesenteronal cells or partly from the hemolymph. However, it was observed that only a small amount of antigen in the tissue from body squash and head squash preparations of orally infected mosquito could be detected by fluorescent staining.

It is interesting that viral antigen was found in the head squash preparations of mosquitoes orally infected with vaccine viruses (dissemination occurs), but little or no transmission was detected. Several findings have suggested that it was a positive correlation between quantity of virus in mosquito and its ability to be transmitted that affected the transmission was detected. Several findings have suggested that it was a positive correlation between quantity of virus in mosquito and its ability to be transmitted that affected the percentage of mosquitoes capable of transmission. Evidence that supports this concept is derived from the result obtained from intrathoracic inoculation in which a high percentage of transmission (57%) of DEN-3 PGMK30F3 from parenterally infected mosquitoes was achieved compared with no transmission from orally infected mosquitoes. This indicated that transmission is, in part, limited to the amount of virus that could escape from the gut to the hemocoel. Although infection and dissemination were successful in mosquitoes parenterally infected with DEN-3 PGMK30F3, the transmission rate was significantly lower than in its parent virus. This was also evident in a study of Japanese encephalitis (2–8) vaccine. It may have been due to a defect at the salivary gland level, apart from a gut barrier demonstrated in various studies. Our findings are also compatible with a study of Culex tarsalis orally infected with low doses of Western equine encephalitis virus. Kramer and others had proposed a mesenteronal and salivary gland infection barrier that was dose-dependent. However, in view of our result, the capability of the virus itself (viral attenuation) might have had a direct effect on the susceptibility and transmissibility in mosquitoes.

The magnitude of viral replication after oral infection in Ae. aegypti appeared to be much less for vaccine viruses than for the parent viruses; this was related to a low dissemination rate. An increase in the virus titer by intrathoracic inoculation indicates a defect at the mesenteronal level in the orally infected mosquito. It is interesting to note that the peak titer of DEN-3 16562 virus after intrathoracic inoculation was at day 14, while that of DEN-3PGMK30F3 virus was at day 21. This is similar to the results obtained with oral infection. Therefore, vaccine viruses had poorer growth than their corresponding parent viruses, in addition to the effect of the route of infection. This correlates well with the observation of stronger fluorescence staining when the vaccine-infected mosquitoes were incubated for a longer period. Additional evidence of a slower growth rate with a lower titer of vaccine virus than the parent virus in mosquitoes comes from the study of DEN-4 PDK-TD3FrhLp3. The precise mechanisms that bring about vector attenuation for vaccine viruses remain unknown. Such knowledge is needed for further investigations.

Acknowledgments: We express our gratitude to the research staff (K. Sinsatitsukul, S. Warasumpati, and K. Kowjatturas) for significant contributions to the research presented herein. The excellent preparation of the mosquitoes used throughout this work by P. Jibkokwai is gratefully acknowledged. We are also grateful to Dr. Warren Broekleman for generous help in correcting the paper.

Financial support: This research was supported by World Health Organization Regional Office for Southeast Asia, Project Identification SN:39, and by the Thai government.

Authors’ address: Nuananong Jirakanjanakit, Mi Mi Khin, Sutee Yoksan, and Nath Bhamarapravati, Center for Vaccine Development, Institute of Science and Technology for Development, Mahidol University at Salaya, Nakornpathom 73170, Thailand.

REFERENCES


