Heterotypic Dengue Infection with Live Attenuated Monotypic Dengue Virus Vaccines: Implications for Vaccination of Populations in Areas Where Dengue Is Endemic

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Background. Because infection with any of the 4 Dengue virus serotypes may elicit both protective neutralizing antibodies and nonneutralizing antibodies capable of enhancing subsequent heterotypic Dengue virus infections, the greatest risk for severe dengue occurs during a second, heterotypic Dengue virus infection. It remains unclear whether the replication of live attenuated vaccine viruses will be similarly enhanced when administered to Dengue-immune individuals.

Methods. We recruited 36 healthy adults who had previously received a monovalent live Dengue virus vaccine 0.6–7.4 years earlier. Participants were assigned to 1 of 4 cohorts and were randomly chosen to receive placebo or a heterotypic vaccine. The level of replication, safety, and immunogenicity of the heterotypic vaccine virus was compared with that of Dengue virus immunologically naive vaccinees.

Results. Vaccine virus replication and reactogenicity after monovalent Dengue virus vaccination in naive and heterotypically immune vaccinees was similar. In contrast to naive vaccinees, the antibody response in heterotypically immune vaccinees was broadly neutralizing and mimicked the response observed by natural secondary Dengue virus infection.

Conclusions. Enhanced replication of these live attenuated Dengue virus vaccines was minimal in heterotypically immune vaccinees and suggests that the further evaluation of these candidate vaccines in populations with preexisting DENV immunity can proceed safely.


Dengue has become the most important arbovirus worldwide, with a 30-fold increase in incidence over the past 50 years. It is estimated that 50 million infections occur annually among the ~2.5 billion persons living in regions of endemicity [1]. Children bear most of the dengue-associated disease burden, which is estimated to be as high as 616,000 disability-adjusted life-years [2].

Dengue viruses (DENVs) are members of the Flavivirus genus of the Flaviviridae family [3]. There are 4 DENV serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Each serotype can cause the full spectrum of dengue illness, which can range from an asymptomatic or mildly symptomatic infection to dengue fever (DF) or to the most severe form of the disease, dengue hemorrhagic fever/shock syndrome (DHF/DSS).

Infection with 1 DENV serotype generates long-term homotypic immunity but is thought to generate only short-term heterotypic immunity [4, 5]. Epidemiologic studies have demonstrated that preexisting immunity to 1 DENV serotype may confer a greater risk of developing more severe disease (DHF/DSS) after subsequent infection with a heterotypic DENV [6–8]. Nonneutralizing, heterotypic antibody is thought to bind to DENV, improving its ability to infect Fcγ-
receptor-bearing cells, leading to enhanced virus production from the greater number of infected cells, a phenomenon
designated antibody-dependent enhancement (ADE) of in-
fec tion [9–11]. The consequence of ADE is a potential 100-fold
increase in viremia, which has been shown to correlate with
more severe disease in patients with DENV infection [12].
Experim entally, ADE has been demonstrated in an AG129
mouse model and in nonhuman primates [13, 14]. For these
reasons, a successful Dengue vaccine should induce long-lived
immunity to each of the 4 serotypes without inducing enhanced
disease in heterotypically immune vaccinees [15]. On the basis
of the success of other live attenuated flavivirus vaccines for
yellow fever and Japanese encephalitis virus, development of
live attenuated Dengue vaccine candidates appears to be an
economical and effective strategy. Nevertheless, several chal-
gen es must be overcome. Because the vaccine will be in-
tr oduced in regions of endemicity with populations that have
preexisting DENV antibody, there is concern that ADE of
vaccine replication could produce a viral load sufficient to cause
disease. In addition, individuals may be at risk for severe disease
if the vaccine fails to induce a balanced immune response to all
serotypes or if antibody titers wane over time. To date, the live
attenuated tetravalent DENV vaccines evaluated in humans
have failed to induce high seroconversion rates to all 4 serotypes
with a single dose [16, 17]. For this reason, the proposed dosing
schedule of a tetravalent DENV vaccine in advanced clinical
evaluation includes 3 doses of vaccine at time 0, 6, and 12
months [17, 18].

Our group has evaluated numerous live attenuated mono-
valent DENV vaccines to determine which candidates, based on
the safety and immunogenicity profile, should be included in
a tetravalent formulation [19–22]. Because of the theoretical
consc erns of enhanced reactogenicity of live DENV vaccines when
administered to Dengue-exposed populations, we evaluated how
the safety, replication, and immunogenicity of 2 of our vaccine
candidates would be altered when administered to persons
with known preexisting heterotypic DENV antibody. For these studies,
preexisting Dengue antibody was elicited by vaccination, which
serves as a surrogate for naturally acquired DENV immunity, and
is considered to be heterotypic when it is elicited by a virus of
another serotype (eg, different envelop protein). Among the 4
groups studied, we observed a small but significant increase in
mean peak virus titer in the group receiving the DENV-2 vaccine
2–7 years after receipt of a DENV-4 vaccine. The level of vaccine
virus replication in heterotypically immune vaccinees remained
low and did not result in an increase in reactogenicity.

METHODS

Regulatory Oversight
This randomized, double-blind, placebo-controlled study was
conducted at the Center for Immunization Research at The
Johns Hopkins Bloomberg School of Public Health under an
investigational new drug application reviewed by the US Food
and Drug Administration. All study documents were approved
by the Western Institutional Review Board and the Johns
Hopkins University Institutional Biosafety Committee. Healthy
adult male and nonpregnant female participants who were
previously flavivirus seronegative and received a monovalent
live attenuated dengue vaccine were recruited among persons
previously enrolled in dengue vaccine trials at the Center for
Immunization Research. Informed consent was obtained in
accordance with the Code of Federal Regulations (CFR21, Part
50).

Study Design and Clinical Monitoring
Healthy persons aged 18–50 years were enrolled if they met the
following eligibility criteria: previous receipt of the DENV-1
vac cine (rDEN1A30) [19], the DENV-2 vaccine (rDENV2A4A30)
[20], or a DENV-4 vaccine (rDEN4A30 or rDEN4A30-200,201)
[21–23]; normal findings during physical examination; negative
for human immunodeficiency virus antibody, hepatitis C virus,
and hepatitis B surface antigen; normal values for complete
blood count, with differential serum aspartate aminotransferase,
alanine aminotransferase, total bilirubin, alkaline phosphatase,
serum creatinine, serum creatine phosphokinase, prothrombin
time, and partial thromboplastin time; and normal urinalysis
results. Female participants were required to have a negative
result of a urine pregnancy test at least 3 days before vaccination
and on the day of vaccination and to agree to use contraception
or abstain from sexual intercourse for the duration of the study.
Participants were enrolled in 1 of 4 heterotypic vaccination
cohorts: participants who had previously received a DENV-4
vaccine received either the DENV-1 or DENV-2 vaccine, par-
ticipants who had previously received a DENV-1 vaccine re-
ceived the DEN2 vaccine, and participants who had previously
received a DENV-2 vaccine received the DENV-1 vaccine [19–
22]. Previous and heterotypic vaccination occurred using the
same vaccine lots prepared for each serotype. Each cohort
consisted of 8–10 participants randomized to receive 10^7 PFU of
the indicated vaccine or placebo (1 or 2 placebo recipients per
cohort) as a single .5 mL subcutaneous injection on study day 0.
Each participant was given a digital thermometer and a diary
card to record their oral temperature 3 times per day for the first
16 days of the study. Participants returned to the clinic every
other day through study day 16 and again on days 21, 28, and 42.
A medical provider performed physical assessments every study
visit, and participants were questioned at each visit about
symptoms of illness. Blood samples were collected at each study
visit for clinical laboratory studies and virology through study
day 28, as described elsewhere [22]. Blood samples for serologic
testing were collected on study days 0, 28, and 42. Adverse events
were graded as mild (easily tolerated), moderate (interferred with
daily activity or required medication), or severe (prevented daily

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activity). Abnormal hematology and serum chemistry findings were also graded as mild, moderate, or severe. Dengue-like illness was defined as infection associated with fever (oral temperature, >38°C, single measurement) and >2 of the following clinical signs: moderate headache lasting ≥12 h, moderate photophobia lasting ≥12 h, or moderate generalized myalgia lasting ≥12 h. Members of the study staff were blinded with regard to each participant’s vaccination status until all participants in a cohort reached study day 42 (completion).

**Virus Quantitation**

Serum virus titers were determined using a standard plaque assay as described elsewhere [19, 20]. In brief, serum dilutions were used to infect duplicate wells of Vero cell monolayers. Virus plaques were identified by immunoperoxidase staining with dengue antibody. Virus was also amplified by inoculating serum directly onto Vero cell monolayers and incubating for 5 days. Tissue culture fluids were then titrated for virus as described.

**Serologic Assessment**

Antibody response to each DENV serotype was determined by 60% plaque reduction neutralization titer (PRNT$_{60}$) assay on Vero cell monolayers as described elsewhere [19, 20, 23]. Seroconversion to each DENV serotype was defined as a ≥4-fold increase in PRNT$_{60}$ to the wild-type DEN virus strains (DEN1 WP, DEN2 NGC, DEN3 Yogyakarta/94, and DEN4 Dominica/81) on study day 28 or 42, compared with the prevaccination titer (day 0). Heterotypic antibody to DENV-1 and DENV-2 was detected by standard enzyme-linked immunosorbent assay (ELISA) against purified whole virus (DEN1-WP or DEN2-NGC).

**Data Analysis**

Baseline characteristics and frequency of vaccine-related adverse events, graded by severity, were compared between vaccine and placebo groups, and were additionally compared with a historical group of participants who received the DENV-1 or DENV-2 vaccine in previous trials [19, 20]. In addition, the mean peak virus titer from each cohort was compared with that attained by participants vaccinated with the same vaccine virus as a primary immunization in a previous study. Statistical significance was determined by analysis of variance and the Tukey-Kramer post-hoc test. Correlation between peak virus titer and duration of the interval between primary and secondary dengue vaccination was also determined. Frequencies were compared using the Fisher exact test.

**RESULTS**

**Demographic Characteristics**

Thirty-six persons aged 24–50 years were enrolled in the study (Table 1). Seven to 8 persons in each cohort received a heterotypic DENV vaccine 7 months–7.4 years after primary DENV vaccination; 6 received placebo (Table 1). There was no statistically significant difference in the mean age (mean ± standard deviation [SD]) of vaccinees (38 ± 1.6 years) and placebo recipients (40 ± 3.8 years). Fifty percent of participants were female; 63% identified as black, 33% as white, and 3% as multiracial. All participants completed the trial.

**Reactogenicity**

There were no serious adverse events reported during this trial. There was no statistically significant difference in the occurrence of solicited adverse events in vaccines, compared with placebo recipients or previously evaluated flavivirus-naive vaccines [19, 20] (Table 2, Supplemental Table 1). Nine vaccinees (30%) and 1 placebo recipient developed a mild maculopapular rash similar to that observed in previous trials with these candidate vaccines [19, 20]. Four vaccinees and 1 placebo recipient developed mild neutropenia (absolute neutrophil count [ANC], 1000–1500 neutrophils/mm$^3$). One participant who received DENV-1 vaccine after primary DENV-4 vaccination developed a grade 3 neutropenia (ANC, <500 neutrophils/mm$^3$). This volunteer developed a grade 1 neutropenia on study day 14 (ANC, 1000 neutrophils/mm$^3$). The ANC decreased to 400 neutrophils/mm$^3$ on study day 16, and by the next study visit at day 21, had returned to 3000 neutrophils/mm$^3$. The participant was viremic on study days 8 and 12, with a peak virus titer of 1.5 log$_{10}$ PFU/
mL, but had no other adverse events related to vaccination other than a moderate headache on study days 13–15.

Viremia
A statistically significant difference in the mean peak titer, onset, and duration of viremia for DENV-1 vaccine was not observed when given as a second, heterotypic vaccination, compared with primary vaccination of naive volunteers (Table 3). Similarly, a statistically significant difference in the mean peak titer, onset, and duration of viremia for DENV-2 vaccine was not observed when it was given after DENV-1 vaccination (Table 3). However, the mean peak titer of DENV-2 vaccine, when given after DEN4, was significantly higher than observed during primary vaccination (1.1 ± .2 log10 PFU/mL vs .5 ± .03 log10 PFU/mL). In addition, the onset of viremia was significantly earlier (day 5 ± 1 vs day 9.2 ± .6). Despite this minor but significant increase in mean peak virus titer, an increase in the reactivity of the vaccine was not observed (Table 2). The magnitude of the peak virus titer in vaccinees correlated positively with the duration of interval between primary and secondary dengue vaccination in cohort 3 (P = .02) and when all cohorts were combined (P = .01). Peak virus titer did not correlate with heterotypic neutralizing antibody titer at the time of vaccination.

Serology
All vaccinees who received DEN-1 vaccine after primary vaccination with a DENV-4 vaccine seroconverted to DEN-1, consistent with seroconversion rates after primary DENV-1 vaccination (Table 4, Supplemental Table 2). However, participants who received DEN-1 vaccine 2–3 years after DENV-2 vaccination had a seroconversion rate to DENV-1 of only 57%, which was significantly lower than that observed for primary DENV-1 vaccination (P < .05, Fisher exact test) and lower than the seroconversion rates observed for the other heterotypic vaccine cohorts (Tables 4 and 5). Of note, these participants had detectable DENV-2 neutralizing antibody at the time of DENV-1 vaccination. In addition, this cohort had lower seroconversion rates to DENV-2, DENV-3, and DENV-4 than did the other cohorts (Table 5). All participants who received the DENV-2 vaccine 6–6.6 years after primary vaccination with DENV-1 or a DENV-4 vaccine seroconverted to DENV-2 (Table 4, Supplemental Table 2). In contrast to primary vaccination, which resulted in seroconversion to only the infecting dengue virus, heterotypic vaccination induced high seroconversion rates to heterotypic dengue viruses, including DENV-3, in all cohorts except the one that received DENV-1 vaccine after DENV-2 vaccine (Table 5). Approximately 63% of vaccinees had heterotypic antibody detected by ELISA on day 42 after primary vaccination that persisted until the time of heterotypic vaccination (Supplemental Table 2). Heterotypic antibody against DENV-1 was undetectable by ELISA after administration of a DENV-4 vaccine; however, such vaccination readily elicited detectable antibody to DENV-2.

DISCUSSION
The development of a live attenuated tetravalent dengue vaccine is an advantageous strategy to protect against dengue disease for several reasons. First, infection with DEN2 4
probably lifelong, protection against disease caused by a homologous serotype [5, 24]. Second, the use of live virus vaccines has been successful for other flaviviruses, such as yellow fever and Japanese encephalitis virus [25, 26]. Third, the high yield and high level of infectivity of our live attenuated DENV vaccines makes them economically feasible vaccine candidates [19–
21]. Because the DENV vaccine candidates examined in the current study have promising safety and immunogenicity profiles, it seemed prudent to examine the effect of preexisting heterotypic immunity on these profiles for at least 2 of our monotypic live attenuated DENV vaccine candidates, including one of the chimeric vaccine candidates.

As an experimental model of vaccine introduction in areas of endemcity, we sought to evaluate whether preexisting heterotypic immunity would enhance the replication and reactogenicity of our live DENV candidate vaccines. Because these experimental studies were conducted in an area where dengue is not endemic in persons formerly flavivirus seronegative, pre-existing immunity was induced by vaccine virus rather than by wild-type DENV. Experimental infection in susceptible adults permits frequent sampling to measure the level and duration of viremia and to compare the mean peak titer, onset, and duration of viremia in heterotypically immune vaccinees and immunologically naive vaccinees. Viremia in humans with DF peaks at 10^8 infectious units/mL of blood and can increase to values up to 10^9 in humans with DHF/DSS [12, 27]. Thus, a relative increase in viremia of nearly 100-fold or an increase in viremia to levels associated with DF in our heterotypic vaccinees would be considered to be problematic. However, we observed only a 4-fold increase in mean peak viral load in only 1 of 4 cohorts (DENV-2 vaccine after DENV-4 vaccine), compared with primary DENV-2 vaccination. The peak viral load was only 10^1

### Table 3. Magnitude, onset, and duration of viremia in persons inoculated with a rDEN1Δ30 as a primary or secondary vaccination and persons inoculated with rDEN2Δ30 as a primary or secondary vaccination

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Previous inoculation</th>
<th>Vaccine dose, log_{10} PFU</th>
<th>No. of volunteers</th>
<th>Percentag e/viremic (no.)</th>
<th>Mean peak titer, \log_{10} PFU/mL ± SE</th>
<th>Mean onset of viremia, day ± SE</th>
<th>Mean duration of viremia, days ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN1Δ</td>
<td>None</td>
<td>3.0</td>
<td>71</td>
<td>61 (43)</td>
<td>1.0 ± .1</td>
<td>10.0 ± .3</td>
<td>3.2 ± .3</td>
</tr>
<tr>
<td>DEN1Δ</td>
<td>DEN4C</td>
<td>3.0</td>
<td>8</td>
<td>63 (5)</td>
<td>1.0 ± .2</td>
<td>8.6 ± .5</td>
<td>3.6 ± .9</td>
</tr>
<tr>
<td>DEN1Δ</td>
<td>DEN2</td>
<td>3.0</td>
<td>7</td>
<td>29 (2)</td>
<td>0.5 ± 0</td>
<td>10.5 ± 3.5</td>
<td>3.0 ± 0</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>None</td>
<td>3.0</td>
<td>40</td>
<td>60 (24)</td>
<td>0.5 ± .03</td>
<td>9.2 ± .6</td>
<td>3.3 ± .6</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>DEN4C</td>
<td>3.0</td>
<td>8</td>
<td>75 (6)</td>
<td>1.1 ± 0.2d</td>
<td>5.0 ± 1.0d</td>
<td>5.0 ± 1.0d</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>DEN1</td>
<td>3.0</td>
<td>7</td>
<td>43 (3)</td>
<td>0.7 ± .2</td>
<td>11.0 ± 2.1</td>
<td>2.7 ± 1.7</td>
</tr>
</tbody>
</table>

**NOTE.**

* Data are from two clinical trials in which volunteers received a single dose of 3 log_{10} PFU of live attenuated DEN1 vaccine candidate rDEN1Δ30.

b Calculated only for volunteers with detectable level of viremia (≥ 5 log_{10} PFU/mL serum).

c Volunteers previously vaccinated with rDEN4Δ30 or rDEN4Δ30-200,201.

d Data are from two clinical trials in which volunteers received a single dose of 3 log_{10} PFU of live attenuated DEN2 vaccine candidate rDEN2/4Δ30.

### Table 4. Neutralizing antibody titers against DEN1 after primary or secondary DEN1 vaccination and against DEN2 after primary or secondary DEN2 vaccination

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Previous inoculation</th>
<th>No. of volunteers</th>
<th>DENV used in assay</th>
<th>GMT on day 42 (range)a</th>
<th>Percentag e seroconversionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN1Δ</td>
<td>None</td>
<td>70</td>
<td>DEN1</td>
<td>128 (&lt;5 – 1242)</td>
<td>93</td>
</tr>
<tr>
<td>DEN1Δ</td>
<td>DEN4f</td>
<td>8</td>
<td>DEN1</td>
<td>452 (132 – 1386)</td>
<td>100</td>
</tr>
<tr>
<td>DEN1Δ</td>
<td>DEN2d</td>
<td>7</td>
<td>DEN1</td>
<td>114 (&lt;10 – 358)</td>
<td>57c</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>None</td>
<td>40</td>
<td>DEN2</td>
<td>104 (16 – 843)</td>
<td>100</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>DEN4f</td>
<td>8</td>
<td>DEN2</td>
<td>74 (21 – 158)</td>
<td>100</td>
</tr>
<tr>
<td>DEN2Δ</td>
<td>DEN1d</td>
<td>7</td>
<td>DEN2</td>
<td>92 (37 – 239)</td>
<td>100</td>
</tr>
</tbody>
</table>

**NOTE.**

a Geometric mean titer and range (reciprocal PRNT60) is calculated for all subjects who received vaccine. Samples evaluated in a separate assay than those used for titers shown in Table 5 and Supplemental Table 2.

b Defined as a ≥ 4-fold rise in serum neutralizing antibody by study day 42 compared with study day 0.

c Seroconversion rate was significantly lower compared to DEN1 vaccine given as a single primary vaccination (P < .05, Fisher exact test).

d The DEN1 vaccine is rDEN1Δ30.

e The DEN2 vaccine is rDEN2/4Δ30.

f Subjects received either rDEN4Δ30 or rDEN4Δ30-200,201 as the DEN4 vaccine.
In addition, the onset of viremia was earlier in this cohort. As expected, this level of viremia was not accompanied by an increase in vaccine reactogenicity. In all 4 cohorts, reactogenicity was comparable between heterotypically immune and immunologically naïve vaccinees, an observation reflecting the low level of replication of each of the vaccine candidates. We would suggest that the attenuating mutations in the vaccine candidates restricted virus replication in target cells, including those expressing the Fcγ receptor, precluding clinically relevant increases in overall vaccine virus replication.

The present study also demonstrated that live attenuated DENV vaccine candidates induced a homotypic and heterotypic antibody response similar to that induced by natural dengue infection. After primary DENV vaccination, participants developed a homotypic neutralizing antibody response with only sporadic, low titer neutralizing antibody observed for heterotypic viruses. This is the pattern seen after infection with wild-type DENV infection [30]. Many of our participants had detectable homotypic neutralizing and heterotypic ELISA antibody induced by their primary immunization at the time of secondary vaccination, some for as long as 7 years. After secondary DENV vaccination, a broad, heterotypic neutralizing antibody response was induced in each of the 4 cohorts. Remarkably, seroconversion rates to DENV-3 ranged from 57% to 100%, despite the lack of exposure to DENV-3 in all participants (Table 5). Even DENV-2 vaccination after DENV-4 vaccination induced a broad heterotypic response, despite these 2 viruses sharing the same nonstructural proteins. Because the level of DENV-2 vaccine replication was comparable (actually slightly higher) in DENV-4 vaccinees and naïve vaccinees, it is clear that the immunity induced by the shared capsid and nonstructural proteins of the DENV-4 vaccine did not decrease the replication of the chimeric DENV-2 vaccine. The ability of secondary

### Table 5. A secondary heterotypic vaccination elicits a broadly neutralizing antibody response to DENV

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Previous DENV administered</th>
<th>Serotype used in neutralization assay</th>
<th>Mean neutralizing antibody titer on indicated day of vaccination (range)</th>
<th>Percentage seroconversiona</th>
<th>Type of antibody response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 42</td>
<td></td>
</tr>
<tr>
<td>DEN1</td>
<td>None</td>
<td>DEN1</td>
<td>&lt; 10</td>
<td>44 (10 – 162)</td>
<td>88 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN2</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>0 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>10 (&lt;10 – 19)</td>
<td>0 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>&lt; 10</td>
<td>10 (&lt;10 – 10)</td>
<td>0 heterotypic</td>
</tr>
<tr>
<td>DEN1</td>
<td>DEN4</td>
<td>DEN1</td>
<td>&lt; 10</td>
<td>264 (91 – 1041)</td>
<td>100 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>20 (&lt;10 – 70)</td>
<td>193 (15 – 1238)</td>
<td>88 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN2</td>
<td>&lt; 10</td>
<td>169 (34 – 417)</td>
<td>75 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>176 (33 – 1112)</td>
<td>75 heterotypic</td>
</tr>
<tr>
<td>DEN1</td>
<td>DEN2</td>
<td>DEN1</td>
<td>&lt; 10</td>
<td>47 (&lt;10 – 178)</td>
<td>57 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN2</td>
<td>27 (11 – 197)</td>
<td>106 (&lt;10 – 603)</td>
<td>71 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>32 (15 – 74)</td>
<td>57 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>&lt; 10</td>
<td>22 (&lt;10 – 95)</td>
<td>43 heterotypic</td>
</tr>
<tr>
<td>DEN2</td>
<td>None</td>
<td>DEN2</td>
<td>&lt; 10</td>
<td>335 (71 – 885)</td>
<td>100 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN1</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>0 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>10 (&lt;10 – 14)</td>
<td>0 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>&lt; 10</td>
<td>10 (&lt;10 – 17)</td>
<td>0 heterotypic</td>
</tr>
<tr>
<td>DEN2</td>
<td>DEN4</td>
<td>DEN2</td>
<td>&lt; 10</td>
<td>367 (212–1287)</td>
<td>100 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>11 (&lt;10 – 34)</td>
<td>130 (11 – 429)</td>
<td>88 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN1</td>
<td>&lt; 10</td>
<td>57 (29 – 297)</td>
<td>63 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>125 (&lt;10 – 363)</td>
<td>88 heterotypic</td>
</tr>
<tr>
<td>DEN2</td>
<td>DEN1</td>
<td>DEN2</td>
<td>&lt; 10</td>
<td>209 (56 – 491)</td>
<td>100 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN1</td>
<td>8 (&lt;10 – 21)</td>
<td>81 (48 – 184)</td>
<td>86 homotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN3</td>
<td>&lt; 10</td>
<td>180 (58 – 633)</td>
<td>100 heterotypic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEN4</td>
<td>6 (&lt;10 – 16)</td>
<td>31 (15 – 68)</td>
<td>57 heterotypic</td>
</tr>
</tbody>
</table>

**NOTE.** a Geometric mean titer (reciprocal PRNT<sub>60</sub>) is presented. A titer of < 10 was assigned a value of 5 for calculation of mean titers. b Defined as a ≥ 4-fold rise in serum neutralizing titer compared with day 0. c One vaccinee had a detectable PRNT<sub>60</sub> of 19 to DEN3. d One vaccinee had a detectable PRNT<sub>60</sub> of 10 to DEN4 (different vaccinee than described in footnote c). e One vaccinee had a detectable PRNT<sub>60</sub> of 14 to DEN3. f One vaccinee had a detectable PRNT<sub>60</sub> of 17 to DEN4 (different vaccinee than described in footnote e).
DENV vaccination to induce a broad heterotypic neutralizing antibody response is encouraging, but the durability of this heterotypic response is unknown.

In populations living in areas where dengue is endemic, which are the principal targets for these vaccines, it has been observed that the majority of dengue disease occurs after the first or second DENV infection, with few third and fourth infections with heterologous DENV serotypes resulting in illness [31]. In these populations, immunity broadens after sequential infection, and protective immunity against all 4 DENV serotypes is likely to be a combination of both homotypic and heterotypic immune responses. Recent observations from a phase 2b live attenuated tetravalent DENV vaccine study suggest that this phenomenon may reduce the number of vaccinations required to achieve immunity to all 4 serotypes [17, 18].

Previous studies from Cuba have reported enhanced disease severity with a longer interval between primary and secondary DENV infection [32]. Although peak virus titers were not reported from the Cuban epidemic of 1997, the authors suggested that decreased avidity of the heterotypic antibody for the DENV-2 virus led to an increased severity of disease by means of ADE. We demonstrated that peak virus titer was positively correlated with the duration between primary DENV vaccination and secondary heterotypic vaccination, recapitulating the Cuban observations.

The correlation between the duration between primary and secondary vaccination remained valid for all sequences of secondary infection, with the exception of DENV-1 vaccination after DENV-2. However, even at the longest durations between vaccine exposures in the current study, the peak titers achieved remained in a safe and acceptable range.

DENVs are known to induce long-lived homotypic antibody but only short-term heterotypic antibody [4, 33]. In the present study, primary vaccination elicited detectable heterotypic antibody in approximately half of the volunteers (Supplementary Table 2). However, the presence or absence of heterotypic antibody did not modify the immunogenicity of the secondary vaccination with regard to either the level of boost in the original homotypic immune response or the level of homologous antibody elicited by the secondary antibody. The fact that the vaccine candidates infected both naive and heterotypic seropositive vaccinees equally well without increased reactogenicity is an indication that the vaccines should be safe for use in areas where dengue is endemic. Of interest, we were able to demonstrate partial heterotypic cross-protection to DENV-1 infection for at least 3 years after primary DENV-2 vaccination. Cohort 2, which received DENV-1 vaccine 2–3 years after DENV-2 vaccine, had a significantly lower rate of infection (57%) with the DENV-1 vaccine than with primary DENV-1 vaccination (86%) (Table 5). All of these participants had detectable DENV-2 neutralizing antibody, but not DENV-1 antibody, at the time of DENV-1 infection (Table 5), suggesting that the decreased infectivity of DENV-1 vaccine in this cohort may be attributable to cross-protective DENV-2 antibody.

The present study provides important observations relevant to the evaluation of DENV vaccines in areas of endemicity. However, there are several limitations to the study. First, we were limited by the number of available participants who had received a DENV vaccine in our previous trials, and thus, the number of persons enrolled in each cohort was small. This also limited our ability to study all possible sequences of DENV vaccine administration, and only 4 such sequences were evaluated. Although all participants in the current study were previously exposed to DENV vaccine, the preexisting antibody titers at the time of heterotypic vaccination ranged from undetectable to easily detectable by either PRNT assay or ELISA. Because it is still unclear what titer of antibody is required for effective neutralization or enhancing virus replication, it is possible that the preexisting antibody titers did not fall within a range functionally capable of enhancement. Second, preexisting immunity was induced by infection with a live attenuated vaccine virus rather than a wild-type virus, although it should be noted that the envelope protein of each vaccine candidate is encoded by sequence derived from wild-type DENV. It is possible that a wild-type DENV would induce a higher level of non-neutralizing heterotypic antibody that more efficiently promotes ADE than the antibody induced by a vaccine virus. It is also possible that heterotypic immunity induced by wild-type infection might provide a higher level of cross-protection against the live attenuated DENV vaccines, thereby decreasing their infectivity and immunogenicity. These 2 questions need to be specifically studied during the testing of these vaccine candidates in regions of endemicity.

Of most importance, the present study provides data that indicate that the current vaccines are highly attenuated both in heterotypically immune and naive persons, providing an experimental framework for the safe evaluation of such vaccines in areas of endemicity. The vaccine candidates evaluated in the present study will be combined in a tetravalent formulation for evaluation in healthy adults in regions where dengue is not endemic before being introduced into regions where dengue is endemic.

Acknowledgments

We thank Priscilla Brooks, Julie McArthur, Jennifer Marron, Dennis Pierro, the members of the Data Safety Monitoring Board, all the support staff at Regulatory Compliance Human Subjects Protection Branch, and all the volunteers, without whom this work would not have been possible.

Funding: This work was supported by the National Institute of Allergy Infectious Diseases Division of Intramural Research, National Institutes of Health.

References


