VACCINATION OF HUMAN VOLUNTEERS WITH MONOVALENT AND TETRAVALENT LIVE-ATTENUATED DENGUE VACCINE CANDIDATES

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Abstract. Four serotypes of monovalent live attenuated dengue virus vaccine candidates were tested for reactogenicity and immunogenicity in 49 flavivirus non-immune adult human volunteers. The four monovalent candidates were then combined into a tetravalent formulation and given to another 10 volunteers. Neutralizing antibody seroconversion rates after a single-dose monovalent vaccination ranged from 53% to 100%. Solicited reactogenicity was scored by each volunteer. A composite index, the Reactogenicity Index, was derived by these self-reported scores. Reactogenicity differed among the four serotype candidates with serotype-1 associated with the most vaccine related side effects. A second dose of monovalent vaccines at either 30 days or 90 days was much less reactogenic but did not significantly increase seroconversion rates. Seroconversion rates in the 10 volunteers who received a single dose of tetravalent vaccine ranged from 30% to 70% among the four serotypes. Similar to the monovalent vaccines, a second dose of the tetravalent vaccine at one month was less reactogenic and did not increase seroconversion. A third dose of the tetravalent vaccine at four months resulted in three of four volunteers with trivalent or tetravalent high-titer neutralizing antibody responses.

INTRODUCTION

Vaccination is the most promising strategy to control the spread of dengue (DEN) infection. Infection with one serotype confers no long-term protection against disease by heterologous serotypes. Secondary infections by heterologous dengue serotypes increase the risk of dengue hemorrhagic fever. Therefore, it is desirable to induce protective immunity against all four serotypes. The World Health Organization has made development of tetravalent dengue vaccine a priority. It has been shown that dengue viruses can be attenuated by serial cell passage in primary dog kidney cells. Up to now, only live attenuated viruses have been formulated as tetravalent vaccines and tested in humans. One such vaccine has been tested in Thailand and the United States. High seroconversion rates were reported in Thailand, but only one of 10 recipients in the United States developed a tetravalent antibody response. The Walter Reed Army Institute of Research (WRAIR) in Washington, District of Columbia and the Center for Vaccine Development (CVD) at the University of Maryland in Baltimore, Maryland have evaluated several live attenuated dengue viruses as vaccines in human volunteers. Based on these small pilot studies a candidate vaccine for each serotype was selected. This report presents results of further testing of these monovalent vaccine candidates, and their tetravalent combination in flavivirus non-immune American adults.

MATERIALS AND METHODS

Study design. Monovalent vaccine candidates were evaluated by randomized, double-blind, Phase 1 clinical trials, followed by an open-label Phase 1 study of the combination of all four serotypes. The monovalent trials evaluated safety and immunogenicity of a single and then two doses given at one or three months apart. The open-label study evaluated the tetravalent vaccine given in two or three doses at 1-4-month intervals.

The single-dose monovalent study was done at CVD. The two-dose monovalent study and the open-label tetravalent study were conducted at WRAIR. The local Institutional Review Boards at CVD or WRAIR and the Human Subjects Research Review Board of the Office of the Surgeon General of the U. S. Army reviewed and approved all studies.

Study population. Volunteers were healthy adults (age range = 18–50 years) recruited by public advertisements. All gave informed consent prior to enrollment and received payment for their participation. Their healthy status was determined by medical history and physical examination as well as normal results on blood and urine tests: complete blood count (CBC), differential count; platelet count; alanine aminotransferase (ALT); aspartate aminotransferase (AST); glucose; creatinine; urea nitrogen; and urinalysis. All volunteers were seronegative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus, as well as for DEN virus serotypes 1–4, Japanese encephalitis virus, St. Louis encephalitis virus and yellow fever viruses by hemagglutination inhibition assay. The single-dose study included 22 volunteers divided into groups of four or five persons each. A control group received yellow fever 17D vaccine YF-VAX® (Connaught Laboratories, Swiftwater, PA). The two-dose study used 31 volunteers divided into four groups who received serotypes 1–4, respectively. Half of each group received the second dose at one month and the other half at three months. In the open-label tetravalent study, 10 volunteers were given two or three doses of the tetravalent vaccine. The first four tetravalent recipients received vaccinations at 0 and 1 months. The latter six tetravalent recipients were similarly vaccinated at 0 and 1 months, but if recipients made less than tetravalent serotype neutralizing antibody responses they were given a third vaccination at four months.

Viruses. The four serotype vaccine candidates, DEN-1(45AZ5 PDK20, FRhL3), DEN-2(S16803 PDK50, FRhL3), DEN-3(CH53489 PDK20, FRhL3), and DEN-4(341750 PDK20, FRhL4), were originally isolated from patients with clinical disease. Viruses from human sera were amplified in
either *Toxorhynchites amboinensis* mosquitoes or cell lines and then underwent serial passages in primary dog kidney (PDK) cells and then in fetal rhesus lung (FRhL) cells. All vaccines were made under Good Manufacturing Practice guidelines and tested as Investigational New Drugs. These candidates were selected based on previous small pilot studies in human volunteers (Kanesa-thasan N and others, unpublished data). Each freeze-dried monovalent vaccine was reconstituted with sterile water and given in a volume of 0.5 mL. The doses of serotypes 1–4 were 10^6, 10^6, 10^5, and 10^3 plaque-forming units (PFU), respectively. The tetravalent vaccine dose was prepared by mixing 0.25 mL of each reconstituted monovalent vaccine and given in a final volume of 1 mL. The dose range of the tetravalent vaccine was 1.1–2.8 x 10^6 PFU. The yellow fever vaccine (YF-VAX) was given per the manufacturer’s recommendation. All vaccinations were given subcutaneously in the upper arm.

**Clinical safety.** Reactions to vaccinations were assessed by combination of daily symptom diaries and frequent periodic physician evaluations during the three weeks after each vaccination. Volunteers were housed in study quarters for close observation for 5–7 days starting on day 7 post-vaccination, a time period during which vaccine reactions and viremia were most likely. Volunteers were examined and queried specifically for subjective feverishness, chills, malaise, headache, eye pain, myalgia, arthralgia, nausea, vomiting, abdominal pain, photophobia, conjunctivitis, rash, pruritis, and any injection site pain. Each symptom was graded on a scale of 0 (none), 1 (mild, did not affect normal activity; did not require medication), 2 (moderate, required medication or change in activity), or 3 (severe, required bed rest or unrelieved by medication). The most common symptoms were grouped into four categories. These categories were 1) headache or retro-orbital pain, 2) myalgia or arthralgia, 3) subjective fever or chills, and 4) gastrointestinal symptoms of nausea, vomiting, or abdominal pain. A symptom index of each category was calculated by the product of the highest symptom grade for each day and the duration of the symptom expressed in days. If a symptom occurred at all during a 24-hour period, it is assigned duration of one day. The Reactogenicity Index (RI) is calculated as the sum of the symptom indices for each of the four categories (i = 4)

\[
RI = \sum (Symptom \ Grade)_i \times (Duration)_i
\]

The RI summarized the vaccine reactions of each volunteer. The symptom category indices and RI allow for quantitative comparison of vaccine reactions among volunteers and vaccine serotypes.

Volunteers were monitored for hematologic and liver abnormalities by serial CBC, platelet counts, and levels of AST and ALT during the study.

Serious adverse reactions were defined as severe illness lacking other likely causes: fever >38.5 °C (101.3 °F) continuously for more than 24 hours or a maximum temperature (T_{max}) >38.5 °C for three consecutive days or a single oral temperature >40 °C (104 °F); neutropenia (<1,000/mL) or thrombocytopenia (<90,000/mL) on two consecutive determinations; or a serum ALT level >5 times the normal level.

**Immunogenicity.** Hemagglutination inhibition assays were performed by the method of Clarke and Cassals. Dengue IgM and IgG were measured by a capture enzyme-linked immunosorbent assay (ELISA) adapted from the method of Innis and others. The assay was performed in Limbro/Titernek (Horsham, PA) enzyme-immunoassay (EIA) 96-well microtiter plates. Pooled DEN-1–4 sucrose acetone–extracted antigens were used. An EIA unit >20 was considered positive. An IgM/IgG ratio ≥1.8 is considered indicative of a primary antibody response. Dengue and yellow fever neutralizing antibodies were measured on days 0 and 30 after each vaccination by a plaque reduction neutralization test (PRNT). The study endpoint determination was the measurement of neutralizing antibody 30 days after vaccination. Neutralizing antibody seroconversion is defined as a 50% reduction in the PRNT at a 1:5 serum dilution (PRNT_{50}). A neutralizing antibody boost was defined as an increase in titer of at least four-fold 30–60 days after revaccination. The viruses used for the dengue PRNT were low-passage parent vaccine viruses. The virus used for the yellow fever PRNT was the commercial 17D vaccine virus amplified in Vero cells.

Viremia was determined on sera from days 3, 7, 9, 11, and 14 after the first and second vaccination. Sera from the last six tetravalent vaccine volunteers were tested for viremia on days 7, 9, 11, and 14 after the first vaccination only. The method used for virus isolation was a delayed plaque method adapted from Yuill and others using LLC-MK₂ or C6/36 cells for amplification and Vero cells for plaque assay. Sera from tetravalent vaccine recipients were also tested by a fluorescein serotype-specific nested reverse transcriptase-polymerase chain reaction (RT–PCR) by a method adapted from Houng and others.

**Data analysis.** The study is mostly descriptive. Comparison of mean RIs between the first and second doses was done by the paired t-test. Comparisons of RIs between different groups were done by the Wilcoxon signed rank test (Mann-Whitney). Comparisons of two proportions were done using the chi-square test or 95% confidence intervals by binomial distribution.

**RESULTS**

**Volunteer retention.** The volunteer characteristics are shown in Table 1. A total of fifty-nine healthy volunteers were given dengue virus vaccines; 49 received monovalent vaccines and 10 received tetravalent vaccine. Four volunteers received a standard subcutaneous dose (0.5 mL) of licensed 17D yellow fever vaccine as control. One DEN-3 monovalent recipient moved away and was lost to follow-up two weeks after his second vaccination. One DEN-4 monovalent recipient who was to receive revaccination also moved away and dropped out after one dose. The second vaccination of volunteer 34 was delayed to day 60 due to his unavailability for day 30 vaccination. Volunteer 38 had an intercurrent illness on day 30, so her revaccination was deferred to four months. A third tetravalent volunteer, volunteer 42, was unavailable for the third vaccination at four months due to work-related travel. Of the latter six volunteers who were to receive a third dose of the tetravalent vaccine, only four received it.

**Reactogenicity.** There were no clinically significant local reactions from vaccinations, and no vaccination affected use of the arm in normal daily activities. The most frequently reported symptoms were headaches, myalgias, and subjective
fear or chills. Symptoms included in the RI accounted for more than 70% of all symptoms.

The volunteers consisted of 31 Caucasians, 25 African-Americans, 1 Hispanic, 1 Asian, and 1 native American. There was no significant difference between the mean RIs of the whites and African-Americans (P = 0.86). Similarly, the reactogenicity did not differ by sex (P = 0.27). Table 2 and Figure 1 compare the reported reactogenicity of each vaccine. Twenty percent of 59 dengue vaccine recipients reported no symptoms with their first vaccination, while 70% of the volunteers were asymptomatic with the second vaccination (P < 0.005). The four volunteers who received a third dose of tetravalent vaccine reported no symptoms associated with it. Figure 1 shows the occurrence of Grade 2 symptoms longer than one day and Grade 3 symptoms of any duration following the first vaccination. The DEN-1 monovalent and tetravalent vaccines had the highest reactogenicity. For example, 25% and 41% of the DEN-1 volunteers complained of subjective fever and myalgias, respectively. After receiving their initial dose of vaccine, five volunteers, one DEN-1, one DEN-4, and three tetravalent recipients, reported one severe grade 3 symptom of either chills, myalgia, headache, or nausea. All Grade 3 symptoms were short-lived, lasting less than one day in duration. No volunteers reported any grade 3 symptoms following revaccination.

Gastrointestinal symptoms occurred in one-third of the volunteers, but they were mild and brief, lasting less than 24 hours. Only one volunteer developed any significant gastrointestinal symptoms (Grade 3 nausea associated with crampy abdominal pain) for one day.

The RI after the first dose of any of the monovalent or tetravalent vaccines ranged from 0 to 35 in this study. The highest RI was recorded in an Asian female tetravalent vaccine recipient and a DEN-3 recipient, developed transient elevation of ALT levels had returned to normal prior to their revaccination.

Eleven volunteers (19%) developed fever (>100.4°F). The breakdown by serotype was 5 of 12 for DEN-1, 1 of 12 for DEN-2, 2 of 13 for DEN-3, 0 of 12 for DEN-4, and 3 of 10 for tetravalent vaccine. The longest fever occurred in a DEN-1 recipient who was febrile for three days with a Tmax of 103.3°F on one occasion. Two other DEN-1 volunteers also had between two and three days of fever with a Tmax of 102.1°F and 101.1°F, respectively. Ten of the 12 episodes of fever occurred following the first vaccination.

Six volunteers (10%), five dengue and one yellow fever 17D recipient, developed transient neutropenia with an absolute neutrophil count (ANC) less than 1,000/mL. The lowest ANC was 288/mL in a DEN-1 volunteer. Neutropenia typically resolved in 2–3 days. Two volunteers, a DEN-1 recipient and a DEN-3 recipient, developed transient elevation of their ALT levels 2–3-fold above the upper range normal level after their first vaccination. The ALT levels had returned to normal prior to their revaccination.

No volunteers experienced serious adverse reactions as pre-defined by the study protocol. No volunteers manifested any evidence of dengue hemorrhagic fever such as thrombocytopenia, hypotension, spontaneous bleeding, or hemococoncentration.

**Viremia.** Viremia by delayed plaque assay was detected in 10 of the 59 volunteers (17%); one received DEN-2, four DEN-3, one DEN-4, and four tetravalent vaccine. No DEN-1 viremia was detected among the 12 DEN-1 monovalent vaccine recipients. Thus, 0%, 8%, 31%, 8%, and 40% of DEN-1, -2, -3, -4, and tetravalent vaccine recipients, respectively, were viremic. All detected viremias occurred after the first dose of vaccine, and none occurred after the second dose of vaccine. To determine the serotype(s) of the viruses causing viremia in the four tetravalent vaccine volunteers (36, 38, 39, and 40), their post-dose sera were tested by a serotype-specific nested

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**Table 1**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of subjects†</th>
<th>Sex</th>
<th>Race</th>
<th>Mean age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN-1</td>
<td>12 (8)</td>
<td>7M/5F</td>
<td>6W/6B</td>
<td>32</td>
</tr>
<tr>
<td>DEN-2</td>
<td>12 (8)</td>
<td>7M/5F</td>
<td>7W/5B</td>
<td>36</td>
</tr>
<tr>
<td>DEN-3</td>
<td>13 (8)</td>
<td>9M/4F</td>
<td>8W/5B</td>
<td>36</td>
</tr>
<tr>
<td>DEN-4</td>
<td>12 (7)</td>
<td>6M/6F</td>
<td>4W/6B/1H1AmI</td>
<td>33</td>
</tr>
<tr>
<td>Tetravalent</td>
<td>10 (10)</td>
<td>4M/6F</td>
<td>6W/3B/1As</td>
<td>29</td>
</tr>
<tr>
<td>YF 17D</td>
<td>4 (0)</td>
<td>3M/1F</td>
<td>3W/1B</td>
<td>30</td>
</tr>
</tbody>
</table>

*W = Caucasian; B = African American; H = Hispanic; AmI = American Indian.*
†Numbers in parentheses are the subset of volunteers who received two doses of vaccine.

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**Table 2**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of volunteers</th>
<th>Dose 1 Mean RI (range)</th>
<th>Dose 2 Mean RI (range)</th>
<th>Dose 3 Mean RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN-1</td>
<td>12</td>
<td>10 (1–23)</td>
<td>1 (0–2)</td>
<td>Not given</td>
</tr>
<tr>
<td>DEN-2</td>
<td>12</td>
<td>3 (0–16)</td>
<td>0 (0–2)</td>
<td>Not given</td>
</tr>
<tr>
<td>DEN-3</td>
<td>13</td>
<td>3 (0–13)</td>
<td>1 (0–5)</td>
<td>Not given</td>
</tr>
<tr>
<td>DEN-4</td>
<td>12</td>
<td>4 (0–15)</td>
<td>1 (0–5)</td>
<td>Not given</td>
</tr>
<tr>
<td>Tetravalent</td>
<td>10</td>
<td>9 (0–35)</td>
<td>2 (0–14)</td>
<td>0</td>
</tr>
<tr>
<td>YF 17D</td>
<td>4</td>
<td>4 (1–10)</td>
<td>Not given</td>
<td>Not given</td>
</tr>
</tbody>
</table>

*DEN = dengue; YF = yellow fever.*
RT-PCR. Only volunteer 39 had detectable viremia by our PCR. DEN-1, -2, and -3 were detected on days 9 and 11. Fever occurred concurrently with viremia only in three tetravalent recipients. The remaining seven tetravalent vaccine volunteers who developed viremia did not have fever. All viremic volunteers developed neutralizing antibody. All but one viremic volunteer developed IgM or IgG antibody detected by the ELISA.

Vaccine immunogenicity. Tables 3 and 4 summarize the antibody responses to monovalent vaccination. Only the first four tetravalent volunteers had sera tested for IgM:lgG by the ELISA. The IgM antibody seroconversion rates after a single vaccination were 75%, 72%, 31%, 42%, and 75% for monovalent serotypes 1, 2, 3, and 4 and the tetravalent vaccine, respectively. Fifty-nine percent of 49 monovalent vaccine recipiepts seroconverted as detected by the IgM ELISA after the first vaccination, half of them by two weeks. Two DEN-3 and one tetravalent recipient seroconverted as detected by the IgM ELISA only after receiving a second vaccination. The IgM antibody level typically peaked by day 30 after the first vaccination. A single exception was in a tetravalent recipient whose IgM antibody level peaked three days after his second vaccination.

The IgG antibody seroconversion rate was low, only 47% overall. Only 23% of the volunteers receiving revaccination showed the expected secondary IgG ELISA antibody response with an IgM:lgG ratio <1.8. Five (9%) of 53 volunteers tested for IgM:lgG antibodies developed a secondary antibody response pattern after their first vaccination. These five volunteers may have had previous exposure to some other

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**Table 3**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Monovalent</th>
<th>First-dose</th>
<th>Seroconversions</th>
<th>Second-dose</th>
<th>Cumulative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>seroconversions</td>
<td>GMT</td>
<td>after second dose</td>
<td>GMT</td>
<td>IgM(+)</td>
</tr>
<tr>
<td>DEN-1</td>
<td>12/12 (100%)</td>
<td>668</td>
<td>—</td>
<td>513</td>
<td>10/12</td>
</tr>
<tr>
<td>DEN-2</td>
<td>11/12 (92%)</td>
<td>112</td>
<td>0/1</td>
<td>559</td>
<td>9/12</td>
</tr>
<tr>
<td>DEN-3</td>
<td>6/13 (46%)</td>
<td>15</td>
<td>2/7</td>
<td>16</td>
<td>6/13</td>
</tr>
<tr>
<td>DEN-4</td>
<td>7/12 (58%)</td>
<td>17</td>
<td>0/5</td>
<td>9</td>
<td>5/12</td>
</tr>
<tr>
<td>YF 17D</td>
<td>4/4 (100%)</td>
<td>2,935</td>
<td>No second dose</td>
<td>—</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* PRNT<sub>50</sub> = 50% plaque reduction neutralization test; GMT = geometric mean titer; DEN = dengue.
† Used 30-day post vaccination titer; used value of 1 for negative titer in calculation.
flaviviruses despite negative hemagglutination inhibition assay results for dengue, St. Louis encephalitis, Japanese encephalitis, and yellow fever viruses, as well as negative results for homologous dengue neutralizing antibody prior to vaccination. This study was conducted prior to the introduction of West Nile virus into the United States. We did not investigate further possible other sources of these prior flavivirus exposures. There was no significant difference between the mean RIs of the five secondary responders when compared with primary antibody responders (9.6 versus 5.8; \( P = 0.19 \)).

The seroconversion rates by PRNT<sub>50</sub> were 100%, 92%, 46%, and 58% after a single dose of monovalent vaccine serotypes 1, 2, 3, and 4, respectively. Neutralizing antibodies were detected more frequently than IgM and IgG antibodies detected by the ELISA. All IgM:IgG seroconverters developed neutralizing antibody, but some neutralizing antibody seroconverters had no IgM:IgG antibody detectable by the ELISA. The second dose of vaccine boosted neutralizing antibody titers in one of eight DEN-1 and three of eight DEN-2 recipients. The second dose at one month boosted the titer of pre-existing neutralizing antibody in only one volunteer (35, DEN-2). A third dose of vaccine was administered. Two volunteers, 33 and 35, developed neutralizing antibody to all four serotypes after a single dose. Two other tetravalent recipients, 39 and 41, seroconverted to all four serotypes after vaccinations at 0, 1, and 4 months. Two others who were vaccinated at four months, 37 and 38, developed trivalent responses. A second dose of the tetravalent vaccine given at one or two months resulted in two additional DEN-1 seroconversions and one DEN-3 seroconversion in volunteers 34, 37, and 42, respectively. A second dose at one month boosted the titer of pre-existing neutralizing antibody in only one volunteer (35, DEN-2). A third dose of tetravalent vaccine at four months resulted in six additional seroconversions, mostly to serotypes 3 and 4. It also boosted titers of pre-existing neutralizing antibody in five of six instances. The overall seroconversion rates after two or three doses of tetravalent vaccines in these 10 volunteers were 100%, 80%, 80%, and 40% for DEN-1, -2, -3, and -4, respectively.

The 19 antibody responders showed either less than a fourfold increase or no change or decrease in titer after revaccination.

Twelve monovalent vaccine recipients did not develop IgM:IgG antibodies detected by the ELISA or neutralizing antibody to homologous virus and are considered non-responders. Of these non-responders, one (8%) received DEN-2, six (46%) DEN-3, and five (42%) DEN-4. Non-responders experienced nearly no reactions. The mean RI for non-responders was significantly less than the mean RI of neutralizing antibody responders (0.9 versus 4.9; \( P < 0.003 \)).

There was no significant difference in neutralizing antibody seroconversion rate when the volunteer responses were stratified by age (79% for those ≥25 years old versus 63% for those <25 years old; \( P = 0.30 \)).

**Tetravalent vaccine immunogenicity.** Neutralizing antibody seroconversion rates for the 29 males and 20 females who received monovalent vaccines were not significantly different (79% versus 75%; \( P = 0.72 \)).

Table 5 shows the summary RI and PRNT<sub>50</sub> antibody results from the 10 tetravalent vaccine volunteers. Three of the first four volunteers received only two vaccinations at 0 and 1 months. The fourth volunteer missed his second vaccination at one month and was vaccinated at two months. The next six volunteers were vaccinated at 0 and 1 months and if an antibody response was incomplete a third vaccination at four months was administered. Two volunteers, 33 and 35, developed neutralizing antibody to all four serotypes after a single dose. Two other tetravalent recipients, 39 and 41, seroconverted to all four serotypes after vaccinations at 0, 1, and 4 months. Two others who were vaccinated at four months, 37 and 38, developed trivalent responses. A second dose of the tetravalent vaccine given at one or two months resulted in two additional DEN-1 seroconversions and one DEN-3 seroconversion in volunteers 34, 37, and 42, respectively. A second dose at one month boosted the titer of pre-existing neutralizing antibody in only one volunteer (35, DEN-2). A third dose of tetravalent vaccine at four months resulted in six additional seroconversions, mostly to serotypes 3 and 4. It also boosted titers of pre-existing neutralizing antibody in five of six instances. The overall seroconversion rates after two or three doses of tetravalent vaccines in these 10 volunteers were 100%, 80%, 80%, and 40% for DEN-1, -2, -3, and -4, respectively.

### Table 4

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Seroconversions after first dose</th>
<th>Seroconversions after second dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>DEN-1</td>
<td>9/12</td>
<td>2/12</td>
</tr>
<tr>
<td>DEN-2</td>
<td>8/11</td>
<td>2/11</td>
</tr>
<tr>
<td>DEN-3</td>
<td>4/13</td>
<td>2/13</td>
</tr>
<tr>
<td>DEN-4</td>
<td>5/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Tetravalent</td>
<td>3/4</td>
<td>2/4</td>
</tr>
</tbody>
</table>

*DEN = dengue.
†One volunteer data were uninterpretable.

### Table 5

<table>
<thead>
<tr>
<th>Dose schedule (months)</th>
<th>RI doses</th>
<th>Post-dose 1</th>
<th>Post-dose 2</th>
<th>Post-dose 3</th>
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<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>33 0, 1</td>
<td>16</td>
<td>0</td>
<td>—†</td>
<td>122</td>
</tr>
<tr>
<td>34 0, 2</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>&lt;5</td>
</tr>
<tr>
<td>35 0, 1</td>
<td>4</td>
<td>0</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>36 0, 1</td>
<td>15</td>
<td>3</td>
<td>—</td>
<td>458</td>
</tr>
<tr>
<td>37 0, 1, 2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>362</td>
</tr>
<tr>
<td>38 0, 4</td>
<td>35</td>
<td>14</td>
<td>—</td>
<td>1,105</td>
</tr>
<tr>
<td>39 0, 1, 2</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>637</td>
</tr>
<tr>
<td>40 0, 1, 2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>149</td>
</tr>
<tr>
<td>41 0, 1, 2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>42 0, 1, 2</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*Vol. = volunteer; PRNT<sub>50</sub> = 50% plaque reduction neutralization test.
†Third dose not given.
DISCUSSION

These vaccine viruses are attenuated in humans. In Simmons’ study of 60 adults experimentally infected with wild-type DEN-1,22 98% developed a fever $>101^\circ F$ for two or more days and overall 52% had fever $>103^\circ F$. In comparison 45AZS PDK20, our DEN-1 vaccine candidate and the most reactogenic of the four serotype vaccines, elicited a fever $>101^\circ F$ in 33% and only 8% developed a fever $>103^\circ F$. To assess reactogenicity in our study, we used volunteer symptom diaries and investigator-elicited symptom queries. Using the symptom grades, we constructed a composite score (the RI) to summarize symptom duration and severity. It is known that volunteer self reports tend to overestimate the number and severity of symptoms, possibly by as much as 10-fold in RI to summarize symptom duration and severity. It is known

Thus, there was no measurable additional immunogenicity achieved with giving a second dose of monovalent DEN-1 or DEN-4 at up to three months. In the case of DEN-1, it had already maximally immunized the volunteers after a single vaccination, while for DEN-4 primary vaccine failure could not be overcome with revaccination.

The PRNT$_{50}$ neutralizing antibody response to monovalent revaccination among seroconverters was similar to that seen with revaccination with other protective live attenuated virus vaccines such as 17D yellow fever and measles.28,29 Eighty percent showed no boosting (four-fold increase) of neutralizing antibody after revaccination, suggesting sterile immunity. Similarly, a second dose of the tetravalent vaccine at one month did not elicit any boosting of titer with the single exception of DEN-2 in volunteer 35. In contrast, boosting was seen in four of six instances among the volunteers who received a third dose at four months (Table 6). This boosting is suggestive of a brisk secondary response to revaccination.

Twelve monovalent volunteers who did not have neutralizing antibody responses to monovalent vaccines also did not show measurable dengue IgM or IgG antibody by the ELISA. All these non-responders received viable virus from the same vial that clearly replicated in other volunteers. These serologic non-responders also did not develop any clinical reactions to the vaccinations. Thus, by all indications there was no evidence of virus replication in these volunteers. These individuals were primary vaccine failures. The mechanism for this nonresponsiveness is unknown but may be due to either over-attenuation of the virus or an effective host innate immunity or both.

The development of neutralizing antibody in vivo is predominantly due to a Th1 type immune response. When peripheral blood mononuclear cells were collected from vaccinees and stimulated with homologous virus, all 17 volunteers who developed an interferon-γ (IFN-γ) response also developed neutralizing antibody. In contrast, of 13 vaccinees who did not have an IFN-γ response, only six developed neutralizing antibody (Gwinn W and others, unpublished data).

Interference among dengue viruses have been described both in vitro and in vivo.1,30 Interference and enhancement can potentially occur when dengue viruses are given in combination such that antibody responses seen with the monovalent vaccine candidates may not extrapolate to the tetravalent situation. Sabin found that simultaneous administration of equal doses of type 1 and type 2 viruses led to suppression or delay of type 1 antibody. When type 2 virus was given followed by type 1 virus, no type 1 antibody was elicited when the interval between administration was six weeks or less.1 Similarly, the Aventis/Mahindol live-attenuated tetravalent dengue vaccine elicited predominantly serotype 3 responses

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**Table 6**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Neutralizing antibody to dengue serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Monovalent 1 dose</td>
<td>12/12 (74–100%)</td>
</tr>
<tr>
<td>Tetravalent 1 dose</td>
<td>7/10 (35–93%)</td>
</tr>
<tr>
<td>Tetravalent 2 doses</td>
<td>9/10 (56–100%)</td>
</tr>
<tr>
<td>Tetravalent 3 doses</td>
<td>4/4 (40–100%)</td>
</tr>
</tbody>
</table>

* Number in parenthesis are 95% confidence intervals using a binomial distribution.
when given in a single dose to non-immune adult volunteers in the United States. Multiple dosing of combination live-attenuated vaccines have been used to circumvent the problem of viral interference. Interference with neutralizing antibody responses among the four serotypes was not seen with any of the four viruses because the seroconversion rate to each serotype did not differ significantly between the monovalent and tetravalent groups (Table 6). However, the number of tetravalent vaccine volunteers in this study may be too small to rule out the presence of such viral interference.

The dose of each virus and the vaccination interval may determine the immunogenicity of the tetravalent vaccine. The doses of type 3 and 4 were 10-fold less than those of types 1 and 2 in the tetravalent vaccine. This disparity was due to viral yield during manufacture. DEN-3 and 4, at the lower dose of 10^7 PFU/mL, may be at a relative replicative disadvantage compared with DEN-1 and 2, both of which contain 10^6 PFU in the tetravalent formulation, resulting in lower immunogenicity. Such dose effects among the four viruses can be potentially complex, involving multiple interactions. In a subsequent study, we used a factorial design to evaluate such effects of variations in the dose and ratios of serotypes on antibody response (Edelman R and others, unpublished data). We are also exploring alternative production strategies to increase titers of the DEN-3 and DEN-4 vaccines. Alternatively, we will evaluate substituting the DEN-4 PDK20 component of the tetravalent vaccine with a lower PDK cell passage level virus.

Four tetravalent vaccine volunteers developed neutralizing antibody to all four serotypes, two after the first dose, and two after a third dose at four months. The second dose of tetravalent vaccine one month after the first did not “fill in” neutralizing antibodies to types 3 and 4 in the bivalent and tetravalent responders. In contrast, four of five volunteers who received revaccination at four months seroconverted to three or more serotypes. The explanation of this difference may be the presence of sufficient cross-reactive neutralizing antibodies one month after initial vaccination to suppress replication of heterotypic viruses in the vaccine. Protection against disease by heterotypic dengue virus was seen in human volunteers given heterotypic virus two months after primary infection. After three months, such protection waned. It is interesting to note that the dose at four months produced the highest titers of neutralizing antibody to DEN-4, the least immunogenic serotype. The DEN-4 GMT was not boosted by the dose at one month, but was boosted eight-fold by the dose at four months. We think this pattern of antibody response probably has more to do with the time interval from the first dose rather than the third dose itself. If so, a longer dosing interval before a second dose of tetravalent vaccine will likely result in improved antibody responses. Our future tetravalent studies will use a 0–6 month vaccination schedule to test this hypothesis. In any case, it is likely that the tetravalent vaccine will require at least two doses.

The delayed plaque method is more sensitive than our PCR in detecting vaccine virus in serum. This was also observed with the Aventis/Mahidol tetravalent vaccine. Without actually detecting all four serotype viremia in the tetravalent volunteers, we cannot be certain that multivalent neutralizing antibodies necessarily indicate replication of all four serotypes. The measured neutralizing antibodies may be cross-reactive and not protective in the long term. Our nested RT-PCR method detected viremia in only one tetravalent volunteer (39) who developed circulating DEN-1, 2-, and -3. In contrast, the Aventis/Mahidol tetravalent vaccine induced DEN-3 viremia in all 10 adults vaccinated, with one volunteer positive concurrently with DEN-4. Previously, our group has reported that mosquitoes feeding on 17 of our monovalent vaccine volunteers resulted in rates of disseminated infection by the four serotype vaccine strains of 0.5%, 0.3%, <0.1%, and <0.1%, respectively. The low rates of viremia by our attenuated viruses preclude the use of virus isolation as evidence of vaccine take. In the absence of measurable viremia, it is difficult to determine vaccine take due to the cross-reactivity of antibodies. Long-term persistence of serotype antibody after tetravalent vaccination may be useful to determine if the different serotypes of neutralizing antibody are the result of individual serotype replication. Conversely, in endemic areas natural boosting may be confounding.

Of the 10 recipients, only two of the tetravalent vaccinees developed neutralizing antibody to all four serotypes after one vaccination. The absence of antibody to all four serotypes may be a risk factor for dengue hemorrhagic fever in the setting of wild-type virus exposure. Incomplete response to tetravalent vaccine raises concern about such potential risk in partially immune individuals. Halstead and Palumbo had shown that rhesus monkeys with trivalent antibody responses to tetravalent dengue virus inoculation were protected when challenged with the fourth virus. The Th1 T cell response to all four serotypes can be measured in tetravalent vaccines, even in the absence of neutralizing antibody (Gwinn W and others, unpublished data). It is unclear if individuals who developed cell-mediated immunity but not the full four serotype neutralizing antibodies after tetravalent vaccination are protected against disease. It seems this question may only be answered by long-term field testing of tetravalent vaccines in endemic areas.

Many more questions remain concerning our tetravalent vaccine. The optimal dose formulation and schedule of vaccinations need to be determined; we need to better understand the effect of interactions among the four serotypes in determining reactogenicity and immunogenicity. Finally, it is clear that we need to do more clinical studies of the tetravalent vaccine in adults as well as children in endemic areas to determine if the vaccine will be useful in preventing both dengue fever as well as dengue hemorrhagic fever.

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