ATYPICAL ANTIBODY RESPONSES IN DENGUE VACCINE RECIPIENTS

N. KANEWA-THASAN, W. SUN, G. V. LUDWIG, C. ROSSI, J. R. PUTNAK, J. A. MANGIAFICO, B. L. INNIS, AND R. EDELMAN

Walter Reed Army Institute of Research, Washington, District of Columbia; United States Army Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland; Department of Medicine and the Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland

Abstract. Eight of 69 (12%) healthy adult volunteers vaccinated with monovalent live-attenuated dengue virus (DENV) vaccine candidates had atypical antibody responses, with depressed IgM:IgG antibody ratios and induction of high-titer hemagglutination-inhibiting and neutralizing (NT) antibodies to all four DENV serotypes. These features suggested flavivirus exposure prior to DENV vaccination, yet no volunteer had a history of previous flavivirus infection, flavivirus vaccination, or antibody to flaviviruses evident before DENV vaccination. Moreover, production of antibody to DENV by atypical responders (AR) was not accelerated compared with antibody responses in the 61 flavivirus-naive responders (NR). Further evaluation revealed no differences in sex, age, race, DENV vaccine candidate received, or clinical signs and symptoms following vaccination between AR and NR. However, viremia was delayed at the onset in AR compared with NR. A comparative panel of all AR and five randomly selected NR found flavivirus cross-reactive antibody after vaccination only in AR. Unexpectedly, six of eight AR had NT antibodies to yellow fever virus (YFV) > 1:10 before vaccination while NR had none (P = 0.04). The AR also universally demonstrated YFV NT antibody titers ≥ 1:160 after DENV vaccination, whereas four of five NR failed to seroconvert (P = 0.02). Yellow fever virus priming broadens the antibody response to monovalent DENV vaccination. The effect of flavivirus priming on the clinical and immunologic response to tetravalent DENV vaccine remains to be determined.

INTRODUCTION

The flaviviruses are separated into eight distinct serogroups, in which the presence of antibody to one flavivirus following natural infection or vaccination (homotypic antibody) does not result in long-term cross-protection against another. Moreover, flavivirus cross-reactive or heterotypic antibodies that are detectable soon after primary infection typically disappear within 3–6 months, leaving only specific antibody responses. In contrast, sequential exposure to two or more flaviviruses induces rapid onset of very broad anamnestic responses with high-titer heterotypic antibodies following the second infection. These responses may be long lasting and protective against subsequent infection with related and unrelated flaviviruses. Other human data suggests cross-protection occurs among group B arboviruses.

The immunologic interactions of two flaviviruses, yellow fever virus (YFV) and dengue virus (DENV), have been studied intensively in humans. Simultaneous administration of attenuated YFV and DENV vaccines to humans modifies fever and general symptoms and increases antibody responses to both viruses. Moreover, initial vaccination with YFV followed by natural DENV infection or administration of an attenuated DENV vaccine candidate boosts antibodies to both flaviviruses. Dengue-specific seroconversion rates and antibody titers following vaccination with attenuated DENV-2 were increased in YFV-immune compared with YFV-naive volunteers. Conversely, DENV infection preceding YFV vaccination may subsequently enhance antibody production to YFV.

In the current study, we observed boosted heterologous anti-DENV and non-DENV flavivirus antibody titers in eight DENV-negative volunteers following vaccination with attenuated monovalent DENV vaccine candidates. Our serologic investigation of these atypical immune responses suggests that DENV-specific immune responses were modified by immunologic memory induced by remote YFV vaccination.

MATERIALS AND METHODS

Study population. Sixty-nine healthy, 18–49-year-old male and non-pregnant female participants in clinical trials of attenuated DENV vaccines were evaluated before and after receipt of monovalent DENV vaccine candidates. Volunteers were predominately urban residents of the greater Washington, DC and Baltimore metropolitan areas. Three studies conducted from 1996 to 1998 at the Walter Reed Army Institute of Research (WRAIR) (Washington, DC) or at the Center for Vaccine Development, University of Maryland School of Medicine (Baltimore MD) were used for the analysis. They include the following vaccine candidates: 1) Aventis Pasteur Phase I study (AvP, 1996) with a total of 20 recipients of AvP attenuated monovalent DENV-1, -2, -3, or -4 vaccines, 2) WRAIR dengue 2-dose Phase I study (D2D, 1999) with a total of 31 recipients of WRAIR attenuated monovalent DENV-1, -2, -3, or -4 vaccines, and 3) WRAIR Phase 2 study (CVD5000, 1999) with a total of 18 recipients of WRAIR attenuated monovalent DENV-1, -2, -3, or -4 vaccines.

Informed consent was obtained from each volunteer in compliance with US 21 CFR Part 50-Protection of Human Subjects. The clinical protocol conformed to all relevant regulatory requirements, including the Declaration of Helsinki (Protocol), Army Regulations 70-25-Use of Volunteers as Subjects of Research, and 40-7-Use of Investigational Drugs in Humans and the Use of Schedule I Controlled Substances. The studies were reviewed and approved by the Human Subject Research Review Board, Office of the Surgeon General, U.S. Army, the WRAIR Human Use Research Committee, and the Institutional Review Board, University of Maryland at Baltimore.

After providing informed consent, volunteers were screened for previous flavivirus exposure through detailed medical history including travel to flavivirus-endemic countries and known vaccination or infection with flaviviruses. All volunteers were also evaluated before vaccination for antibodies to DENV-1, -2, -3, or -4, YFV, Japanese encephalitis...
virus (JEV), and St. Louis encephalitis (SLE) virus by hemagglutination-inhibition (HAI) assay and to DENV by a plaque-reduction neutralization test (PRNT). Volunteers confirmed to be seronegative for flavivirus antibodies were selected to receive a single dose of attenuated monovalent DENV-1, -2, -3, or -4 candidate vaccine.

Dengue virus isolation. Serum specimens were obtained for virus isolation on days 2 through 21 following vaccination and stored at -70°C. Serum was thawed and inoculated onto LLC-MK2 cell (AvP) or C6/36 mosquito cell (D2D, CVD5000) monolayers for amplification.13 Culture fluid harvests were subsequently assayed for the presence of virus by plaque assay on confluent Vero cells.14 No direct titration of virus from serum specimens was performed in these studies.

Flavivirus serology. Serum specimens were obtained from volunteers at designated time periods before vaccination and from 7 to 60 days after DENV vaccination. Serum specimens were stored at -20°C until used in antibody assays described below.

Hemagglutination-inhibition assay. The HAI assay was performed with 4–8 units of individual flavivirus antigens: DENV-1, DENV-2, DENV-3, DENV-4, YFV, JEV, and SLE virus.15 Serum specimens were extracted with 25% kaolin to remove inhibitors. After a 1:10 dilution with kaolin, serum specimens were incubated at room temperature for 20 minutes and centrifuged prior to the hemagglutination reaction with fresh goose red blood cells. An HAI antibody titer ≥1:10 was considered seropositive.

IgM/IgG antibody assay. The DEN IgM and IgG enzyme-linked immunosorbent assays (ELISAs) were performed at WRAIR and used standardized methods that captured IgM or IgG onto a solid phase, after which a mixture of all four DENV antigens produced in mouse brains were applied and allowed to react.16 Results of both assays were considered seropositive at values > 20 units, as defined by a reference serum. The ELISAs performed at the United States Army Institute for Infectious Diseases (USAMRIID) (Fort Detrick, Frederick, MD) were done using flavivirus antigens bound onto a solid phase to detect total specific IgG antibody, using serum specimens tested at a 1:100 dilution.17 These assays were standardized with an optical density > 0.19 (mean plus 3 SD of negative serum controls) established as the threshold value for seropositivity.

Plaque reduction neutralization test. This assay was performed using serial four-fold dilutions of heat-inactivated serum and DENV-1 through -4, SLE, or YFV viruses, with exogenous complement added to the virus diluent.18 After virus absorption at 35°C for one hour, confluent Vero cell monolayers were overlaid with 0.6% agarose/medium 199 mixture and incubated at 35°C for six days. Plaques were stained with a second overlay containing neutral red stain, counted, and the 50% PRNT (PRNT<sub>50</sub>) titer was determined. A PRNT antibody titer ≥1:10 was considered seropositive for any flavivirus. Homologous DENV PRNTs were performed at WRAIR before and after vaccination for all volunteers; PRNTs for heterologous antibody to DENV were performed only in the AvP study. The PRNTs for SLE and YFV viruses were performed at USAMRIID as part of a retrospective evaluation in 13 selected volunteers (see Serologic Evaluation Panel in the Results).

Statistical analysis. Data for vaccine recipients were entered into a computer database constructed using a standard-ized database entry program (Microsoft Access version 7.0; Microsoft Corporation, Redmond, WA). In addition, serologic and virologic results from WRAIR laboratory notebooks were forwarded to the volunteer’s study chart and recorded in the database in such a way as to preserve the anonymity of each volunteer.

Demographic and clinical features and viremia were compared between the atypical responders and the flavivirus-naive recipient group using chi-square tests (Glanz SA, 1988, Primer on Biostatistics. Version, 1.0, New York: McGraw-Hill, 1988). A Student’s t-test was used to compare mean values of variables calculated for the atypical and naive responders. Findings were considered significant if a P value < 0.05 was achieved.

RESULTS

Antibody responses following vaccination with attenuated dengue viruses. Seropositive DENV-specific IgM antibody responses were evident in flavivirus-naive responders (NR), with a peak 21 days after vaccination (Figure 1a). No DENV-specific IgG antibodies were detected. In eight atypical responders (AR), the pattern of antibody responses was reversed (Figure 1b): DENV-specific IgG antibody responses exceeded IgM antibody production, and were significantly higher than IgG antibodies observed in the other 61 DENV vaccine recipients (NR in Figure 1a). While the increases in DENV IgM antibodies were equivalent in AR and NR on days 14 and 21, the DENV IgM response was significantly higher in the eight AR by day 30 (mean ± SEM = 99.8 ± 14.3 versus 45.1 ± 6.1 units; P = 0.003). The onset of either DENV IgM or IgG antibody responses was not accelerated in the AR compared with the NR.

Weak DENV HAI antibody responses were apparent after monovalent DENV vaccination in NR (Figure 2a). There were few seropositive antibody responses, which were typically strongest against the infecting serotype. In the eight AR, the HAI antibody responses were significantly boosted for all four DENV serotypes following vaccination with monovalent DENV vaccine (Figure 2b). Broadly cross-reactive HAI antibody responses after DENV vaccination are consistent with prior flavivirus exposure, yet in contrast, the onset of HAI antibody responses was not accelerated in AR compared with NR, as one would expect in a typical anamnestic response.

In the AvP study, neutralizing antibodies were measured to the vaccine virus type (homologous PRNT) and to other DENV types (heterologous PRNTs) after vaccination with monovalent DENV-1, -2, -3, or -4 vaccine (Table 1).11 Thirteen NR volunteers generated only homologous neutralizing antibody by day 28 after DENV vaccination, while three AR generated both homologous and heterologous DENV neutralizing antibodies following vaccination that persisted to day 180 in two of these volunteers and to day 120 in the other. One DENV-4 vaccine recipient developed low-titer DENV-2 and DENV-3 neutralizing antibodies, but these disappeared after day 60 following vaccination. One DENV-3 vaccinee developed borderline positive (titer = 1:10) DENV-1 antibody detectable only on day 28. Unlike classic, high-titered anamnestic responses, the three AR generated low-titered homologous geometric mean neutralizing antibody titers comparable to the NR group: 490 versus 631, respectively, on
Comparison of naive and atypical responders. There were no significant differences in age distribution, racial characteristics, serotype, and manufacturer of DENV vaccine between the NR and AR (Table 2). However, sex was exclusively male among the AR versus 59% of the NR group (\( P = 0.07 \)).

Frequency of signs of fever and rash, or frequency of moderate to severe symptoms of headache, myalgias, and eye symptoms, revealed no differences between the NR and AR. Duration of signs or symptoms after DENV vaccination were also similar in the AR and NR.

Comparison of viremia after vaccination with attenuated DENV among the three studies used for this analysis showed that similar proportions of volunteers in the AR and NR groups had virus isolated from their serum specimens after vaccination (50% and 25%, respectively; \( P = 0.28 \)), and the proportions of viremic serum specimens among all serum specimens collected from the AR and NR were also similar (12 of 83 [14%] versus 57 of 624 [9%], respectively; \( P = 0.18 \)). However, in a subset analysis of the CVD study, the number of viremic serum specimens were significantly higher from the two AR volunteers compared with 16 NR (4 of 16 versus 4 of 159, respectively; \( P = 0.004 \)).

Viremia occurred earlier in NR compared with AR (Table 3): peak viremia was observed on days 8–10 after vaccination in NR, while AR had a delayed peak on days 12 and 13. The proportion of volunteers with viremia was significantly higher in the AR on day 12 after vaccination (60% versus 9%; \( P = 0.02 \)). The duration of viremia in the AR and NR was similar (\( P = 0.43 \)).

**Serologic evaluation panel.** A comparative panel of serum specimens was created to evaluate possible pre-vaccination flavivirus exposures among the AR and NR DENV candidate vaccine recipients. Representative NR volunteers were randomly selected from each study: two each from D2D and AvP, and one from CVD 5000. Serum specimens collected from the NR and AR volunteers before DENV vaccination

---

**FIGURE 1.** Dengue IgM and IgG enzyme-linked immunosorbent assay antibody responses after vaccination with attenuated monovalent dengue vaccine viruses. a, Mean ± SEM dengue IgM and IgG responses in 61 flavivirus-naive responders (NR) after dengue vaccination on day 0. Development of dengue IgM antibodies with a peak 21 days after vaccination is evident, while no dengue IgG antibody responses are detected. b, Mean ± SEM dengue IgM and IgG responses after vaccination in the eight atypical responders (AR). Dengue IgG antibody responses predominate over IgM responses, but the onset of dengue IgG responses occurs concurrently with large increases in IgM antibody.

---

**FIGURE 2.** Dengue hemagglutination-inhibiting (HAI) antibody responses after vaccination with attenuated monovalent dengue vaccine viruses. a, Dengue HAI antibody reciprocal geometric mean titer (GMT) responses in 61 flavivirus-naive responders (NR) after dengue vaccination on day 0, with antiserotype responses to DENV-3 and DENV-4 only exceeding the seropositive threshold (GMT > 1: 10) after vaccination. b, HAI antibody GMT responses in the eight atypical responders (AR) are dramatically increased to all four serotypes, despite vaccination with only a single attenuated type virus. The kinetics of development of HAI antibodies are similar in the two groups.
Homologous and heterologous neutralizing antibody responses following monovalent dengue (DENV) vaccination*

<table>
<thead>
<tr>
<th>Volunteer no.</th>
<th>Vaccine D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvP01</td>
<td>DENV-1</td>
<td>0, 0, 0, 0, 0, †</td>
<td>–‡</td>
<td>–</td>
</tr>
<tr>
<td>AvP16</td>
<td>DENV-1</td>
<td>0, 440, 270, 530, 1900</td>
<td>0, 450, 220, 105, 70</td>
<td>0, 510, 130, 70, 45</td>
</tr>
<tr>
<td>AvP19</td>
<td>DENV-1</td>
<td>0, 290, 940, 600, 250</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AvP22</td>
<td>DENV-1</td>
<td>0, 0, 0, 0, 0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AvP29</td>
<td>DENV-1</td>
<td>0, 40, 0, 0, 0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AvP05</td>
<td>DENV-2</td>
<td>–</td>
<td>0, 1,320, 720, 340, 60</td>
<td>–</td>
</tr>
<tr>
<td>AvP06</td>
<td>DENV-2</td>
<td>–</td>
<td>0, 850, 2,110, 490, 165</td>
<td>–</td>
</tr>
<tr>
<td>AvP08</td>
<td>DENV-2</td>
<td>–</td>
<td>0, 1,300, 1,300, 270, 300</td>
<td>–</td>
</tr>
<tr>
<td>AvP27</td>
<td>DENV-2</td>
<td>0, 75, 40, 140, 130</td>
<td>0, 300, 480, 160, 290</td>
<td>0, 50, 110, 30, 20</td>
</tr>
<tr>
<td>AvP31</td>
<td>DENV-2</td>
<td>–</td>
<td>0, 290, ND, 360, 170</td>
<td>–</td>
</tr>
<tr>
<td>AvP10</td>
<td>DENV-3</td>
<td>0, 10, 0, 0, 0</td>
<td>–</td>
<td>0, 370, 80, 30, 290</td>
</tr>
<tr>
<td>AvP15</td>
<td>DENV-3</td>
<td>–</td>
<td>–</td>
<td>0, 1,560, 2,660, 90, 220</td>
</tr>
<tr>
<td>AvP25</td>
<td>DENV-3</td>
<td>–</td>
<td>–</td>
<td>0, 390, 120, 200, 190</td>
</tr>
<tr>
<td>AvP26</td>
<td>DENV-3</td>
<td>–</td>
<td>–</td>
<td>0, 1,640, 1,500, 350, 1,800</td>
</tr>
<tr>
<td>AvP28</td>
<td>DENV-3</td>
<td>–</td>
<td>–</td>
<td>0, 640, ND, 210, 230</td>
</tr>
<tr>
<td>AvP32</td>
<td>DENV-4</td>
<td>–</td>
<td>0, 45, 10, 0, 0</td>
<td>0, 40, 0, 0</td>
</tr>
<tr>
<td>AvP33</td>
<td>DENV-4</td>
<td>0, 125, 60, 0, 0</td>
<td>0, 160, 55, 0, 0</td>
<td>0, 140, 40, 20, 0</td>
</tr>
<tr>
<td>AvP35</td>
<td>DENV-4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AvP37</td>
<td>DENV-4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AvP39</td>
<td>DENV-4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Data from the 20 monovalent vaccine recipients participating in the AvP96 study.[11] Heterologous anti-DENV plaque reduction neutralization tests (PRNTs) were not performed in the other two studies. Bold numbers indicate neutralizing antibody titers from atypical responders (AR). ND = not done.
† Homologous neutralizing antibody titers at day 0 (pre-vaccination), and days 28, 60, 120, and 180 post-vaccination with monovalent DENV vaccine. Seroconversion after vaccination defined as a homologous DENV PRNT titer $\geq 1:10$; a titer < 1:10 is shown as 0.
‡ Heterologous neutralizing antibody titers <1:10 for all sampled days (days 0, 28, 60, 120, and 180). Heterologous DENV PRNT titers are shown if seropositive (titer $\geq 1:10$ on any day post-vaccination).

DISCUSSION

We observed atypical DENV antibody responses and delayed viremia in eight of 69 volunteers following vaccination with attenuated DENV. These findings suggested previous flavivirus exposure despite medical history and serology screening for previous travel or vaccination. Unlike classic mean titer $[\text{GMT}_{30}] = 575$ versus 813, respectively; $P = 0.28$). However, six of eight AR had detectable NT antibody titers to YFV (range $40–640$) before vaccination while four of five NR from the evaluation panel had YFV titers $< 1:10$ ($P = 0.04$). After DENV vaccination, all eight AR demonstrated titers $\geq 160$ while four NR failed to seroconvert (YFV NT antibody titer $\geq 1:10$) ($P = 0.02$). One of the five NR had a small increase in titer to 40 after DENV vaccination.

### Table 2

Comparison of flavivirus-naive (NR) and atypical (AR) dengue vaccine (DENV) recipients*

<table>
<thead>
<tr>
<th>Demographics</th>
<th>NR (n = 61)</th>
<th>AR (n = 8)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age decade (second/third/fourth/fifth)</td>
<td>18:28/14:1</td>
<td>1:3:3:1</td>
<td>0.30</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>36:25</td>
<td>8:0</td>
<td>0.07</td>
</tr>
<tr>
<td>Race (African American:Caucasian:Hispanic:Other)</td>
<td>34:24:1:2</td>
<td>2:5:0:1</td>
<td>0.40</td>
</tr>
<tr>
<td>Dengue vaccine type (DENV-1:DENV-2:DENV-3:DENV-4)</td>
<td>15:14:17:15</td>
<td>2:3:12</td>
<td>1.00</td>
</tr>
<tr>
<td>Vaccine product (AvP:WRAIR)</td>
<td>17:44</td>
<td>3:5</td>
<td>0.88</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of volunteers with $T_{\text{max}} &gt; 38.5^\circ C$</td>
<td>3</td>
<td>2</td>
<td>0.18</td>
</tr>
<tr>
<td>No. of volunteers with rash</td>
<td>15</td>
<td>3</td>
<td>0.74</td>
</tr>
<tr>
<td>No. of volunteers with moderate or severe headache†</td>
<td>21</td>
<td>4</td>
<td>0.64</td>
</tr>
<tr>
<td>No. of volunteers with moderate or severe myalgia‡</td>
<td>7</td>
<td>1</td>
<td>0.61</td>
</tr>
<tr>
<td>No. of volunteers with moderate or severe eye symptoms§</td>
<td>5</td>
<td>2</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* AVP = Aventis Pasteur; WRAIR = Walter Reed Army Institute of Research; $T_{\text{max}}$ = maximum temperature.
† Presence requiring medication or bedrest for relief.
‡ Symptom requiring medication or bedrest for relief.
§ Presence of photophobia, conjunctivitis, or retro-orbital eye pain.
secondary flavivirus infections, these enhanced heterotypic antibody responses were not accelerated in appearance and did not result in anamnestic boosting of antibody titer to infecting DENV.19 Furthermore, these individuals had concurrent DENV IgM increases comparable to flavivirus-naive volunteers. These responses are also clearly different from experience with secondary infections, in which DENV-specific IgM is usually reduced compared with IgG responses.20

To our knowledge, this is the first report of delayed viremia and sustained broadly cross-reactive antibody responses in individuals following DENV vaccination. Typically, serologic responses to DENV vaccination measured by HAI, ELISA, or NT are primarily if not exclusively against the infecting DENV vaccine virus, and are not associated with an increase in DENV-specific IgG measured by ELISA (Figure 1a).11 We observed similar patterns of atypical responses (delayed viremia, IgG >> IgM antibodies, broadly cross-reactive HAI and NT antibodies) among volunteers in three studies conducted at different times and sites. Despite minor differences in study design, all volunteers were similarly screened and evaluated after vaccination. Our retrospective evaluation was unbiased because the NR and AR in each study were only identified later by their antibody responses after vaccination.

Initial efforts revealed no flavivirus exposure in these eight AR. An evaluation panel of paired serum specimens demonstrated boosted heterotypic serologic responses to flaviviruses by HAI, ELISA (Table 4), and NT antibodies (Table 5) following vaccination of the AR. These responses were absent in five NR randomly selected from the three studies. Furthermore, the antibodies detected by the HAI or ELISA confirmed lack of pre-vaccination antibodies to flavivirus in the AR, excluding apparent pre-exposure to SLE and Powassan viruses, the only flaviviruses previously known to circulate in North America.21 Only the YFV PRNT assay was found to discriminate between AR and NR before DENV vaccination, proving that PRNT assays are most sensitive for detecting previous exposure to YFV compared with the HAI or ELISA.22 Even here, two of the eight NR did not have detectable pre-existing NT antibodies to YFV (Table 5); thus; the PRNT is far from perfect in predicting previous YFV exposure.

Prior YFV exposure may be an important confounder of antibody responses to DENV vaccination, as first demonstrated by Schlesinger and others.24 Unrecalled YFV exposure through vaccination more than 20 years previously may be present in some volunteers in the United States (8 of 69 [12%] based on participants recruited at our centers), and should be considered in screening certain populations before flavivirus vaccination. Our experience suggests that the NT antibody to YFV be the most discriminating assay for prior infection because of the documented persistence of NT antibody to YFV over decades.23 Although the USAMRIID YF ELISA showed near seropositive antibody titers in the two AR with the highest NT antibody titers to YFV (> 160) before DENV vaccination, more sensitive ELISAs or other assays will be required to replace the screening YFV PRNT assay.

It is clear that antibody responses to attenuated flavivirus vaccines, particularly to YFV 17D vaccine, may be enhanced by previous flavivirus exposure. Cross-reactive HAI and complement-fixing antibodies to flavivirus, as well as heterologous NT antibodies, are commonly observed after YFV infection or vaccination in individuals with prior flavivirus exposure.10,24 Wiseman and Sweet showed in JEV-immune subjects that the pattern of induction of NT antibodies to YFV following YFV vaccination was similar in time of appearance and in magnitude of antibody response to that observed in flavivirus-naive individuals.25 Sweet and others did not observe a significant difference in incidence of YFV vaccine viremia between JEV-immune and flavivirus-naive subjects following vaccination, and onset of viremia only was delayed slightly (not significant).20 Our data, when compared with the results of Sweet and others, may reflect differences in sampling, immune memory conferred by natural infection.

### Table 4

Evaluation panel: mean flavivirus IgG antibody responses by enzyme-linked immunosorbent assay (ELISA) in the flavivirus-naive (NR) and atypical (AR) responder groups following dengue virus (DENV) vaccination

<table>
<thead>
<tr>
<th>Group</th>
<th>DENV-1</th>
<th>DENV-2</th>
<th>DENV-3</th>
<th>DENV-4</th>
<th>SLE</th>
<th>JE</th>
<th>POW</th>
<th>YF</th>
<th>TBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR (n = 5)</td>
<td>0.02*</td>
<td>0.03</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>AR (n = 8)</td>
<td>0.79*</td>
<td>1.01</td>
<td>1.58</td>
<td>0.27</td>
<td>1.33</td>
<td>1.40</td>
<td>0.46</td>
<td>1.45</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* Mean optical densities (ODs) from IgG ELISA for antibodies to DENV-1, DENV-2, DENV-3, DENV-4, SLE, JE, POW, YF, and TBE viruses. Post-vaccination (day 30) serum specimens were tested at 1:100 dilutions. An OD < 0.20 was determined as negative by United States Army Medical Research Institute of Infectious Diseases.

† Mean fold increase in OD ELISA OD after vaccination (day 30) compared to pre-vaccination (day 0) values. The formula used to calculate change was (ODpost = ODpre)/ODpre. Pre-vaccination (day 0) serum specimens showed uniformly negative antibody responses for all volunteers (data not shown); pre-vaccination serum specimens with an average OD of 0.00 were not calculable and are indicated by –.
with JEV versus remote vaccine-induced memory in our subjects, or between the tempo of YFV and DENV viremia after infection. Interestingly, with inactivated flavivirus vaccines such as TBE virus, the effect of prior YFV immunization may be different after vaccination, in which only early-high titer IgG responses to TBE virus were found without appreciable IgM responses.27

Our vaccinated subjects illustrate the time course for evolution of viremia and DENV-specific IgM and IgG antibodies after experimental infection with attenuated DENV vaccine viruses. Viremia in all vaccine recipients preceded the appearance of DENV-specific antibody. In the AR group, onset of viremia occurred immediately prior to the development of augmented IgG and IgM antibody responses to DENV. Unfortunately, we cannot comment on the magnitude of viremia associated with this burst of antibody production because virus titers were not directly measured. Similar timing of appearance of DENV-specific HAI, ELISA, and NT antibodies in the AR after DENV infection suggest that these responses are qualitatively different from those found in a typical case of dengue fever due to a secondary (anamnestic) DENV infection. We consider this phenomenon to be an example of immunologic priming by earlier YFV exposure leading to enhanced antibody responses to DENV.

Why DENV vaccination elicits atypical antibody responses in individuals primed by previous YFV vaccination is not clear, but stimulation of cross-reactive memory T cells that have been previously stimulated with YFV virus may be involved.28 Recall responses based on related YFV antigens may induce secondary responses to DENV, a process perhaps central medially by interferon-γ, as observed in heterologous influenza infection.29 It would be intriguing to look at the immunologic profile of T and B cells obtained from YFV-immune individuals at serial time points following DENV vaccination to see if there is earlier production of interferon-γ, or possibly diversion towards a Th2 type response with enhanced secretion of interleukin-4, compared with flavivirus-naïve recipients.

Could YFV vaccination potentiate the effects of DENV infection?29 Prior YFV vaccination increased neutralizing and enhancing antibody responses after DENV vaccination, without augmenting clinical signs or symptoms.9,31 In our studies, six of eight AR were probably remotely immunized with YFV vaccine and had no increased reactivity after DENV vaccination. Because of the frequency of YFV vaccination, early attention should be paid to each new DENV vaccine candidate on the impact of prior YFV exposure and the range of clinical responses, particularly fever, following vaccination.8,32

Whether cross-reactive antibodies produced after sequential immunization with flaviviruses are able to protect against subsequent DENV infection is uncertain and controversial.33,34 Halstead and Palumbo concluded protection against heterologous challenge DENV was equivalent or better in monkeys previously given a combination of flaviviruses rather than sequentially immunized.35 In humans, the experience of Wiseman and others is important in suggesting that development of cross-reactive antibodies after sequential immunization did not correlate with protection against DENV-1 viral challenge.36 These data have been used to justify the current strategy of tetravalent vaccination using a combination of four attenuated DENV.

### References


3. Makino Y, Tadano M, Saito M, Maneekarn N, Sittisombut N,


