Safety and Immunogenicity of a Tetravalent Live-attenuated Dengue Vaccine in Flavivirus Naive Children


Abstract. A live-attenuated tetravalent dengue virus (DENV) vaccine candidate has been well tolerated and immunogenic in healthy, US flavivirus naive adult volunteers. We conducted a pilot, safety, and immunogenicity trial of the vaccine candidate in healthy Thai children (6–7 years of age) to prepare for its eventual evaluation in Thai infants. In an uncontrolled, open clinical trial, the investigational vaccine was administered on study Days 0 and 180 to seven volunteers residing in Bangkok without neutralizing antibodies to DENV1–4 or to Japanese encephalitis virus (JEV). Clinical and laboratory safety assessments were completed during the 30 days after each vaccine dose, and immunogenicity was determined at Day 30. In this study, the vaccine was well tolerated with no serious adverse events or alert laboratory values. One volunteer experienced fever (38.2°C, <2 days) and associated DENV4 vaccine viremia 7 days after Dose 2. One month after Dose 2, six volunteers in the per-protocol analysis exhibited a tetravalent neutralizing antibody response with DENV1–4 geometric mean titers of 55, 475, 350, and 171, respectively. Ten weeks (~75 days) after Dose 2, five of the six volunteers continued to exhibit a tetravalent neutralizing antibody profile; one volunteer’s DENV4 PRNT50 titer fell below the assay cut-off (29 → <10); (clinicaltrials.gov NCT00384670).

INTRODUCTION

Dengue is the febrile illness caused by infection with any of four antigenically distinct dengue viruses (DENV1, DENV2, DENV3, DENV4). Infection outcomes vary and include asymptomatic seroconversion, a mild febrile illness, dengue fever (DF), and life-threatening dengue hemorrhagic fever (DHF). Dengue is an expanding public health problem in the tropics and subtropics driven by population growth, increased urbanization, international travel, and uncontrolled vector populations. An estimated 2.5 billion people are at risk for dengue, with 100 million DENV infections and 25,000 deaths occurring annually.2–4

Control of dengue by widespread vaccination has been a priority of the World Health Organization (WHO).3 The Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline (GSK) Biologics are co-developing a live-attenuated tetravalent DENV vaccine to protect children and adults against dengue.

The rationale for developing and testing the WRAIR/GSK tetravalent dengue vaccine candidate has been described previously.5,6 As of 2002, before this study began, the WRAIR/GSK tetravalent dengue vaccine candidates had been safely administered to 174 healthy adults living in the United States5 (Edelman and others, unpublished data). A well-tolerated and immunogenic tetravalent formulation had been identified.

Immunization of young children at risk for dengue is a potentially important disease control strategy. The age at which public health authorities will initiate routine dengue vaccination will depend on local factors such as age-specific prevalence of maternal dengue antibodies, age-specific disease risk, and the complexity of the existing pediatric vaccination schedule, including licensed flavivirus vaccines, as well as a vaccine’s attributes such as the number of doses required and its safety and efficacy profile. Therefore, safety and efficacy must be shown in children with a wide range of ages.

We began evaluation of our tetravalent dengue vaccine candidate in children living in Thailand because their risk of dengue is among the highest in the world. Moreover, Thailand’s infrastructure for performing dengue vaccine clinical trials is well developed.6,7 Ultimately, we envision conducting vaccine evaluations in three distinct populations: first, in school children without antibody evidence of past flavivirus vaccination or infection, then in infants in the second year of life without antibody evidence of past flavivirus vaccination or infection, and finally, in children of all ages regardless of prior flavivirus immunologic priming. The sequence of trials was designed to minimize risk to study volunteers and to maximize information regarding vaccine immunogenicity, which is best evaluated in immunologically naïve recipients.

Herein, we report a pilot open-label, safety, and immunogenicity trial of the candidate tetravalent dengue vaccine, the first step in the sequence of planned trials described above. We found the vaccine was well tolerated and satisfactorily immunogenic.

MATERIALS AND METHODS

Study design. We conducted the open-label Phase I/II trial in accordance with good clinical practice guidelines, the provisions of the Declaration of Helsinki, and regulations of both the United States and Thailand. The institutional review boards of the Phramongkutklao Hospital (Royal Thai Army), Thai Ministry of Public Health, and the Office of the Surgeon General of the US Army approved the study protocol. The US Army Medical Materiel Development Activity and GSK...
monitored the conduct of the trial and the veracity of the
data. An independent data and safety monitoring board
monitored adverse events (AE). A parent or guardian of each
subject provided written informed consent before participa-
tion.

Role of the sponsors. The study was designed by the
WRAIR and GSK. Investigators in Thailand collected the
data; statisticians at GSK analyzed the data according to a
pre-specified and mutually approved plan. All the authors
wrote the manuscript and vouch for the accuracy and com-
pleteness of the article.

Study subjects. Volunteers were recruited from students
6–9 years of age attending the Wat Samian Naree School in
Bangkok, Thailand, who were in good health and who had
never received a Japanese encephalitis (JE) vaccine. After
parental written informed consent was obtained, 89 children
were screened by medical history, physical examination, and
laboratory testing. Screening tests included a complete blood
count (CBC, including white blood cell differential and plate-
let count), alanine aminotransferase (ALT), aspartate ami-
notransferase (AST), hepatitis B surface antigen, antibody to
human immunodeficiency virus (HIV), and antibody to hepa-
titis C virus. Those with normal test results were further
screened to exclude those with hemagglutination-inhibiting
(HI) or neutralizing antibodies to any DENV type or to JE
virus (JEV).

Vaccines. The development of a live-attenuated DENV
vaccine against each of the four DENV types has been de-
scribed elsewhere.10 Monovalent vaccine lots (Table 1) were
manufactured by the Salk Institute, Government Services Di-
vision, Swiftwater, PA, at the third fetal rhesus monkey lung
(FRhl) cell culture passage, using viral supernatants clarified
after harvest, aliquoted in vials, and freeze-dried. To prepare
the investigational tetravalent DENV vaccine for administra-
tion, freeze-dried monovalent vaccines were rehydrated and
mixed in equal volumes as a tetravalent vaccine in sterile glass
vials. The viral content for each DENV type vaccine strain
contained in the tetravalent formulation is listed in Table 1.
Viral concentrations for each strain were chosen based on the
results of previous Phase I clinical trials (Edelman and others,
unpublished data).11 Two doses of tetravalent vaccine (1 mL
each) were administered subcutaneously ~6 months apart.

As part of the clinical trial, catch-up immunization against
JE was provided. Two doses of JE vaccine were administered
~14 days apart, beginning 1 month after the second dose of
DENV vaccine. The JE vaccine (Beijing strain), approved by
the Thai Food and Drug Administration (FDA), was manu-
factured by Thailand’s Government Pharmaceutical Organi-
zation (GPO).

Adverse events. Volunteers were evaluated at visits 2, 4, 10,
14, 21, and 30 days after each dengue vaccination and by

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>DENV strains, PDK passage, and viral concentration of each tetravalent preparations retained from each day of vaccine dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>PDK passage</td>
</tr>
<tr>
<td>DENV1 (45AZ5)</td>
<td>PDK 27</td>
</tr>
<tr>
<td>DENV2 (S16803)</td>
<td>PDK 50</td>
</tr>
<tr>
<td>DENV3 (CH53489)</td>
<td>PDK 20</td>
</tr>
<tr>
<td>DENV4 (341750)</td>
<td>PDK 6</td>
</tr>
</tbody>
</table>

Telephone follow-up 1, 3, 5, 6, 7, 9, 13, and 18 days after each
dengue vaccination. Parents were requested to consult an in-
vestigator for any illness their child experienced throughout
the study period. Investigators asked subjects about any AE at
all study visits and performed a history-directed physical
examination for rash, mucosal or conjunctival hemorrhage,
conjuvtical injection, lymphadenopathy, hepatomegaly, or
splenomegaly. Absolute neutrophil counts (ANCs), platelets,
and serum ALT and AST levels were determined on study
Days 0, 10, 30, 180, 190, and 210. Alert laboratory values were
defined as follows: ANC < 1,000 cells/mm³, platelets <
100,000 cells/mm³, ALT level > 2.5 times the upper limit of
normal (≥ 30 U/L), and AST level > 2.5 times the upper limit
of normal (≥ 40 U/L). A test for dengue viremia was per-
formed routinely 10 days after each dengue vaccination and
in case of any febrile illness consistent with dengue.

Study volunteers and their parents recorded oral tempera-
ture and the intensity of injection site and general symptoms
on diary cards for a period of 21 days after each dengue
vaccination and 7 days after each JE vaccination. Injection
site symptoms included pain, redness, and swelling; general
symptoms included fatigue, headache, photophobia, retro-
orbital pain, abdominal pain, nausea and/or vomiting, pruri-
tus, and muscle and/or joint ache.

Serious AE (which were defined as medically significant
events, including those resulting in hospitalization, disability,
or death) were recorded throughout the study. Adverse
events were coded with the use of the Medical Dictionary for
Regulatory Activities (http://www.meddrumsso.com).

We performed an interim analysis of available safety data
up to 21 days after Dose 1 of dengue vaccine. Results were
reviewed by an independent Data Monitoring Committee
(DMC) in Thailand, local and US Army IRBs, and the US
FDA before administration of a second vaccine dose.

Assays for immune response, viremia, and vaccine potency.
Volunteers were screened for the presence of HI antibodies
and neutralizing antibodies to DENV and JEV by the plaque
reduction neutralization test (PRNT50) according to the stan-
dard methods of the Armed Forces Research Institute of
Medical Sciences (AFRIMS; Bangkok); a titer of ≥ 1:10 was
considered positive.12,13 JE vaccine immunogenicity against
the Nakayama JEV strain was also characterized at AFRIMS.

To characterize DENV vaccine immunogenicity, DENV
neutralizing antibodies on the day of vaccination and ~30 days
after each vaccination were measured in the Division of Viral
Diseases, WRAIR (Silver Spring, MD). The endpoint was a
50% PRNT titer; a result ≥ 10 was considered positive. The
assay was performed in Vero cells [Vero-81; American Type
Culture Collection (ATCC), Manassas, VA] without comple-
ment and with virus reference strains DENV1 (WP74),
DENV2 (S16803), DENV3 (CH53489), and DENV4 (TVP360).

Evidence of natural infection with DENV or JEV during
the study period was evaluated through passive surveillance
of febrile illnesses and by performing IgM/IgG capture EIA
on the day of each vaccination and 30 days after each vacci-
nation.14 Case confirmation was attempted using HAI,
PRNT50, and detection of wild-type dengue viremia.

Detection of DENV RNA was accomplished by qualitative
nested RT-PCR. Viral RNA was extracted from volunteer
serum using the TRIzol reagent (Invitrogen, Life Technology,
Carlsbad, CA), according to the manufacturer’s instructions.
Viral genomic RNA was converted to cDNA by reverse transcriptase (RT) according to previously described method. The cDNA was used in the first round as a template to amplify the 511-bp DNA fragment from the region between capsid and pre-membrane (prM) genes by AmpliTaq DNA polymerase using conserved primers to all four DENV types. The type-specific DNA fragments for DENV1–4 were amplified by AmpliTaq DNA polymerase using the DNA fragment (the product of the first-round PCR) as a template and mixed primer pairs (one forward primer DENV1 and four reverse primers: TS1, TS2, TS3, and TS4) in the nested PCR (the second round PCR). The type-specific DNA fragments amplified by the nested PCR were electrophoresed in 15% agarose gel, followed by staining of the gel with ethidium bromide. The specific DNA bands for each DENV type were visualized under ultraviolet light.

DENV viremia was measured by titration in *Toxorhynchites splendens* (Tx) mosquitoes, as previously described. Serum specimens were diluted 10-fold to 10⁻⁴; 0.318 μL from each dilution was injected into 20 Tx. *splendens* mosquitoes. Injected mosquitoes were reared for 14 days at 30°C. Antigen was detected in mosquito heads by indirect immunofluorescent staining with polyvalent anti-DENV1–4 hyperimmune mouse ascitic fluid (HMAF). The number of median mosquito infectious doses per milliliter (MID₅₀/mL) was calculated by probit analysis (v12; SPSS, Chicago, IL).

Each volunteer blood sample containing DENV had a partial genomic sequence (E gene) analysis performed to characterize it as a vaccine virus or wild-type virus. Monovalent and tetravalent vaccines served as controls. RNA was extracted directly from the volunteer serum sample. Cycle sequencing reactions were performed on purified DNA fragments using the DYEnamic ET dye terminator sequencing kit (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden), according to the manufacturer’s instructions. The sequencing products were cleaned by standard precipitation before sequencing on a MegaBACE 500 automated DNA sequencer (Amersham Pharmacia Biotech). Overlapping nucleic acid sequences were combined for analysis and edited with the aid of Sequencher software (Gene Code Corp., Ann Arbor, MI). The maximum likelihood (ML) phylogenetic tree for each E gene sequence was estimated [PAUP*, Phylogenetic Analysis Using Parsimony (*and other methods), version 4; Sinauer Associates, Sunderland, MA].

Dengue vaccine potency was confirmed using an immunofocus assay (IFA) modified from a previously described protocol. Briefly, each residual DENV vaccine formulation sample was serially diluted in 2% fetal bovine serum (FBS)-Eagle’s minimal essential medium (EMEM) and used to inoculate confluent Vero cells seeded in 24-well plates. After 1 hour of virus absorption, the wells were overlaid with 2× EMEM supplemented with FBS, 1-glutamine, non-essential amino acids (NEAA), streptomyacin, neomycin, Fungizone, and carboxymethyl-cellulose (CMC) (0.75% wt/vol). After a 5-day incubation, the plates were fixed, permeabilized, and blocked. Virus-specific infected cells (foci) were detected using diluted DENV1–4 monoclonal antibodies (1F1, 3H5, 5D4, and 1H10), goat-anti-mouse-AP, and BCIP/NBT as a substrate in DENV mono-specific designated plates. The foci were manually counted and final titers were defined as a given number of infectious foci units per unit volume expressed as focus forming units per milliliter (FFU/mL). Only dilutions that developed between 10–100 foci/well were used. The final titers were log-transformed.

**Data analysis.** Because most children of an age eligible for this study in Thailand were expected to have antibodies to either DENV or JEV, the specified sample size was 5–10 volunteers, enough to provide a qualitative assessment of vaccine safety.

The primary safety analysis was performed on the total vaccinated cohort. The overall percentage of volunteers reporting an AE after vaccine administration (21 days for solicited AEs after each dengue vaccination, 7 days for solicited AEs after JE vaccination, and 30 days after any vaccine dose for spontaneously reported symptoms) were tabulated with exact 95% confidence intervals (CIs), by type of AE, by intensity (any grade and Grade 3), and by relationship to vaccination. The proportion of volunteers with abnormal findings at the physical examination reported up to 30 days after each dengue vaccination was tabulated with exact 95% CI.

The primary analysis of immunogenicity was based on the per-protocol cohort, which included all evaluable volunteers (i.e., those who met all eligibility criteria, who complied with the procedures defined in the protocol, with no elimination criteria during the study) and for whom data concerning immunogenicity endpoint measures were available (volunteers for whom assay results were available for at least one laboratory test after dengue vaccination). The percent of the cohort that seroconverted to any dengue type 30 days after Dose 2 of dengue vaccine was calculated with 95% exact CIs. Seroconversion was defined as the appearance of neutralizing antibodies in the serum of volunteers seronegative before vaccination. The geometric mean titer (GMT) calculations were performed by taking the anti-log of the mean of the log titer transformations, with a value of 5 given to titers < 1: 10. The proportion of subjects with neutralizing antibodies to each DENV type and JE vaccine were calculated with exact 95% CI. Seroconversion rates and GMTs (with exact 95% CIs) for each DENV vaccine type were calculated on blood samples taken 30 days after DENV doses. Seroconversion rate (with exact 95% CIs) for JE vaccine, 30 days after JE vaccine Dose 2, was also calculated.

Data analysis was performed with the use of SAS software (version 8.2) and ProcStatXact 5 with Windows NT 4.0.

**RESULTS**

**Study population.** Investigators recruited volunteers without a history of JE vaccination. Of 89 children screened, 41 (46.1%) had no DENV1–4 or JEV antibodies by HI; of these, 21 (51.2%) also had no neutralizing antibodies. Clinical laboratory abnormalities (*N* = 9) and refusal to participate (*N* = 4) eliminated 13 additional subjects. Of eight potential volunteers, seven (five girls and two boys) were enrolled and vaccinated; one volunteer, febrile on the day of vaccination, was excluded. Their mean age was 6.6 years (range, 6–7 years).

All volunteers completed the study having received all doses of DENV and JE vaccines. One volunteer was eliminated from the per-protocol immunogenicity analysis because of a sub-clinical DENV2 infection between screening and vaccine dose 1 (Day 0). The infection was identified by an IgM to DENV (41 enzyme immunoassay [EIA] units) and neutralizing antibody to DENV2 (1:126) detected in Day 0 serum.
**Vaccine safety.** All volunteers returned a symptom diary after each study vaccine dose. The incidence of solicited injection site symptoms is presented in Table 2. After dengue vaccine, injection site swelling was the most frequently reported local symptom (9 of 14 doses; 64.3%). Local symptoms lasted no more than 2 days and were similar between dengue vaccine doses. One case of Grade 3 redness was reported on Day 0 (25 mm) and Day 1 (30 mm) after dengue vaccine Dose 1. The same volunteer reported Grade 3 swelling (30 mm) on the day of dengue vaccine Dose 2. After JE vaccination, the incidence of injection site symptoms appeared to be less than after dengue vaccine: 3 of 14 doses (21.4%), with no Grade 3 reports.

The incidence of solicited general symptoms reported after dengue and JE vaccination is presented in Table 3. Although there were no Grade 3 solicited general symptoms reported after dengue vaccine, all general symptoms, except for photophobia, were reported in at least one volunteer after dengue vaccine Dose 1. Headache was reported by five of seven (71.4%) volunteers, associated with 8 of 14 dengue vaccine doses (57.1%). Fever (≥ 37.5°C) was reported by four of seven (57.1%) volunteers, associated with 7 of 14 (50%) dengue vaccine doses. Fatigue and muscle and/or joint aches were reported by three of seven (42.9%) volunteers, associated with 6 of 14 (42.9%) and 5 of 14 (35.7%) dengue vaccine doses, respectively. All but two volunteers graded their general symptoms after dengue vaccine as mild; symptoms were reported less frequently after dengue vaccine Dose 2 compared with Dose 1. Two volunteers reported Grade 2 general symptoms after dengue vaccination. One volunteer reported unilateral (left) Grade 2 retro-orbital pain on study Day 2 after dengue vaccine Dose 1. This report of pain was confirmed by the volunteer developing a recurrence of an infected, unilateral meibomian cyst shortly after vaccination. A second volunteer experienced a temperature of 38.2°C on Day 7 and 38.5°C on Day 14 after dengue vaccine Dose 2 (see illness description below). There were no Grade 3 solicited general symptoms reported after JE vaccine administration.

Headache was the most frequently reported general symptom, reported by three of seven (42.9%) volunteers, associated with 3 of 14 JE vaccine doses (21.4%).

The most frequently occurring examination finding after dengue vaccination was painless lymphadenopathy. Lymphadenopathy detected during follow-up safety visits was not distinguished from the baseline lymphadenopathy appreciated during a physical examination completed the day of, and immediately before, vaccination. The majority of lymphadenopathy was cervical and/or inguinal in location and was reported in six of seven volunteers (85.7%) and was associated with 10 of 14 doses (71.4%). One volunteer developed a focal and localized rash (upper back), not typical of a dengue rash, on Day 4 after dengue vaccine Dose 1. A second volunteer developed a generalized rash 10 days after dengue vaccine Dose 2 and after a brief febrile illness (see case history below). In the entire cohort, there were no reports or findings of conjunctival hemorrhage, hepatomegaly, or splenomegaly.

Among all seven volunteers, 9 of 14 (64.3%) dengue vaccine doses were associated with at least one spontaneously reported symptom within 30 days of vaccination. None of the spontaneous reports were considered by investigators to be related to vaccination, and none were Grade 3 in severity. These AEs were predominantly upper respiratory ailments (bronchitis, rhinorrhea, nasal congestion).

One of seven subjects was immunologically primed by recent DENV2 infection when administered dengue vaccine Dose 1. The profile of symptoms reported after Dose 1 in this volunteer was similar to that of immunologically naive volunteers.

No alert laboratory values (see Adverse Events) were reported during the study. One volunteer (14.3%) had an increased ALT (48 U/L) and AST (41 U/L) value measured 1 month after dengue vaccine Dose 1. After Dose 2, the same volunteer again had elevations in AST (maximum value 51 U/L) and ALT (maximum value 71 U/L); the volunteer was diagnosed with non-alcoholic fatty liver disease. A second volunteer had a mildly elevated AST level (44 U/L) measured 10 days after dengue vaccine Dose 2.

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### Table 2

<table>
<thead>
<tr>
<th>Dose</th>
<th>DENV (N = 7)</th>
<th>JE (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Any Grade 3</td>
<td>Any Grade 3</td>
</tr>
<tr>
<td>Dose 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>4 (57.1)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Redness</td>
<td>4 (57.1)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Swelling</td>
<td>5 (71.4)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Dose 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>3 (42.9)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Redness</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Swelling</td>
<td>4 (57.1)</td>
<td>1 (14.3)</td>
</tr>
</tbody>
</table>

**Values are N (%).** Schedule: vaccination with DENV (Months 0 and 6) and JE (Months 7 and 7.5). Total vaccinated cohort = seven volunteers; total number of doses = 14. Severity of pain was graded as follows: 0 (no pain), 1 (minor reaction to touch), 2 (cries on touch), and 3 (spontaneously painful).

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### Table 3

**Incidence of any and grade 3 solicited general symptoms during 21 days after dengue vaccination and 7 days after JE vaccination (total vaccinated cohort)**

<table>
<thead>
<tr>
<th>Symptom</th>
<th>DENV (N = ?)</th>
<th>JE (N = ?)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Any Grade 3</td>
<td>Any Grade 3</td>
</tr>
<tr>
<td>Dose 1 Temperature ≥ 37.5°C</td>
<td>4 (57.1)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>3 (42.9)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Headache</td>
<td>5 (71.4)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Retro-orbital pain</td>
<td>2 (28.6)</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Muscle/joint aches</td>
<td>3 (42.9)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Photophobia</td>
<td>0</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>2 (28.6)</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Dose 2 Temperature ≥ 37.5°C</td>
<td>3 (42.9)</td>
<td>0</td>
</tr>
<tr>
<td>Fatigue</td>
<td>3 (42.9)</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>3 (42.9)</td>
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<td>0</td>
</tr>
<tr>
<td>Pruritus</td>
<td>2 (28.6)</td>
<td>1 (14.3)</td>
</tr>
</tbody>
</table>

Values are N (%). Elevated temperature was graded 1 (≥ 37.5°C and ≤ 38.0°C), 2 (> 38.0°C and ≤ 39.0°C), and 3 (> 39°C). Severity ratings for other symptoms were graded as follows: 1 (easily tolerated), 2 (symptoms that interfered with normal activities), and 3 (symptoms that prevented normal activities).
There were no serious AEs during the study. All emergency room visits and physician office visits throughout the study were related to routine care or common acute illnesses, except for one febrile illness episode (see case history below).

Case history of febrile illness after dengue vaccine. One volunteer (D003-05) experienced a febrile illness with accompanying DENV4 vaccine viremia (4.02 log{MID}{sub}{50}/mL; 95% CI, 3.44, 4.39) after dengue vaccine Dose 2. At the time of vaccine Dose 2, this volunteer had a monovalent neutralizing antibody to DENV2. A maximum temperature of 38.2°C was measured on study Day 7 after vaccination and was accompanied by mild headache, retro-orbital pain, and fatigue. When examined 8 days after vaccination, there was bilateral conjunctival injection and a positive tourniquet test (> 10 petechiae in a 2.5-cm{sup}2 area); the ANC was 2,930 cells/mm{sup}3, the platelet count was 273,000 cells/mm{sup}3, the AST was 44 U/L, and the ALT was 30 U/L. By Day 10 after vaccination, the symptoms had resolved and the volunteer returned to school. Subsequently, a macular, erythematous, and non-pruritic rash appeared. Four days after the resolution of illness (14 days after vaccination), the volunteer became febrile again (38.5°C) and reported cough, rhinitis, and anorexia. Physical examination and chest x-ray were consistent with bronchitis; the hematocrit was 41.9%, the ANC was 5,090 cells/mm{sup}3, the platelet count was 434,000 cells/mm{sup}3, the AST was 44 U/L, and the ALT was 53 U/L. The volunteer was treated with an antibiotic and returned to school 2 days later (16 days after vaccination).

Vaccine immunogenicity. In the per-protocol cohort (N = 6), the seroconversion rate 30 days after dengue vaccine Dose 1 was 0%, 50%, 16.7%, and 50% for DENV1, –2, –3, and –4, respectively. Two of six volunteers (33.3%) had no detectable neutralizing antibodies, two of six (33.3%) had a monovalent antibody, one of six (16.7%) volunteers had bivalent antibodies, and one of six (16.7%) volunteers had trivalent antibodies. Between 30 and 180 days after dengue vaccine Dose 1, there was a general rise in neutralizing antibodies; by Day 180, 50% of volunteers had monovalent antibodies, 33.3% had trivalent antibodies, and 16.7% had tetravalent antibodies. One month after dengue vaccine Dose 2, all volunteers in the per-protocol cohort had tetravalent neutralizing antibodies. Volunteer D003-04 had lost DENV4 antibodies when evaluated –75 days after Dose 2.

The pattern of differential response between DENV2 and –4 compared with DENV1 and –3 is reflected in the GMTs to each vaccine type at all time points (Table 4). After two doses of dengue vaccine, GMTs of neutralizing antibodies were highest to DENV2 and DENV3. Seventy-five days after dengue vaccine Dose 2, GMTs had declined for all DENV types except DENV1; the reciprocal titers were 56, 107, 37, and 37. The priming effect of Dose 1 and the profound booster effect of administering Dose 2 were reflected in the geometric mean fold rise comparing 30 days after Dose 2 to 30 days after Dose 1; the response ratios for DENV1–4 were 11, 30, 58, and 6, respectively.

Volunteer D003-01 experienced an asymptomatic DENV2 infection before dengue vaccine Dose 1 (day of vaccination DENV1–4 PRNT{sub}{50} titers: < 10, 126, < 10, and < 10). Thirty days after Dose 1, the subject had a monovalent antibody response; DENV1–4 PRNT{sub}{50} titers were < 10, 151, < 10, and < 10, respectively. Thirty days after Dose 2, the subject had seroconverted to all but DENV1; DENV2–4 PRNT{sub}{50} titers were 2,000, 93, and 126, respectively.

Dengue vaccine elicited no neutralizing antibodies to JEV; therefore, all volunteers were antibody negative when administered their first JE vaccine dose 30 days after dengue vaccine Dose 2. One month after a second JE vaccine dose, two of six (33.3%) volunteers (D003-02/-03) had JE neutralizing antibodies > 1:10 (GMT 9); the use of the heterologous Nakayama JEV strain in the PRNT{sub}{50} assay may have confounded this evaluation (Table 5). However, there is evidence from the testing of well-characterized sample sets that PRNT results vary only between 2- and 3-fold when using different challenge viruses (Alan Barrett, personal communication). Additionally, antibodies to JEV resulting from vaccination with a particular strain have been shown to be cross-reactive against all genotypes and protective against homotypic and heterotypic challenge.

Viremia. Circulating DENV4 RNA and DENV4 viremia were detected by nested RT-PCR and mosquito inoculation, respectively, in three volunteers (Table 6). In volunteers D003-06 and D003-07, viremia occurred after Dose 1 and was either asymptomatic or associated with 2 days of body temperature elevated to 37.8°C. In volunteer D003-05, viremia occurred after Dose 2 and was associated with a fever of 38.2°C and mild symptoms (see case history above). The quantity of viremia was determined in MID{sub}{50}/mL; this assay could not distinguish virus type as performed. Sequence analysis of the envelope (E) gene sequences showed all viral isolates were of vaccine origin.

Vaccine potency. The potency of vaccines administered to

### Table 4

<table>
<thead>
<tr>
<th>Vaccine Potency</th>
<th>Reciprocal 50% plaque-reduction neutralization titers in flavivirus-naive children administered tetravalent dengue vaccine at 0 and 6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 days after Dose 1</td>
</tr>
<tr>
<td></td>
<td>D1 D2 D3 D4</td>
</tr>
<tr>
<td><strong>Volunteer</strong></td>
<td></td>
</tr>
<tr>
<td>D003-02</td>
<td>&lt;10 &lt;10 &lt;10 &lt;10</td>
</tr>
<tr>
<td>D003-03</td>
<td>&lt;10 &lt;10 &lt;10 10&lt;10</td>
</tr>
<tr>
<td>D003-04</td>
<td>&lt;10 &lt;10 &lt;10 10&lt;10</td>
</tr>
<tr>
<td>D003-05</td>
<td>&lt;10 &lt;10 &lt;10 &lt;10&lt;10</td>
</tr>
<tr>
<td>D003-06</td>
<td>&lt;10 &lt;10 &lt;10 10&lt;10</td>
</tr>
<tr>
<td>D003-07</td>
<td>&lt;10 &lt;10 &lt;10 10&lt;10</td>
</tr>
<tr>
<td><strong>GMT</strong></td>
<td>&lt;10 &lt;10 &lt;10 &lt;10</td>
</tr>
<tr>
<td>95% CI</td>
<td>NA 4.67 4.11 3.35</td>
</tr>
<tr>
<td>D003-01F</td>
<td>&lt;10 &lt;10 &lt;10 &lt;10</td>
</tr>
</tbody>
</table>

* Geometric mean titer (95% CI) in the per-protocol cohort.
† Excluded from per-protocol cohort because of DENV2 infection just before vaccination.
NA, not applicable.
DENV4 vaccine viremia was detected after vaccination: two instances after Dose 1 (measured Day 10) and one after Dose 2 (measured post-vaccination Day 8). Of the two volunteers experiencing DENV4 viremia after Dose 1, one was completely asymptomatic, and the other reported a maximum temperature of 37.8°C (Days 10 and 11) without other symptoms. The volunteer experiencing viremia after Dose 2 had a fever of 38.2°C and associated symptoms, but the symptoms were mild and short lived. In previous adult studies of the same vaccine formulation, 11 of 43 (25.5%) vaccine recipients had measurable DENV4 viremia; 5 of 11 (45.4%) were accompanied by mild to moderate symptoms (unpublished data).

The measurement of DENV-neutralizing antibodies needs to be validated as a surrogate endpoint for effective immunization when evaluating live dengue vaccine candidates. Although the minimum antibody response indicating protection is unknown, seroconversion is assumed to indicate activation of immunologic memory associated with recovery from infection by the vaccine viruses administered. The immunogenicity observed in this study was similar to that observed in earlier studies in flavivirus-naive adults administered two doses of DENV vaccine, subcutaneously, at Months 0 and 6. Most vaccine recipients developed neutralizing antibodies to DENV2 and DENV4 but failed to develop trivalent and tetravalent responses after one vaccine dose. Subsequently, neutralizing antibody titers to all types gradually increased between Doses 1 and 2. The candidate dengue vaccine elicited a tetravalent neutralizing antibody response in all volunteers in the per-protocol cohort (Figure 1) when measured 1 month after Dose 2 administration. This response is similar to that observed in a previous adult study where 88% of two-dose recipients (subcutaneous, study Months 0 and 6) responded by developing trivalent or tetravalent neutralizing antibodies. In this study, when measured ~75 days after Dose 2, all subjects had retained a tetravalent neutralizing antibody profile except one: subject D003-4 had a decline in DENV4-neutralizing antibody below the cut-off of the assay (29 → < 10). This finding may point to the ability of the PRNT assay to measure cross-reactive antibody or to the shorter durability of neutralizing antibodies generated by highly attenuated DENV. In one DENV-primed volunteer, recent infection did not seem to affect the immune response to the two-dose vaccination course, although no anamnestic response was observed after Dose 1.

Variation in GMTs across the DENV types has been observed in other dengue vaccine trials conducted by these authors and others.11,20,21 Without a neutralizing antibody correlate of protection, it is uncertain whether a balanced tetravalent response is required to protect against infection with any

<table>
<thead>
<tr>
<th>Vaccine dose</th>
<th>DENV type</th>
<th>Isolate sequence</th>
<th>Viremia level bioassay (log10 mosquito ID50/mL) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D003-06</td>
<td>DENV4</td>
<td>Vaccine</td>
<td>4.78 (3.07–5.78)</td>
</tr>
<tr>
<td>D003-07</td>
<td>DENV4</td>
<td>Vaccine</td>
<td>3.96 (3.43–4.40)</td>
</tr>
<tr>
<td>D003-05</td>
<td>DENV4</td>
<td>Vaccine</td>
<td>4.02 (3.44–4.39)</td>
</tr>
</tbody>
</table>

**Table 5**

Reciprocal 50% plaque-reduction neutralization titers after JE primary immunization (two doses at 2 weeks apart) administered 1 and 1.5 months after primary dengue immunization

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Day of JE Dose 1</th>
<th>30 days after JE Dose 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D003-02</td>
<td>&lt; 10</td>
<td>41</td>
</tr>
<tr>
<td>D003-03</td>
<td>&lt; 10</td>
<td>17</td>
</tr>
<tr>
<td>D003-04</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>D003-05</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>D003-06</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>D003-07</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>GMT</td>
<td>&lt; 10</td>
<td>9</td>
</tr>
<tr>
<td>95% CI</td>
<td>NA</td>
<td>3–23</td>
</tr>
</tbody>
</table>

* Excluded from per-protocol cohort because of DENV2 infection just before vaccination. NA, not applicable.

Volunteers was determined using tetravalent vaccine preparations retained from Dose 1 and 2 study visits. Potencies for Doses 1 and 2 were similar; DENV1–4 potencies in log10 FFUs per milliliter were 6.1–6.0, 6.7–6.3, 5.1–4.9, and 6.3–6.0, respectively.

**DISCUSSION**

This first evaluation of the WRAIR/GSK tetravalent dengue vaccine in children found that the vaccine was well tolerated and highly immunogenic.

There were no serious adverse events or alert laboratory values (see Adverse Events). Local and general symptoms typically were reported as mild, short in duration, and more often occurred after Dose 1 than Dose 2. There was no increase in the severity of reactogenicity after vaccination of a volunteer who had recently been infected with DENV2. There was no increase in reactogenicity observed after the administration of an inactivated JE vaccine subsequent to two doses of tetravalent live dengue vaccine. Extemporaneous formulation of tetravalent vaccine from monovalent components achieved the expected viral concentrations for each type.

Dengue vaccine injection site symptoms were mild and transient. Swelling was the most common sign, possibly related to the 1.0-mL injection volume. The current WRAIR/GSK dengue vaccine is administered as a 0.5-mL dose.

The most commonly reported general symptoms after dengue vaccine were mild and transient headache, fever, fatigue, muscle and/or joint aches, and pruritus. Painless cervical and inguinal lymphadenopathy, was the most common physical examination finding after dengue vaccination. There was one episode of a rash typical of that seen with dengue.

Clinical laboratory tests after dengue vaccination revealed no ANC or platelet counts below the range of normal. Mild transaminase abnormalities were observed in two volunteers after dengue vaccination; neither volunteer was found to have hepatomegaly on serial physical examinations.

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Variation in GMTs across the DENV types has been observed in other dengue vaccine trials conducted by these authors and others.11,20,21 Without a neutralizing antibody correlate of protection, it is uncertain whether a balanced tetravalent response is required to protect against infection with any
of the serotypes or whether unique antibody thresholds for protection exist for each serotype. We consider the development of neutralizing antibodies to at least three DENV types after vaccination as sufficient to protect against all DENV types based on the epidemiologic observation that an individual is unlikely to sustain dengue illness more than twice after natural infection\textsuperscript{23} (Gibbons and others, unpublished data). Whether immune responses after vaccination with attenuated viruses are similar to natural infection can only be confirmed in a controlled clinical endpoint trial.

This pilot pediatric study extends the previous work of the WRAIR and GSK to develop a tetravalent live-attenuated DENV vaccine and has enabled a subsequent study of the dengue vaccine candidate administered to infants in the second year of life.\textsuperscript{23}

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Disclaimer: The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the official views of the United States Army, Royal Thai Army, or the US Department of Defense.

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