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PHASE I TRIAL OF 16 FORMULATIONS OF A TETRAVALENT LIVE-ATTENUATED DENGUE VACCINE

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Abstract. Laboratory-attenuated strains of each of the four dengue serotypes previously tested as monovalent vaccines in volunteers were combined and tested for immunogenicity, safety, and reactogenicity in 16 dosage combinations. Tetravalent vaccines made using combinations of high (10^{5-6} plaque-forming units [PFU]/dose) or low ($10^{3.5-4.5}$ PFU/dose) dosage formulations of each of the four viruses were inoculated in 64 flavivirus non-immune adult volunteers to determine which, if any, formulation raised neutralizing antibodies in at least 75% of volunteers to at least three of four dengue serotypes following one or two inoculations. Such formulations, if safe and sufficiently non-reactogenic, would be considered for an expanded Phase II trial in the future. Formulations 1–15 were each inoculated into three or four volunteers (total = 54) on days 0 and 28. Formulation 16 was tested in 10 volunteers, five volunteers inoculated on days 0 and 30, one volunteer on days 0 and 120, and four volunteers on days 0, 30, and 120. Blood was drawn for serologic assays immediately before and one month after each vaccination, and for viremia assay on day 10 after each vaccination. The 16 formulations were safe, but variably reactogenic after the first vaccination, and nearly non-reactogenic after the second and third vaccinations. Reactogenicity was positively correlated with immunogenicity. Similar proportions of volunteers seroconverted to dengue-1 (69%), dengue-2 (78%), and dengue-3 (69%), but significantly fewer volunteers seroconverted to dengue-4 (38%). The geometric mean 50% plaque reduction neutralization test titers in persons who seroconverted were significantly higher to dengue-1 (1:94) than to dengue-2 (1:15), dengue-3 (1:10), and dengue-4 (1:2). Seven formulations met the serologic criteria required for an expanded trial, and three of these were sufficiently attenuated clinically to justify further testing.

INTRODUCTION

Prevention of dengue fever through widespread vaccination represents a priority of the World Health Organization and the United States Army.[1] A tetravalent vaccine is needed to immunize against all four dengue virus (DEN) serotypes, and to reduce the possibility of inducing severe dengue fever that theoretically could follow sequential immunization with monovalent vaccines. Underlying the rationale for a tetravalent vaccine was the initial demonstration in rhesus monkeys that primary-type immune responses can be elicited by the simultaneous inoculation of the four DEN serotypes.[2] Several Walter Reed Army Institute of Research (WRAIR) vaccine candidates, tested under investigational new drugs (INDs) filed between 1989 and 1998, have performed well in Phase 1 trials conducted at the University of Maryland[3] and at WRAIR.[4,5] The best four monovalent components for a tetravalent vaccine have been identified.

The design of the current trial was influenced by the possibility that one or more serotypes in the tetravavent mixture could either enhance or suppress the replication, immunogenicity, or reactogenicity of other vaccine serotypes. Limited data in rhesus monkeys suggest that the dose ratio of two serotypes of simultaneously inoculated dengue viruses affects the degree of protection obtained after rechallenge.[2] Another example of viral interaction is the trivalent Sabin oral polio vaccine in which serotype 2 interferes with serotypes 1 and 3.[6] The current design was also influenced by previous monovalent dengue vaccine trials, in which the vaccine was injected twice to explore the possibility that two vaccinations would be more immunogenic than one.[5] A dose interval of one month was chosen as a rapid, practical vaccination schedule for military personnel. In the first clinical trial of the tetravalent DEN 1–4 vaccine, one formulation was administered twice at an interval of one month with acceptable reactogenicity but with poor booster effect. Several of these volunteers later safely received a third vaccination three months after the second injection and a booster effect was noted.[5] The current study was designed to provide additional clinical and serologic data on the safety, reactogenicity, and immunogenicity of 15 other tetravalent vaccine formulations. The 15 formulations consisted of either high or low doses of the following four strains of DEN virus attenuated by passage in primary dog kidney (PDK) cell culture: DEN-1 (45AZ5) PDK 20, DEN-2 (S16803) PDK 50, DEN-3 (CH53489) PDK 20, and DEN-4 (341750) PDK 20 (Table 1). The high doses (~1×10^6 plaque-forming units [PFU] for DEN-1 and DEN-2 and ~1×10^5 PFU for DEN-3 and DEN-4) and low doses (~$1 \times 10^{4.5}$ PFU for DEN-1 and DEN-2 and ~$1 \times 10^{3.5}$ PFU for DEN-3 and DEN-4) were chosen by consensus of WRAIR and University of Maryland investigators based on previous experience with other attenuated multivalent vaccines, and by the titers of available vaccine candidates bottled and approved for trial by the Food and Drug Administration. As reported below, screening of all 16 tetravalent vaccine formulations in 64 flavivirus non-immune adult volunteers has permitted us to select two or three formulations for future expanded trial.

MATERIALS AND METHODS

Vaccine. The origins of the four serotype vaccine candidates have been described.[3–5,7,8] The four DEN vaccines administered under this protocol as a tetravalent vaccine (Table 1) were initially administered as monovalent vaccines in separate, initial Phase I trials.[3–5] The DEN-1, -2, and -3 vaccines were manufactured at the Salk Institute (Swiftwater, PA). The DEN-4 vaccine was produced at the Pilot Bioproduction Facility, WRAIR (Forest Glen, Silver Spring, MD). The vac-

TABLE 1

Composition of the 16 formulations of the live-attenuated tetravalent dengue (DEN) vaccine and the number of volunteers immunized with each formulation*

Formulation	Mean viral titer (PFU/mL) measured in tetravalent vaccine†	Vaccine strain and high (10^{5-6} PFU) or low ($10^{3.5-4.5}$ PFU) dose‡				
		DEN-1 (45AZ5) PDK 20 (H, 10^6 PFU); (L, $10^{4.5}$ PFU)	DEN-2 (S16803) PDK 50 (H, 10^6 PFU); (L, $10^{4.5}$ PFU)	DEN-3 (CH53489) PDK 20 (H, 10^5 PFU); (L, $10^{3.5}$ PFU)	DEN-4 (341750) PDK 20 (H, 10^5 PFU); (L, $10^{3.5}$ PFU)	Number of volunteers
1	5.5×10^4	L	L	L	L	3
2	5.3×10^5	H	L	L	L	4
3	9.9×10^5	L	H	L	L	3
4	1.5×10^5	L	L	L	H	3
5	7.1×10^4	L	L	H	L	4
6	6.6×10^5	L	H	L	H	3
7	5.2×10^5	H	L	H	L	4
8	6.5×10^5	L	H	H	L	4
9	1.2×10^5	L	L	H	H	4
10	1.3×10^6	H	L	L	H	4
11	1.1×10^6	H	H	L	L	3
12	6.2×10^5	L	H	H	H	4
13	4.3×10^5	H	L	H	H	4
14	1.2×10^6	H	H	L	H	4
15	9.2×10^5	H	H	H	L	3
16§	1.8×10^6	H	H	H	H	10
	Total	H = 8, L = 8	H = 8, L = 8	H = 8, L = 8	H = 8, L = 8	

* PFU = plaque-forming units; PDK = primary dog kidney; H = high; L = low.
† Calculated by adding the virus concentration in the residuals of each single-dose tetravalent vaccine vial after vaccination on days 0 and 28 / number of volunteers immunized with each formulation on those two days.
‡ Measured virus concentration of each DEN serotype by high and low dose.
§ Formulation 16 was tested at the Walter Reed Army Institute of Research.[5]

cines were lyophilized for stability and stored at –20°C in a controlled-access room at the Pilot Bioproduction Facility. The vaccines contain 50 μg/mL of neomycin base, 5.5% lactose, and 1.9 g% of human serum albumin. The manufacturing information for each DEN vaccine was included in a separate IND. All four INDs were combined into a single IND (7047) for testing of the tetravalent vaccine.

Preparation and inoculation of vaccines. The 16 tetravalent DEN vaccine formulations were prepared under Good Manufacturing Practice conditions on the day of inoculation at the Pilot Bioproduction Facility. Freeze-dried monovalent vaccines were rehydrated with sterile water for injection and formulated as tetravalent vaccines in sterile, eight-dose (8.0 mL) glass vials. The high dose of each monovalent vaccine was undiluted vaccine, while the low dose was a 1:32 dilution of the same vaccine. Vaccine preparations were formulated on ice, and formulations 1–15 were transported immediately to the University of Maryland (College Park, MD) (UMCP) for inoculation. The high dose of all four serotypes (formulation 16) was tested first at WRAIR as described.[5] Volunteers were inoculated subcutaneously over the deltoid muscle with 1.0 mL of vaccine. Following vaccination, unused vaccines were immediately transported back to WRAIR to determine viral titer. The viral composition, inoculum size, and number of volunteers immunized with each of the 16 formulations are summarized in Table 1. The final injected concentrations of tetravalent vaccine formulations ranged from 5.5×10^4 to 1.8×10^6 PFU/mL.

Volunteers. Fifty-four healthy male and female volunteers between the ages of 18 and 45 years were recruited at UMCP. The 10 volunteers recruited and immunized at WRAIR have been described.[5] The results for all 64 volunteers are combined in the current report. Volunteers were recruited by posted and published advertisements followed by verbal presentation of the study design and protocol. Of 64 volunteers, 37 (58%) were men, 39 were Caucasian, 15 were African-American, 7 were Asian, and 3 were Hispanic. Their mean age was 23.6 years, with an age range of 19–45 years.

Their status of good health was determined by a normal medical history, vital signs, a physical examination if the medical history suggested any abnormality, and clinical laboratory screening. Screening included hemoglobin; hematocrit; white blood cell, differential, and platelet counts; alanine aminotransferase (ALT); aspartate aminotransferase (AST); glucose; creatinine; urea nitrogen; hepatitis B surface antigen; antibody to human immunodeficiency virus; antibody to hepatitis C virus; and urinalysis. Persons allergic to neomycin, streptomycin, gentamicin, or similar antibiotics were excluded. The volunteers were seronegative for DEN 1–4, Japanese encephalitis, St. Louis encephalitis, and yellow fever by hemagglutinin inhibition assay. They denied prior yellow fever and Japanese encephalitis vaccination, having had a flavivirus infection, and travel to dengue-endemic areas. Women had a negative pregnancy test result one hour before vaccination, and were instructed not to become pregnant for the duration of the study. All volunteers scored 70% or better on a written examination designed to insure they were familiar with all aspects of the clinical trial.

Clinical protocols were reviewed and approved by the Institutional Review Boards of the University of Maryland at Baltimore, the University of Maryland at College Park, and the Office of the Surgeon General of the United States Army. Written, informed consent was obtained from all volunteers.

Study design. This single-blinded, outpatient Phase I/II trial was conducted in 54 healthy young adult volunteers at the University of Maryland at College Park, and in 10 volunteers at WRAIR.[5] A similar clinical protocol was used at both study sites. Volunteers did not know which vaccine formulation was injected. The 54 volunteers at UMCP were divided into 15 groups of 3–4 volunteers (Table 1) and each group

given a different formulation. The cohorts, initially immunized on 10/15/98, 10/21/98, and 1/19/99, consisted of 15, 16, and 23 volunteers, respectively. The second vaccination was administered 28 days later. Fifty-three of the volunteers at UMCP were inoculated on days 0 and 28; one was immunized on day 0 only. As previously described, the 10 volunteers at WRAIR were immunized with high dose formulation 16; nine of the 10 were immunized on days 0 and 28; four of these nine subjects received a third dose on day 120; one volunteer was immunized on days 0 and 120.[5]

Reactions to vaccines were assessed by a combination of daily symptom diaries maintained by each volunteer for 21 days after each vaccination, by telephone reports to the research nurse, and by regularly scheduled visits to the study physician on days 10, 15, and 28 after each vaccination. Volunteers took their oral temperature daily for 21 days in the mornings and evenings using a digital thermometer and recorded the results. After each vaccination, blood was drawn for clinical chemistries on days 0, 10, 15, and 28 and for DEN antibody assays on days 0 and 28. Serum was frozen 10 days after each vaccination for virus assay.

Clinical reactions. The clinical reactions to a vaccine formulation were defined in two ways: as safe or unsafe and as non-reactogenic or reactogenic. The criterion unsafe was met if any volunteer experienced any one of four criteria: 1) any severe clinical illness not explained by a diagnosis unrelated to DEN vaccination; 2) an oral temperature ≥38.5°C (101.3°F) for four determinations over a 24-hour period, a maximum daily oral temperature ≥38.5°C on three successive days, or a temperature exceeding 40°C (104.0°F) on any individual determination; (3) thrombocytopenia (fewer than 90,000 platelets/mm^3 on two consecutive determinations, e.g., days 10 and 15) or neutropenia (absolute neutrophil count <1,000/mm^3 on two consecutive determinations); and 4) serum ALT levels more than five times normal (normal range = 0–45 U/L) on three or more successive days that was otherwise unexplained. Each symptom was graded on a scale of 0 to defined as 0 = none; 1 = mild (did not affect normal activity, no medication required); 2 = moderate (required medication or change in activity); and 3 = severe (required bedrest, and or unrelieved by medication). Systemic reactions were analyzed for each subject by the Reactogenicity Index (RI), defined as the sum of total days of 1) feeling hot or a temperature ≥100°F (feverish or fever), 2) rash, 3) chills, 4) headache, 5) anorexia, 6) nausea and/or vomiting, 7) stomach ache 8) myalgia, 9) arthralgia, 10) eye symptoms (photophobia, redness or painful movement), and 11) itching at sites other than the injection site. If a symptom occurred at any time during a 24-hour period, it was assigned a duration of one day.

The RI allowed a quantitative comparison of vaccine reactions among subjects and vaccine formulations. The RI criteria used to grade formulations 1–15 included the duration of the 11 symptoms or signs (*vida supra*), but not their intensity. The intensity (grades 1–3) of any symptom was expressed in narrative fashion. The more intense reactions (grades 2 and 3) interfered with the daily activities of school, work, recreation, or sleep. In contrast, the RI criteria used by Sun and others to grade formulation 16 described the duration of nine major symptoms, included their intensity.[5] The RI formula used to grade formulation 16 was proportional to, but yielded somewhat lower values than the RI used to grade formulations 1–15. Febrile and non-febrile symptoms were treated if clinically indicated with analgesics without antipyretic properties. The dose and duration of all medications used were recorded in the Volunteer Symptom Diary.

The identification of a vaccine formulation that was too reactogenic was reached by a consensus of study investigators. Thus, a vaccine formulation could be safe but too reactogenic, and so unsuited for expanded study.

Laboratory procedures. *Routine clinical assays.* Venous blood was drawn for a complete blood count, differential counts, and ALT and AST levels on days 0, 10, 15, 28, 38, 43, and 56 in a commercial laboratory accredited by the American College of Pathology.

Serology. The study end point determination was measurement of the 50% plaque reduction neutralization test ($PRNT_{50}$) titer against DEN serotypes 1–4 28 days after the first and second vaccination.[9] The four DEN strains used in the PRNT were the parents of the four stains used in the vaccine formulations. Neutralization was defined as 50% reduction in plaques at a minimum serum dilution of 1:5. Complement was not added to the neutralization mixture.

Virus isolation by delayed plaque assay. Ten milliliters of venous blood obtained 10 days after each vaccination was allowed to clot at 4°C for ≤2 hours. The serum was separated by low-speed centrifugation, divided into aliquots, frozen, labeled, and stored at −70°C before shipment to WRAIR for virus isolation. Frozen serum was thawed and 0.20 mL was inoculated onto the C6/36 clone of *Aedes albopictus* mosquito cell monolayers and incubated at 30°C for 14 days. Supernatant culture fluid was then harvested and assayed for virus by plaques that formed seven days after the supernatant was applied to a Vero cell monolayer overlayed with methyl red agar. The direct plaque method for determination of virus in serum was not used because of low yield in preliminary studies.

Virus detection by Taqman assay. We used a fluorogenic, reverse transcriptase–polymerase chain reaction (RT-PCR) system (Taqman assay) to determine the serotype of circulating dengue vaccine strains.[10] This assay can detect each of the four dengue serotypes at similar low detection limits (20–50 PFU/mL of serum). Briefly, the viral RNA was routinely extracted from 0.10 mL of serum, followed by reverse transcription. The RT reactions were performed on RNA extracted from the equivalent of 1.6 μL of serum, according to the PE *Taq* RT reaction kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). Using serotype-specific, 3′-noncoding region-based primers and an fluorescent reporter dye, 6-carboxy-fluorescein (FAM)–labeled oligonucleotide probe, a total of 40 cycles of amplification was carried out using the ABI 7700 instrument (Perkin Elmer-Applied Biosystems). Positive identification was made when the two replicates of each serum specimen yielded C^t values less than 40 cycles. The viral titer (PFU/mL) for each dengue type was predicted and estimated from the C^t cycle number using a plasmid standard curve.[10] We assayed serum specimens from all 54 volunteers 10 days after the first vaccination (formulations 1–15), and from 53 of these volunteers 10 days after the second vaccination (day 38). Volunteers vaccinated with formulation 16 were assayed by the Taqman assay and are presented elsewhere.[5]

Data analysis. This was a descriptive study. The design ensured that if three of the 3–4 volunteers per group develop

neutralizing antibody after either of two vaccinations, the upper 95% confidence interval for infection exceeds 95% for that vaccine formulation. At least three of the four DEN serotypes in a vaccine formulation must induce antibody in at least three volunteers for that formulation to be considered for further study. Relationships between categorical variables were examined using either Fisher's exact tests, or replicated goodness-of-fit G-tests, as appropriate. Inter-group comparisons of continuous variables were examined using Wilcoxon's rank-sum tests. Comparisons of PRNT titers against pairs of DEN types were performed using Wilcoxon's signed ranks tests. Comparison of RIs by low and high dose DEN formulations were performed using Wilcoxon's rank-sum tests. Relationships between continuous variables were evaluated using Spearman's correlation coefficients. Two-sided null hypotheses were evaluated at 5% throughout. Data analysis was performed using SAS software (SAS, Inc., Cary, NC).

RESULTS

Volunteer retention. Fifty-four volunteers were immunized once, and 53 of them were immunized a second time according to protocol. One volunteer (9-3) refused the second vaccination because she had an intercurrent illness on the day of vaccination. All 54 volunteers were followed for side effects, and all of the 378 scheduled serum specimens were obtained on time according to protocol. The retention of the 10 volunteers studied at WRAIR has been described.[5]

Serologic responses. *Seroconversion rates.* The neutralizing antibody responses induced by each of the 16 vaccine formulations after the first and second vaccinations, together with the cumulative responses, are summarized in Tables 2–5. Seven of the first 15 formulations, (2, 5, 10, 11, 13, 14, 15) were considered superior because they met study criteria by eliciting antibody to at least three of four DEN serotypes in at least three (≥75%) volunteers in a group of 3–4 individuals (Table 2). Nine formulations were considered inferior because they failed to meet study criteria for immunogenicity (Table 4). The high dose formulation (16) induced a trivalent response in only three of the nine individuals immunized at 0 and 1 months, and in the one person immunized at 0 and 4 months (Table 4).[5] There were no significant differences in the seroconversion rates by race or sex for all formulations.

Although seven formulations induced a trivalent response in 100% of the individuals, no formulation induced a tetravalent response in every individual. The most robust tetravalent antibody responses noted (75% of the volunteers) were induced by formulations 2 and 14 (Table 2). In 64 volunteers, 38 (59%) seroconverted to three DEN serotypes, and 16 (25%) seroconverted to all four serotypes.

When all 16 formulations were combined, the rates of seroconversion to DEN-1, DEN-2, and DEN-3 were similar (69%, 78%, and 69%, respectively), but significantly fewer volunteers seroconverted to DEN-4 (38%; $P < 0.001$) (Table 6). Although significantly more volunteers seroconverted to DEN-1, -2, or -3 than to DEN-4, these serotypes in the vaccine did not appear to interfere with DEN-4. For example, after the first vaccination (Table 7), 7 (58%) of 12 volunteers seroconverted after the 10^5 dose of monovalent DEN-4 vaccine[5] compared with 17 (47%) of 36 volunteers administered the 10^5 dose of DEN-4 in the tetravalent vaccine. The rates of seroconversion after the first vaccination were also similar in the monovalent and tetravalent formulations for the three other DEN serotypes, suggesting no heterologous interference between serotypes. We did not compare the geometric mean titers (GMTs) in the monovalent and tetravalent trials because the $PRNT_{50}$ assays were performed differently, with complement added to the serum assayed in the monovalent vaccine studies but not in the tetravalent vaccine studies.

We next calculated the seroresponse rate to each DEN serotype as a function of the virus concentration (high or low) in the vaccine formulations (Table 8). Of the four serotypes, only DEN-1 showed a relationship between concentration and response rate, with the high concentration being significantly more immunogenic than the low concentration. The high and low concentrations of DEN-2, DEN-3, and DEN-4 gave similar seroconversion rates.

Geometric mean titers. Although the PRNT titers of seropositive individuals within a group often varied considerably (see ranges in Tables 3 and 5), the GMTs within serotypes were similar among the seven acceptable formulations (Table 3). Although the rate of seroconversion to DEN-1 was no greater than to other serotypes, the DEN-1 GMT was significantly higher than the DEN-2 GMT, and the DEN-3 GMT was significantly higher than the DEN-4 GMT (by post-hoc analysis, Table 6).

Serologic response to the second vaccination. We compared day 28 and day 56 DEN neutralizing antibody titers for evidence of a booster effect after the second vaccination (Tables 3 and 5). An antibody titer increase was defined as a $PRNT_{50}$ titer increase from <1:5 to ≥1:5, or a ≥4-fold increase in titer to one or more serotypes; an antibody titer decrease was defined as a ≥4-fold decrease, or loss of detectable antibody against 1–3 of the vaccine serotypes. After 62 volunteers were re-vaccinated on day 28, 25 (40%) had an antibody titer increase and 28 (45%) had a titer decline. In addition, of 248 viral assays represented in the day 56 data set (62 volunteers × 4 serotypes), nearly equal numbers of sera showed an increase (17%) as a decrease (19%). Thus, re-vaccination at one month offered no discernable benefit.

TABLE 2

Neutralizing antibody responses induced by seven superior (serologically acceptable)*formulations of tetravalent dengue (DEN) vaccine in volunteers receiving each formulation who seroconverted to at least three DEN serotypes and to all four serotypes

	Number of volunteers with neutralizing antibody † against					
	≥3 Serotypes (%)			4 Serotypes (%)		
Formulation	Day 28	Day 56	Cumulative	Day 28	Day 56	Cumulative
2	4/4	1/4	4/4 (100)	3/4	1/4	3/4 (75)
5	4/4	3/4	4/4 (100)	1/4	0/4	1/4 (25)
10	2/4	3/4	4/4 (100)	0/4	1/4	1/4 (25)
11	2/3	1/3	3/3 (100)	0/3	0/3	0/3
13	3/4	4/4	4/4 (100)	2/4	1/4	2/4 (50)
14	1/4	4/4	4/4 (100)	1/4	3/4	3/4 (75)
15	0/3	3/3	3/3 (100)	0/3	0/3	0/3

* Formulation achieved the study objective; namely, at least three of the four DEN serotypes in the vaccine formulation induced antibody in three or more volunteers (≥75%). The composition of each formulation is shown in Table 1.

† Number of volunteers in each formulation who achieved a 50% plaque reduction neutralization test ($PRNT_{50}$) titer ≥1:5, divided by number of volunteers in the group. Calculations based on a $PRNT_{50}$ titer ≥1:5 reached 28 days after the first vaccination (day 28), 28 days after the second vaccination (day 56), and cumulative seroconversions (total number of volunteers who seroconverted after the first or second vaccination).

TABLE 3

Neutralizing antibody responses induced by seven superior (serologically acceptable)* formulations of tetravalent dengue (DEN) vaccine with geometric mean titers† and range of peak titers elicited by each formulation against each of the four DEN serotypes 28 days after vaccinations 1 and 2

	Reciprocal of the geometric mean titer											
	DEN-1			DEN-2			DEN-3			DEN-4		
Formulation	Day 28	Day 56	Range‡	Day 28	Day 56	Range†	Day 28	Day 56	Range‡	Day 28	Day 56	Range‡
2	1,189	1,197	530–3,100	12	6	5–168	25	3	5–51	5	2	0–20
5	1,197	275	616–1,978	17	5	5–94	5	5	0–10	2	2	0–5
10	1,538	843	565–9,133	6	9	5–156	33	32	44–180	1	2	0–5
11	935	177	385–2,750	11	17	10–82	4	2	5–10	1	1	0
13	1,665	598	828–3,277	5	3	0–138	9	7	5–92	24	35	26–184
14	13	939	1,009–1,544	5	13	0–86	7	19	5–475	2	9	5–23
15	26	176	31–600	5	17	5–31	4	10	5–59	1	1	0

*Serologically acceptable is defined in the first footnote in Table 2.
† For calculation of geometric mean titer, a titer <1:5 was expressed as 1:1.
‡ Range of peak 50% plaque reduction neutralization test titers in volunteers on day 28 or day 56 showing the higher of the two titers against each serotype.

Clinical reactions to formulations 1–15. *Local reactions.* Twenty-four (44%) of 54 individuals first vaccinated and 14 (26%) of 53 re-vaccinated reported one or more local symptoms and signs at the injection site (pain, tenderness, or erythema) ($P = 0.07$). Between 0 and 2 volunteers of the 3–4 individuals administered each of the first 15 formulations had local reactions. The one exception was formulation 2, in which all four volunteers developed a reaction after the first vaccination, but none after the second vaccination. Reactions were generally mild; they began on days 0–1 and persisted for 1–3 days. One volunteer developed delayed pain, redness, swelling, and pruritis on days 12, 14, and 15 after the first vaccination, but did not develop a reaction to the second vaccination. One volunteer developed signs of local inflammation that persisted for seven days. No reactions required treatment, and none interfered with activity or sleep.

Systemic reactions. Viremia and systemic reactions reported by volunteers are summarized in Table 9. The seven superior formulations (2, 5, 10, 11, 13, 14, 15) varied in their mean RI from a high of 36.5 (formulation 2) to a low of 2.0 (formulation 15). The RIs of individual volunteers are shown in Figure 1a for the seven superior formulations and in Figure 1b for the nine inferior formulations. As a group, the superior formulations were more reactogenic, although three formulations (13, 14, 15) appeared to provide an acceptable balance of immunogenicity and reactogenicity. The RIs were similar between African-Americans and Caucasians among the 10 formulations administered to both races ($P = 0.38$, by a binomial test).

The clinical reactions reported as interfering with daily activities (seen with 6 of 15 formulations) are described in Table 10, together with viremia results. Of 54 University of Maryland volunteers, 13 (24%) curtailed school, work, recreation, or sleep. Their symptoms lasted 1–3 days, and anti-pyretics/analgesics (principally acetaminophen) when administered, provided some relief. All reactions occurred after the first vaccination. Five of these volunteers were afebrile; however, their symptoms were similar in type, intensity, and duration to the seven volunteers who were febrile (Table 10). Formulation 2 was the most reactogenic, with rash persisting eight days in one volunteer and myalgia for seven days in another. No symptoms were reported by any of the 54 University of Maryland volunteers after day 19. Three of 10 volunteers immunized with formulation 16 at WRAIR experienced some loss of activity on the inpatient study ward.[5]

Twelve (22%) of 54 Maryland vaccinees developed fever ≥100.0°F. These individuals received six formulations; three of four administered formulations 2, 7, and 10 were febrile, and one of four administered formulations 5, 12, and 13 were febrile. Febrile episodes began 7–16 days after the first vaccination and lasted 1–4 days. The highest fever recorded was 102.2°F. Morbidity among febrile volunteers varied. Four febrile persons reported no loss of activity, despite the fact that in one such individual the fever lasted four days.

Association of reactogenicity and immunogenicity. There was a positive relationship between the RI and seroconversion to the four DEN serotypes among 54 volunteers immunized with formulations 1–15 (Spearman's r = 0.35, $P = 0.01$) (Figure 2).

Effect of vaccine dose on reactogenicity. Reactogenicity indices in high dose and low dose formulations (1–15) of the four DEN serotypes were compared with determine if high dose preparations of a particular serotype were more reactogenic (Table 11). We found that high dose formulations had

TABLE 4

Neutralizing antibody responses induced by nine inferior (serologically unacceptable)* formulations of tetravalent dengue (DEN) vaccine in volunteers receiving each formulation who seroconverted to at least three, and to all four DEN serotypes

	Number of volunteers with neutralizing antibody† against					
	≥3 Serotypes (%)			4 Serotypes (%)		
Formulation	Day 28	Day 56	Cumulative	Day 28	Day 56	Cumulative
1	1/3	1/3	2/3 (66%)	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	0/3	0/3	0/3	0/3	0/3
6	0/3	0/3	0/3	0/3	0/3	0/3
7	2/4	2/4	2/4 (50%)	2/4	1/4	2/4 (50%)
8	1/4	0/4	1/4 (25%)	0/4	0/4	0/4
9	1/4	2/4	2/4 (50%)	1/4	0/4	1/4 (25%)
12	0/4	1/4	1/4 (25%)	0/4	1/4	1/4 (25%)
16‡	3/10	3/10	4/10 (40%)	2/10	2/10	2/10 (20%)

* Formulation did not achieve study objective; namely, less than three of the four DEN serotypes in the vaccine formulation induced antibody in three or more volunteers (≥75%). The composition of each formulation is shown in Table 1.
† Number of volunteers in each formulation who achieved $PRNT_{50}$ titer of ≥1:5, divided by number of volunteers in the group. Calculations based on a 50% plaque reduction neutralization test titer ≥1:5 reached 28 days after the first vaccination (day 28), 28 days after the second vaccination (day 56), and cumulative seroconversions (total number of volunteers who seroconverted after the first or second vaccination).
‡ Data from Sun and others.[5]

TABLE 5

Neutralizing antibody responses induced by nine inferior (serologically unacceptable)* formulations of tetravalent dengue (DEN) vaccine with geometric mean titers† and range of peak titers elicited by each formulation against each of the four DEN serotypes 28 days after vaccinations 1 and 2

	Reciprocal of the geometric mean titer											
	DEN-1			DEN-2			DEN-3			DEN-4		
Formulation	Day 28	Day 56	Range‡	Day 28	Day 56	Range	Day 28	Day 56	Range	Day 28	Day 56	Range
1	7	34	0–780	3	7	5–67	2	3	0–5	1	1	0
3	1	1	0	3	4	0–46	1	1	0	1	1	0
4	1	1	0	9	3	0–66	1	1	0	1	2	0–5
6	1	1	0	13	4	5–212	6	2	0–167	1	2	0–5
7	897	398	519–1,700	4	2	0–48	30	28	5–157	3	2	0–20
8	7	5	0–1,775	19	12	5–148	4	2	0–33	1	1	0
9	56	28	0–4,625	12	14	0–353	29	3	0–193	7	4	0–219
12	1	4	0–167	6	29	0–260	1	2	0–5	1	2	0–31
16§	41	26	0–1,105	12	11	0–820	12	12	0–306	3	2	0–118

*Serologically unacceptable is defined in the first footnote in Table 4.
† For calculation of geometric mean titer, a titer <1:5 was expressed as 1:1.
‡ Range of peak 50% plaque reduction neutralization test titers in volunteers on day 28 or day 56 showing the higher of the two titers against each serotype.
§ Data from Sun and others.[5]

no greater reactogenicity. Unexpectedly, the low dose formulations of DEN-2 and DEN-4 were significantly more reactogenic than their high dose counterparts (Table 11), and these two formulations induced the highest mean RIs among all formulations. Among volunteers who received low dose DEN-2 formulations, the RIs were significantly higher in the subset who received high dose DEN-1 compared with low dose DEN-1 ($P = 0.04$, by Wilcoxon's rank sum test). The same phenomenon occurred in volunteers who received low dose DEN-3 ($P = 0.03$) and low dose DEN-4 ($P = 0.02$). In contrast, this difference in reactogenicity between recipients of high and low dose DEN-1 was not seen in formulations with high dose DEN-2, DEN-3, or DEN-4 ($P \geq 0.59$).

Clinical laboratory results. *Vaccine safety.* No clinically important abnormal laboratory tests results were noted in any volunteer. One volunteer (15-1) met the criteria of unsafe absolute neutropenia (800/mm^3 and 900/mm^3 on day 10 and day 15), but his baseline absolute neutrophil count (ANC) was borderline low (1,300/mm^3), and he was asymptomatic. No volunteer met the definitions of unsafe thrombocytopenia or elevation of the ALT level. With the exception of one afebrile volunteer (9-1, Table 10) who developed transient elevation of the ALT level (171 U/L on day 15, 55 U/L on day 22, and 19 U/L on day 28), no other volunteer developed an elevated ALT level (defined as >2 times normal: 45 U/L × 2).

Absolute neutrophil counts, platelet counts, and vaccine reactogenicity. Among formulations, there was no statistically significant correlation between mean RI and maximum percent fall from baseline in the mean ANC ($P > 0.2$) or the mean platelet count ($P > 0.3$, by Spearman's test). However, an association did exist between higher RIs and change of ANCs and platelet counts from baseline (Figure 3). For example, combining mean maximum % ANC changes from baseline for the combined first and second vaccinations (15 formulations × 2 vaccinations = 30 formulation data points), five (83%) of six formulations with a mean RI ≥10 developed a ≥32% mean decrease in the ANC, whereas of only 3 (13%) of 24 formulations with mean RI <10 developed a ≥32% decrease in the ANC ($P = 0.002$, by Fisher's exact test) (Figure 3a). Similarly, for platelet counts, three (50%) of six formulations with mean RI ≥10 developed a ≥14% mean decrease, whereas only 1 (4%) of 24 formulations with a mean RI < 10 developed a ≥14% decrease ($P = 0.02$, by Fisher's exact test) (Figure 3b). Because considerable variation existed among the RIs of the 54 volunteers and their decreases in ANCs and platelets counts, the RI was unreliable as a predictor of decreases in the ANC and platelet count for any single volunteer.

Viremia. *Delayed plaque assay.* After the first vaccination, viremia was detected in 1–4 volunteers given 11 of 16 formulations. Altogether, 30 (47%) of 64 volunteers were viremic; 28 were viremic only after the first vaccination, 2 were viremic only after the second vaccination, and 2 were viremic after both vaccinations (Table 9).

TABLE 6

Neutralizing antibody response to the dengue (DEN) serotypes in the tetravalent vaccine with the number (%) of 64 volunteers seroconverting* and maximum geometric mean titers (GMTs) to the four DEN serotypes, combining high and low formulations of each serotype

	DEN-1			DEN-2			DEN-3			DEN-4		
	Day 28	Day 56	Cum.†	Day 28	Day 56	Cum.†	Day 28	Day 56	Cum.†	Day 28	Day 56	Cum.†
Number (%) Seroconverting‡	38 (59%)	43 (68%)	44 (69%)	41 (64%)	40 (63%)	50 (78%)	34 (53%)	36 (57%)	44 (69%)§	16 (25%)	20 (32%)	24 (38%)§
Peak GMTs in 64 volunteers	1:50	1:49	1:94¶	1:8	1:8	1:15¶	1:7	1:5	1:10#	1:2	1:2	1:2#

* 50% plaque reduction neutralization test ($PRNT_{50}$) ≥ 1:5 assayed 28 days after the first or second vaccination administered on study day 0 and day 28. Two volunteers were immunized on day 0, but not on day 28.
† Cumulative (Cum.) seroconversions = total number of volunteers who seroconverted after the first or second vacination; the GMT was calculated by using the higher titer on day 28 or day 56.
‡ Number and percent of volunteers in each formulation who achieved a $PRNT_{50}$ titer ≥1:5. Calculations based on a $PRNT_{50}$ titer ≥1:5 reached 28 days after the first vaccination (day 28) and 28 days after the second vaccination (day 56).
§ Significantly more voolunteers serovonverted to DEN-3 than to DEN-4 ($P < 0.001$). There were no significant differences in percent of volunteers seroconverting to DEN-1, DEN-2, or DEN-3.
¶ DEN-1 GMT is significantly higher than DEN-2 GMT ($P < 0.001$).
# DEN-3 GMT is significantly higher than DEN-4 GMT ($P = 0.003$).

TABLE 7

Seroconversion rates* to each dengue (DEN) serotype induced by the monovalent and tetravalent vaccines after the first vaccination

DEN serotype	Inoculation dose (plaque-forming units)	Number (%) of volunteer seroconversions† induced by	
		Monovalent vaccine‡	Tetravalent vaccine
DEN-1	10^6	12/12 (100)	34/36 (94)
DEN-2	10^6	11/12 (92)	27/34 (79)
DEN-3	10^5	6/13 (46)	26/37 (70)
DEN-4	10^5	7/12 (58)	17/36 (47)

* Serum 50% plaque reduction neutralization test titer ≥1:5 28 days after the first vaccination only. Most monovalent vaccines were only administered once.
† Seroconversion rate for each serotype, monovalent vs tetravalent = not significant ($P \geq 0.18$, Fisher's exact test).
‡ Data from Sun and others.[5]

Taqman assay. Altogether, 24 (44%) of 54 volunteers (formulations 1–15) were viremic by delayed plague assay on day 10 (Table 9); of these 24, only five were positive in the Taqman assay (four sera contained DEN-1, and one sera contained DEN-4) (Table 12). After the second vaccination, four (8%) of 53 vaccinees were viremic by delayed plague assay (day 38); none of these four were Taqman positive. The Taqman assay result was positive in two vaccinees on day 38 (both with DEN-2) whose sera were virus negative by the delayed plaque assay (Table 12). Volunteer 7-4 was the only person viremic by the Taqman assay after both vaccinations. The estimated viral titers by the Taqman assay were generally low in all vaccinees (range = 2–3,000 PFU/mL of serum) (Table 12).

Association of viremia and clinical reactions (formulations 1–15). The presence of viremia was positively correlated with clinical reactions following the first vaccination. Of 54 persons, 13 reported curtailed activities of whom 11 (85%) were viremic (Table 10). In contrast, only 13 (32%) of 41 volunteers with no or mild reactions were viremic ($P = 0.001$). Only four (8%) of 53 volunteers re-immunized on day 28 were viremic, and none had dengue symptoms.

Association of viremia with antibody response (formulations 1–15). After the first vaccination, 44 volunteers developed antibody, of which 20 (45%) were viremic. Not all viremic volunteers (formulations 1–15) developed DEN antibody. Two volunteers after the first vaccination with formulation 3 and one volunteer after the second vaccination with formulation 4 did not develop antibody by days 28 or 56.

DISCUSSION

The objective of this Phase I trial was achieved. We identified several tetravalent vaccine formulations for an expanded Phase II trial. Seven of the 16 formulations satisfied our study criteria and induced antibody to at least three of four DEN serotypes in at least three volunteers in a group of 3–4 individuals (Table 2). However, no formulation induced a tetravalent response in every individual when administered twice at a 28-day interval. In fact, there was no consistent pattern of antibody response after the second vaccination. Antibody was as likely to increase as to decrease. These results underscore the inconsistency and unpredictability of the tetravalent antibody response engendered by two vaccinations given 28 days apart. In contrast, five vaccinees at WRAIR re-vaccinated after a three- or four-month interval (four at 0, 1, 4 months, and one at 0 and 4 months) sustained a robust booster response.[5]

TABLE 8

Cumulative seroresponse rate* to homologous dengue (DEN) virus serotypes induced by high and low doses of four DEN serotypes in vaccine formulations 1–16†

Serotype	High dose ($10^{5.6}$ PFU)	Low dose ($10^{3.5–4.5}$ PFU)	P‡
DEN-1	34/36† (94%)	10/28 (36%)	<0.001
DEN-2	27/34 (79%)	23/30 (77%)	1.00
DEN-3	26/37 (70%)	18/27 (67%)	0.79
DEN-4	17/36 (47%)	7/28 (25%)	0.08

* Serum of 64 volunteers assayed by 50% plaque reduction neutralization test ($PRNT_{50}$) 28 days after the first and second vaccination.
† Values are the number of responders ($PRNT_{50}$ titer ≥1:5) to homologous DEN serotype/number immunized. PFU = plaque-forming units.
‡ By Fisher's exact test.

The poor booster response at one month could be attributed to the phenomenon of heterotypic immunity, first observed by Albert Sabin, who reported that immunity induced by a DEN-1 challenge of volunteers down-regulates subsequent disease induced by DEN-2 challenge.[11] The heterotypic immunity lasts approximately three months and then is lost. In our trial, responses to the first vaccination may have interfered with replication of vaccine virus in the second dose one month later, which may explain why relatively few volunteers were viremic after the second inoculation (four individuals) compared with the first (28 individuals). This relative lack of viral replication after re-vaccination is consistent with vaccination affording cross-reactive immunity, which surely contributed to the unsatisfactory antibody booster response. The transience of heterotypic immunity observed by Sabin, together with the observation of Sun and others,[5] suggest that re-vaccination after a three or more month interval would provide better immunogenicity than two vaccinations over one month.

Because the interactions of the four DEN serotype vaccines combined in a single formulation are difficult to predict based on responses to the monovalent vaccines, the current study was designed to detect any immunologic or clinical interactions among the four DEN serotypes. As described in the Introduction, the four serotypes were administered for practical reasons combined into high concentration, low concentration, and mixed high and low concentration formulations. In fact, we anticipated that one or more serotype(s) in the dengue vaccine mixture would dominate and suppress the replication and resulting immunogenicity of less dominant serotype(s). This assumption was based on results of a previous trial of the Thai tetravalent vaccine candidate being developed by Aventis Pasteur, which showed a predominant monotypic DEN-3 antibody response, thought to be caused by interference with DEN-1, DEN-2, and DEN-4 by DEN-3 in the vaccine mixture.[12] Moreover, the combination of high and low doses of each of the four serotype monovalent vaccine candidates in the current trial was based on the unproven assumption that the lower dose of a dominant DEN serotype(s) may allow replication of the higher dose of the less robust serotype(s), in a manner similar to that found with the trivalent Sabin oral polio vaccine. A balanced "take" against all three serotypes of the Sabin vaccine was achieved only when the dose of the more robust serotype 2 polio virus was reduced compared with the less robust serotypes 1 and 3.[6]

We believe that the RIs are accurate. The college students were highly motivated, and they were schooled to record all signs and symptoms irrespective of perceived cause. They me-

thodically recorded their temperature twice daily and their symptoms daily in a clinical diary used successfully in other DEN vaccine trials. In every case, the diary was audited by telephone on day 7, before the onset of most dengue symptoms, and by detailed, face-to-face interview with a study physician on days 10 and 15, during the period of most frequent and intense DEN symptoms. If symptoms started or persisted after day 15, the volunteer was instructed to call or visit the clinic on day 21. A final face-to-face audit was conducted on day 28 on all volunteers. For all these reasons, we are confident that the RI is accurate. An important advantage of the outpatient setting was that it permitted us to record how symptoms interfered with the daily routine of sleep, school, work and recreation in a real-world outpatient setting, unbiased by the intense scrutiny associated with hospitalization (Table 10).

The seven superior formulations (Figure 1a and Table 9) varied markedly in their mean RIs. Three superior formulations (13, 14, 15) appeared to provide an acceptable balance of immunogenicity and reactogenicity, although one of four volunteers who received formulation 14 curtailed work for one day. All 16 formulations fulfilled the clinical and laboratory criteria of being safe. Nevertheless, among all formulations, 13 (25%) of 54 Maryland volunteers were forced to curtail time at school, work, recreation, or sleep. Their reactions began usually 7–16 days after vaccination, lasted 1–3 days, and anti-pyretics/analgesics provided some relief. All reactions occurred after the first vaccination. No volunteer reported residual symptoms after 28 and 56 days of follow-up. Three of 10 volunteers immunized with formulation 16 at WRAIR experienced some curtailment of normal daily activity on the inpatient study ward.[5] Although a significantly higher incidence of severe dengue occurred among white persons in Cuba compared with black persons during an epidemic,[13] we found the RIs to be similar in African-American and Caucasian volunteers administered these attenuated viruses.

For the past 20 years, many monovalent, live DEN vaccine candidates representing the four serotypes have been evaluated in pre-clinical and clinical studies by United States Army and University of Maryland investigators[3–5,7,8,14–18] Most candidates were either under-attenuated and made volunteers too ill or over-attenuated and failed to infect and immunize people. Thai DEN vaccine candidates appear to be immunogenic and relatively free of side effects.[12,19–22] In the current study of tetravalent vaccines, reactogenicity was again correlated with immunogenicity (Figures 1 and 2). Predictably, viremia was commonly associated with curtailed activities (Table 10). The DEN-1 vaccine has been shown previously to be the most reactogenic of the four monovalent vaccines.[5] In this study, no clear association existed between the dose of DEN-1 or DEN-3 and reactogenicity (Table 11). In contrast, a paradoxical inverse relationship existed between high and low dose formulations of DEN-2 and DEN-4, such that low doses were associated with significantly greater reactogenicity than the high dose formulations (Table 11). A possible explanation for this paradox may be the interaction of other serotypes with DEN-1; low doses of DEN-2, DEN-3, and DEN-4 were more reactogenic when combined with high dose DEN-1, suggesting they were unable to dampen the reactogenicity caused by high dose DEN-1. Our conclusion from this experience is that the interaction of DEN serotype, strain, and dose and is complex, making it difficult to predict clinical outcome based on experience with monovalent vaccines.

The DEN-1 strain induced significantly higher GMTs compared with other DEN serotypes (Tables 3 and 6), but the proportion of persons seroconverting to DEN-1 was no greater (Table 6). The DEN-1 strain was unique in that the high dose formulations were more immunogenic than the low dose formulations, whereas the other serotypes showed no dose effect (Table 8). The DEN-4 strain was significantly less immunogenic than the other serotypes (Table 6), but there was no evidence that the other serotypes interfered with DEN-4 in the tetravalent vaccine formulations (Table 7). In-

TABLE 9
Systemic reactions and viremia induced by 16 formulations of tetravalent dengue vaccine

Formulation	No. of volunteers	Mean reactogenicity index (RI) (range)*		No. (%) with severe reactions†	No. viremic 10 days after	
		First vaccination	Second vaccination		First vaccination	Second vaccination
1-LLLL	3	6.3 (5–7)	0.7 (0–2)	0	0	1
2-HLLL‡	4	36.5 (32–43)	0.0	4 (100)	3	1
3-LHLL	3	3.3 (0–7)	1.3 (0–3)	0	3	0
4-LLLH	3	3.7 (1–6)	3.0 (0–5)	0	0	1
5-LLHL‡	4	19.5 (0–42)	0.0	2 (50)	4	0
6-LHLH	3	3.7 (1–9)	1.7 (0–5)	0	1	0
7-HLHL	4	31.5 (21–48)	0.7 (0–2)	3 (75)	4	0
8-LHHL	4	3.3 (1–6)	0.3 (0–1)	0	0	0
9-LLHH	4/3§	11.8 (3–26)	5.7 (0–17)	1 (25)	2	0
10-HLLH‡	4	16.3 (3–43)	0.3 (0–1)	2 (50)	2	0
11-HHLL‡	3	23.3 (14–31)	0.7 (0–2)	0	3	1
12-LHHH	4	7.3 (0–14)	1.5 (0–6)	0	0	0
13-HLHH‡	4	7.5 (0–18)	1.8 (0–5)	0	1	0
14 HHLH‡	4	4.5 (0–18)	2.5 (0–10)	1 (25)	1	0
15-HHHL‡	3	2.0 (0–4)	0.7 (0–1)	0	0	0
16-HHHH¶	10	8.0 (0–44)≠	1.9 (0–14)≠	3 (30)**	4	0

* Total days of 1) feeling hot or a temperature ≥100° F (feverish or fever), 2) rash, 3) chill, 4) headache, 5) anorexia, 6) nausea or vomiting, 7) stomach ache, 8) myalgia, 9) arthralgia, 10) eye symptoms (photophobia, redness, or painful movement), and (11) itching other than injection site.
† Grades 2 or 3, defined as loss of time from school, work, recreation, or sleep. Data shown for first vaccination. No severe reactions occurred after the second vaccination.
‡ Serologically acceptable formulation (defined in the first footote in Table 2).
§ One volunteer was not vaccinated a second time.
¶ Data from Sun and others.[5] The RI criteria used to grade formulation 16 was different than the RI criteria used to grade formulations 1–15.
≠ Data on nine volunteers immunized at 0 and 1 months.
** Three volunteers required bed rest or experienced interference with sleep while housed on the closed study ward at the WRAIR.

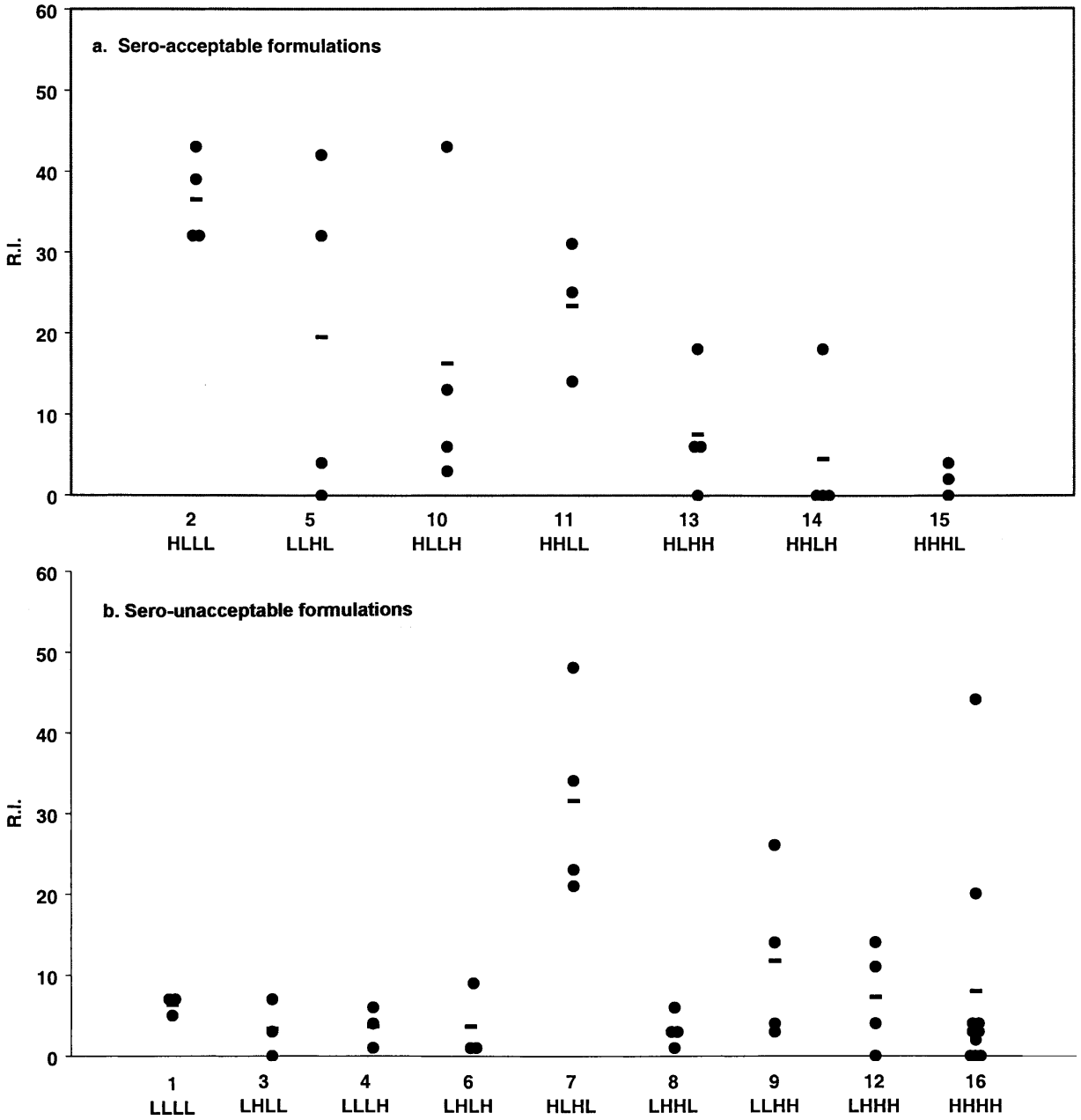

FIGURE 1. Reactogenicity indices (R.I.s) of 64 volunteers immunized with 16 dengue vaccine formulations. The R.I.s are those associated with the first vaccination of seven superior (**a**) and nine inferior formulations (**b**). The mean R.I. for each formulation is indicated by the horizontal bar. The R.I. criteria used to grade formulations 1–15 was different than the criteria used to grade formulation 16.[5]

creasing the DEN-4 inoculum seemed to provide no immunizing advantage because high and low dose DEN-4 formulations provided similarly poor seroresponse rates (Table 8). The properties of the DEN-4 strain itself, or the high PDK-passage of the strain (Table 1) may have contributed to its weak immunogenicity. Preliminary results do suggest that DEN-4 seroconversions and antibody titers can be boosted considerably by re-immunizing volunteers at four months rather than at one month.[5] Moreover, we have tested a lower-passaged DEN-4 strain (PDK-6 instead of PDK-20) as a component of another tetravalent vaccine formulation, and found it was associated with a more robust DEN-4 antibody response (Cunningham D and Sun W, unpublished data).

It is important to review the limits of the DEN virus neutralization ($PRNT_{50}$) assay. We selected a PRNT titer ≥1:5 as a measure of immunogenicity and to justify further clinical testing. It remains to be tested if a neutralizing titer ≥1:5 will protect against natural DEN infection in field trials. In fact, the protective $PRNT_{50}$ titer in humans is unclear. Moreover, although neutralizing antibody is the best available surrogate for DEN infection and is highly correlated with homologous protection against disease in those recovered from DEN infection, there is no proof that neutralizing antibody is absolutely necessary to afford protection from disease. Indeed, non-neutralizing monoclonal antibodies can protect mice against lethal DEN challenge, possibly by binding complement and by yet unknown mechanisms.[23,24] In an unpublished DEN challenge trial, a volunteer vaccinated with the tetrava-

TABLE 10
Clinical reactions that interfered with normal activity* after the first vaccination of dengue (DEN) formulations 1–15

Volunteer no.-Formulation	Reactogenicity index†	Fever ≥ 100.0°F on post-vaccination day		Viremia‡
2-1-HLLL	34	11, 12, 13	Days 12, 13—Attended class, but unable to study secondary to fever symptoms.	Yes
2-2	43	None	Day 12—Unable to sleep more than 3 hours secondary to myalgia and sore throat; Day 13—Unable to work secondary to chest pains, headache, myalgia, and sore throat	No
2-3	39	16	Day 18—Missed evening work secondary to photophobia and headache	Yes
2-4	32	14, 15	Days 14, 15—Unable to exercise secondary to fever symptoms	Yes
5-2-LLHL	42	None	Days 11, 12—Missed classes secondary to headache, nausea, myalgia, arthralgia, and pruritis	Yes
5-4	32	11, 12	Days 11, 12—Missed classes secondary to fever symptoms	Yes
7-1-HLHL	23	11, 12, 13	Day 11—Lost night of work secondary to fever symptoms	Yes
7-2	21	10, 11, 12	Days 9, 10, 11—Daytime bedrest needed for fatigue	Yes
7-4	48	None	Days 10, 11—Incapacitated by chills, headache, nausea, stomach ache, myalgia, arthralgia, photophobia, eye pain, and pruritis	Yes
9-1-LLHH	26	None	Days 10, 11—Extra bedrest needed for myalgia and arthralgia	Yes
10-3-HLLH	43	15	Days 14, 15—Missed classes and could not study secondary to fever symptoms	No
10-4	13	13, 14	Day 14—Missed classes and work secondary to fever symptoms	Yes
14-2-HHLH	18	None	Day 13—Curtailed work secondary to chills, headache, feverish, nausea, and arthralgia	Yes

* No loss of activity after second vaccination.
† The reactogenicity Index is defined as total days of 1) feverish or fever, 2) rash, 3) chills, 4) headache, 5) anorexia, 6) nausea or vomiting, 7) stomach ache, 8) myalgia, 9) arthralgia, 10) eye symptoms (photophobia, redness, or pain), and 11) itching other than injection site.
‡ DEN viremia present on day 10.

lent vaccine, but who lacked detectable DEN-1 antibody at the time of subsequent DEN-1 challenge, was still protected. Conversely, in the same vaccine challenge trial, a DEN-3 titer of 1:16 induced by the tetravalent vaccine did not protect against illness caused by DEN-3 challenge (Sun W, unpublished data). Finally, the absence of neutralizing antibody after the first vaccination may not predict immunity after re-vaccination, particularly if the individual has been immunogically primed by the first dose. Thus, a highly attenuated but poorly immunogenic DEN vaccine may be safe and protective after two doses, if properly spaced.

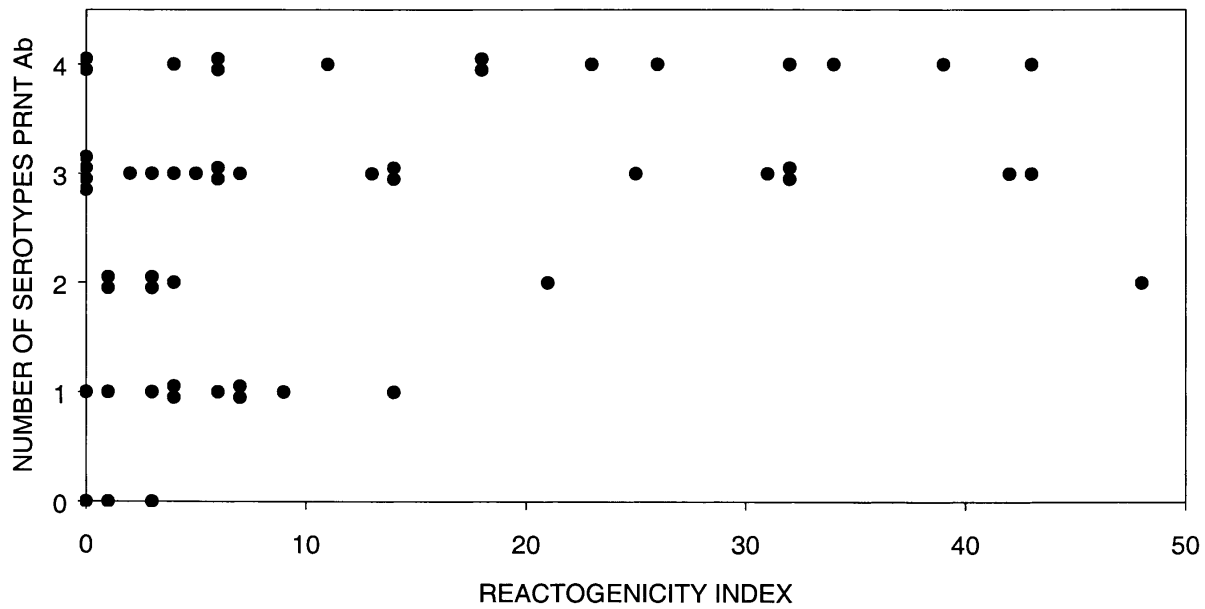

FIGURE 2. Association of individual reactogenicity indices after the first vaccination and seroconversion (number of volunteers seroconverting to 1, 2, 3, or 4 dengue serotypes after the first vaccination) in vaccine formulations 1–15. The association between reactogenicity and immunogenicity was significant (r = 0.35, $P = 0.01$, by Spearman's test). PRNT Ab = plaque reduction neutralization test antibody.

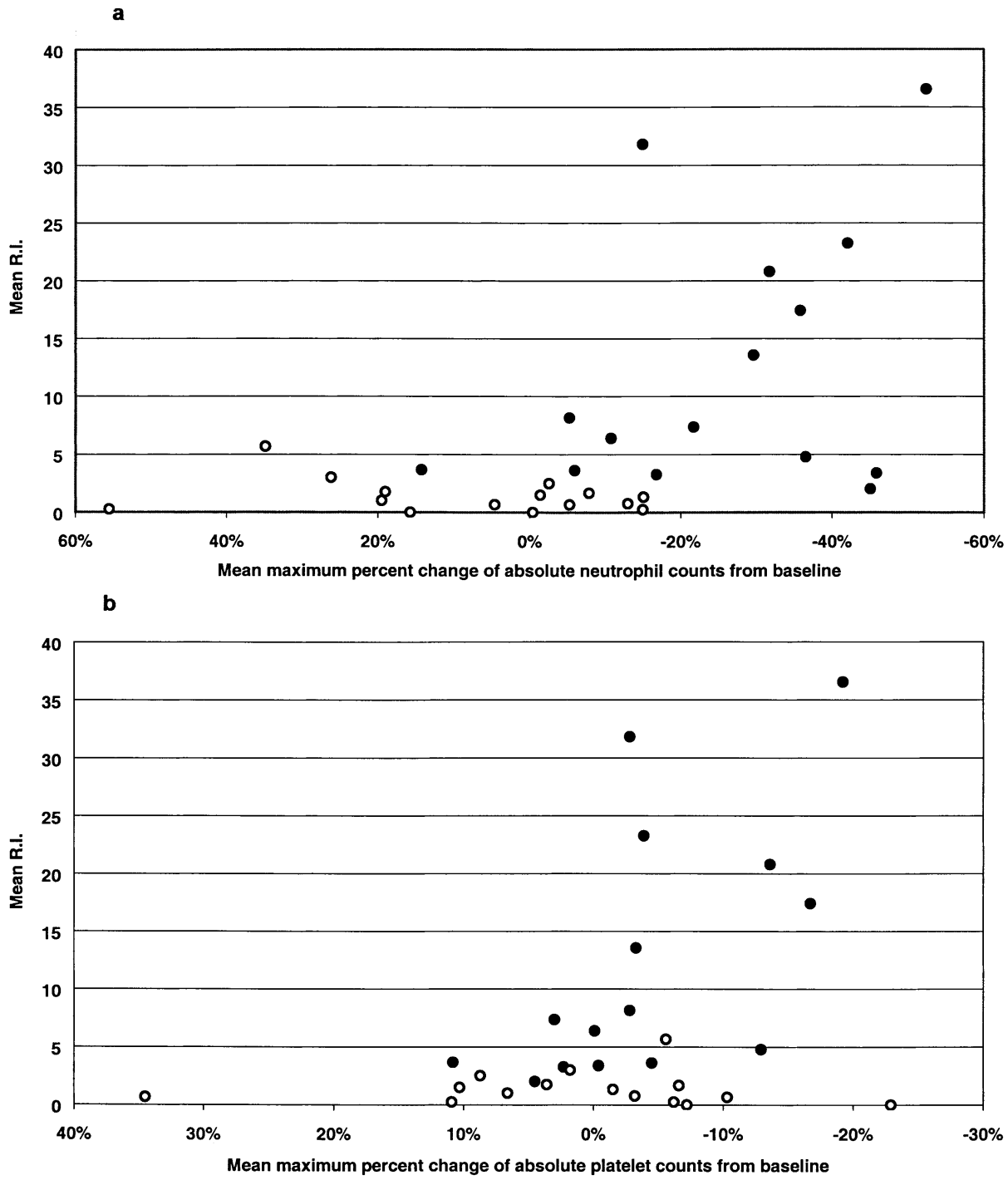

FIGURE 3. Association of the mean reactogenicity index (R.I.) for dengue vaccine formulations 1–15. **a**, The mean maximum percent change of absolute neutrophil counts from baseline. **b**, The mean maximum percent change of platelet counts from baseline. Change from baseline of absolute neutrophil counts and platelet counts are shown after the first vaccination (●) and second vaccination (○).

Complement was not used in the PRNT assays in this trial. In the monovalent DEN vaccine trials, guinea pig complement tended to enhance cross-reactive neutralizing antibodies induced by monovalent vaccines against heterospecific DEN strains.[4,5] Thus, to enhance the specificity of the serologic response against each of the four serotypes in the tetravalent vaccine formulations (and to obtain a more accurate estimate of the immunogenicity of each dengue strain), we did not use complement in the PRNT assays of serum obtained from tetravalent-vaccinated volunteers.

The results of the Taqman assay were disappointing. Only seven (7%) of the 107 post-vaccination serum samples were positive by this assay, suggesting that the level of viremia was too low for ready detection of DEN virus by extraction of viral RNA from 1.6 μL of serum (Table 12). In contrast, 23 sera (21%) were positive by delayed plaque assay but not by the Taqman assay. Two sera (2%) were positive by the Taqman assay but not by the delayed plaque assay. Five (5%) were positive by both the delayed plaque and Taqman assays. Thus, in our hands, the sensitivity of the Taqman assay was

TABLE 11

Mean reactogenicity indices* (range) induced by high- and low-dose dengue vaccine formulations 1–15†

Type	High dose ($10^{5–6}$ PFU)	Low dose ($10^{3.5–4.5}$ PFU)	P‡
DEN-1	15.0 (0–48)[2]	7.8 (0–42)	0.27
DEN-2	7.0 (0–44)	17.4 (0–48)	<0.001
DEN-3	11.1 (0–48)	13.0 (0–43)	0.47
DEN-4	8.1 (0–44)	16.7 (0–48)	0.03

* Reactogenicity Index (RI) after the first vaccination. The RI is defined in Table 9.
† Values are the range of RIs experienced by individuals who received those formulations. PFU = plaque-forming units.
‡ By Mann-Whitney test.

low compared with that of the delayed plaque assay, probably due to the low volume of serum tested per reaction.

The occurrence of viremia in this trial is a minimal estimate based on one time point. The selection of day 10 viremia was somewhat arbitrary and was based on our experience with monovalent DEN vaccines, in which viremia occurred between days 7 and 14. We were restricted to one time point so as not to burden the college students with additional clinic visits, and to ease the burden on the virus isolation facilities at WRAIR. The selection of days 10 and 15 for determination of platelet and neutrophil counts was also guided by our experience with monovalent DEN vaccines, where day 15 was the nadir for these counts. The values may not reflect precisely the temporal changes after vaccination with tetravalent vaccines. With this caveat in mind, none of the volunteers developed unsafe levels of thrombocytopenia or neutropenia after tetravalent vaccination.

Many important questions about selected tetravalent vaccine formulations need to be answered. First, we are vaccinating more flavivirus-negative, adult volunteers to confirm that formulations 13, 14, and another, new formulation are safe, well-tolerated, and immunogenic. Second, another study will carefully evaluate the safety and reactogenicity of selected formulations in flavivirus antibody-positive volunteers. Third, DEN-enhancing antibody needs to be sought in vaccinees.[25] Finally, since infants often respond to wild DEN infection with few to no symptoms, suitable tetravalent vaccine formulations should be independently evaluated in infants and young children, the optimal target group of endemic dengue through vaccination. Such trials are planned.

TABLE 12

Dengue (DEN) virus serotypes and titers detected in sera of vaccinees by a serotype-specific 3′-based fluorogenic RT-PCR (Taqman) assay*

Formulation-volunteer no.†	Serotype		Virus titer (2 replicates) PFU/ML	Delayed plague assay§	
	Day 10‡	Day 38‡		Day 10	Day 38
7-2	DEN-1	Neg	33, 16	Pos	Neg
7-3	DEN-4	Neg	2, 14	Pos	Neg
7-4	DEN-1		670, 3,000	Pos	
		DEN-2	350, 200		Neg
11-3	DEN-1	Neg	22, 8	Pos	Neg
12-4	Neg	DEN-2	130, 120	Neg	Neg
14-2	DEN-1	Neg	12, 13	Pos	Neg

* Taqman assay described in the Materials and Methods and in Houng and others.[10] RT-PCR = reverse transcriptase–polymerase chain reaction; PFU = plaque-forming units; Neg = negative; Pos = positive.
† 54 volunteers vaccinated with tetravalent DEN vaccine formulations 1–15 (3–4 persons per formulation) on day 0; 53 of the 54 volunteers were re-vaccinated on day 28.
‡ Taqman assay conducted on serum collected 10 days after the first vaccination (day 0) and second vaccination (day 28). The Taqman assay result was negative on serum obtained from the remaining volunteers.
§ Delayed plaque assay for presence of circulating virus described in the Materials and Methods.

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