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

Anna P. Durbin,[1] Alexander Schmidt,[2] Dan Elwood,[1] Kimberli A. Wanionek,[1] Janece Lovchik,[1] Bhavin Thumar,[1] Brian R. Murphy,[2] and Stephen S. Whitehead[1]

[1]Center for Immunization Research, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore; and [2]Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland

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

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

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

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

Clinical trials registration: NCT00458120 (http://www.clinicaltrials.gov/ct2/show/NCT00458120).

Dengue has become the most important arbovirus worldwide, with a 30-fold increase in incidence over the past 50 years. It is estimated that 50 million infections occur annually among the ~2.5 billion persons living in regions of endemicity [1]. Children bear most of the dengue-associated disease burden, which is estimated to be as high as 616,000 disability-adjusted life-years [2]. Dengue viruses (DENVs)are members of the Flavivirus genus of the *Flaviviridae* family [3]. There are 4 DENV serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Each serotype can cause the full spectrum of dengue illness, which can range from an asymptomatic or mildly symptomatic infection to dengue fever (DF) or to the most severe form of the disease, dengue hemorrhagic fever/shock syndrome (DHF/DSS).

Infection with 1 DENV serotype generates long-term homotypic immunity but is thought to generate only short-term heterotypic immunity [4, 5]. Epidemiologic studies have demonstrated that preexisting immunity to 1 DENV serotype may confer a greater risk of developing more severe disease (DHF/DSS) after subsequent infection with a heterotypic DENV [6–8]. Nonneutralizing, heterotypic antibody is thought to bind to DENV, improving its ability to infect Fcγ-

Received 9 August 2010; accepted 23 September 2010.

Potential conflicts of interest: none reported.

Presented in part: A portion of these data were presented at a meeting of the Pediatric Dengue Vaccine Initiative held in June 2009 in Asilomar, California.

Reprints or correspondence: Dr Anna P Durbin, 624 N Broadway, Rm 251, Baltimore, MD 21205 (adurbin@jhsph.edu).

The Journal of Infectious Diseases 2011;203:327–334

1537-6613/2011/2033-0001$15.00

DOI: 10.1093/infdis/jiq059

receptor-bearing cells, leading to enhanced virus production from the greater number of infected cells, a phenomenon designated antibody-dependent enhancement (ADE) of infection [9–11]. The consequence of ADE is a potential 100-fold increase in viremia, which has been shown to correlate with more severe disease in patients with DENV infection [12]. Experimentally, ADE has been demonstrated in an AG129 mouse model and in nonhuman primates [13, 14]. For these reasons, a successful dengue vaccine should induce long-lived immunity to each of the 4 serotypes without inducing enhanced disease in heterotypically immune vaccinees [15]. On the basis of the success of other live attenuated flavivirus vaccines for yellow fever and Japanese encephalitis virus, development of live attenuated dengue vaccine candidates appears to be an economical and effective strategy. Nevertheless, several challenges must be overcome. Because the vaccine will be introduced in regions of endemicity with populations that have preexisting DENV antibody, there is concern that ADE of vaccine replication could produce a viral load sufficient to cause disease. In addition, individuals may be at risk for severe disease if the vaccine fails to induce a balanced immune response to all serotypes or if antibody titers wane over time. To date, the live attenuated tetravalent DENV vaccines evaluated in humans have failed to induce high seroconversion rates to all 4 serotypes with a single dose [16, 17]. For this reason, the proposed dosing schedule of a tetravalent DENV vaccine in advanced clinical evaluation includes 3 doses of vaccine at time 0, 6, and 12 months [17, 18].

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

METHODS

Regulatory Oversight

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

Study Design and Clinical Monitoring

Healthy persons aged 18–50 years were enrolled if they met the following eligibility criteria: previous receipt of the DENV-1 vaccine (rDEN1Δ30) [19], the DENV-2 vaccine (rDEN2/4Δ30) [20], or a DENV-4 vaccine (rDEN4Δ30 or rDEN4Δ30-200,201) [21–23]; normal findings during physical examination; negative for human immunodeficiency virus antibody, hepatitis C virus, and hepatitis B surface antigen; normal values for complete blood count, with differential serum aspartate aminotransferase, alanine aminotransferase, total bilirubin, alkaline phosphatase, serum creatinine, serum creatine phosphokinase, prothrombin time, and partial thromboplastin time; and normal urinalysis results. Female participants were required to have a negative result of a urine pregnancy test at least 3 days before vaccination and on the day of vaccination and to agree to use contraception or abstain from sexual intercourse for the duration of the study. Participants were enrolled in 1 of 4 heterotypic vaccination cohorts: participants who had previously received a DENV-4 vaccine received either the DENV-1 or DENV-2 vaccine, participants who had previously received a DENV-1 vaccine received the DEN2 vaccine, and participants who had previously received a DENV-2 vaccine received the DENV-1 vaccine [19–22]. Previous and heterotypic vaccination occurred using the same vaccine lots prepared for each serotype. Each cohort consisted of 8–10 participants randomized to receive 10^3 PFU of the indicated vaccine or placebo (1 or 2 placebo recipients per cohort) as a single .5 mL subcutaneous injection on study day 0. Each participant was given a digital thermometer and a diary card to record their oral temperature 3 times per day for the first 16 days of the study. Participants returned to the clinic every other day through study day 16 and again on days 21, 28, and 42. A medical provider performed physical assessments every study visit, and participants were questioned at each visit about symptoms of illness. Blood samples were collected at each study visit for clinical laboratory studies and virology through study day 28, as described elsewhere [22]. Blood samples for serologic testing were collected on study days 0, 28, and 42. Adverse events were graded as mild (easily tolerated), moderate (interfered with daily activity or required medication), or severe (prevented daily

Table 1. Cohort demographic characteristics

Cohort	Vaccine	Previous inoculation	No. of volunteers	Mean Age, years	White, %	Female, %	Vaccination interval, years	
							Mean	Range
1	DEN1[a]	DEN4[b]	8	41.0	62	50	3.6	1.3 – 7.5
2	DEN1[a]	DEN2[c]	7	38.4	0	28	2.5	2.1 – 3.0
3	DEN2[c]	DEN4[b]	8	38.2	12	50	4.5	0.6 – 6.6
4	DEN2[c]	DEN1[a]	7	32.7	28	43	2.7	1.9 – 3.6
Placebo	Diluent	Various	6	40.2	67	83	3.3	0.6 – 6.1

NOTE. [a] DEN1 vaccine is rDEN1Δ30.

[b] Volunteers previously vaccinated with rDEN4Δ30 or rDEN4Δ30-200,201.

[c] DEN2 vaccine is rDEN2/4Δ30.

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

Virus Quantitation

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

Serologic Assessment

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

Data Analysis

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

RESULTS

Demographic Characteristics

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

Reactogenicity

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

Table 2. Clinical and virological response of volunteers inoculated with attenuated dengue vaccines rDEN1Δ30 or rDEN2/4Δ30 given as a primary or secondary vaccination

					Percentage with indicated clinical sign (no.)						
Vaccine	Previous inoculation	Vaccine dose, log_{10} PFU	No. of participants	Percentag eviremic (no.)	Fever	Macular/ maculopapular rash	Arthralgia	Myalgia	Neutropenia[a]	Thrombo-cytopenia[b]	Increase in ALT level
DEN1[c]	None	3.0	71	60 (43)	1 (1)	32 (23)	3 (2)	13 (9)	44 (31)	1 (1)	1 (1)
DEN1	DEN4[e]	3.0	8	63 (5)	0	0	0	0	25 (2)	0	12[d] (1)
DEN1	DEN2	3.0	7	29 (2)	0	0	0	14 (1)	29 (2)	0	0
DEN2[f]	None	3.0	40	63 (25)	0	32 (13)[g]	2 (1)	5 (2)	28 (11)	0	12 (5)
DEN2	DEN4[e]	3.0	8	75 (6)	0	75 (6)[g]	0	0	13 (1)	0	0
DEN2	DEN1	3.0	7	43 (3)	0	43 (3)	0	0	0	0	0
Placebo	none	n/a	6	0	17 (1)	17 (1)	0	0	17 (1)	0	0

NOTE. Percentage of subjects with an increase in ALT level as defined as ⩽ 1.25 times the upper limit of normal.[a] Neutropenia is defined as an absolute neutrophil count ⩽ 1500 neutrophils/mm^3.

[b] Defined as a platelet count <120,000 platelets/mL.

[c] Data are from 2 clinical trials in which volunteers received a single dose of 3 log_{10} PFU of live attenuated DEN1 vaccine candidate rDEN1Δ30.

[d] ALT level elevation was determined to be unrelated to vaccination.

[e] Volunteers previously vaccinated with rDEN4Δ30 or rDEN4Δ30-200,201.

[f] Data are from 2 clinical trials in which volunteers received a single dose of 3 log_{10} PFU of live attenuated DEN2 vaccine candidate rDEN2/4Δ30.

[g] Frequency of rash statistically greater following inoculation of rDEN2/4Δ30 as a secondary vaccine compared to its use as a primary vaccine ($P < .05$, Fisher exact test).

mL, but had no other adverse events related to vaccination other than a moderate headache on study days 13–15.

Viremia

A statistically significant difference in the mean peak titer, onset, and duration of viremia for DENV-1 vaccine was not observed when given as a second, heterotypic vaccination, compared with primary vaccination of naive volunteers (Table 3). Similarly, a statistically significant difference in the mean peak titer, onset, and duration of viremia for DENV-2 vaccine was not observed when it was given after DENV-1 vaccination (Table 3). However, the mean peak titer of DENV-2 vaccine, when given after DEN4, was significantly higher than observed during primary vaccination (1.1 ± .2 log_{10} PFU/mL vs .5 ± .03 log_{10} PFU/mL). In addition, the onset of viremia was significantly earlier (day 5 ± 1 vs day 9.2 ± .6). Despite this minor but significant increase in mean peak virus titer, an increase in the reactogenicity of the vaccine was not observed (Table 2). The magnitude of the peak virus titer in vaccinees correlated positively with the duration of interval between primary and secondary dengue vaccination in cohort 3 ($P = .02$) and when all cohorts were combined ($P = .01$). Peak virus titer did not correlate with heterotypic neutralizing antibody titer at the time of vaccination.

Serology

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

DISCUSSION

The development of a live attenuated tetravalent dengue vaccine is an advantageous strategy to protect against dengue disease for several reasons. First, infection with DENV induces long-lived,

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

Vaccine	Previous inoculation	Vaccine dose, $\log_{10}$ PFU	No. of volunteers	Percentag eviremic (no.)	Mean peak titer, [b] $\log_{10}$ PFU/mL ± SE	Mean onset of viremia,day ± SE	Mean durationof viremia,days ± SE
DEN1[a]	None	3.0	71	61 (43)	1.0 ± .1	10.0 ± .3	3.2 ± .3
DEN1	DEN4[c]	3.0	8	63 (5)	1.0 ± .2	8.6 ± .5	3.6 ± .9
DEN1	DEN2	3.0	7	29 (2)	0.5 ± 0	10.5 ± 3.5	3.0 ± 0
DEN2[d]	None	3.0	40	60 (24)	0.5 ± .03	9.2 ± .6	3.3 ± .6
DEN2	DEN4[c]	3.0	8	75 (6)	1.1 ± 0.2[d]	5.0 ± 1.0[d]	5.0 ± 1.0
DEN2	DEN1	3.0	7	43 (3)	0.7 ± .2	11.0 ± 2.1	2.7 ± 1.7

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

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

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

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

[e] Statistically significant difference when compared to rDEN2/4Δ30 given as a single primary vaccination ($P < .01$, Tukey-Kramer post-hoc test).

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

As an experimental model of vaccine introduction in areas of endemicity, we sought to evaluate whether preexisting heterotypic immunity would enhance the replication and reactogenicity of our live DENV candidate vaccines. Because these experimental studies were conducted in an area where dengue is not endemic in persons formerly flavivirus seronegative, preexisting immunity was induced by vaccine virus rather than by wild-type DENV. Experimental infection in susceptible adults permits frequent sampling to measure the level and duration of viremia and to compare the mean peak titer, onset, and duration of viremia in heterotypically immune vaccinees and immunologically nave vaccinees. Viremia in humans with DF peaks at $\sim 10^6$ infectious units/mL of blood and can increase to values up to 10^8 in humans with DHF/DSS [12, 27]. Thus, a relative increase in viremia of nearly 100-fold or an increase in viremia to levels associated with DF in our heterotypic vaccinees would be considered to be problematic. However, we observed only a 4-fold increase in mean peak viral load in only 1 of 4 cohorts (DENV-2 vaccine after DENV-4 vaccine), compared with primary DENV-2 vaccination. The peak viral load was only 10^1

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

			Neutralizing antibody response		
Vaccine	Previousinoculation	No. of volunteers	DENV used in assay	GMT on day 42(range)[a]	Percentag eseroconversion[b]
DEN1[d]	None	70	DEN1	128 (<5 – 1242)	93
DEN1[d]	DEN4[f]	8	DEN1	452 (132 – 1386)	100
DEN1[d]	DEN2[e]	7	DEN1	114 (<10 – 358)	57[c]
DEN2[e]	None	40	DEN2	104 (16 – 843)	100
DEN2[e]	DEN4[f]	8	DEN2	74 (21 - 158)	100
DEN2[e]	DEN1[d]	7	DEN2	92 (37 - 239)	100

NOTE. [a] Geometric mean titer and range (reciprocal $PRNT_{60}$) is calculated for all subjects who received vaccine. Samples evaluated in a separate assay than those used for titers shown in Table 5 and Supplemental Table 2.

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

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

[d] The DEN1 vaccine is rDEN1Δ30.

[e] The DEN2 vaccine is rDEN2/4Δ30.

[f] Subjects received either rDEN4Δ30 or rDEN4Δ30-200,201 as the DEN4 vaccine.

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

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

NOTE. [a] Geometric mean titer (reciprocal $PRNT_{60}$) is presented. A titer of < 10 was assigned a value of 5 for calculation of mean titers.

[b] Defined as a ⩾ 4-fold rise in serum neutralizing titer compared with day 0.

[c] One vaccinee had a detectable $PRNT_{60}$ of 19 to DEN3.

[d] One vaccinee had a detectable $PRNT_{60}$ of 10 to DEN4 (different vaccinee than described in footnote c).

[e] One vaccinee had a detectable $PRNT_{60}$ of 14 to DEN3.

[f] One vaccinee had a detectable $PRNT_{60}$ of 17 to DEN4 (different vaccinee than described in footnote e).

PFU/mL, well below the level seen in humans with DF [12, 28, 29]. In addition, the onset of viremia was earlier in this cohort. As expected, this level of viremia was not accompanied by an increase in vaccine reactogenicity. In all 4 cohorts, reactogenicity was comparable between heterotypically immune and immunologically naive vaccinees, an observation reflecting the low level of replication of each of the vaccine candidates.[12, 28, 29]. We would suggest that the attenuating mutations in the vaccine candidates restricted virus replication in target cells, including those expressing the Fcγ receptor, precluding clinically relevant increases in overall vaccine virus replication.

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

DENV vaccination to induce a broad heterotypic neutralizing antibody response is encouraging, but the durability of this heterotypic response is unknown.

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

Previous studies from Cuba have reported enhanced disease severity with a longer interval between primary and secondary DENV infection [32]. Although peak virus titers were not reported from the Cuban epidemic of 1997, the authors suggested that decreased avidity of the heterotypic antibody for the DENV-2 virus led to an increased severity of disease by means of ADE. We demonstrated that peak virus titer was positively correlated with the duration between primary DENV vaccination and secondary heterotypic vaccination, recapitulating the Cuban observations. The correlation between the duration between primary and secondary vaccination remained valid for all sequences of secondary infection, with the exception of DENV-1 vaccination after DENV-2. However, even at the longest durations between vaccine exposures in the current study, the peak titers achieved remained in a safe and acceptable range.

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

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

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

Acknowledgments

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

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

References

1. WHO. Dengue haemorrhagic fever: Diagnosis, treatment, prevention control, 3rd ed. Geneva, Switzerland: World Health Organization, 2009.

2. Mathers CD, Ezzati M, Lopez AD. Measuring the burden of neglected tropical diseases: The global burden of disease framework. PLoS Negl Trop Dis **2007**; 1:e114.
3. Burke DS, Monath TP. Flaviviruses. In Knipe DM, Howley PM eds: Fields virology, 4th ed. Vol 1. Baltimore, MD: Lippincott Williams Wilkins, **2001**; 1043–125.
4. Sabin A. Research on dengue during World War II. Am J Trop Med Hyg **1952**; 1:30–50.
5. Papaevangelou G, Halstead SB. Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? Am J Trop Med Hyg **1977**; 80:46–51.
6. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg **1988**; 38:172–80.
7. Guzman MG, Kouri GP, Bravo J, Soler M, Vazquez S, Morier L. Dengue hemorrhagic fever in Cuba, 1981: A retrospective seroepidemiologic study. Am J Trop Med Hyg **1990**; 42:179–84.
8. Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg **1989**; 40:444–51.
9. Halstead SB. Dengue. Lancet **2007**; 370:1644–52.
10. Halstead SB, Chow J, Marchette NJ. Immunologic enhancement of dengue virus replication. Nat New Biol **1973**; 243:24–26.
11. Halstead SB, O'Rourke EJ. Dengue viruses mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med **1977**; 146:201–17.
12. Vaughn DW, Green S, Kalayanarooj S, et al. Dengue viremia titer, antibody response pattern, virus serotype correlate with disease severity. J Infect Dis **2000**; 181:2–9.
13. Williams KL, Zompi S, Beatty PR, Harris E. A mouse model for studying dengue virus pathogenesis and immune response. Ann N Y Acad Sci **2009**; 1171(suppl 1);E12–23.
14. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ. Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci U S A **2007**; 104:9422–7.
15. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol **2007**; 5:518–28.
16. Sun W, Edelman R, Kanesa-Thasan N, et al. Vaccination of human volunteers with monovalent tetravalent live-attenuated dengue vaccine candidates. Am J Trop Med Hyg **2003**; 69:24–31.
17. Lang J. Recent progress on sanofi pasteur's dengue vaccine candidate. J Clin Virol **2009**; 46(suppl 2);S20–4.
18. Morrison D, Legg TJ, Billings CW, Forrat R, Yoksan S, Lang J. A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naive adults. J Infect Dis **2010**; 201:370–7.
19. Durbin AP, McArthur J, Marron JA, et al. The live attenuated dengue serotype 1 vaccine rDEN1Delta30 is safe highly immunogenic in healthy adult volunteers. Hum Vaccin **2006**; 2:167–73.
20. Durbin AP, McArthur JH, Marron JA, et al. rDEN2/4Delta30(ME), A live attenuated chimeric dengue serotype 2 vaccine is safe highly immunogenic in healthy dengue-naive adults. Hum Vaccin **2006**; 2:255–60.
21. Durbin AP, Whitehead SS, McArthur J, et al. rDEN4 Delta 30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, highly infectious in healthy adult volunteers. J Infect Dis **2005**; 191:710–8.
22. McArthur JH, Durbin AP, Marron JA, et al. Phase I clinical evaluation of rDEN4Delta30-200,201: A live attenuated dengue 4 vaccine candidate designed for decreased hepatotoxicity. Am J Trop Med Hyg **2008**; 79:678–84.
23. Durbin AP, Karron RA, Sun W, et al. Attenuation immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. Am J Trop Med Hyg **2001**; 65:405–13.
24. Imrie A, Meeks J, Gurary A, et al. Antibody to dengue 1 detected more than 60 years after infection. Viral Immunol **2007**; 20:672–5.
25. Halstead SB, Jacobson J. Japanese encephalitis vaccines. In Plotkin S, Orenstein WA, Offit PA eds: Vaccines, 5th ed. Vol 1. Philadelphia, PA: Saunders-Elsevier, **2008**; 311–352.
26. Monath T, Cetron M, Teuwen DE. Yellow fever vaccine. In Plotkin S, Orenstein WA, Offit PA eds: Vaccines, 5th ed. Vol 1. Philadelphia, PA: Saunders-Elsevier, **2008**; 959–1055.
27. Endy TP, Nisalak A, Chunsuttitwat S, et al. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis **2004**; 189:990–1000.
28. Kalayanarooj S, Vaughn DW, Nimmannitya S, et al. Early clinical laboratory indicators of acute dengue illness. J Infect Dis **1997**; 176:313–21.
29. Murgue B, Roche C, Chungue E, Deparis X. Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996-1997 dengue-2 outbreak in French Polynesia. J Med Virol **2000**; 60:432–8.
30. Innis BL. Antibody responses to dengue virus infection. In Gubler DJ, Kuno G eds: Dengue and dengue hemorrhagic fever. Cambridge, MA: CAB International, **1997**; 221–43.
31. Gibbons RV, Kalanarooj S, Jarman RG, et al. Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions dengue hemorrhagic fever, and serotype sequences. Am J Trop Med Hyg **2007**; 77:910–3.
32. Guzman MG, Kouri G, Valdes L, Bravo J, Vazquez S, Halstead SB. Enhanced severity of secondary dengue-2 infections: Death rates in 1981 and 1997 Cuban outbreaks. Rev Panam Salud Publica **2002**; 11:223–7.
33. Papapanagiotou J, Kyriazopoulou V, Antoniadis A, Batikova M, Gresikova M, Sekeyova M. Haemagglutination-inhibiting antibodies to arboviruses in a human population in Greece. Zentralbl Bakteriol Orig A **1974**; 228:443–6.