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Phase 1 Trial of the Dengue Virus Type 4 Vaccine Candidate rDEN4Δ30-4995 in Healthy Adult Volunteers

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Abstract

rDEN4Δ30-4995 is a live attenuated dengue virus type 4 (DENV4) vaccine candidate specifically designed as a further attenuated derivative of the rDEN4Δ30 parent virus. In a previous study, 5 of 20 vaccinees who received 10^5 plaque-forming units (PFU) of rDEN4Δ30 developed a transient elevation of the serum alanine aminotransferase (ALT) level and an asymptomatic maculopapular rash developed in 10 of 20. In the current study, 28 healthy adult volunteers were randomized to receive 10^5 PFU of rDEN4Δ30-4995 (20) or placebo (8) as a single subcutaneous injection. The vaccine was safe, well-tolerated, and immunogenic. An asymptomatic generalized maculopapular rash and elevations in ALT levels were observed in 10% of the rDEN4Δ30-4995 vaccinees. None of the rDEN4Δ30-4995 vaccinees became viremic, yet 95% developed a four-fold or greater increase in neutralizing antibody titers. Thus, rDEN4Δ30-4995 was demonstrated to be safe, highly attenuated, and immunogenic. However, an asymptomatic localized erythematous rash at the injection site was seen in 17/20 rDEN4Δ30-4995 vaccinees. Therefore, alternative DENV4 vaccine strains were selected for further clinical development.

INTRODUCTION

Dengue has emerged as the world's most important mosquito-borne viral infection. The World Health Organization estimates that there are 50–100 million cases of dengue annually in the 2.5 billion people at risk for infection.[1] These infections result in hundreds of thousands of hospitalizations and approximately 20,000 deaths each year. Children bear the brunt of the

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dengue-associated burden of disease, which is estimated to be as high as 616,000 disability-adjusted life years.[2]

There are four antigenically distinct serotypes of dengue virus (DENV), namely DENV1, DENV2, DENV3, and DENV4. All of them are capable of causing the full spectrum of dengue disease, which ranges from an undifferentiated febrile illness to classic dengue fever to life-threatening dengue hemorrhagic fever/dengue shock syndrome.[3] Although long-term homotypic immunity is generated after infection with a single DENV serotype,4 heterotypic protection is less durable.5¯7 Pre-existing immunity to one DENV serotype has been identified as a risk factor for more severe disease upon a secondary, heterotypic infection.[8–10] For these reasons, an effective dengue vaccine should induce long-lived protective immunity against all four DENV serotypes simultaneously, be attenuated and immunogenic in both DENV-negative persons and in persons with various levels of immunity to one or more DENVs, and exhibit an acceptable safety profile.

Encouraged by the success of the live attenuated yellow fever vaccine, several live attenuated dengue vaccine candidates are being evaluated in clinical trials.[11–13] However, formulating a live attenuated tetravalent dengue vaccine that is sufficiently attenuated for each of the monovalent components and immunogenic for all four DENV serotypes has been challenging. Components of several live attenuated vaccines that were attenuated and immunogenic in animal models as monovalent candidates exhibited either insufficient attenuation or insufficient immunogenicity in a tetravalent formulation.[14–18] Attenuation phenotypes that have been identified in preclinical studies such as temperature sensitivity, small-plaque phenotype, or reduced level or frequency of viremia in non-human primates, have not consistently predicted a satisfactory level of infectivity, attenuation, or immunogenicity in humans.[12,13,19–22] Therefore, it seems prudent to develop a panel of attenuated vaccine candidates for each DENV serotype and to identify in phase 1 studies the best four monovalent candidates for inclusion in a tetravalent vaccine.

The dengue vaccine development strategy pursued by the Laboratory of Infectious Diseases is based on the introduction of an attenuating 30-nucleotide deletion mutation (Δ30) into the 3′ untranslated region (UTR) of DENV1 and DENV4, and on chimerization of DENV2 and DENV3 with rDEN4Δ30.[23] Additional approaches for developing an appropriately attenuated DENV3 vaccine are based on the use of more extensive deletions in the 3′-UTR and on replacement of the 3′-UTR of DEN3 with that derived from DEN4Δ30.24

We have previously reported the safety and immunogenicity of rDEN4Δ30, a highly immunogenic DEN4 vaccine that was safe and well tolerated by volunteers, with minimal reactogenicity. rDEN4Δ30 was tested at doses ranging from 10^5 to 10^1 plaque-forming units (PFU).25,26 At the highest dose, 25% of vaccinees developed a transient increase in alanine aminotransferase (ALT) levels, and 50% developed an asymptomatic maculopapular rash.25 In response to the observed elevation in serum ALT levels in some vaccinees at the 10^5 PFU dose, it was decided to further attenuate rDEN4Δ30 by the introduction of previously identified mutations that reduced replication in human liver cells.

Two additional vaccine candidates were created: rDEN4Δ30-200,201, which encodes two charge-to-alanine substitutions at amino acid residues 200 and 201 of the nonstructural 5 (NS5) protein[27] and rDEN4Δ30-4995, which encodes a single residue change at amino acid 158 of NS3. This NS3 mutation was originally identified as one that promoted efficient virus replication in Vero cells *in vitro* and was attenuating *in vivo*.[28] In preclinical studies, rDEN4Δ30-200,201 and rDEN4Δ30-4995 were more attenuated than rDEN4Δ30 in severe combined immunodeficiency (SCID) mice implanted with a human tumor cell line (SCID-HuH-7 mice) and in rhesus macaques.[29,30] Although both viruses were highly attenuated in

rhesus monkeys, both induced moderate levels of neutralizing antibodies and prevented replication of challenge DENV4. We previously reported that compared with rDEN4Δ30, rDEN4Δ30-200,201 exhibited increased attenuation, as shown by a reduced frequency of rash, ablation of elevation of ALT levels and viremia in dengue-negative humans, and retention of sufficient immunogenicity.31 rDEN4Δ30 and rDEN4Δ30-200,201 were passaged and manufactured in Vero cells, and both viruses acquired Vero cell adaptation mutations during passage *in vitro* ; virus titers greater than 10^7 PFU/mL were achieved during manufacture of the clinical trials material. Because the 4995 mutation promoted efficient virus replication in Vero cells, thereby decreasing the likelihood of additional Vero cell adaptation mutations, and because it decreased replication in human liver cells, it was selected as a second further attenuated DENV4 vaccine candidate for clinical evaluation.

We present the results of a phase 1 clinical trial of the further attenuated DENV4 live attenuated vaccine candidate rDEN4Δ30-4995. When administered at a dose of 10^5 PFU, rDEN4Δ30-200,201 and rDEN4Δ30-4995 are more attenuated in humans than rDEN4Δ30, as indicated by the absence of detectable viremia, reduced frequency and magnitude of liver enzyme abnormalities, and reduced immunogenicity. However, in contrast to rDEN4Δ30 and rDEN4Δ30-200,201, rDEN4Δ30-4995 induced local erythema at the site of inoculation, not unlike the local reactogenicity described for experimental wild-type DENV infection by Sabin in 1952.[5]

MATERIALS AND METHODS

Study population

This phase 1, randomized, double-blind, placebo-controlled study was conducted at Vanderbilt University Medical Center under an investigational new drug application (BB-IND 12977) reviewed by the U.S. Food and Drug Administration. The clinical protocol, protocol amendments, informed consent form, consent to photography, advertisements, and other study-related documents were reviewed and approved by the Institutional Review Boards of Vanderbilt University and the Johns Hopkins Bloomberg School of Public Health. The clinical protocol was also reviewed and approved by the Vanderbilt University and Johns Hopkins University Institutional Biosafety Committees. The study was sponsored by the Regulatory Compliance and Human Subjects Protection Branch of the National Institute of Allergy and Infectious Diseases (NIAID), and oversight was provided by the NIAID data safety monitoring board. Healthy adult male and non-pregnant female volunteers were recruited from the metropolitan Nashville area. Informed consent was obtained from each volunteer in accordance with the Code of Federal Regulations, Title 21, Part 50.

Healthy adult male and non-pregnant female volunteers between the ages of 18 and 50 were enrolled if they met the following eligibility criteria: normal findings during physical examination; negative for antibodies to all four DENV types, Saint Louis encephalitis virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, and human immunodeficiency virus; negative for hepatitis C virus and hepatitis B surface antigen; and normal values for complete blood count with differential, serum aspartate aminotransferase, ALT, total bilirubin, alkaline phosphatase, creatinine, creatine phosphokinase, prothrombin time, partial thromboplastin time, and urinalysis. Female volunteers were required to have a negative result on a urine pregnancy test at least three 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.

Study design and clinical monitoring

Twenty-eight healthy adult volunteers meeting the above eligibility criteria were enrolled in this double-blind, randomized, placebo-controlled study. On study day 0, volunteers were randomly assigned to receive 10^5 PFU of rDEN4Δ30-4995 vaccine virus or placebo (a safety-tested lot of Leibovitz L-15 medium that was also used as vaccine diluent), given as a single 0.5-mL subcutaneous dose in the deltoid region of the upper arm. Twenty volunteers received vaccine and eight volunteers received placebo. For intensive safety monitoring, clinical, virologic, hematologic, and chemistry assessments were performed every other day from day 0 through day 16 and days 21 and 28, as previously described.[31] DEN4-specific antibody titers were determined in serum obtained on study days 0, 28, 42, and 180. All adverse events were graded for severity and relationship to vaccine as previously described.31 Study staff remained blinded to vaccination status until all volunteers completed study day 42.

Vaccine virus

The rDEN4Δ30-4995 vaccine virus is a live attenuated recombinant virus derived from the rDEN4Δ30 vaccine virus.25 The parent rDEN4Δ30 vaccine virus contains a 30-nucleotide deletion in the 3′-UTR of the genome.26 rDEN4Δ30-4995 was created using site-directed mutagenesis to generate a U to C change at nt 4995 encoding a Ser to Pro amino acid change at residue 158 of NS3 in the full-length cDNA copy of rDEN4Δ30, i.e., plasmid p4Δ30.[28] To increase genetic stability, the Ser (UCA) to Pro (CCA) substitution at residue 158 of NS3 was altered to a Ser (UCA) to Leu (CUU) substitution.[30] rDEN4Δ30-4995 containing the Ser to Leu substitution exhibited decreased replication in SCID-HuH-7 mice and reduced viremia in rhesus monkeys in comparison with rDEN4Δ30, making it a suitable candidate for further evaluation as an attenuated dengue type 4 vaccine.30

The seed virus for the production of the rDEN4Δ30-4995 vaccine was produced in the Laboratory of Infectious Disease at NIAID. The clinical trial material was produced under current Good Manufacturing Practices conditions at Charles River Laboratories (Malvern, PA) and stored at −70 ± 10°C in an NIAID contract facility. Before administration, the vaccine ($10^{7.2}$ PFU/mL) was thawed and diluted to $10^{5.3}$ PFU/mL with safety tested Leibovitz L-15 medium and 0.5 mL was drawn up in a 1-mL syringe labeled with the volunteer number. The L-15 medium was identity and safety tested (amino acid composition, general safety, sterility, bacteriostasis/fungistasis, and pH) and shown to be free of adventitious agents. The L-15 vaccine diluent was also used as placebo.

Virus quantitation

Viremia was determined using a standard plaque assay as previously described.[26] Briefly, serum was diluted 10-fold in tissue culture medium and placed in duplicate wells of Vero cells. After virus adsorption (1 hour at 37°C) and addition of a methylcellulose overlay, the cells were incubated for five days at 37°C. Virus plaques were identified by immunoperoxidase staining with antibody against DENV4. Attempts to amplify and detect virus were made by inoculating plasma or serum directly onto Vero cells and incubating for five days, followed by titration as described above.

Serologic assessment

The antibody response to DENV4 was determined by 60% plaque reduction neutralization titer ($PRNT_{60}$) assay on Vero cells as previously described.[26] The $PRNT_{60}$ was determined for sera collected from volunteers on study days 0, 28, 42, and 180. Seroconversion to DENV4 was defined as a ≥ 4-fold increase in serum neutralizing antibody titer to wild-type DEN4 parent virus (DEN4 strain 814669, Dominica 1981) at study day 28 or 42, compared with the pre-vaccination $PRNT_{60}$ titer. The limit of detection in this assay was a $PRNT_{60}$ titer of 1:5. The

twelve volunteers with the highest titers to DENV4 were examined for titers to each of the other dengue serotypes. Day 42 antibody titers were also compared for each of the candidate DENV4 vaccines (rDEN4Δ30-4995, rDEN4Δ30- 200,201 and rDEN4Δ30) in a single assay to permit a comparison of the immunogenicity of each vaccine.

Cytokine profiles

Serum cytokine concentrations were determined using a cytokine bead assay (Becton Dickinson, San Jose CA) for interleukin-2 (IL-2), IL-6, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor, and interferon-γ. Assays were performed according to the manufacturer's instructions. Briefly, serum samples collected on days 0, 2, 4, 8, 12, and 28 were thawed and 0.05 mL of serum or cytokine standard mixture was added to a mixture of 0.05 mL of each capture bead. After incubating for 1 hour, 0.05 mL of mixed phycoerythrin detection reagent was added and incubated for 2 hours. Testing reagents were aspirated through the filter plate bottom and beads were resuspended in 0.15 mL of assay buffer. The cytokine bead assay was performed in a 96-well filter plate and analyzed on a custom LSRII flow cytometer with a high throughput sampler platform (Becton Dickinson). Data was analyzed using FCAP software version 1.0 (Becton Dickinson) to obtain values in picograms per milliliter. Data are expressed with concentrations below the lower limit of detection recorded as half of that value for the individual cytokine.

Data analysis

The purpose of this phase 1 study was to evaluate adverse event rates and immune responses in a group of healthy DENV-negative volunteers to determine the suitability of the rDEN4Δ30-4995 vaccine as the DENV4 component of a tetravalent dengue vaccine formulation. Baseline characteristics and frequency of vaccine-related adverse events, graded by severity, were compared between vaccine and placebo groups and with the results of historic studies with the rDEN4Δ30 parent vaccine virus at a dose of 10^5 PFU.[25] The durability of the antibody response was determined by measurement of the $PRNT_{60}$ at study day 180. The number of vaccinees infected with vaccine virus was determined. Infection was defined as the presence of vaccine virus in the serum of a vaccinee and/or seroconversion. Cytokine responses were analyzed in comparison to baseline cytokine concentrations immediately prior to vaccination, as previously described.[32] The choice of cytokines to be measured was based on previous experience with informative responses to vaccinia and yellow fever vaccines.[32] The primary analysis of cytokine data was performed using a paired *t*-test between day 0 (pre-vaccination) and each subsequent day sampled. The vaccine recipient with a documented influenza B virus infection was excluded from this analysis.

RESULTS

Demographics

Twenty-eight healthy volunteers 20–50 years of age were enrolled in this study. Twenty volunteers received vaccine and eight received placebo. Mean ages for vaccine and placebo groups were 34 and 32 years, respectively. There were no significant differences in sex or ethnicity between the vaccine group and the placebo group. Seventy one percent (13 of 20) of the vaccinees were female. Two volunteers self-identified as African American, one as Hispanic, and 25 as Caucasian. All 28 volunteers completed the study through day 180.

Local reactogenicity

Injection site tenderness, erythema, and induration were assessed 30 minutes post-vaccination and at each follow up study visit through study day 12. Seventeen of 20 vaccinees developed a non-tender, non-itchy, erythematous rash at the site of inoculation with a median onset of

four days post-inoculation. In many persons, the rash was visible for up to one week. A representative rash is shown in Figure 1. None of the placebo recipients developed local erythema (Table 1).

Solicited adverse events

All recipients tolerated the vaccine well. None of the volunteers experienced a systemic illness with fever, myalgia, or other dengue-associated symptoms and no severe adverse events were reported. Ten of twenty vaccine recipients reported having a headache. This was similar in frequency to placebo recipients (three of eight, Table 1). The next most common solicited adverse events were rash and fatigue/malaise. A mild maculopapular rash developed in three volunteers (two vaccinees and one placebo). The rash appeared on the abdomen, chest, back, and proximal upper extremities. In one vaccine recipient, the rash was generalized. The rash was non-pruritic and similar in character to that observed in recipients of rDEN4Δ30 vaccine.[25,26] The most prominent rash was seen in a vaccinee who had received an inactivated tick-borne encephalitis virus (TBEV) vaccine more than 20 years ago. In all cases, the rash resolved within 10 days of onset. Three vaccinees reported four episodes of fatigue and/or malaise. One volunteer reported two episodes of mild fatigue, lasting from study day four through nine and 12 through 14. The other two episodes of fatigue, one mild and one moderate, lasted one day each, starting one day 1 and on day 7, respectively. A mild transient neutropenia (absolute neutrophil count = 1,430 cells/mm^3) developed in one vaccinee on study day 4. A transient elevation of the ALT level developed in two vaccine recipients during the course of the study. One had a level of 59 IU/L on day 4 and the second had a level of 110 IU/L on day 10 accompanying an intercurrent influenza B virus infection (Table 1).

Viremia

None of the vaccine recipients had detectable viremia during the 16 day acute follow-up period in spite of sampling every other day, which included a virus amplification step. The limit of detection in this assay was 0.5 $\log_{10}$ PFU/mL.

Cytokine responses

To examine potential etiologies for the observed local reactogenicity, serum cytokine levels were determined immediately prior to vaccination and on days 2, 4, 8, 12, and 28 post-vaccination for IL-2, IL-6, IL-8, IL-10, MCP-1, tumor necrosis factor, and interferon-γ. Cytokine concentrations in rDEN4Δ30-4995 recipients were compared with those in rDEN4Δ30-200,201 vaccinees because the two vaccines exhibited a comparable degree of attenuation in humans yet differed in their local reactogenicity. All samples had been stored at −80°C until assayed. Most of the cytokine responses in vaccinees differed little from baseline, and there were no significant differences in cytokine responses between recipients of rDEN4Δ30-4995 and rDEN4Δ30-200,201. The chemokine MCP-1 was the only cytokine for which a significant elevation compared with day 0 was detected. The MCP-1 concentrations were higher only on day 2 compared with day 0 for rDEN4Δ30-4995 ($P = 0.0047$, by paired *t*-test) and rDEN4Δ30-200,201 ($P = 0.0032$, by paired *t*-test). The mean day 2 serum MCP-1 concentration was 130 pg/mL in rDEN4Δ30-4995 recipients and 62 pg/mL in rDEN4Δ30-200, 201 recipients.

Serologic responses

Nineteen of 20 vaccinees included in the day 28 and day 42 serologic analyses seroconverted to DENV4. The one volunteer who did not seroconvert had an acute virus infection with clinical symptoms and influenza B virus shedding on days 10–17 of the study. The reciprocal geometric mean $PRNT_{60}$ (95% confidence interval) at study day 28 was 150 (74–227) and at study day 42 was 126 (46–206) (Figure 2). When run simultaneously in the same assay, the geometric

mean antibody titer induced by rDEN4Δ30- 4995 at study day 42 was lower than that induced by the rDEN4Δ30 parent virus and similar to that induced by rDEN4Δ30-200,201 (Table 2). Twelve of 19 seroconverted volunteers maintained their antibody titer through study day 180. However, the mean titer decreased to 47 (range 5–476) (Figure 2). A maculopapular rash developed in the volunteer who had received TBEV vaccine as a child in Germany (> 20 years ago), and this person had higher dengue antibody titers than the other volunteers (reciprocal titer = 715 on day 42). When the 12 volunteers with the highest DENV4 antibody titers were tested for heterotypic antibody responses, only the volunteer with a TBEV vaccine history had heterotypic $PRNT_{60}$ responses. The day 28 and day 42 $PRNT_{60}$ responses in this person were 83 and 33 against DENV1, 14 and 82 against DENV2, and 73 and 25 against DENV3, respectively. All other vaccinees had $PRNT_{60}$ titers against DENV1, DENV2, and DENV3 ≤ 1:5.

DISCUSSION

A live attenuated tetravalent dengue vaccine based on a genetically stable attenuating deletion in the 3′-UTR of each of the four vaccine components is a promising approach to reducing the burden of disease caused by DENV.[23] To this end, several monovalent live attenuated dengue vaccine candidates have been developed and evaluated in phase 1 clinical trials to determine which candidates would be the most appropriate for inclusion in a tetravalent DENV vaccine formulation.[25,26,31,33,34] rDEN4Δ30 was the first of these live attenuated dengue vaccines tested in humans at doses ranging from 10^1 PFU to 10^5 PFU.[25,26] The vaccine was found to be safe, highly infectious, and immunogenic at all doses tested. Ninety-five to 100% of vaccine recipients became infected and 70% of the vaccinees given a dose of 10^5 PFU developed low-level viremia, with a mean serum virus titer of $10^{1.6}$ PFU/mL, i.e., at least 1,000-fold lower than infectivity titers typically seen in wild-type DENV infection.[8,35] At 10^5 PFU, the most frequently observed reactogenicity events were an asymptomatic rash in 50%, neutropenia in 15%, and transient elevation of ALT levels in 25% of the volunteers. Asymptomatic rash and neutropenia were observed at similar frequencies at all dose levels tested, but the elevation of serum ALT levels was dose dependent, occurring with the highest frequency at 10^5 PFU.[25] Transient elevations in serum liver enzyme levels have also been observed in vaccinees infected with other live attenuated DENV vaccine candidates,[13,36,37] but the levels observed in vaccinees were generally much lower than those observed in humans experiencing dengue fever or dengue hemorrhagic fever/dengue shock syndrome.[38,39]

In an attempt to reduce the mild liver toxicity associated with high-dose administration of rDEN4Δ30, a series of further attenuated rDEN4Δ30 viruses were generated.[28,29,40] The first of these further attenuated DEN4 vaccines to be evaluated in phase 1 clinical trials was rDEN4Δ30-200,201, a mutant virus that, in addition to Δ30 deletion in the 3′-UTR, contained a paired charge-to-alanine substitution in the NS5 protein.29 Compared with rDEN4Δ30, viremia in rDEN4Δ30-200,201 infected SCID-HuH-7 mice and rhesus monkeys was reduced in titer, and in rhesus monkeys the frequency of viremia was also reduced.[28] When rDEN4Δ30-200,201 was administered to 20 healthy adult volunteers, elevations in ALT levels were not observed, and the frequency of a maculopapular asymptomatic rash was reduced from 50% (rDEN4Δ30) to 20%. Although viremia was not detectable with rDEN4Δ30-200,201, all vaccinees seroconverted to DENV4.[31]

An additional further attenuated DEN4 vaccine, rDEN4Δ30-4995, was evaluated in a first-in-human phase 1 trial. In contrast to rDEN4Δ30-200,201, rDEN4Δ30-4995 contains a single amino acid substitution in the protease domain of the NS3 protein.[40] The originally described Ser-to Pro substitution at nt 4995 was selected for fitness in Vero cells, the cell substrate used for vaccine manufacture, suggesting that this attenuating mutation would likely be stable during replication in Vero cells.[28] Although the vaccine virus contains a Ser-to-Leu instead of the Ser-

to-Pro substitution in the 4995 position, the mutation was found to be stable, at least during the generation of the experimental lot and during vaccine manufacture. rDEN4Δ30-4995 containing the Ser-to-Leu substitution in the 4995 position was found to be attenuated in SCID-HuH-7 mice and in rhesus monkeys.[30]

To evaluate the clinical properties of the rDEN4Δ30-4995 vaccine and the parental rDEN4Δ30 vaccine, we compared the incidence and severity of adverse events and the frequency and magnitude of viremia in recipients of 10^5 PFU of either vaccine.25 Similar to what was observed in the rDEND30-200,201 trial,[31] none of 20 volunteers vaccinated with rDEN4Δ30-4995 had detectable viremia at any time point during the study, but 14 of 20 recipients of rDEN4Δ30 developed viremia. A maculopapular rash developed in 2 (10%) rDEN4Δ30-4995 vaccinees compared with 20% of the rDEND30-200,201 vaccinees and 50% of the rDEN4Δ30 vaccinees. One of the two had received a TBEV vaccine approximately 20 years before this study. This volunteer had the highest neutralizing antibody titer to DENV4, was the only volunteer of 12 tested who had heterotypic antibody to each of the other three DENV serotypes, and was the only volunteer in which a generalized non-pruritic rash developed. Although this observation was only made in one volunteer, it suggests that heterotypic immunity from exposure to another flavivirus may have played a role in these findings since higher-titer homotypic antibody and induction of heterotypic antibody titers has been described after secondary flavivirus infection[41,42] and after heterotypic flavivirus vaccination.43 Our TBEV vaccinated volunteer had a TBEV $PRNT_{60}$ titer of 1:151 on study day 0 and 1:234 on study day 28.

The apparent reduction in the observed since frequency of transient serum ALT elevation from 5 of 20 (DEN4Δ30) to 1 of 19 (DEN4Δ30-4995) infected volunteers (the one DEN4D30-4995 volunteer who did not seroconvert to DENV4 shed influenza B virus, a known cause of elevation of ALT levels during days 10–17 and had one elevated ALT value on day 10) suggests that the 4995 mutation might attenuate the replication of the virus in liver cells of the vaccine recipients, confirming previous observations made in the SCID-HuH-7 mouse model of dengue liver tropism.[28] It is also possible that the 4995 mutation reduced DENV replication at peripheral sites, e.g., in monocytes, because none of the volunteers became viremic. Although viremia was not detectable in the rDEN4Δ30-4995 vaccinees, the vaccine virus infected almost all vaccines, as shown by a 95% seroconversion rate.

Serum dengue virus neutralizing antibody titers in rDEN4Δ30-4995 recipients were directly compared with those in volunteers who received 10^5 PFU of rDEN4Δ30 or rDEN4Δ30-200,201 in our previous trials.[25,31] The $PRNT_{60}$ induced by rDEN4Δ30-4995 at study day 42 was lower than that induced by rDEN4Δ30 but comparable to that induced by rDEN4Δ30-200,201. However, the antibody titer induced by rDEN4Δ30-4995 decreased over the six- month period of follow-up with only 63% (12 of the 19 vaccinees) having a measurable $PRNT_{60}$ titer at six months. In the previously reported rDEN4Δ30-200,201 study, the mean $PRNT_{60}$ titer decreased from 79 on day 42 to 22 on day 180, yet 17 of 18 volunteers who seroconverted still had detectable antibody titers on day 180.[31] A direct comparison of day 180 sera from rDEN4Δ30-200,201 and rDEN4Δ30-4995 vaccinees in a single assay was not performed.

The local erythematous rash at the injection site observed in 17 of 20 vaccinees was not seen with the parent virus rDEN4Δ30, the rDEN4Δ30-200,201 vaccine, or any other live attenuated DEN vaccine containing the Δ30 mutation. The observed local reactogenicity did not seem to be related to the vaccine diluent because placebo recipients, who received vaccine diluent, did not show erythema at the injection site and because this diluent has been used without incident in previous trials. In addition, an unusually high particle-to-infectivity ratio was ruled out as a

cause of the local reaction. The genome equivalent-to-PFU ratio was 50 for rDEN4Δ30-4995 and 100 for rDEN4Δ30-200,201.

In 1952, Sabin described that in experimental dengue infection "intracutaneous injection of 10 or more mouse infectious doses of dengue virus was regularly followed after an interval of 3 to 5 days by local edema and erythema, 1 to 4 cm in diameter."[5] He further noted that when the generalized dengue rash appeared, these areas were spared and "stood out as blanched zones surrounded by the diffuse rash." Because the local reaction after rDEN4Δ30-4995 vaccination was an erythema of similar size that developed within several days after inoculation, we believe that it may be similar in appearance and possibly in pathogenesis to the erythema Sabin described decades ago.

Systemic cytokine profiles obtained after vaccination did not provide insight into an explanation of this local reaction because a mild elevation in MCP-1 levels on day 2 did not segregate with development of injection site erythema and was not unique to rDEN4Δ30-4995 recipients. Much higher elevations of MCP-1 levels and other inflammatory cytokines have been reported in patients at the time of severe dengue disease and were interpreted as a correlate of dengue infection of human mononuclear cells.[44] In clinical dengue disease, MCP-1 concentrations well above those observed in this trial were found to be associated with marked thrombocytopenia and hypotension.[45] The overall lack of alteration in proin-flammatory cytokine levels with the rDEN4Δ30-200,201 and rDEN4Δ30-4995 vaccines may be considered a further manifestation of their attenuation.

Based on the lack of detectable viremia, reduced frequency of elevation of ALT levels, and comparable immunogenicity, rDEN4Δ30-200,201 and rDEN4Δ30-4995 may be acceptable for inclusion into a tetravalent dengue vaccine formulation. However, because rDEN4Δ30-4995 caused local reactogenicity in most vaccine recipients and rDEN4Δ30-200,201 and rDEN4Δ30 did not, we will not pursue further clinical development of rDEN4Δ30-4995 at this time. rDEN4Δ30, given at a dose of 10^3 PFU, will likely be selected for inclusion in the tetravalent vaccine formulation, with rDEN4Δ30-200,201 serving as a backup candidates should rDEN4Δ30 turn out to be under-attenuated in the tetravalent formulation or in specific populations such as young children, who are the ultimate target for a dengue vaccine in the developing world.

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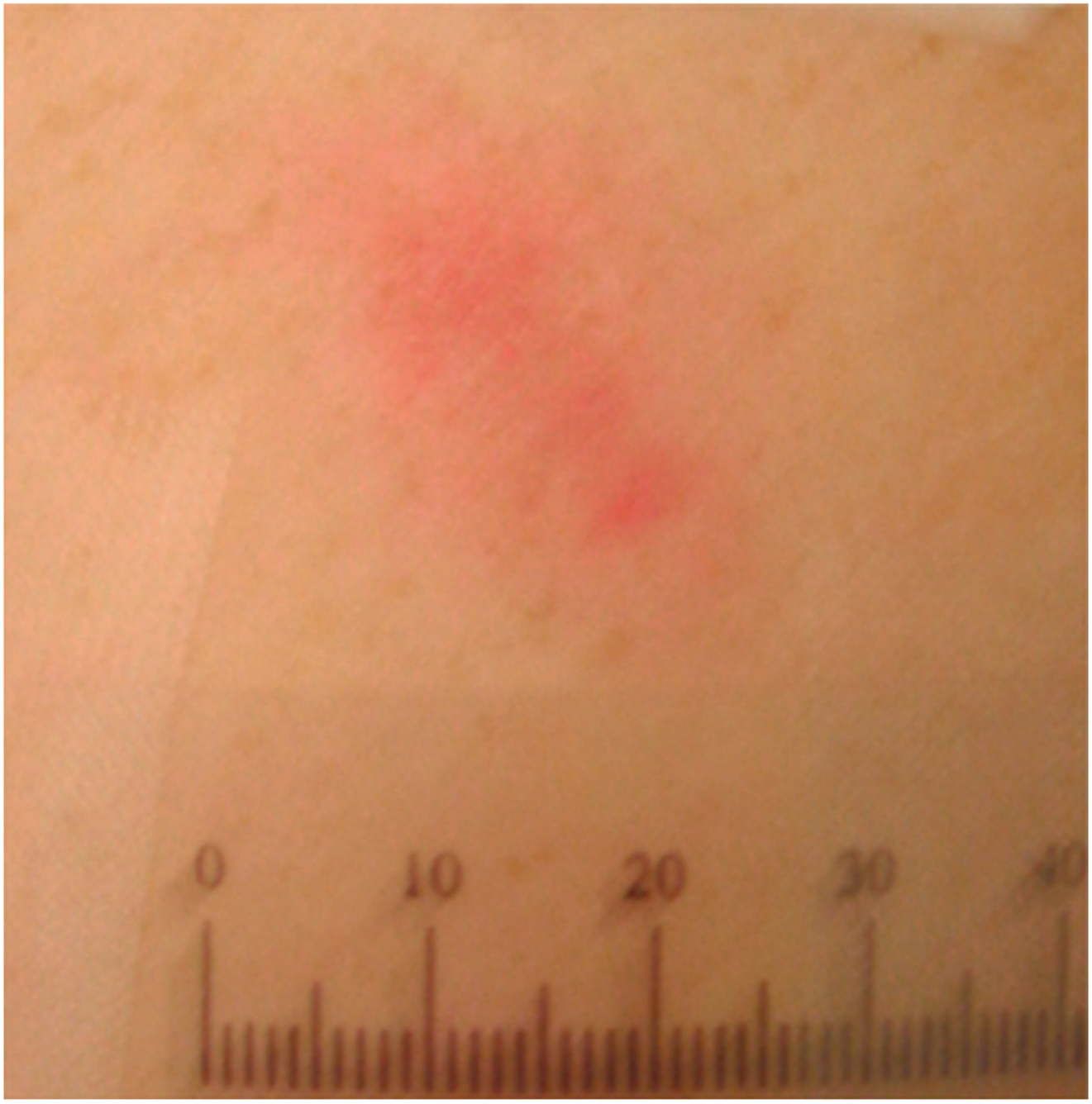

FIGURE 1.
Typical localized rash at the injection site (left deltoid area) on day 5 after receipt of the rDEN4Δ30-4995 vaccine.

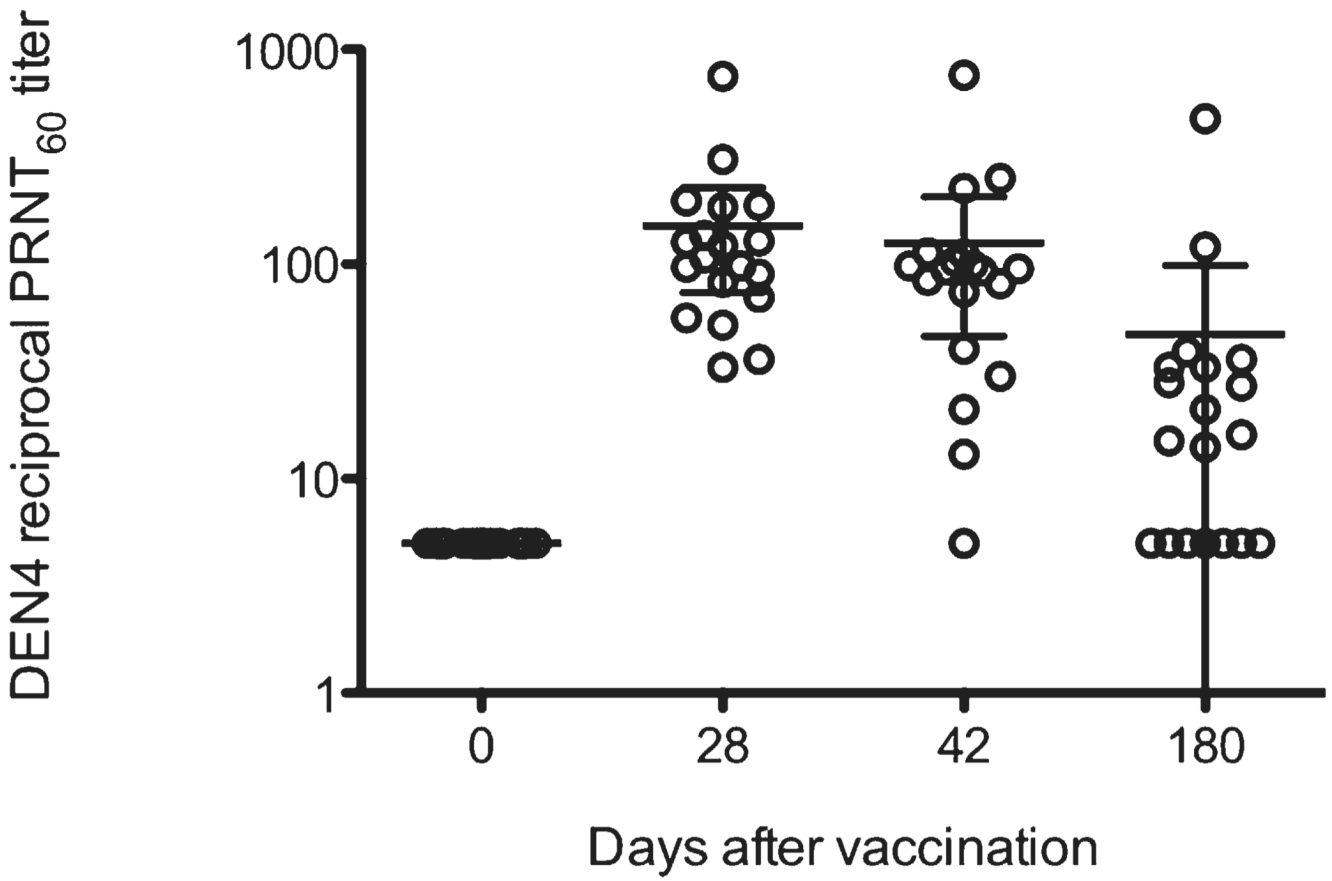

FIGURE 2.
Magnitude and duration of serum neutralizing antibody responses after receipt of rDEN4Δ30-4995 vaccine. Individual values, mean reciprocal titer, and 95% confidence intervals are indicated.

TABLE 1

Clinical summary of volunteers inoculated with rDEN4Δ30-4995*

Inoculum	Dose (log_{10} PFU)	No. of volunteers	No. (%) volunteers with indicated adverse event†						
			Systemic illness‡	Injection site erythema	Headache	Rash	Fatigue and/or malaise	Neutropenia§	Elevated ALT level¶
rDEN4Δ30-4995	5	20	0	17 (85)	10 (50)#	2 (10)#	3 (15)#	1 (5)	2 (10)#
Placebo	–	8	0	0	3 (38)	1 (13)	0	0	0

*PFU = plaque-forming units; ALT = alanine aminotransferase.

†All solicited adverse events were mild or moderate in severity.

‡Defined as ≥ 2 of the following symptoms lasting ≥ 2 days: headache, malaise, vomiting, arthralgia/malaise, nausea, or photophobia. There was no significant difference between vaccinees and placebo recipients in the occurrence of any of the individual solicited symptoms used to define systemic illness.

§Defined as an absolute neutrophil count (ANC) <1,500 cells/mm^3. The one neutropenic volunteer had an ANC of 1,430 on day 4.

¶Defined as a value 1.25-fold above the upper limit of normal of 40 IU/L.

#One vaccinee had a maximum ALT level of 59 IU/L on day 4, and another vaccinee had a maximum ALT level of 110 IU/L on day 10. The latter volunteer had clinical influenza, shed influenza B virus during days 10–17, and did not seroconvert to dengue virus type 4.

TABLE 2

Immunogenicity of rDEN4Δ30-4995 despite its enhanced attenuation phenotype*

Vaccine candidate (10^5 PFU)	% of volunteers with viremia	Mean ± SE peak virus titer (log_{10} PFU/mL serum)†	% Seroconversion‡	Reciprocal geometric mean serum neutralizing antibody titer (range) at day 42§
rDEN430-4995	0	< 0.5	95¶	52 (7–715)
rDEN4Δ30#	70	1.6 ± 0.1	100	156 (23–772)
rDEN4Δ30-200,201**	0	< 0.5	100	84 (9–382)

*PFU = plaque-forming units.

†Calculated for viremic volunteers only. The lower limit of detection is $10^{0.5}$ PFU/mL.

‡Defined as a ≥ 4-fold rise increase in serum 60% plaque reduction neutralizing antibody titer ($PRNT_{60}$) to dengue virus type 4 (DENV4) on day 28 or day 42.

§$PRNT_{60}$ was < 10 for all volunteers on study day 0. For direct comparison of reciprocal geometric mean titers, day 42 serum samples from vaccinees receiving either rDEN4Δ30-4995, rDEN4Δ30-200,201, or rDEN4Δ30 were evaluated in a single assay.

¶The only volunteer who failed to seroconvert to DENV4 had clinical influenza and shed influenza B virus during days 10–17.

#Historical data.[25]

**Historical data.[31]