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ATTENUATION AND IMMUNOGENICITY IN HUMANS OF A LIVE DENGUE VIRUS TYPE-4 VACCINE CANDIDATE WITH A 30 NUCLEOTIDE DELETION IN ITS 3′-UNTRANSLATED REGION

ANNA P. DURBIN, RUTH A. KARRON, WELLINGTON SUN, DAVID W. VAUGHN, MARY J. REYNOLDS, JOHN R. PERREAULT, BHAVIN THUMAR, RUHE MEN, CHING-JUH LAI, WILLIAM R. ELKINS, ROBERT M. CHANOCK, BRIAN R. MURPHY, AND STEPHEN S. WHITEHEAD

Center for Immunization Research, Johns Hopkins School of Public Health, Baltimore, Maryland; Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; Walter Reed Army Institute of Research, Silver Spring, Maryland

Abstract. The recombinant dengue virus type-4 vaccine candidate 2AΔ30 was attenuated in rhesus monkeys due to an engineered 30-nucleotide deletion in the 3′-untranslated region of the viral genome. A clinical trial to evaluate the safety and immunogenicity of a single dose of 2AΔ30 was conducted with 20 adult human volunteers. The vaccine candidate was well tolerated and did not cause systemic illness in any of the 20 volunteers. Viremia was detectable in 14 volunteers at a mean level of 1.6 $\log_{10}$ plaque-forming units/ml of serum, although all 20 volunteers seroconverted with a seven-fold or greater increase in serum neutralizing antibody titer on day 28 post-vaccination (mean titer = 1:580). A mild, asymptomatic, macular rash developed in 10 volunteers, and a transient elevation in the serum level of alanine aminotransferase was noted in five volunteers. The low level of reactogenicity and high degree of immunogenicity of this vaccine candidate warrant its further evaluation and its use to create chimeric vaccine viruses expressing the structural genes of dengue virus types 1, 2, and 3.

INTRODUCTION

Dengue virus is a positive-sense RNA virus belonging to the *Flavivirus* genus of the family Flaviviridae. Dengue virus is widely distributed throughout the tropical and semitropical regions of the world and is transmitted to humans by mosquito vectors. Dengue virus is a leading cause of hospitalization and death in children in at least eight tropical Asian countries.[1] There are four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) that annually cause an estimated 50–100 million cases of dengue fever and 500,000 cases of the more severe form of dengue virus infection known as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).[2] This latter disease is seen predominately in children and adults experiencing a second dengue virus infection with a serotype different than that of their first dengue virus infection and in primary infection of infants who still have circulating dengue-specific maternal antibody.[3–5] A vaccine is needed to lessen the disease burden caused by dengue virus, but none is licensed. Because of the association of more severe disease with secondary dengue virus infection, a successful vaccine must induce immunity to all four serotypes. Immunity is primarily mediated by neutralizing antibody directed against the envelope (E) glycoprotein, a virion structural protein. Infection with one serotype induces long-lived homotypic immunity and a short-lived heterotypic immunity.[6] Therefore, the goal of immunization is to induce a long-lived neutralizing antibody response against DEN-1, DEN-2, DEN-3, and DEN-4, which can best be achieved economically using live attenuated virus vaccines. This is a reasonable goal since a live attenuated vaccine has already been developed for the related yellow fever virus, another mosquito-borne flavivirus present in tropical and semitropical regions of the world.[7]

Several live attenuated dengue vaccine candidates have been developed and evaluated in humans or non-human primates. The first live attenuated dengue vaccine candidates were host range mutants developed by serial passage of wild-type dengue viruses in the brains of mice and selection of mutants attenuated for humans.[8–10] Although these candidate vaccine viruses were immunogenic in humans, their poor growth in cell culture discouraged further development. Additional live attenuated DEN-1, DEN-2, DEN-3, and DEN-4 vaccine candidates have been developed by serial passage in tissue culture[11–16] or by chemical mutagenesis.[17] It has proven very difficult to achieve a satisfactory balance between attenuation and immunogenicity for each of the four serotypes of dengue virus using these approaches and to formulate a tetravalent vaccine that is safe and satisfactorily immunogenic against each of the four dengue viruses.[16,18]

Two major advances using recombinant DNA technology have recently made it possible to develop additional promising live attenuated dengue virus vaccine candidates. First, methods have been developed to recover infectious dengue virus from cells transfected with RNA transcripts derived from a full-length cDNA clone of the dengue virus genome, thus making it possible to derive infectious viruses bearing attenuating mutations that have been introduced into the cDNA clone by site-directed mutagenesis.[19] Second, it is possible to produce antigenic chimeric viruses in which the structural protein coding region of the full-length cDNA clone of dengue virus is replaced by that of a different dengue virus serotype or from a more divergent flavivirus.[20–23] These techniques have been used to construct intertypic chimeric dengue viruses that have been shown to be effective in protecting monkeys against homologous dengue virus challenge.[24] A similar strategy is also being used to develop attenuated antigenic chimeric dengue virus vaccines based on the attenuation of the yellow fever vaccine virus or the attenuation of the cell-culture passaged dengue viruses.[25,26] The present study examines the level of attenuation for humans of a DEN-4 mutant bearing a 30-nucleotide deletion (Δ30) introduced into its 3′-untranslated region by site-directed mutagenesis and that was found previously to be attenuated for rhesus monkeys.[27] Additional studies were carried out to examine whether this Δ30 mutation present in

TABLE 1

Nucleotide and amino acid differences of dengue type-4 wt and attenuated (*att*) viruses and of the p4 plasmid used to generate the recombinant rDEN4 and rDEN4Δ30 viruses

		Nucleotide and amino acid differences*						
Nucleotide position	Gene/ region	814669 wt virus	2A wt virus	2AΔ30 *att* virus	p4 plasmid	rDEN4 wt virus	rDEN4Δ30 *att* virus	Comment
447–452	prM	TTGTCA	TTGTCA	TTGTCA	CTCAGC	CTCAGC	CTCAGC	Add *Bbv*CI site (silent)†
2348	E	A (ser)	A (ser)	A (ser)	G (ser)	G (ser)	G (ser)	Add *Xho*I site (silent)†
2440	E	T (val)	T (val)	T (val)	C (ala)	C (ala)	C (ala)	*Xho*I site co-mutant†
4172	NS2B	**G/A (leu)**	G (leu)	G (leu)	G (leu)	G (leu)	G (leu)	814669 mutant (silent)‡
4307	NS2B	**T (asn)**	C (asn)	C (asn)	C (asn)	C (asn)	C (asn)	814669 mutant (silent)‡
4353	NS2B	A (ile)	A (ile)	A (ile)	A (ile)	G (val)	A (ile)	rDEN4 mutant‡
5826	NS3	**A (thr)**	C (pro)	C (pro)	C (pro)	C (pro)	C (pro)	814669 mutant‡
6195	NS3	A (lys)	A (lys)	A (lys)	A (lys)	G (glu)	A (lys)	rDEN4 mutant‡
7153	NS4B	T (val)	T (val)	C (ala)	T (val)	T (val)	T (val)	2AΔ30 mutant‡
7163	NS4B	A (leu)	A (leu)	A (leu)	A (leu)	A (leu)	T (phe)	rDEN4Δ30 mutant‡
7295	NS4B	C (ser)	C (ser)	C (ser)	C (ser)	C (ser)	T (ser)	rDEN4Δ30 mutant (silent)‡
7630	NS5	**G (arg)**	A (lys)	A (lys)	A (lys)	A (lys)	A (lys)	814669 mutant‡
7730	NS5	T (ser)	T (ser)	T (ser)	A (ser)	A (ser)	A (ser)	Remove *Xba*I site (silent)†
8308	NS5	A (lys)	A (lys)	G (arg)	A (lys)	A (lys)	A (lys)	2AΔ30 mutant‡
8534	NS5	**A (val)**	G (val)	G (val)	G (val)	G (val)	G (val)	814669 mutant (silent)‡
9318–20	NS5	AGA	AGA	AGA	CGG	CGG	CGG	Add *Sac*II site (silent)†
10157	NS5	C (his)	C (his)	C (his)	C (his)	T (his)	C (his)	rDEN4 mutant (silent)‡
10452	3′ UTR	C	C	C	C	T	C	rDEN4 mutant‡
10475	3′ UTR	G	G	–	G	G	–	Δ30 co-mutant§
10478–507	3′ UTR			Δ30			Δ30	Δ30 mutation¶

* Bold-faced sequence changes represent differences between 814669 wt virus and its recombinant derivative 2A wt virus. Underlined sequence changes represent differences between 2A wt virus and its derivatives 2AΔ30, p4 plasmid, rDEN4, or rDEN4Δ30.

† Engineered mutation designed to create or remove restriction enzyme recognition site in cDNA plasmid.

‡ Mutation is presumed to have occurred during amplification of virus in tissue culture.

§ Deletion of nucleotide 10475 occurred during construction of Δ30 mutation in 2A cDNA plasmid.

¶ Engineered deletion mutation designed to remove 30 nucleotides of the 3′ untranslated region of the genome.

the DEN-4 vaccine candidate was the major determinant of its attenuation for monkeys. It was found that the Δ30 mutation was indeed the major determinant of attenuation for monkeys, and that it specified a satisfactory balance between attenuation and immunogenicity for humans.

MATERIALS AND METHODS

Viruses and cells. The wild type (wt) DEN-4 virus strain 814669 (Dominica, 1981), originally isolated in *Aedes pseudoscutellaris* (AP61) cells, was previously plaque-purified in LLC-MK2 cells and amplified in C6/36 cells as described.[28]

For further amplification, the C6/36 suspension was passaged two times in Vero (WHO) cells maintained in minimal essential medium (MEM)-E (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Viruses derived from RNA transfection or used for clinical lot development were grown in Vero (WHO) cells maintained in serum-free medium VP-SFM (Life Technologies).

Construction of DEN-4 deletion mutants. A 30-nucleotide deletion was previously introduced into the 3′-untranslated region of the 2A cDNA clone of wild-type DEN-4 strain 814669 as described.[27] This deletion removes nucleotides 10478–10507, and was originally designated 3′d 172–143, signifying the location of the deletion relative to the 3′ end of the viral genome. In this paper, this deletion is referred to as Δ30. The full-length 2A cDNA clone has undergone several subsequent modifications to improve its ability to be genetically manipulated. As previously described, a translationally silent *Xho*I restriction enzyme site was engineered near the end of the E region at nucleotide 2348 to create clone 2A-*Xho*I.[20] In this study, the viral coding sequence of the 2A-*Xho*I cDNA clone was further modified using site-directed mutagenesis to create clone p4 (Table 1): a unique *Bbv*CI restriction enzyme site was introduced near the C-prM junction (nucleotides 447–452), an extra *Xba*I restriction enzyme site was ablated by mutation of nucleotide 7730, and a unique *Sac*II restriction enzyme site was created in the nonstructural protein 5 (NS5) region (nucleotides 9318–9320). Each of these engineered mutations is translationally silent and does not change the amino acid sequence of the viral polypeptide. Also, several mutations were made in the vector region of clone p4 to introduce or ablate additional restriction sites. The cDNA clone p4Δ30 was generated by introducing the Δ30 mutation into clone p4. This was accomplished by replacing the *Mlu*I-*Kpn*I fragment of p4 (nucleotides 10403–10654) with that derived from plasmid 2AΔ30 containing the 30-nucleotide deletion. The cDNA clones p4 and p4Δ30 were subsequently used to generate recombinant viruses rDEN4 and rDEN4Δ30, respectively.

Generation of viruses. Full-length RNA transcripts were synthesized from cDNA clones 2A and 2AΔ30 using SP6 RNA polymerase as previously described.[19,27] The reaction to generate full-length RNA transcripts from cDNA clones p4 and p4Δ30 was modified and consisted of a 50-μl reaction mixture containing 1 μg of linearized plasmid, 60 units of SP6 polymerase (New England Biolabs, Beverly, MA), 1× RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol), 0.5 mM $m^7G(5')ppp(5')G$ cap analog (New England Biolabs), 1 mM of each nucleotide triphosphate, 1 unit of pyrophosphatase (New England Biolabs), and 80 units of RNAse inhibitor (Roche, Indianapolis, IN). This reaction mixture was incubated at 40°C for 90 min and the resulting transcripts were

purified using RNeasy mini kit (Qiagen, Valencia, CA). For transfection of Vero cells, purified transcripts (1 μg) were mixed with 12 μl of DOTAP liposome reagent (Roche) in saline containing 20 mM HEPES buffer (pH 7.6) and added to cell monolayer cultures in a six-well plate. After 5–17 days, tissue culture medium was harvested, clarified by centrifugation, and virus was amplified in Vero cells. The presence of virus was confirmed by plaque titration. It should be noted that during the course of transfection and amplification of 2AΔ30 to create the vaccine lot, the virus underwent six passages entirely in Vero cells. The remaining viruses (rDEN4 and rDEN4Δ30) were passaged five times in Vero cells to generate the virus suspension used for sequence analysis and studies in rhesus monkeys.

Vaccine production. An aliquot of clarified tissue culture fluid containing vaccine candidate 2AΔ30 was sent to DynCorp (Rockville, MD) for amplification of virus in Vero cells and production of a vaccine lot. For vaccine production, 2AΔ30-infected tissue culture supernatant was harvested, SPG buffer added (final concentration: 218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM monobasic potassium phosphate, and 7.2 mM dibasic potassium phosphate, pH 7.2), and the virus suspension was clarified by low-speed centrifugation. To degrade residual Vero cell DNA, the vaccine suspension was treated with Benzonase endonuclease (American International Chemical, Natick, MA) (100 U/ml) and incubated for 1 hr at 37°C, followed by high-speed centrifugation (17,000 × *g* for 16 hr). The resulting virus pellet was gently rinsed with MEM-E, resuspended in MEM-E containing SPG, sonicated, distributed into heat-sealed ampules, and stored frozen at −70°C. Final container safety testing confirmed microbial sterility, tissue culture purity, and animal safety. The 2AΔ30 vaccine lot (designated DEN4–9) has a titer of 7.48 $\log_{10}$ plaque-forming units (PFU)/ml, with a single dose of 5.0 $\log_{10}$ PFU/ml containing < 1 pg/ml of Vero cell DNA and < 0.001 U/ml of Benzonase endonuclease.

Sequence of cDNA clones and viral genomes. The nucleotide sequence of the viral genome region of cDNA plasmids 2A and p4 was determined on a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using vector-specific and DEN-4-specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems). The nucleotide sequence of the genomes of the parental wild-type DEN-4 strain 814669 and of recombinant viruses 2A wild-type, 2AΔ30 (vaccine lot), rDEN4, and rDEN4Δ30 was also determined. Viral RNA was extracted from virus preparations and serum samples using the QIAamp Viral RNA mini kit (Qiagen). Reverse transcription (RT) was performed using random hexamers and the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). Overlapping polymerase chain reaction (PCR) fragments of approximately 2,000 basepairs were generated using optimized DEN-4 specific primers and Advantage cDNA polymerase (ClonTech, Palo Alto, CA). Both strands of purified PCR fragments were sequenced directly using dye-terminator reactions as described above and results were assembled into a consensus sequence. To determine the nucleotide sequence of the viral RNA 5′ and 3′ regions, the 5′ cap nucleoside of the viral RNA was removed with tobacco acid pyrophosphatase (Epicentre, Madison, WI), followed by circularization of the RNA using RNA ligase (Epicentre). The RT-PCR was performed as described and a cDNA fragment spanning the ligation junction was sequenced using DEN-4 specific primers. GenBank accession numbers have been assigned as follows (virus: accession number): 814669: AF326573, 2AΔ30: AF326826, rDEN4: AF326825, and rDEN4Δ30: AF326827.

Human vaccine recipients. Twenty healthy adult volunteers were recruited by the Johns Hopkins School of Hygiene and Public Health Center for Immunization Research (CIR) located in Baltimore, Maryland. The clinical protocol was reviewed and approved by the Joint Committee for Clinical Investigation of the Johns Hopkins University School of Medicine and informed consent was obtained from each volunteer. Volunteers were enrolled in the study if they met the following inclusion criteria: 18–45 years of age; no history of chronic illness; normal results on a physical examination; negative for antibodies to human immunodeficiency virus, negative for hepatitis B surface antigen, and negative for antibodies to hepatitis C virus; no stool occult blood; and normal values for a complete blood cell count (CBC) with a differential count, hematocrit, platelet count, serum creatinine, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, bilirubin, prothrombin time, partial thromboplastin time, and urinalysis. Female volunteers were required to have a negative result on a urine pregnancy test prior to vaccination and on the day of vaccination and to agree to use contraception or abstain from sexual intercourse for the duration of the study. Volunteers also lacked serologic evidence of prior flavivirus infection as defined by a hemagglutination-inhibition antibody titer < 1:10 to DEN-1, DEN-2, DEN-3, DEN-4, St. Louis encephalitis virus, Japanese encephalitis virus, or yellow fever virus and a plaque-reduction neutralization titer < 1:10 to DEN-4 and yellow fever virus.

Studies in humans. Volunteers were immunized in three successive cohorts of four, six, and 10 volunteers to assess the safety of the vaccine. In this study, an illness was defined as the following: dengue virus infection associated with a platelet count < 90,000/mm^3, a serum ALT level > 4 times normal, an oral temperature > 38°C for two successive days, or headache and/or myalgia lasting two successive days. Systemic illness was defined as the occurrence of fever > 38°C for two consecutive days, or any two of the following for at least two consecutive study days: headache, malaise, anorexia, and myalgia/arthralgia. The trials were conducted between October and April, a time of low mosquito prevalence, to reduce the risk of transmission of vaccine virus from the volunteers to the community.

On the day of vaccination, vaccine candidate 2AΔ30 was diluted to 5.3 $\log_{10}$ PFU/ml in sterile saline for injection (USP), and each volunteer was injected subcutaneously with 0.5 ml containing 5.0 $\log_{10}$ PFU of vaccine into the left deltoid region. Volunteers were given a home diary card on which they were to record their temperature twice a day for days 0–5 post-vaccination. The volunteers returned to the clinic each day for examination by a physician and their diary cards were reviewed. The injection site was evaluated for erythema, induration, and tenderness. Clinical signs and symptoms such as headache, rash, petechiae, lymphadenopathy, hepatomegaly, abdominal tenderness, anorexia, nausea, fatigue, myalgia, arthralgia, eye pain, and photophobia were

assessed daily. Symptoms were graded as mild (no need for treatment or a change in activity), moderate (treatment needed or change in activity noted, yet still able to continue daily activity) or severe (confined to bed). Blood was drawn for a CBC with a differential count and for virus quantitation on days 0, 2, and 4. Volunteers were admitted to the inpatient unit at the CIR on the sixth day after immunization. The study physician evaluated all volunteers each day by physical examination and interview. The volunteers had their blood pressure, pulse, and temperature recorded four times a day. Blood was drawn each day for a CBC with a differential count and for virus quantitation and every other day for ALT measurement. Volunteers were confined to the inpatient unit until discharge on study day 15. On study days 28 and 42, volunteers returned for physical examination and blood was drawn for virus quantitation (day 28) and for serum antibody measurement (day 28 and 42).

Virus quantitation and amplification. Serum was obtained for detection of viremia and titration of virus in positive specimens. For these purposes, 8.5 ml of blood was collected in a serum separator tube and incubated at room temperature for less than 30 min. Serum was decanted into 0.5-ml aliquots, rapidly frozen in a dry ice/ethanol bath, and stored at −70°C. Serum aliquots were thawed and serial 10-fold dilutions were inoculated onto Vero cell monolayer cultures in 24-well plates. After a 1-hr incubation at room temperature, the monolayers were overlaid with 0.8% methylcellulose in OptiMEM (Life Technologies) supplemented with 5% FBS. Following incubation at 37°C for four days, virus plaques were visualized by immunoperoxidase staining. Briefly, cell monolayers were fixed in 80% methanol for 30 min and rinsed with antibody buffer (5% nonfat milk in phosphate-buffered saline). Rabbit polyclonal DEN-4 antibodies were diluted 1:1,000 in antibody buffer and added to each well followed by a 1-hr incubation at 37°C. Primary antibody was removed and the cell monolayers were washed twice with antibody buffer. Peroxidase-labeled goat-anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted 1:500 in antibody buffer and added to each well, followed by a 1-hr incubation at 37°C. Secondary antibody was removed and the wells were washed twice with phosphate-buffered saline. Peroxidase substrate (4-chloro-1-naphthol in H_2O_2) was added to each well and visible plaques were counted.

For amplification of virus in serum samples, a 0.3-ml aliquot of serum was inoculated directly onto a single well of a six-well plate of Vero cell monolayers and incubated at 37°C for seven days. Cell culture fluid was then assayed for virus by plaque assay as described above.

Serology. Hemagglutination-inhibition assays were performed as previously described.[29] Plaque-reduction neutralization titers were determined by a modification of the technique described by Russell and others.[30] Briefly, test sera were heat inactivated (56°C for 30 min) and serial two-fold dilutions beginning at 1:10 were made in OptiMEM supplemented with 0.25% human serum albumin. The rDEN4Δ30 virus, diluted to a final concentration of 1,000 PFU/ml in the same diluent, was added to equal volumes of the diluted serum and mixed well. The virus/serum mixture was incubated at 37°C for 30 min. Cell culture medium was removed from 90% confluent monolayer cultures of Vero cells on 24-well plates and 50 μl of virus/serum mixture was transferred onto duplicate cell monolayers. Cell monolayers were incubated for 60 min at 37°C and overlaid with 0.8% methylcellulose in OptiMEM supplemented with 2% FBS. Samples were incubated at 37°C for four days after which plaques were visualized by immunoperoxidase staining as described above, and a 60% plaque-reduction neutralization titer was calculated.

Studies in rhesus monkeys. Evaluation of the replication and immunogenicity of wt virus 814669, and recombinant viruses 2A wt, 2AΔ30 (vaccine lot), rDEN4, and rDEN4Δ30 in juvenile rhesus monkeys was performed as previously described.[27] Briefly, dengue virus–seronegative monkeys were injected subcutaneously with 5.0 $\log_{10}$ PFU of virus diluted in L-15 medium (Quality Biological, Gaithersburg, MD) containing SPG buffer. A dose of 1 ml was divided between two injections in each side of the upper shoulder area. Monkeys were observed daily and blood was collected on days 0–10 and 28, and processed for serum, which was stored frozen at −70°C. Titer of virus in serum samples was determined by plaque assay in Vero cells as described above. Neutralizing antibody titers were determined for the day 28 serum samples as described above. A group of monkeys inoculated with either 2AΔ30 (n = 4) or wild-type virus 814669 (n = 8) were challenged on day 42 with a single dose of 5.0 $\log_{10}$ PFU/ml of wild-type virus 814669 and blood was collected for 10 days. Husbandry and care of rhesus monkeys was in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals.

RESULTS

Construction and characterization of DEN-4 wt and deletion mutant viruses. The nucleotide and deduced amino acid sequences of the previously described wild-type 814669 virus, the DEN-4 2A wt virus derived from it (designated 2A wt), and the 2AΔ30 vaccine candidate derived from 2A wt virus were first determined (Table 1). Sequence analysis showed that the wt 814669 virus used in this study had apparently accumulated two missense mutations (nucleotides 5826 and 7630) and three silent mutations during its passage and amplification since these mutations were not described in previously published reports of the viral sequence (GenBank accession number M14931) and were not present in the 2A cDNA derived from the virus. Sequence comparison between viruses 2A wt and vaccine lot 2AΔ30 revealed that 2AΔ30 accumulated two missense mutations (nucleotides 7153 and 8308) and also confirmed the presence of the 2AΔ30 mutation (nucleotides 10478–10507), as well as an additional deletion of nucleotide 10475, which occurred during the original construction of the Δ30 mutation.[27] This sequence analysis revealed significant sequence divergence between the biologically derived wt 814669 virus and its recombinant 2A wt derivative and between the 2A wt and 2AΔ30 virus. Since the 2A wt and 2AΔ30 viruses differed at nucleotides other than the deletion mutation, the attenuation phenotype previously reported for 2AΔ30[27] could not be formally ascribed solely to the Δ30 mutation and may have been specified by the mutations at nucleotides 7153, 8308, 10475, or the Δ30 deletion.

TABLE 2

Deletion of 30 nucleotides in the 3′ untranslated region of dengue type 4 viruses 2AΔ30 and rDEN4Δ30 attenuates the viruses for rhesus monkeys

Virus*	No. of monkeys	No. of monkeys with viremia	Mean no. of viremic days per monkey†	Mean peak virus titer (log_{10}PFU/ml ± SE)	Geometric mean serum neutralizing antibody titer (reciprocal dilution)	
					Day 0	Day 28
814669 wt	8	8	3.6	1.8 ± 0.10	<10	532
2A wt	2	2	4.5	1.9 ± 0.20‡	<10	442
2AΔ30	6	5	1.2	0.8 ± 0.11‡	<10	198
rDEN4	4	4	3.8	1.5 ± 0.15§	<10	446
rDEN4Δ30	4	2	0.8	0.7 ± 0.03§	<10	223

* Groups of rhesus monkeys were inoculated subcutaneously with 5.0 log_{10}PFU (plaque-forming units) of the indicated virus in a 1 ml dose. Serum was collected daily for 10 days and on day 28. Virus titer was determined by plaque assay in Vero cells.

† Viremia was not detected in any monkey after day 5.

‡ Significant difference, unpaired *t*-test, $P < 0.01$.

§ Significant difference, unpaired *t*-test, $P < 0.005$.

To determine whether the Δ30 mutation was responsible for the observed attenuation of 2AΔ30, a second pair of viruses, one with and one without the Δ30 mutation, were produced for evaluation in monkeys. A new DEN-4 cDNA vector construct, designated p4, was derived from the 2A-*Xho*I cDNA clone and translationally silent mutations were introduced to add or ablate several restriction enzyme sites (Table 1). These sites were added to facilitate the future genetic manipulation of this wt DEN-4 cDNA by the introduction of other attenuating mutations if needed. The sequence of the genomic region of the p4 cDNA plasmid was identical to that of the 2A wt virus except for the engineered restriction site changes and a point mutation at nucleotide 2440, which was introduced during the original mutagenesis of the 2A cDNA plasmid to create the *Xho*I site.[20] The Δ30 mutation and the neighboring deletion at nucleotide 10475 were co-introduced into the p4 plasmid by replacing a short restriction fragment with one derived from the cDNA clone of 2AΔ30. The RNA transcripts derived from the p4 cDNA clone and from its Δ30 derivative each yielded virus (designated rDEN4 and rDEN4Δ30, respectively) following transfection of Vero cells. Sequence analysis of the rDEN4 virus revealed that during its passage and amplification in Vero cells it accumulated two missense mutations (nucleotides 4353 and 6195), a silent mutation (nucleotide 10157), and a point mutation in the 3′-untranslated region (nucleotide 10452). In addition to containing the Δ30 and the accompanying deletion at nucleotide 10475, rDEN4Δ30 had also accumulated a missense mutation (nucleotide 7163) and a silent mutation (nucleotide 7295).

Parental wt 814669 virus and recombinant viruses 2A wt, 2AΔ30, rDEN4, and rDEN4Δ30 each replicate in Vero cells to a titer exceeding 7.0 log_{10} PFU/ml, and their replication is not temperature sensitive at 39°C.

Virus replication, immunogenicity, and efficacy in monkeys. Groups of rhesus monkeys were inoculated with wt DEN-4 814669, 2A wt, rDEN4, 2AΔ30, and rDEN4Δ30 to assess the level of restriction of replication specified by the Δ30 mutation. Serum samples were collected daily and titer of virus present in the serum was determined by plaque enumeration on Vero cell monolayer cultures. Monkeys inoculated with wt 814669 virus or its recombinant counterparts (2A wt or rDEN4) were viremic for 3–4 days with a mean peak virus titer of nearly 2 log_{10} PFU/ml (Table 2). Monkeys inoculated with virus 2AΔ30 or rDEN4Δ30 had a lower frequency of viremia (83% and 50%, respectively), were viremic for only approximately one day, and the mean peak titer was 10-fold lower (Table 2). Monkeys inoculated with DEN-4 814669, 2A wt, or rDEN4 viruses developed high levels of neutralizing antibody, with mean titers between 442 and 532, consistent with their presumed wt phenotype. Monkeys inoculated with 2AΔ30 or rDEN4Δ30 developed a lower level of neutralizing antibody, with mean titers of 198 and 223, respectively. The decrease in neutralizing antibody titer in response to 2AΔ30 and rDEN4Δ30 is consistent with the attenuation phenotype of these viruses. Monkeys inoculated with either 2AΔ30 (n = 4) or wt 814669 virus (n = 8) were challenged after 42 days with wt virus 814669. Dengue virus was not detected in any serum sample collected for up to 10 days following virus challenge, indicating that these monkeys were completely protected following immunization with either wt virus or vaccine candidate 2AΔ30.

Since DEN-4 814669, 2A wt, and rDEN4 each manifest the same level of replication and immunogenicity in rhesus monkeys, it is reasonable to conclude that the identified sequence differences between these presumptive wt viruses that arose during passage in tissue culture or during plasmid construction do not significantly affect their level of replication *in vivo.* Similarly, the comparable level of attenuation of 2AΔ30 and rDEN4Δ30 indicates that the mutations shared by these viruses, namely, the Δ30 mutation and its accompanying nucleotide 10475 deletion mutation, are probably responsible for the attenuation of these viruses rather than their incidental sequence differences.

Clinical response to immunization with 2AΔ30. The 2AΔ30 vaccine candidate was administered subcutaneously at a dose of 10^5 PFU to 20 seronegative volunteers. Each of the vaccinees was infected and the virus was well tolerated by all vaccinees (Table 3). Viremia was detected in 70% of the vaccinees, was present only at low titer (Table 3), and did not extend beyond day 11 (Figure 1A).

None of the 20 vaccinees reported soreness or swelling at the injection site. Mild erythema (1–3 mm) around the injection site was noted on examination of eight volunteers 30 min post-vaccination, which resolved by the next day in seven of those volunteers and by the third day in the remaining volunteer. Mild tenderness to pressure at the vaccination site

TABLE 3

Dengue-4 candidate vaccine virus 2AΔ30 is well-tolerated and induces an immunologic response in both viremic and nonviremic volunteers

No. subjects	Viremia		No. subjects with:			Serum antibody response		
							Geometric mean serum neutralizing antibody titer‡ (range)	
	Detectable	Mean peak titer (log_{10}PFU/ml)	Fever ≥ 38°C	Systemic illness*	Rash	% Seroconversion†	Day 28	Day 42
6	No	<0.5	0	0	0	100	426 (128–955)	323 (84–1261)
14	Yes	1.6 ± 0.2	1	0	10	100	662 (74–2521)	450 (45–1261)

* Systemic illness is defined as ≥ 2 of the following symptoms lasting ≥ 2 days and associated with infection: headache, malaise, anorexia, myalgia/arthralgia, nausea/vomiting, or photophobia.
† Seroconversion is defined as a greater than 4-fold rise in serum neutralizing antibody titer by day 28, compared with the pre-vaccination titer, which was < 10 for each volunteer.
‡ Reciprocal titer. Geometric mean titer for all 20 volunteers: <10 (day 0), 580 (day 28), 407 (day 42).

was noted in two volunteers and lasted a maximum of 48 hr. During physical examination, 10 volunteers (50%) were noted to a have a very mild dengue-like erythematous macular rash (truncal distribution), which occurred with greatest frequency on day 10 (Table 3 and Figure 1B). None of the volunteers noted the rash themselves, and it was asymptomatic in each instance. Rash was seen only in vaccinees with detectable viremia. Volunteers did not develop systemic illness. Seven volunteers noted an occasional headache that was described as mild, lasting less than 2 hr, and was not present in any volunteer on two consecutive days. One volunteer reported fever of 38.6°C and 38.2°C without accompanying headache, chills, eye pain, photophobia, anorexia, myalgia, or arthralgia as an outpatient the evening of day 3 and day 5, respectively. However, this volunteer was afebrile when evaluated by the study staff on the morning of days 3, 4, 5, and 6. All other temperature measurements recorded by the volunteer or study staff were normal. Although tourniquet tests were not performed, two volunteers were noted to have petechiae at the site of the blood pressure cuff after a blood pressure measurement was performed (one on day 6, the other on days 7 and 10). Both of these volunteers had normal platelet counts at that time and throughout the study.

Significant hematologic abnormalities were not seen in any vaccinee. Three vaccinees with presumed benign ethnic neutropenia manifested an absolute neutrophil count (ANC) less than 1,500/mm^3. These three volunteers had baseline ANCs that were significantly lower than the remaining 17 volunteers and which did not decrease disproportionately relative to the other volunteers. Two of the three volunteers who became neutropenic never had detectable viremia. A mild increase in ALT levels was noted in four volunteers (Figure 2), and a more significant increase in ALT level (up to 238 IU/L) was noted in one volunteer. These ALT elevations were transient, were not associated with hepatomeg-

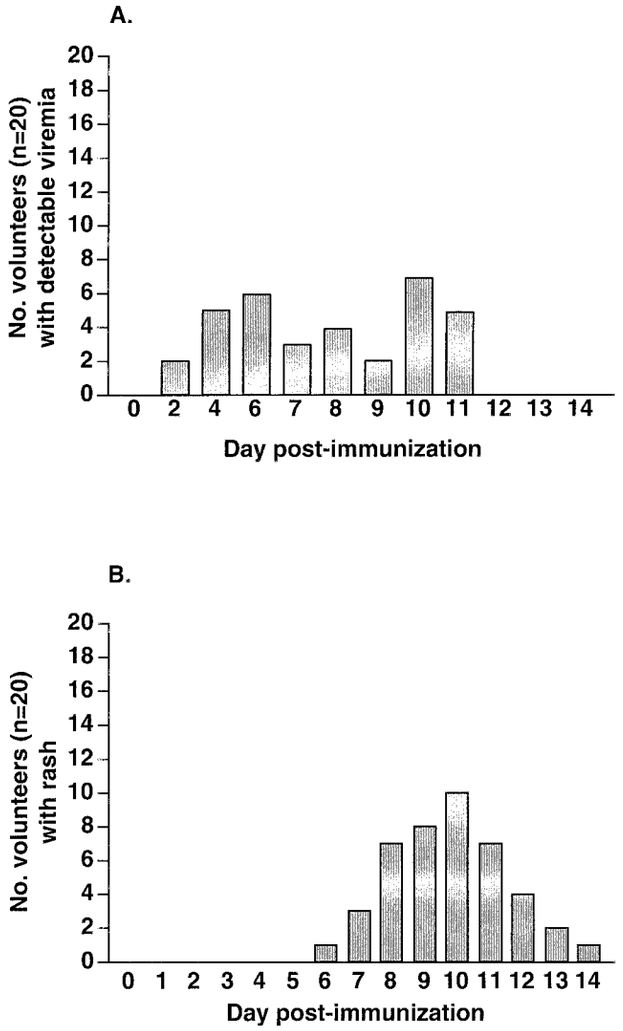

FIGURE 1. Occurrence of viremia (**A**) and rash (**B**) in 20 volunteers immunized with dengue virus type-4 (DEN-4) vaccine candidate 2AΔ30.

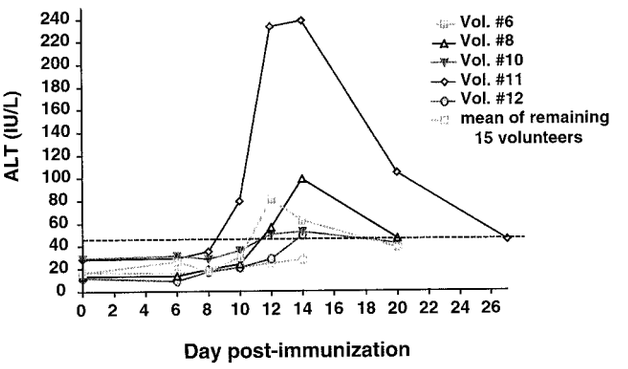

FIGURE 2. Serum alanine aminotransferase (ALT) levels of five volunteers who experienced elevation in their ALT level compared with the mean ALT level of the remaining 15 volunteers. The dotted horizontal line represents the upper limit of the normal ALT level.

aly, and were completely asymptomatic in each of the five volunteers. Elevated ALT values returned to normal by day 26 post-vaccination. The volunteer with the high ALT value was also noted to have an accompanying mild elevation in AST on day 14 (104 IU/L) which also returned to baseline by day 26 post-vaccination. This volunteer did not have an associated increase in levels of lactate dehydrogenase, bilirubin, or alkaline phosphatase.

Serologic response of humans to immunization with 2AΔ30. Each of the 20 vaccinees developed a significant increase in serum neutralizing antibody titer against DEN-4 by day 28 (Table 3). The level of serum neutralizing antibody was similar in viremic (1:662) and non-viremic vaccinees (1:426). The DEN-4 neutralizing antibody titers of both groups had not changed significantly by day 42 (Table 3).

Genetic stability of the Δ30 mutation. The RT-PCR and sequence analysis of viral RNA isolated from serum samples (n = 6) collected from volunteers 6–10 days post-vaccination confirmed the presence of the Δ30 mutation and neighboring deletion at nucleotide 10475.

DISCUSSION

The use of recombinant cDNA technology is greatly facilitating the development of live attenuated vaccine candidates for dengue viruses and for other flaviviruses.[20–23,25,26,31] One approach currently being pursued is the construction of antigenic chimeric viruses that possess the nonstructural genes of the attenuated yellow fever vaccine and the structural proteins of one of the four types of dengue virus.[25] Such chimeric viruses bear the genes for prM and E proteins of dengue virus and all other sequences from the yellow fever vaccine virus, including regions that possess some, but not all, of the attenuating mutations of the yellow fever vaccine virus.[26] A yellow fever-DEN-2 chimeric virus was attenuated, immunogenic, and protective in rhesus monkeys.[25] This approach is also being used to generate a candidate vaccine for Japanese encephalitis virus; however, satisfactory attenuation was not provided by the yellow fever virus vector alone.[31] Satisfactory attenuation required the presence of additional attenuating mutations in the E protein of Japanese encephalitis virus.[26] A second approach is to generate an antigenic chimeric dengue virus vaccine candidate (DEN-2/DEN-1) using an attenuated DEN-2 vaccine virus as a vector for the C-prM-E structural proteins of DEN-1.[22] The markers of attenuation of the DEN-2 vaccine candidate were shown not to be present in its C-prM-E structural protein,[32] and the DEN-2/DEN-1 chimeric virus possessed the *in vitro* phenotypes of the DEN-2 vaccine candidate. However, the attenuation phenotype of the DEN-2/DEN-1 chimeric virus in animals has not been reported. A third approach to the development of live attenuated vaccine candidate for dengue viruses is represented by the present study in which a novel attenuating deletion mutation (Δ30) was introduced into a wt DEN-4 virus, and the attenuating effect of this mutation was evaluated in rhesus monkeys and humans.

The Δ30 deletion mutation (along with the neighboring one nucleotide deletion) was found to be responsible for attenuation of the 2AΔ30 mutant virus for rhesus monkeys. Sequence analysis of wt DEN-4 and mutant viruses indicated that *in vitro* passage of virus gave rise to several incidental mutations. Therefore, it was not possible to immediately conclude that the attenuation phenotype of the 2AΔ30 deletion mutant virus was specified by the deletion itself since the mutant and wt virus differed at several other loci. The occurrence of a high frequency of such incidental mutations in dengue viruses passaged *in vitro* has been described previously.[33] To define the contribution of the Δ30 deletion mutation to the observed attenuation in rhesus monkeys, two sets of independently-derived wt DEN-4 and Δ30 deletion mutant viruses bearing different incidental mutations were made and evaluated for their level of replication and immunogenicity in rhesus monkeys. Each of the two mutants bearing the Δ30 deletion exhibited a similar degree of restriction of replication and decrease in immunogenicity compared with their respective wt DEN-4 parents, strongly suggesting that the mutations shared by these viruses, namely, the Δ30 mutation and its accompanying deletion mutation at nucleotide 10475, were most likely responsible for the attenuation of these viruses rather than their incidental sequence differences. The only missense mutation present in a structural gene is nucleotide 2440 (Val to Ala) in the E protein transmembrane hydrophobic region. It is unlikely that such a mutation would contribute to attenuation.

The DEN-4 vaccine candidate virus 2AΔ30 was well tolerated, safe, and immunogenic in human volunteers. The vaccine virus infected each of the 20 volunteers enrolled in the study and produced few clinical symptoms. None of the volunteers experienced symptoms that affected their daily activities or that required treatment. During the period of viremia, 50% of the volunteers developed an asymptomatic, transient rash. Interestingly, the rash was seen only in volunteers with detectable viremia. The appearance of such a rash is not unexpected, since rash has been reported in several studies that evaluated other live attenuated dengue vaccine candidates.[12,15,16,34,35] Following immunization with 2AΔ30, the level of viremia was low (between 0.5 and 2.3 $\log_{10}$ PFU/ml). Since we did not evaluate the virulence of the wt DEN-4 virus in humans, a direct determination of the relative attenuation level of 2AΔ30 is not possible. However, the observation that the vaccinees without detectable viremia failed to develop a rash yet developed a vigorous neutralizing antibody response suggests that this vaccine virus could be even further attenuated by the addition of other attenuating mutations if indicated.

The only laboratory abnormality detected in vaccinees was an asymptomatic, transient elevation of serum ALT values in five volunteers. This ALT elevation was mild in four of the five volunteers and moderate in one volunteer and was observed on days 12–14. Liver function abnormalities, particularly elevations in serum ALT and AST values, occur routinely in natural dengue infections.[36] Kuo and others noted that 82.2% of 270 dengue fever patients had elevations in serum ALT values, with 11.1% of these patients achieving ALT levels 10-fold higher than the upper limit of normal, consistent with acute hepatitis.[36] Elevations in ALT levels have also been observed in clinical trials of other candidate dengue vaccines.[14,16,35,37] In our study, elevations in ALT levels were not accompanied by hepatomegaly or any other sign or symptom and returned to normal in all affected volunteers by day 26 post-immunization. During natural dengue virus

infection of humans, dengue virus antigens are detected in hepatocytes, which most likely represent the source of elevated serum ALT values.[38,39] The low levels and low frequency of ALT elevations in the 2AΔ30 vaccinees compared with those observed during natural dengue virus infections indicate that the 2AΔ30 vaccine candidate is partially attenuated for the human liver. This observation provides the support for our decision to continue evaluation of this vaccine candidate in humans.

It has been reported that primary infection with DEN-4 results in little or only mild disease, suggesting that DEN-4 is naturally attenuated in comparison with other dengue virus types.[40] The relatively low level of replication of wt DEN-4 strain 814669 in monkeys is consistent with this suggestion. Although it is clear that the Δ30 mutation confers a reduction in duration and level of viremia in rhesus monkeys, an additional component of attenuation in monkeys and humans may be attributable to the natural attenuation of DEN-4. The relative contribution of each of these factors to the attenuation of 2AΔ30 remains undefined; however, such questions can be addressed experimentally in the future.

We are currently exploring the use of the 2AΔ30 virus derivative rDEN4Δ30 as an attenuated vector for the generation of antigenic chimeric viruses bearing the structural proteins (C, prM, and E) of DEN-1, DEN-2, or DEN-3. A tetravalent vaccine comprised of the three chimeric viruses and the rDEN4Δ30 vaccine candidate virus would provide protection against all four serotypes of dengue, which is essential for any dengue vaccine because of the need to reduce sensitization that might lead to DHF/DSS during subsequent infection. The attenuated rDEN4Δ30 virus appears to be an attractive vector for the generation of chimeric viruses for several reasons. First, the mutation responsible for attenuation is a 30-nucleotide deletion in the 3′-untranslated region, thus ensuring that all of the structural proteins expressed by the vaccine candidate virus and its antigenic chimeric derivatives are authentic wt proteins. The wt properties of the structural proteins of the dengue virus vaccine candidates should permit efficient infection of host cells and minimize differences in infectivity among components of a tetravalent vaccine. Large differences in the 50% human infectious dose (HID_{50}) of dengue virus vaccine candidates attenuated by passage in cell culture have been observed, and the vaccine candidates with authentic E proteins are the most infectious.[22] Second, deletion mutations, in general, are more stable than point mutations and reversion of the attenuation phenotype specified by the mutation is unlikely. Correction of attenuating deletion mutations has been observed *in vivo*,[41] but this occurred only late in the course of a persistent infection with a human immunodeficiency virus and is unlikely to occur during an acute infection that is typical of the dengue viruses. In this study, the 30-nucleotide deletion remained unaltered in vaccine virus detected up to 10 days post-vaccination. Third, this 30-nucleotide deletion would be common to each chimeric virus derived from the parent rDEN4Δ30. Therefore, due to the presence of identical viral replicative machinery, each chimeric virus serotype would be expected to have a similar attenuation phenotype as well as a similar level of replication. This should minimize interference between the chimeric virus serotypes when they are given as a tetravalent formulation, thereby providing uniform infectivity and immunogenicity. And fourth, since the rDEN4Δ30 vector is common to all the chimeric viruses of the tetravalent formulation, recombination between the vaccine serotypes would not lead to loss of the attenuating mutation or reversion to a wt phenotype. Recombination between components of the trivalent polio vaccine has been observed,[42] and naturally occurring recombinant dengue viruses have been described.[43]

In summary, the DEN-4 vaccine candidate virus 2AΔ30 was well tolerated and induced an acceptable neutralizing antibody response in each of the 20 volunteers in this initial Phase I clinical trial. The high degree of immunogenicity and very mild reactogenicity demonstrated in this trial is encouraging and warrants further evaluation of this vaccine candidate. Specifically, placebo-controlled, dose-ranging studies have been planned to determine the HID_{50} and to determine whether the mild clinical signs and mild laboratory abnormalities described are dose related.

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Authors' addresses: Anna P. Durbin, Ruth A. Karron, Mary J. Reynolds, John R. Perreault, and Bhavin Thumar, Johns Hopkins School of Public Health, Hampton House, 624 N. Broadway, Baltimore, MD 21205. Wellington Sun and David W. Vaughn, Department of Virus Diseases, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910. Ruhe Men, Ching-Juh Lai, William R. Elkins, Robert M. Chanock, Brian R. Murphy, and Stephen S. Whitehead, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 7, Room 100, 7 Center Drive, Bethesda, MD 20892.

Reprint requests: Stephen S. Whitehead, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 7, Room 100, 7 Center Drive, Bethesda, MD 20892.

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