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Recombinant, Live-Attenuated Tetravalent Dengue Virus Vaccine Formulations Induce a Balanced, Broad, and Protective Neutralizing Antibody Response against Each of the Four Serotypes in Rhesus Monkeys

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Three tetravalent vaccine (TV) formulations of previously described monovalent dengue (DEN) virus vaccine candidates were compared to a tetravalent formulation of wild-type DEN viruses (T-*wt*) for replication in SCID mice transplanted with human liver cells (SCID-HuH-7) or for replication and immunogenicity in rhesus monkeys. TV-1 consists of recombinant DEN1, -2, -3, and -4, each with a 30-nucleotide deletion in the 3′ untranslated region (Δ30). TV-2 consists of rDEN1Δ30, rDEN4Δ30, and two antigenic chimeric viruses, rDEN2/4Δ30 and rDEN3/4Δ30, both also bearing the Δ30 mutation. TV-3 consists of rDEN1Δ30, rDEN2Δ30, rDEN4Δ30, and a 10-fold higher dose of rDEN3/4Δ30. TV-1 and TV-2 were attenuated in SCID-HuH-7 mice with minimal interference in replication among the virus components. TV-1, -2, and -3 were attenuated in rhesus monkeys as measured by duration and peak of viremia. Each monkey immunized with TV-1 and TV-3 seroconverted to the four DEN components by day 28 with neutralization titers ranging from 1:52 to 1:273 and 1:59 to 1:144 for TV-1 and TV-3, respectively. TV-2 induced low antibody titers to DEN2 and DEN3, but a booster immunization after 4 months increased the neutralizing antibody titers to greater than 1:100 against each serotype and elicited broad neutralizing activity against 19 of 20 DEN subtypes. A single dose of TV-2 induced protection against wild-type DEN1, DEN3, and DEN4 challenge, but not DEN2. However, two doses of TV-2 or TV-3 induced protection against DEN2 challenge. Two tetravalent formulations, TV-2 and TV-3, possess properties of a successful DEN vaccine and can be considered for evaluation in clinical trials.

The mosquito-borne dengue (DEN) viruses, members of the *Flaviviridae* family, contain a single-stranded positive-sense RNA genome (36). A single polypeptide is cotranslationally processed by viral and cellular proteases generating three structural proteins (C, M, and E) and at least 7 nonstructural proteins. The genome organization of the DEN viruses is 5′ UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-UTR-3′ (UTR–untranslated region, C–capsid, prM–membrane precursor, E–envelope, NS–nonstructural). There are four DEN virus serotypes (DEN1, DEN2, DEN3, and DEN4) which circulate in tropical and subtropical regions of the world inhabited by more than 2.5 billion people (12). Annually, there are an estimated 50 to 100 million DEN virus infections and hundreds of thousands of cases of the more severe and potentially lethal DEN hemorrhagic fever/shock syndrome, with children bearing much of the disease burden (13). DEN viruses are endemic in at least 100 countries and cause more human disease than any other mosquito-borne virus. In at least eight Asian countries, the DEN viruses are a leading cause of hospitalization and death in children (45). Unfortunately, many countries affected by DEN viruses have very limited financial resources for healthcare, and the economic burden of DEN disease is considerable (1, 45). An economical vaccine that prevents disease caused by the DEN viruses is a global public health priority.

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The cost effectiveness, safety, long-term immunity, and efficacy associated with the live-attenuated vaccine against yellow fever virus, another mosquito-borne flavivirus, serves as a model for the feasibility of a live-attenuated DEN virus vaccine (31). However, the development of a live-attenuated DEN virus vaccine has been complicated by several factors. First, it has been difficult to develop monovalent vaccines against each of the four serotypes that exhibit a satisfactory balance between attenuation and immunogenicity (25, 26). Second, an effective live-attenuated DEN virus vaccine must consist of a tetravalent formulation of components representing each serotype because multiple serotypes typically cocirculate in a region, each DEN serotype is capable of causing disease, and the introduction of additional serotypes is common (18, 37, 42). In addition, the association of increased disease severity (DEN hemorrhagic fever/shock syndrome) in previously infected persons undergoing an infection by a different DEN virus serotype necessitates a vaccine that will confer long-term protection against all four serotypes (19). Third, it has been difficult to formulate a tetravalent vaccine (TV) with low reactogenicity that induces a broad neutralizing antibody response against each DEN virus serotype (16, 26, 39, 41). Fourth, a DEN vaccine must confer protection against a wide range of genetically diverse subtypes which are dispersed around the world and can be readily introduced into a new region by international travel (18, 37). Fifth, a DEN virus vaccine must be produced economically so that it can be made available to populations that need it most.

We have tried to address these issues as part of a program to generate a live-attenuated tetravalent DEN virus vaccine. To maximize the likelihood that suitable vaccine candidates would be identified, monovalent vaccine candidates for DEN1 to -4 were generated by two distinct recombinant methods and found to be attenuated and immunogenic in mouse and rhesus monkey models (2, 3, 9, 43, 44). In one method, deletion of 30 contiguous nucleotides from the 3′ UTR of wild-type cDNA clones of DEN1 to -4 was used to generate vaccine candidates. Specifically, the deletion of nucleotides 10478 to 10507 of the 3′ UTR (Δ30) of recombinant wild-type DEN4 yielded a vaccine candidate, rDEN4Δ30, which is safe, attenuated, and immunogenic in rhesus monkeys and humans (9). Incorporation of the Δ30 mutation into infectious cDNA clones of DEN1 and DEN2, but not DEN3, wild-type virus at a site homologous to that in DEN4 attenuated these viruses for rhesus monkeys (2, 3, 43). Using a second method, antigenic chimeric viruses were generated by replacing wild-type M and E structural genes of rDEN4Δ30 with those from DEN2 or DEN3, and the resulting chimeric viruses were attenuated and immunogenic in rhesus monkeys (2, 44). Importantly, these vaccine candidates retain wild-type structural proteins to maximize infectivity, thereby decreasing the potential for virus interference. In addition, immunity is induced by an authentic wild-type E protein that will likely increase the magnitude and breadth of the neutralizing antibody response.

We have also described a set of point mutations which may be used to further attenuate vaccine candidates if evaluation as a monovalent vaccine or a component of a tetravalent formulation demonstrates that further attenuation is required. Such mutations are capable of attenuating wild-type rDEN4 for suckling mice (4, 6, 20), for SCID mice transplanted with human liver cells (SCID-HuH-7) (5), for rhesus monkeys (22), or for mosquitoes (21). Since these mutations are in the nonstructural gene regions of DEN4, they can also be used to modify the attenuation phenotype of antigenic chimeric viruses with the DEN4 background. In addition, mutations identified in rDEN4 might be imported into conserved sites of cDNA clones for other DEN serotypes in an attempt to transfer desired phenotypes, as has been demonstrated for rDEN2Δ30 (3). Finally, to enhance replication in Vero cells and minimize the cost of manufacture, a panel of Vero cell adaptation mutations have been identified and incorporated into vaccine candidates (7). Thus, using the Δ30 mutation, intertypic chimerization, and the set of modifying point mutations, we have been able to generate and characterize attenuated DEN vaccine candidates for each serotype that can now be combined as tetravalent formulations.

In the present study, three TV formulations, TV-1, -2, and -3, of the aforementioned monovalent DEN virus vaccine candidates were compared to a tetravalent formulation of wild-type DEN viruses, T-*wt*, for replication in SCID-HuH-7 mice or for replication and immunogenicity in rhesus monkeys. Attenuation and virus interference were assessed in SCID-HuH-7 mice for TV-1, TV-2, and T-*wt*. In rhesus monkeys, attenuation, virus interference, and neutralizing antibody responses were evaluated for each formulation and compared to T-*wt*. In addition, the timing and effect of booster vaccinations were determined, and the breadth of the neutralizing antibody response was tested against divergent DEN subtypes. Analysis of these results and protection studies with rhesus monkeys indicates that two tetravalent formulations can be considered for evaluation in humans.

TABLE 1. Tetravalent formulations evaluated in SCID-HuH-7 mice and rhesus monkeys

Tetravalent formulation and virus	Reference	Dose administered to rhesus monkeys (PFU)	Antigenic chimeric vaccine?
T-*wt*			
DEN1 Nauru/74 (WP)	43	10^5	
DEN2 Tonga/74	3	10^5	
DEN3 Sleman/78	2	10^5	
DEN4 Dominica/81	9	10^5	
TV-1			
rDEN1Δ30	43	10^5	No
rDEN2Δ30	3	10^5	No
rDEN3Δ30	2	10^5	No
rDEN4Δ30	9	10^5	No
TV-2			
rDEN1Δ30	43	10^5	No
rDEN2/4Δ30(ME)	44	10^5	Yes
rDEN3/4Δ30(ME)	2	10^5	Yes
rDEN4Δ30	9	10^5	No
TV-3			
rDEN1Δ30	43	10^5	No
rDEN2Δ30	3	10^5	No
rDEN3/4Δ30(ME)	2	10^6	Yes
rDEN4Δ30	9	10^5	No

MATERIALS AND METHODS

Cells and viruses. Vero cells (African green monkey kidney) were propagated in OptiPro SFM (Invitrogen, Grand Island, NY) supplemented with 4 mM L-glutamine (Invitrogen). HuH-7 cells (human hepatoma) were maintained in D-MEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 0.05 mg/ml gentamicin (Invitrogen).

A tetravalent formulation of wild-type viruses, termed T-*wt*, and three TV formulations, termed TV-1, -2, and -3, were evaluated in this study. The components, dosage, and references describing the viruses used in these formulations are listed in Table 1.

Studies with SCID-HuH-7 mice. Four- to six-week-old SCID mice [Tac:Icr:Ha(ICR)-*Prkdc*^{scid}] (Taconic, Germantown, NY) were injected intraperitoneally with 10^7 HuH-7 human hepatoma cells suspended in 0.2 ml of phosphate-buffered saline as previously described (5). Tumors were detected in the peritoneum, and groups of mice were infected by direct inoculation of the tumor with 10^4 PFU of a monovalent virus or 10^4 PFU of each component of a tetravalent formulation (total of 4×10^4 PFU) in 0.05 ml of Opti-MEM (Invitrogen). On day 7 postinfection, serum was obtained from cardiac blood and stored at −70°C. Virus titer in serum samples was determined by plaque assay in Vero cells with detection by immunohistochemistry with a flavivirus-specific monoclonal antibody, as well as serotype-specific quantitative PCR (qPCR) as described below.

Serotype-specific qPCR. qPCR was used to measure the levels and identities of viruses in serum samples from SCID-HuH-7 mice and rhesus monkeys infected with monovalent or tetravalent virus preparations. Viral RNA was isolated from 0.04 ml of sample (serum from SCID-HuH-7 mice or tissue culture supernatant from Vero cell passage of virus present in monkey serum) using a Qiamp Viral RNA Mini Kit (QIAGEN, Valencia, CA). cDNA was prepared from viral RNA samples using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), and 0.005 ml was used as the template for a qPCR using the Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and fluorogenic Lux primers (Invitrogen). Reactions were amplified in an Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA).

For quantitation of DEN virus replication, standard curves for the concentration of viral genomes were generated using plasmid cDNA for each genome at 10-fold dilutions, representing a range of 1 ng (6.1×10^7 genome equivalents) to 10^{-6} ng (61 genome equivalents) of plasmid cDNA per reaction containing

0.0015 ml of original sample. The limit of detection was 61 genome equivalents per reaction and was equivalent to 4.6 $\log_{10}$ genome equivalents/ml of sample tested. Primers were designed to amplify regions of the M or E structural gene and were confirmed for the ability to distinguish between DEN1 WP, DEN2 NGC and Tonga/74, DEN3 Sleman/78, and DEN4 Dominica/81 (data not shown). To establish the specificity of each primer set, four qPCRs were performed using 1 ng (6.1×10^7 genome equivalents) of DEN1, -2, -3, and -4 plasmid cDNA per reaction. Primer sets detected the plasmid (serotype) from which they were designed and did not detect plasmids of other serotypes, with a limit of detection of 61 genome equivalents per reaction.

qPCR was used to quantitate the level of serotype-specific virus replication in SCID-HuH-7 mice. In monkeys, the peak virus titer in serum was at the lower limit of detection for the qPCR assay, so this assay was not useful to quantitate DEN virus for this species. However, the qPCR assay was used to estimate the relative level of virus replication for each component of the TV by identification of clonal populations of virus isolated from monkey serum by terminal dilution in Vero cells (see below).

Studies with rhesus monkeys. The tetravalent formulations were evaluated in rhesus macaques using established methods (9). DEN virus seronegative monkeys were injected subcutaneously with tetravalent formulations of viruses (Table 1) delivered in 1 ml of L-15 medium (Invitrogen) or with a mock inoculum. Serum was collected on days 0 to 6, 8, and 28 after inoculation and stored at −70°C. Virus titer was determined for each serum sample from day 0 to day 8 by plaque assay in Vero cells. Serum samples from days that were positive for virus replication in any monkey from a given group, as determined by plaque assay, were subjected to clonal virus isolation. For this isolation, 0.2 ml of serum was diluted into 2.5 ml of tissue culture medium and 0.025 ml was inoculated onto each well of a 96-well plate of Vero cells. Virus-positive wells were detected by immunohistochemistry, and supernatants were stored at −70°C. The serotype of each virus clone was identified using qPCR as described above.

Serum neutralizing antibody titer was determined for samples collected on days 0 and 28 by plaque reduction neutralization assay against DEN1 WP, DEN2 NGC, DEN3 Sleman/78, and DEN4 Dominica/81. The 60% plaque reduction neutralization titers ($PRNT_{60}$) are expressed as geometric means. In addition, the breadth of the neutralizing antibody titer against each DEN serotype was evaluated for selected sera using multiple members of each serotype.

The effect of booster immunization on the neutralizing antibody response was tested by immunizing groups of monkeys with TV-2 at day 0 and then boosting with the same formulation at 1 month or 4 months. Serum was collected on day 0 and at 1, 2, 3, 4, and 5 months postinoculation, and the neutralizing antibody titer against each serotype was determined for each serum sample. In an additional two-dose experiment, a group of monkeys were inoculated with TV-3 at day 0 and boosted at 4 months. Serum was collected at day 0 and at 4 and 5 months and tested in an individual plaque reduction neutralization assay against each DEN serotype.

Three efficacy studies were performed with rhesus monkeys. In the first study, groups of four monkeys inoculated with a single dose of TV-2 or with placebo were challenged on day 42 with DEN1 WP, DEN2 NGC, DEN3 Sleman/78, or DEN4 Dominica/81. Serum was collected on days 0 to 6 and 28 after challenge, and virus titer was determined for each serum sample from day 0 to day 6 by plaque assay with Vero cells. The serum antibody response following challenge was assessed by measuring the neutralizing antibody titer on the day before challenge and on day 28 postchallenge. In the second and third efficacy studies, groups of monkeys immunized with TV-2 or TV-3, respectively, and boosted at 4 months were challenged with DEN2 NGC at 5 months postimmunization. Serum was collected on days 0 to 8 after challenge, and the virus titer was determined by plaque assay in Vero cells. The antibody response following challenge was not tested in the latter two studies.

RESULTS

Tetravalent formulations evaluated in this study. Two TV formulations (TV-1 and TV-2) were initially compared to a tetravalent formulation of wild-type viruses (T-*wt*). Each of the six separate components of the two TV formulations has previously been evaluated for replication in SCID-HuH-7 mice and rhesus monkeys and for immunogenicity in rhesus monkeys (2, 3, 9, 43, 44). TV-1 consists of four viruses generated from full-length cDNA clones: rDEN1Δ30, rDEN2Δ30, rDEN3Δ30, and rDEN4Δ30 (Table 1). T-*wt* consists of the DEN virus isolates from which the full-length cDNA clones were derived: DEN1 Nauru/74 (Western Pacific), DEN2 Tonga/74, DEN3 Sleman/78, and DEN4 Dominica/81. TV-2 consists of rDEN1Δ30, rDEN4Δ30, and two antigenic chimeric viruses, rDEN2/4Δ30 and rDEN3/4Δ30. The M and E proteins of rDEN2/4Δ30 and rDEN3/4Δ30 are derived from DEN2 New Guinea/44 (NGC prototype) and DEN3 Sleman/78, respectively. A third TV was next formulated, consisting of 10^5 PFU each of rDEN1Δ30, rDEN2Δ30, and rDEN4Δ30 and 10^6 PFU of rDEN3/4Δ30, and studied in rhesus monkeys.

Replication levels of tetravalent formulations in SCID-HuH-7 mice. The SCID-HuH-7 mouse model has previously been used to evaluate the level of replication of DEN virus vaccine candidates administered as monovalent vaccines (2, 5, 22). Using this model, attenuated vaccine candidates have been identified, and attenuation phenotypes observed in SCID-HuH-7 mice have been confirmed in rhesus monkeys. Examination of the virus replication data derived from SCID-HuH-7 mice and rhesus monkeys reveals a significant correlation in the relative level of virus attenuation, making the SCID-HuH-7 mouse model an attractive tool for the evaluation of attenuated vaccine candidates administered as monovalent vaccines or as a tetravalent formulation. In addition, the absolute virus titers are much higher in the SCID-HuH-7 mice than in monkeys, which allows greater sensitivity in determining the magnitude of attenuation and the occurrence of interference in the replication of viruses administered as a tetravalent formulation. It should be noted that attenuated replication in either the SCID-HuH-7 or rhesus monkey model may not correctly predict virus attenuation in humans, although the rhesus model is believed to be the most reliable. As an initial evaluation of these parameters, tetravalent formulations TV-1, TV-2, and T-*wt* were assessed for replication in SCID-HuH-7 mice following inoculation with a dose of 10^4 PFU of each virus component. Relative growth of each serotype component in T-*wt* or TV tetravalent formulations was determined in SCID-HuH-7 mice by serotype-specific qPCR and was compared to that in mice administered the same amount of a monovalent virus.

Comparison of the level of viremia of each component in a monovalent versus a tetravalent formulation indicated that interference or enhancement in replication of any component in the tetravalent formulation had not occurred (Table 2). In the group inoculated with T-*wt*, the replication of individual components was equivalent or only slightly reduced compared to that observed for groups inoculated with a monovalent virus. The reduction in replication of DEN2 and DEN3 in mice receiving T-*wt* or the monovalent component was 0.7 $\log_{10}$ PFU/ml or 0.5 $\log_{10}$ PFU/ml, respectively, indicating that only a fivefold or smaller reduction in replication occurred for any of the four DEN wild-type viruses when inoculated together in the tetravalent formulation. This result demonstrates that enhancement or interference in replication among DEN viruses in multivalent formulations appears to be of low magnitude. It should be noted that a lack of virus interference in an animal model does not necessarily preclude interference in humans. Likewise, for both TV-1 and TV-2, the replication of individual vaccine components was equivalent or only minimally reduced compared to that observed for mice inoculated with monovalent viruses, and the largest decrease in replication was ob-

TABLE 2. TV-1 and -2 are attenuated in SCID-HuH-7 mice compared to a formulation of wild-type DEN viruses and do not show interference among individual components

Inoculum[a]	*n*	Virus titer (fold reduction) in serum determined by:				
		Plaque assay in Vero cells (log_{10} PFU/ml of serum)	Serotype-specific quantitative PCR (log_{10} genome equivalents/ml of serum)[b]			
			DEN1	DEN2	DEN3	DEN4
DEN1	9	8.0 ± 0.3	9.9 ± 0.3	ND[c]	ND	ND
DEN2	9	6.3 ± 0.3	ND	9.1 ± 0.3	ND	ND
DEN3	10	6.2 ± 0.3	ND	ND	8.7 ± 0.3	ND
DEN4	7	7.5 ± 0.3	ND	ND	ND	9.4 ± 0.3
T-*wt*	13	8.2 ± 0.2	9.9 ± 0.2	8.4 ± 0.2	8.2 ± 0.2	9.3 ± 0.2
rDEN1Δ30	7	5.1 ± 0.3	8.4 ± 0.2	ND	ND	ND
rDEN2Δ30	5	5.1 ± 0.2	ND	7.6 ± 0.2	ND	ND
rDEN3Δ30	8	5.5 ± 0.3	ND	ND	8.1 ± 0.3	ND
rDEN4Δ30	8	6.3 ± 0.3	ND	ND	ND	8.9 ± 0.3
TV-1	12	6.6 ± 0.2 (1.6)[d]	7.9 ± 0.3 (2.0)[e]	7.0 ± 0.3 (2.1)[e]	8.4 ± 0.4 (0.3)[e]	8.4 ± 0.4 (1.0)[e]
rDEN1Δ30	7	5.5 ± 0.2	8.2 ± 0.2	ND	ND	ND
rDEN2/4Δ30	12	4.9 ± 0.3	ND	8.2 ± 0.3	ND	ND
rDEN3/4Δ30	9	5.4 ± 0.2	ND	ND	8.0 ± 0.1	ND
rDEN4Δ30	7	6.1 ± 0.4	ND	ND	ND	8.8 ± 0.3
TV-2	11	6.2 ± 0.3 (2.0)[d]	8.0 ± 0.3 (1.9)[e]	8.3 ± 0.3 (0.8)[e]	7.7 ± 0.3 (1.0)[e]	8.4 ± 0.2 (1.0)[e]

[a] SCID-HuH-7 mice were inoculated with an individual component virus (10^4 PFU) or a tetravalent formulation (10^4 PFU of each virus), and serum was collected on day 7.
[b] Viral RNA was isolated from serum and used in a qPCR assay with serotype-specific primers as described in Materials and Methods.
[c] ND, not detected. The limit of detection was <4.6 log_{10} genome equivalents/ml of serum.
[d] Reduction in mean virus titer (log_{10} PFU/ml of serum) of TV formulation compared to that of the T-*wt* formulation.
[e] Reduction in genome equivalents of each component of the TV formulation compared to that of the respective wild-type component of the T-*wt* formulation.

served for rDEN2Δ30 in TV-1 (0.6 log_{10} PFU/ml reduction). Thus, a reduction in the level of replication of an attenuated virus in a TV formulation versus the wild-type virus from which it was derived can be largely ascribed to the attenuating mutations in the virus and not to interference among the four components of the TV.

The overall level of attenuation of the set of four viruses in TV-1 and TV-2 versus the four viruses in T-*wt* was first assessed by comparing the virus titer in each group of SCID-HuH-7 mice by plaque assay in Vero cells. The total virus titer of T-*wt* was 8.2 log_{10} PFU/ml, while TV-1 (6.6 log_{10} PFU/ml) and TV-2 (6.2 log_{10} PFU/ml) were 40-fold and 100-fold reduced, respectively. Thus, the total level of virus replication for each of the two TV formulations was less than that of the wild-type virus, indicating that TV-1 and TV-2 were attenuated compared to T-*wt*.

Attenuation of the four individual viruses in TV-1 and TV-2 was assessed by quantitation of their replication by serotype-specific qPCR. In Table 2, the reduction in virus titer is indicated for each vaccine component of TV-1 and TV-2 compared to that of the corresponding wild-type monovalent group. For example, wild-type DEN2 replicated to 9.1 log_{10} genome equivalents/ml of serum as a monovalent inoculation while rDEN2Δ30 replicated to 7.0 log_{10} genome equivalents/ml of serum in TV-1 (a 2.1 log_{10} reduction). For TV-1, the rDEN1Δ30 and rDEN2Δ30 components were 100-fold restricted, rDEN4Δ30 was 10-fold restricted, but rDEN3Δ30 was not restricted compared to the value of the corresponding wild-type monovalent group. The rDEN3Δ30 virus, which was previously found to not be attenuated in SCID-HuH-7 mice (2), was examined here to see if its behavior as a monovalent vaccine would also be observed when administered as a tetravalent formulation. These results confirm the lack of attenuation of rDEN3Δ30 and the inability of a tetravalent formulation to largely affect the replication of an individual component. For TV-2, the rDEN1Δ30 component was nearly 100-fold restricted, and rDEN2/4Δ30, rDEN3/4Δ30, and rDEN4Δ30 were each approximately 10-fold restricted compared to the value of the corresponding wild-type monovalent group.

Comparison of replication and immunogenicity of TV-1 and TV-2 to the T-*wt* formulation in rhesus monkeys. The overall level of attenuation of the set of four viruses in TV-1 and TV-2 compared to that of the four viruses in T-*wt* was next assessed by evaluation of the total virus titer in rhesus monkeys immunized with a tetravalent formulation (Table 3). Monkeys inoculated with T-*wt* developed a mean peak virus titer of 2.0 log_{10} PFU/ml and a mean duration of viremia of 5.8 days. Both TV-1 and TV-2 had reduced replication as measured by mean peak virus titer and mean duration of viremia. Despite the lack of attenuation of rDEN3Δ30 when administered as a monovalent vaccine in monkeys and compared to wild-type rDEN3 (2), its inclusion in the TV-1 formulation resulted in an acceptable level of replication in rhesus monkeys. This may possibly indicate a differing level of interference observed in virus replication in SCID-HuH-7 mice versus rhesus monkeys since no interference was seen in mice.

The relative level of replication of each virus component in serum samples from rhesus monkeys infected with T-*wt*, TV-1, and TV-2 was determined. Since the virus titers that are achieved in rhesus monkeys by attenuated DEN viruses are near the limit of detection for qPCR (approximately 1.0 log_{10} PFU/ml or <4.6 log_{10} genome equivalents/ml of serum), this method was not used to quantitate virus replication. Instead, the relative level of replication of each of the four virus components was estimated by determining the proportion of each virus in the serum of monkeys administered a tetravalent formulation. Monkey serum containing virus was diluted and

TABLE 3. Replication and immunogenicity in rhesus monkeys following a single dose of T-*wt* or TV-1, -2, or -3

Tetravalent formulation (n)[a]	Virus replication determined by plaque assay in Vero cells		Relative level of replication of each component of tetravalent formulation[b]					Geometric mean serum neutralizing antibody titer on day 28 (% seroconversion)[d]			
	Mean no. of viremic days/monkey	Mean peak virus titer (log_{10} PFU/ml of serum)	Total no. of virus clones analyzed (days)[c]	% of each serotype among virus isolates (% of monkeys with at least one clone of indicated virus)							
				DEN1	DEN2	DEN3	DEN4	DEN1	DEN2	DEN3	DEN4
T-*wt* (4)	5.8	2.0 ± 0.2	155 (1–8)	69 (100)	12 (100)	8 (75)	11 (100)	289 (100)	113 (100)	243 (100)	241 (100)
TV-1 (6)	1.5	1.2 ± 0.1	44 (1–4)	5 (33)	5 (33)	56 (83)	34 (50)	52 (100)	67 (100)	273 (100)	79 (100)
TV-2 (16)	1.3	1.3 ± 0.1	125 (1–3)	9 (50)	7 (50)	19 (56)	65 (94)	57 (81)	16 (13)	18 (19)	126 (100)
TV-3 (6)	1.5	1.4 ± 0.1	214 (1–4)	12 (100)	9 (100)	11 (83)	68 (100)	59 (100)	117 (100)	91 (100)	144 (100)

[a] Groups of rhesus monkeys were inoculated subcutaneously with each formulation in a 1-ml dose. Serum was collected for days 0 to 6, 8, and 28. n = number of monkeys per group.

[b] Virus clones were isolated in Vero cells by terminal dilution of serum shown by plaque assay to contain virus. RNA was isolated from each virus clone and reverse transcribed, and the serotype was determined in a qPCR assay with serotype-specific primers as described in Materials and Methods.

[c] Virus clones were isolated from daily serum samples from days on which virus was detectable by plaque assay.

[d] Plaque reduction (60%) neutralizing antibody titers (reciprocal dilution) were determined using DEN1 Western Pacific, DEN2 New Guinea C prototype, DEN3-Sleman/78, and DEN4 Dominica/81. Percent seroconversion was defined as the percentage of monkeys with a fourfold or greater increase in neutralizing antibody titer after immunization.

used to infect Vero cells, and clonal populations of virus were isolated. The serotype of each virus clone was determined by qPCR. Of 498 culture supernatants yielding virus, only 40 contained more than one DEN serotype, indicating that our clonal selection procedure was largely successful. The percentage of each serotype represented among the total number of virus clones was calculated and is presented in Table 3. For T-*wt*, TV-1, and TV-2, replication of each serotype component was detected although at various levels and frequencies among inoculated monkeys. Replication of each serotype was detected in T-*wt*-inoculated monkeys, with the exception of one monkey that had no detectable DEN3 replication. DEN1 virus accounted for 69% of virus clones isolated from serum samples of monkeys infected with T-*wt*, while DEN2, -3, and -4 each accounted for about 10% of the virus clones. Thus, for replication of the wild-type virus mixture, DEN1 was the predominant component.

Replication in monkeys immunized with TV-1 was dominated by rDEN3Δ30 and rDEN4Δ30 with 56% and 34% of the virus clones, respectively. The high prevalence of rDEN3Δ30 was anticipated based on its low level of attenuation when administered as a monovalent vaccine to rhesus monkeys. In monkeys administered TV-2, rDEN4Δ30 (65%) and rDEN3/4Δ30 (19%) were the predominant viruses, with each of the viruses being detected. rDEN4Δ30 replication was found in almost 100% of the monkeys, while the other three components were found in approximately 50% of the monkeys. Although relative levels of replication did vary widely among vaccine components, evidence for replication of each tetravalent component exists, supporting the likelihood that immunity to each component would develop. For both TV-1 and TV-2, replication was attenuated compared to T-*wt*, and importantly, replication of TV-2 was dominated by rDEN4Δ30, which is already known to be safe in humans (9, 10).

The immunogenicity of T-*wt*, TV-1, and TV-2 was assessed by determination of serotype-specific neutralizing antibody titers in postinfection serum (Table 3). The geometric mean neutralizing antibody titers induced by inoculation with T-*wt* were greater than 1:225 against DEN1, DEN3, and DEN4, whereas for DEN2 the mean titer was 1:113. These values were similar to those observed by a monovalent inoculation of these viruses (2, 22, 43, 44), and all monkeys seroconverted to each serotype (defined as a fourfold or greater increase in antibody titer). The antibody response elicited by inoculation with T-*wt* was evenly distributed against the four wild-type viruses despite the dominant replication of DEN1 observed by clonal analysis and qPCR (Table 3).

Not surprisingly, the neutralizing antibody titers induced by TV-1 on day 28 were highest to DEN3 (1:273) and ranged from 1:52 to 1:79 for DEN1, -2, and -4. Although each of the six monkeys infected with TV-1 seroconverted to each serotype, further study of TV-1 was curtailed because three lines of evidence suggested that the rDEN3Δ30 component in it was under attenuated. Specifically, the rDEN3Δ30 component was (i) underattenuated as a monovalent vaccine (2), (ii) the dominant component of TV-1 in terms of level of replication (Table 3), and (iii) equally as immunogenic as wild-type DEN3 (Table 3).

The neutralizing antibody response on day 28 induced by TV-2 was the most unbalanced of any formulation tested. Seroconversion to DEN1 and DEN4 was 81% and 100%, respectively, with geometric mean antibody titers of 1:57 for DEN1 and 1:126 for DEN4. However, seroconversion was below 15% to DEN2 or DEN3, with geometric mean titers below 1:20. In this case, immunization with the two chimeric viruses, rDEN2/4Δ30 and rDEN3/4Δ30, as part of the TV-2 tetravalent formulation resulted in significantly lower antibody induction compared to administration of the chimeric viruses as monovalent vaccines (2, 44).

Protection from DEN1, -3, and -4 challenges after a single dose of TV-2 while protection from DEN2 challenge requires two doses. The ability of a single immunization with TV-2 to confer protection against a wild-type virus challenge was evaluated. Sixteen monkeys immunized with TV-2 (described in Table 3) were divided into groups of four and, along with groups of two mock-inoculated monkeys, challenged with DEN1 WP, DEN2 NGC prototype, DEN3 Sleman/78, or DEN4 Dominica/81 on day 42 after immunization (Table 4). All mock-immunized monkeys developed viremia following challenge at a duration and level expected for wild-type infection. While immunized monkeys challenged with DEN1, DEN3, and DEN4 were completely protected, as indicated by

TABLE 4. Viremia and neutralizing antibody levels in TV-2-immunized monkeys following challenge

Immunizing virus (day 0)	Challenge virus[a] (day 42)	% of monkeys with viremia after challenge	Mean peak virus titer (log_{10} PFU/ml of serum)	Immunization (day 28) and postchallenge (day 70) geometric mean serum neutralizing antibody titer (reciprocal dilution)[b] against:							
				DEN1		DEN2		DEN3		DEN4	
				Day 28	Day 70	Day 28	Day 70	Day 28	Day 70	Day 28	Day 70
TV-2	DEN1	0	<1.0	55	352[c]	12	15	25	101[e]	133	167
None (mock infection)	DEN1	100	2.1 ± 0.3	<10	324[d]	<10	16	<10	27	<10	24
TV-2	DEN2	75	2.0 ± 0.5	51	65	16	304[d]	15	92[e]	93	160
None (mock infection)	DEN2	100	1.9 ± 0.4	<10	22	<10	477[d]	<10	48	<10	49
TV-2	DEN3	0	<1.0	62	50	19	28	15	252[d]	142	247
None (mock infection)	DEN3	100	2.4 ± 0.4	<10	29	<10	10	<10	244[d]	<10	28
TV-2	DEN4	0	<1.0	63	27	21	99[c]	16	28	142	286
None (mock infection)	DEN4	100	2.6 ± 0.4	<10	11	<10	29	<10	19	<10	311[d]

[a] Sixteen rhesus monkeys previously inoculated with TV-2 (Table 2) were divided into four groups of four monkeys, and two mock-immunized monkeys were tested per group. Monkeys were challenged on day 42, and serum was collected on days 0 to 6 postchallenge and analyzed by plaque assay in Vero cells. For neutralization assay, postchallenge serum was collected on day 70 (28 days following virus challenge).
[b] Plaque reduction (60%) neutralizing antibody titers were determined using the challenge strains in footnote *d* of Table 3.
[c] Seventy-five percent of rhesus monkeys had a four-fold or greater increase in antibody titer after challenge.
[d] One hundred percent of rhesus monkeys had a four-fold or greater increase in antibody titer after challenge.
[e] Fifty percent of rhesus monkeys had a four-fold or greater increase in antibody titer after challenge.

the lack of viremia following challenge, three of four monkeys became viremic after a challenge with DEN2. The monkey which developed no viremia did not have a significantly higher neutralizing antibody titer compared to the geometric mean. As a monovalent vaccine, rDEN2/4Δ30 was protective, indicating that the lack of protection likely resulted from its formulation in a TV (44). After a single dose of TV-2, the antibody responses to DEN2 and DEN3 on day 28 were at similarly low levels. However, monkeys were completely protected against a DEN3 challenge, unlike that observed with DEN2, for undefined reasons.

The neutralizing antibody levels against each serotype in TV-2- and mock-immunized monkeys were evaluated before and after challenge with each wild-type DEN virus (Table 4). Animals challenged with DEN1 or DEN3 virus developed an enhanced antibody response to the challenge virus, suggesting that reinfection had occurred with these two DEN serotypes despite an absence of viremia. As expected, monkeys challenged with DEN2 had a rise in titer and serological responses. Monkeys immunized with TV-2 had low rises in titer to DEN4 following a challenge with this virus, perhaps reflecting the high prechallenge antibody titers. Interestingly, after a challenge, there were three examples of increased antibody titers against a nonhomologous DEN serotype among TV-2-immunized monkeys (Table 4). For example monkeys challenged with DEN1 or DEN2 had a greater than fourfold increase in postchallenge neutralization titer against DEN3. In addition, after a DEN4 challenge, the mean titer against DEN2 increased from 1:21 to 1:99. The duration of this apparent cross-reactive antibody response is unknown.

The serotype specificity of the antibody responses was also characterized in this study by evaluating the antibody titers against each serotype following challenge of the mock-inoculated control monkeys. Only infection with DEN2 NGC resulted in a fourfold or greater increase in antibody to a nonhomologous serotype (1:48 against DEN3 and 1:49 against DEN4). However, these cross-reactive responses were 10-fold lower than the titer against the homologous serotype (1:477 against DEN2). Infection with DEN1, DEN3, or DEN4 did not induce a 4-fold increase in geometric mean titer against any nonhomologous serotype, and any increase in titer was approximately 10-fold less than that observed against the homologous serotype. These results indicate that the considerably lower neutralization antibody titers induced by the vaccine viruses in our studies would unlikely induce detectable cross-reactive antibodies. It is reasonable to conclude that the neutralization titers measured here are predominantly serotype specific.

To overcome the suboptimal immunogenicity of the DEN2 and DEN3 components of TV-2 following a single dose, a second dose was given and the effect of timing of administration of the second dose on immunogenicity was explored. TV-2 was administered to two groups of four monkeys, with a second dose delivered after 1 month or 4 months. Serum samples were collected monthly for 5 months and evaluated to determine the effect of the timing of the booster dose on the magnitude of the neutralizing antibody response (Fig. 1). The antibody titers to DEN1, DEN3, and DEN4 at day 28 were similar to those previously observed (Table 3), while the response to DEN2 was approximately threefold higher for unexplained reasons. Following booster immunization at 1 month, the antibody titer did not increase against any of the four serotypes. Importantly, over the next 4 months, the level of antibody response was maintained and did not decrease. In contrast, a booster immunization at 4 months increased the mean neutralizing antibody titer against each serotype. Fifty percent and 100% of the monkeys had a fourfold or greater increase in antibody titer against DEN2 and DEN3, respectively. While no monkey had a fourfold or greater increase in antibody titer against DEN1 or DEN4 after a 4-month booster immunization, 50% of monkeys had a twofold or greater increase in antibody against DEN1 or DEN4. Importantly, the unacceptably low response to DEN3 was increased to a geometric mean titer of 1:148.

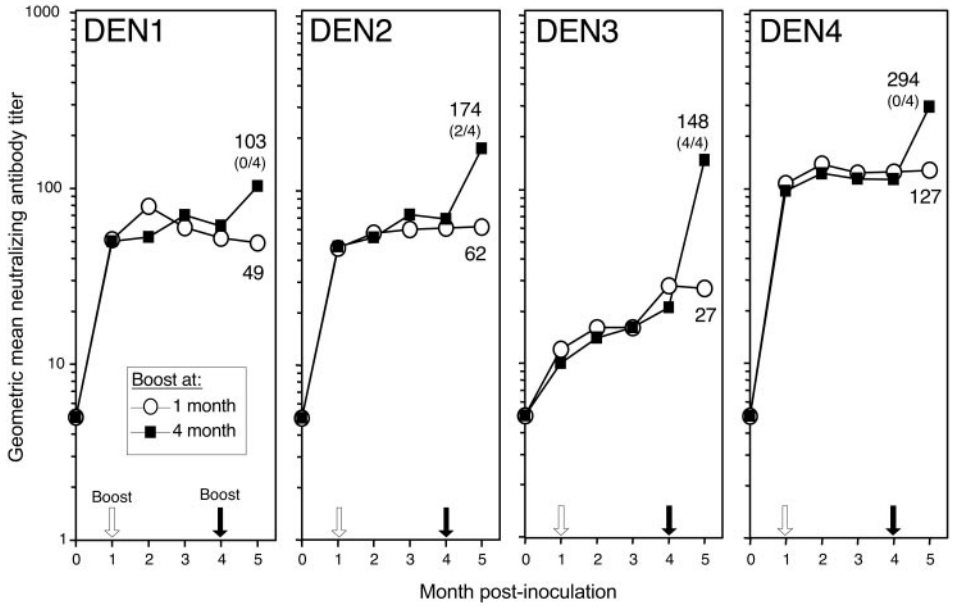

FIG. 1. Serum neutralizing antibody response to TV-2 given in two doses at either 1 or 4 months. Eight rhesus monkeys were inoculated with TV-2 and boosted at 1 month ($n = 4$) or 4 months ($n = 4$). Serum was collected on day 0 and at 1, 2, 3, 4, and 5 months postinoculation, and all samples were tested in four individual plaque reduction neutralization assays against a single DEN serotype (DEN1 WP, DEN2 NGC, DEN3 Sleman/78, and DEN4 Dominica/81). The reciprocal dilution of the geometric mean of the $PRNT_{60}$ for each group is shown. For the group of monkeys that received the second dose of vaccine at 4 months, the titer and ratio of animals with a fourfold further increase in titer above the titer from the 4-month serum sample (in parentheses) are presented. None of the animals receiving a second immunization at 1 month had a further fourfold rise in titer; however, their antibody titers were maintained near peak levels over the subsequent 4-month period of observation.

Thus, a TV administered in two doses at a 4-month interval, but not at a 1-month interval, induced antibody titers in rhesus monkeys that were >1:100 to each serotype.

Since the single immunization with TV-2 protected against DEN1, -3, and -4 challenges but not a DEN2 challenge, the ability of a two-dose (0 and 4 months) immunization with TV-2 to confer protection against DEN2 challenge was assessed (Table 5). One month after booster immunization, mock-immunized and immunized monkeys were challenged. All mock-inoculated control animals developed viremia, while monkeys immunized and boosted with TV-2 were completely protected against DEN2, as indicated by the lack of detectable viremia.

Replication, immunogenicity, and protection induced by a new vaccine formulation, TV-3, in rhesus monkeys. Based on results indicating that (i) rDEN3Δ30 was underattenuated in rhesus monkeys as a monovalent vaccine and a dominant component in terms of replication in TV-1 (Table 3), (ii) rDEN2/4Δ30 and rDEN3/4Δ30 were weakly immunogenic after one dose of TV-2 (Table 3), and (iii) the lack of protection against DEN2 after a single dose of TV-2 (Table 4), a third TV formulation was tested in rhesus monkeys. TV-3 consists of 10^5 PFU each of rDEN1Δ30, rDEN2Δ30, and rDEN4Δ30 with 10^6 PFU of the chimeric virus rDEN3/4Δ30 (Table 1). In monovalent studies, rDEN3/4Δ30 was found to be considerably more attenuated than rDEN3Δ30 and less immunogenic than rDEN3Δ30, so a 10-fold higher dose of this component was administered (2). Monkeys inoculated with TV-3 developed a mean peak virus titer of 1.4 $\log_{10}$ PFU/ml and a mean duration of viremia of 1.5 days, which was similar to that of both TV-1 and TV-2 and reduced from that of T-*wt* (Table 3). Replication of TV-3 was dominated by the rDEN4Δ30 virus, accounting for 68% of the virus clones, despite administration of the 10-fold higher dose of rDEN3/4Δ30. Evidence for replication of each serotype was detected in

TABLE 5. Two doses of TV-2 or TV-3 induce protection from DEN2 virus challenge

Immunization		No. of monkeys	% of monkeys with detectable viremia after DEN2 challenge[a]	Mean peak virus titer ($\log_{10}$ PFU/ml of serum)
Dose 1	Dose 2			
TV-2[b]	TV-2	4	0	<1.0
None (mock infection)	None (mock infection)	2	100	2.4 ± 0.3
TV-3[c]	TV-3	6	0	<1.0
None (mock infection)	None (mock infection)	2	100	2.5 ± 0.4

[a] Rhesus monkeys were challenged with 10^5 PFU of DEN2 NGC prototype. Serum was collected for days 0 to 6, 8, and 10, and virus was detected by plaque assay in Vero cells. The limit of detection was 1.0 $\log_{10}$ PFU/ml.
[b] Four monkeys were inoculated with TV-2, boosted at 4 months (Fig. 1), and challenged at 5 months.
[c] Six monkeys were inoculated with TV-3, boosted at 4 months (Fig. 2), and challenged at 5 months.

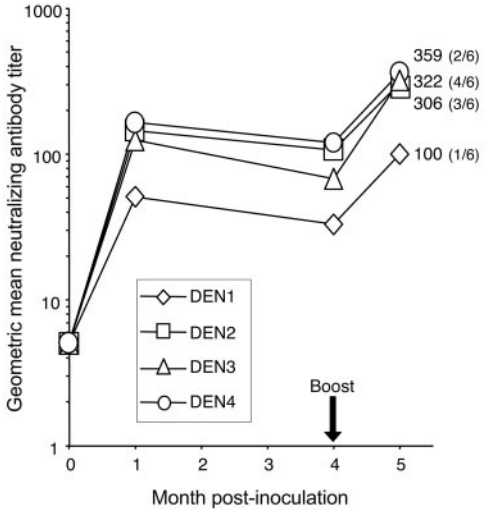

FIG. 2. Effect of 4-month boost with TV-3 on neutralizing antibody response. Six rhesus monkeys were inoculated with TV-3 and boosted after 4 months. Serum was collected on day 0 and at 1, 4, and 5 months postinoculation, and all samples were tested in a plaque reduction neutralization assay against DEN1 WP, DEN2 NGC, DEN3 Sleman/78, and DEN4 Dominica/81. The reciprocal dilution of the geometric mean of the $PRNT_{60}$ against each serotype is shown. Numerical values are indicated for neutralization titers achieved 1 month after booster immunization, and the ratio of animals with a fourfold further increase in titer above the titer from the 4-month serum sample are presented (in parentheses).

each monkey, with the exception of one monkey which had no detectable rDEN3/4Δ30 replication.

Immunization with a single dose of TV-3 resulted in the most balanced neutralizing antibody response against the four DEN serotypes with 100% seroconversion (Table 3). There was less than threefold variability of geometric mean titers, ranging from 1:59 to 1:144 against DEN1 and DEN4, respectively. Based on the efficacy of the 4-month boost of TV-2, a booster immunization at 4 months was also evaluated for monkeys inoculated with TV-3. In Fig. 2, the booster immunization was found to significantly increase geometric mean titers to 1:100 for DEN1 and >1:300 for DEN2, DEN3, and DEN4. In addition, stability of the antibody response was demonstrated by the maintenance of consistent antibody levels from 1 month to 4 months prior to the second dose. Taken together with the results of the TV-2 booster immunizations, these results indicate the effectiveness of a booster immunization given at 4 months.

The antibody levels induced by a single dose of TV-3 against each serotype have been associated with protection from a wild-type virus challenge in this study (Table 3 and 4) or previous studies including a DEN2 challenge (44). Nevertheless, since TV-2 required two doses to confer protection against DEN2 NGC the ability of two doses of TV-3 to confer protective immunity to DEN2 was assessed (Table 5). One month after booster immunization, mock-treated and immunized monkeys were challenged with DEN2. All mock-inoculated control animals developed viremia, while monkeys immunized and boosted with TV-3 were completely protected. These results indicate that two doses of TV-2 or TV-3 should confer protection from each serotype.

Neutralizing antibody responses induced by two doses of TV-2 are cross-reactive to divergent DEN subtypes. The neutralizing antibody response against genetically, temporally, and geographically diverse DEN1 to -4 subtypes was next evaluated. Broad neutralizing antibody activity against the diversity of circulating DEN viruses is essential for protection from disease. The postboost serum samples of the group of rhesus monkeys boosted at 4 months with TV-2 (Fig. 1) and the day 28 serum samples from monkeys immunized with a single dose of T-*wt* (Table 3) were tested in a neutralization assay against four virus strains from each serotype described in Table 6 and the parent virus for each vaccine candidate. The E protein is the major protective antigen of the DEN viruses, and the amino acid divergence of the E protein for these selected viruses reflects the range described for the members of DEN virus serotypes in previous epidemiological studies (27, 28, 37).

For monkeys immunized with T-*wt* or TV-2 (4-month boost), seroconversion (fourfold increase in antibody titer) was observed for all monkeys against each DEN1 and DEN4 subtype (Fig. 3). One monkey immunized with a single dose of T-*wt* failed to seroconvert to two DEN2 subtypes, Taiwan/87 and Sri Lanka/90, but all monkeys immunized with TV-2 seroconverted to each DEN2 subtype. Neutralizing antibody levels against the DEN1, DEN2, and DEN4 subtypes induced by two doses of TV-2 were similar or in some cases greater than those induced by a single dose of T-*wt*, indicating that the breadth and level of antibodies induced by the vaccine candidate were similar after two doses to those induced by wild-type infection.

TABLE 6. Divergent DEN virus strains used to test breadth of neutralizing antibody response induced by immunization with TV-2 formulation

Serotype (% relatedness of E protein for 5 strains) and virus	Genotype	% relatedness of E protein vs TV-2 E	Accession no.	Reference
DEN1 (93)				
Western Pacific	4	100	AY145121	43
Thailand 16007	2	97	AF180817	23
Jamaica/77	5	97	D00501	8
Peru IQT-6152	5	97	AY780643	This study
Puerto Rico/94	5	96	AY780642	This study
DEN2 (93)				
NGC prototype	1[a]	100	AY243467	44
Taiwan/87	2[a]	96	L10052	29
Sri Lanka/90	4	95	L10049	29
Tonga/74	5[b]	95	AY744147	3
Trinidad/53	5[b]	95	L10053	29
DEN3 (94)				
Sleman/78	1	100	AY648961	2
Fiji/92	1	99	L11422	28
Thailand/87	2	97	L11442	28
Sri Lanka/91	3	96	L11438	28
Puerto Rico/77	4	96	L11434	28
DEN4 (96)				
Dominica/81	2	100	AF326573	9
Puerto Rico/86	2	99	U18436	27
Indonesia/73	2	98	U18428	27
Thailand/85	1	97	AY780644	This study
Philippines/84	1	97	U18435	27

[a] Member of the Asian genotype.
[b] Member of the American genotype.

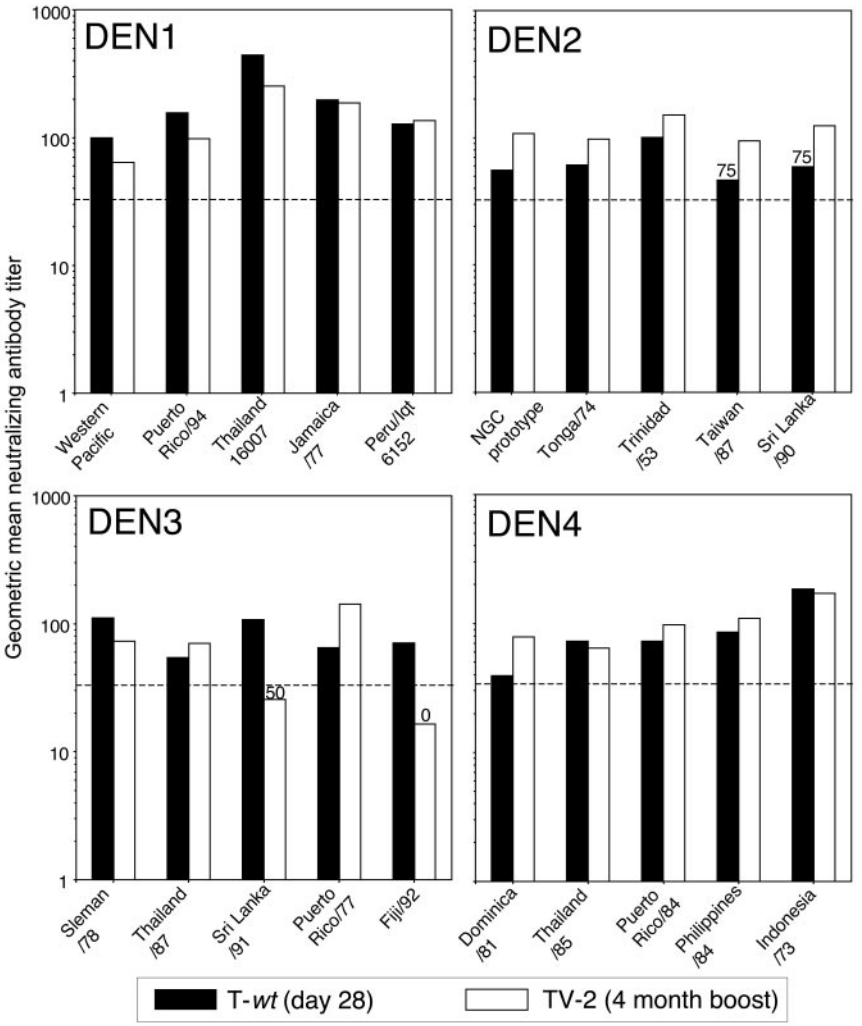

FIG. 3. Two doses of an attenuated tetravalent DEN virus vaccine (TV-2) induce a broadly reactive serum neutralizing antibody response at levels comparable to that of a single dose of tetravalent wild-type virus (T-*wt*). The 5-month serum samples of the four rhesus monkeys boosted at 4 months with TV-2 (Fig. 1) and the 1-month serum samples from the four monkeys immunized with a single dose of T-*wt* (Table 3) were evaluated in individual plaque reduction neutralization assays against five DEN subtypes from each serotype. Viruses are described in Table 6. The reciprocal dilution of the geometric mean of the $PRNT_{60}$ for each group is shown. The dashed line indicates a fourfold increase in neutralizing antibody titer (1:32) above the limit of detection (1:8). Percent seroconversion (percentage of monkeys with greater than fourfold increase in neutralizing antibody titer) was 100% unless indicated numerically above individual bars.

The antibody response against the DEN3 subtypes induced by two doses of TV-2 was not as broadly neutralizing compared to that induced by T-*wt*. Each monkey inoculated with T-*wt* seroconverted to each DEN3 virus, and all monkeys immunized with TV-2 seroconverted against DEN3 Sleman/78, Thailand/87, and Puerto Rico/77. However, only 50% of the monkeys immunized with TV-2 seroconverted against DEN3 Sri Lanka/91 and no monkeys seroconverted against DEN3 Fiji/92. Nevertheless, the neutralization activity against 19 of 20 DEN subtypes tested demonstrates a strong breadth of antibody response induced by the TV-2 formulation.

DISCUSSION

A successful live-attenuated tetravalent DEN virus vaccine must exhibit a balance between attenuation and immunogenicity for each of the four virus components, but this has been difficult to achieve. A major problem with previously evaluated TVs has been their capacity to cause a DEN-like illness in vaccinees (11, 26, 38). This reactogenicity appears to be related to a high level of replication of at least one of the components of the TV. Since DEN viruses that replicate to high levels during natural DEN virus infection cause more severe disease (14, 15, 30, 32, 42), it is not surprising that this also occurs for the live-attenuated virus vaccine candidates. DEN virus vaccine strains that were dominant in replication in the TV may have appeared to be underattenuated when tested as a monovalent vaccine (26, 41), indicating that the three subdominant members of the TV exert little effective interference in replication. Our first TV, TV-1, also appeared to follow these rules. Replication in rhesus monkeys of each of the viruses in TV-1 was characterized by predominance of rDEN3Δ30, a virus that was underattenuated as a monovalent vaccine candidate (2). Since the level of replication of rDEN3Δ30 was the same as its

wild-type parent, and its level of immunogenicity exceeded that of other vaccine candidates, rDEN3Δ30 was considered underattenuated and eliminated from further evaluation. Nevertheless, a formulation such as TV-1, derived from attenuated DEN1, DEN2, DEN3, and DEN4 viruses in their native genetic confirmation has one major advantage, the induction of cell-mediated immunity to the full spectrum of proteins present in each of the four DEN viruses, in addition to neutralizing antibody against each serotype. In order to take advantage of this property, we are currently attenuating rDEN3Δ30 to a level suitable for use as a DEN3 component of a second-generation TV-1 formulation. Antigenic chimeric DEN viruses in which the structural proteins of an attenuated DEN virus or other flavivirus are replaced by those of a DEN virus from a different serotype would not induce this broad-based cell-mediated immune response and may be less desirable (2, 17, 24).

These findings indicate that it is essential to select viruses that are satisfactorily attenuated as monovalent vaccines for inclusion in a TV. Each of the individual vaccine viruses in TV-2 and TV-3 was selected because they were clearly restricted in replication in rhesus monkeys or in SCID-HuH-7 mice (2, 3, 9, 43, 44). The overall level of replication of the viruses in TV-2 and TV-3 in SCID-HuH-7 mice and rhesus monkeys was restricted in magnitude and duration compared to that of the viruses in a tetravalent formulation of wild-type strains. The rDEN4Δ30 vaccine component in both TV-2 and TV-3 replicated to the highest level. This was an encouraging observation because rDEN4Δ30 has already been established as highly attenuated and safe in humans when administered at doses of 10^1 to 10^5 PFU (9, 10). In a study of 79 human volunteers, the rDEN4Δ30 virus was found to be largely nonreactogenic and failed to induce a systemic illness in any vaccinee (10). Viremia and rash were found in only about 50% of vaccinees, and the mean serum virus titer ranged from 0.6 to 1.6 $\log_{10}$ PFU/ml, which is considerably lower than the viremia levels typically reached in symptomatic DEN virus infections (6.0 to 8.0 $\log_{10}$ PFU/ml) (42). Thus, the other DEN1, DEN2, and DEN3 components in either TV-2 or TV-3, which replicated to a lower level than rDEN4Δ30, should be even less reactogenic than rDEN4Δ30. However, this needs to be confirmed in clinical trials.

A second major problem encountered previously in the development of tetravalent DEN vaccine candidates has been the induction of unequal levels of neutralizing antibodies against the four DEN serotypes, with levels to some components being unacceptably low (16, 26, 39, 41). This unbalanced immunogenicity could potentially result from (i) inclusion of vaccine components that are over- or underattenuated, (ii) interference in virus replication among vaccine components, or (iii) antigenic competition among vaccine components. In the case of the three TV formulations tested here, antigenic competition would appear to have been eliminated as a cause of unequal immunogenicity since immunization with T-*wt* induced strong neutralizing antibody responses to each of the four serotypes. This demonstrates that the monkeys can produce high titers of antibodies to the four E proteins following a single dose of four wild-type viruses in a tetravalent preparation. As described above, rDEN3Δ30 induced the highest levels of neutralizing antibodies in TV-1. This dominance most likely reflected its underattenuation, as the neutralizing antibody responses to the other vaccine components were largely preserved. Underattenuation of a DEN vaccine component of a TV accompanied by a high level of immunogenicity has been observed before. In the Mahidol University/Aventis Pasteur vaccine, the DEN3 component was the most reactogenic of the four serotypes when evaluated as a monovalent vaccine and was also the dominant component in a TV since it achieved the highest levels of viremia and neutralizing antibodies compared to the other three components (38, 39). This DEN3 strain even dominated replication and induced the highest level of antibody when tested at a dose of 10 50% tissue culture infective doses, 100-fold lower than that of the DEN1, -2, or -4 component (38). Maintenance of the neutralizing antibody response to rDEN1Δ30, rDEN2Δ30, and rDEN4Δ30 suggests that these vaccine candidates could compete effectively with the rDEN3Δ30 virus in the nonhuman primate host.

Monkeys immunized with a single dose of TV-2 also demonstrated an unequal neutralizing antibody response, characterized by low responses to the rDEN2/4Δ30 and rDEN3/4Δ30 chimeric virus vaccine components. Since the monovalent preparations of rDEN2/4Δ30 and rDEN3/4Δ30 induced satisfactory levels of antibodies, the low levels of antibodies to the DEN2 and DEN3 viruses in monkeys immunized with TV-2 likely reflect a decrease in their level of virus replication when formulated in a tetravalent versus a monovalent vaccine preparation. Interestingly, this apparent interference in virus replication among the components of TV-2 was not observed in SCID-HuH-7 mice. Immunization with the chimeric viruses as monovalent vaccines induced geometric mean neutralizing antibody titers of greater than 1:50 with 100% seroconversion (2, 44), but in TV-2, titers were <1:20 and seroconversion was low. Our chimeric DEN2 and DEN3 components differ from the overattenuated DEN4 component of the Walter Reed Army Institute of Research TV, which was weakly immunogenic even when delivered as a monovalent vaccine (11, 41). The rDEN2/4Δ30 and rDEN3/4Δ30 viruses are our most attenuated monovalent vaccine candidates, and it is reasonable to suggest that it is their inherent growth restriction that makes them less competitive in a TV and thus susceptible to interference by the more dominant viruses in the vaccine. It is important to note that replication of these vaccine candidates in humans might be slightly greater than that in rhesus monkeys since humans are the natural host and support more vigorous replication of DEN viruses than nonhuman primates. This increased replicative capacity in humans could result in increased immunogenicity of rDEN2/4Δ30 and rDEN3/4Δ30.

After study of TV-1 and -2, a third tetravalent formulation, TV-3, was made that induced a balanced antibody response to each of the four serotypes following a single dose. This was achieved by making two changes to the formulation of TV-2, yielding TV-3. First, the weakly immunogenic rDEN2/4Δ30 component was replaced with the less attenuated rDEN2Δ30 virus. This illustrates the value of having several DEN vaccine candidates for each serotype with differing levels of attenuation that can be interchanged in a TV to achieve a balanced immune response. Second, the weakly immunogenic rDEN3/4Δ30 vaccine candidate was delivered at a 10-fold higher dose in TV-3. Increasing the dose of a poorly immunogenic component of a TV appears to augment its immunogenicity (11, 38).

Previous studies of live-attenuated tetravalent DEN vaccines in humans have similarly concluded that to induce sufficient immunity against each DEN serotype, multiple doses of vaccine are necessary (38, 41), and a satisfactory antibody response to TV-2 was achieved by administration of a second dose of vaccine. Similarly, multiple immunizations of the live-attenuated, trivalent poliovirus vaccine are required to achieve protective immunity to each of the poliovirus serotypes. A balanced immune response against the three poliovirus strains is achieved, in part, by immune restriction of the dominant poliovirus type 2 strain in the booster doses (35). Our results indicate that a second dose of tetravalent DEN vaccine, either TV-2 or TV-3, induced 100% seroconversion from baseline levels and geometric mean neutralization titers of greater than 1:100 against each serotype. To augment the immune response, the timing of administration of the second dose was critical. It was found that a 1-month boost was ineffective in increasing the antibody response, whereas a 4-month boost was highly effective. This result may be explained by homotypic exposure to the TV components or by the short-term heterotypic immunity induced by the DEN viruses, first described by Sabin (40). In this early study, immunization with DEN1 was found to decrease illness following a DEN2 challenge, but only for a brief time (1 to 3 months), at which time individuals would possess only homotypic immunity and be sensitive to a heterologous challenge (40). Previous studies of TVs in humans have also demonstrated that a 1-month boost was ineffective at augmenting immunity while boosts performed after longer intervals were effective (11, 41). Thus, a short time interval (1 month) between initial and booster immunizations appears to not be an option for tetravalent DEN vaccines. However, evidence exists which suggests that during this period before optimal immunity is reached after a second dose, vaccine-induced heterotypic immunity might prevent infection or modify disease associated with infection (40).

We were somewhat surprised that a second dose of vaccine given at 4 months would be able to replicate sufficiently to increase the antibody response since the monkeys were largely resistant to replication of wild-type virus upon challenge. Even animals with a vigorous primary response developed an increased antibody response following a second dose given at 4 months. Based on the immunization experience with live-attenuated measles virus vaccine (34), we thought that the immunity induced by the first vaccine dose would be sufficient to restrict replication of the attenuated vaccine viruses given after 4 months, but this was not the case. It is clear that attenuated DEN viruses can initiate infections in an immune host to a level sufficient to boost the immune response, but not to a level that would result in detectable generalized infection.

A single dose of TV-2 was found to confer protection from DEN1, DEN3, and DEN4, but not DEN2. However, two doses of the TV were able to induce protection against DEN2. Importantly, enhanced replication was not observed after a challenge with any of the four wild-type DEN viruses despite neutralizing antibodies being at minimally effective levels for the DEN2 and DEN3 viruses. Just as the second immunization of TV-2 and TV-3 resulted in increased neutralizing antibody titers, challenge with wild-type viruses resulted in increased antibody responses. Such increases in antibody levels following a wild-type virus challenge of tetravalent-vaccine-inoculated monkeys in the absence of detectable viremia have been observed previously, suggesting a lack of sterilizing immunity conferred by DEN vaccines (17). This ability to readily infect and immunize in the presence of prior immunity appears to be unlike other virus vaccines, such as those for mumps and measles, that are poorly immunogenic even in the presence of low levels of passively acquired antibodies (34). There are three important implications for the use of a tetravalent DEN vaccine as a consequence of the ability to increase antibody responses in the presence of existing neutralizing antibody. First, vaccination with two doses of highly attenuated vaccines should be as effective as vaccination with one dose of a marginally attenuated vaccine. This property serves to foster the development of vaccines that are viewed as safer based on their high level of attenuation. Second, in addition to booster immunizations, natural DEN infection that is effectively controlled by vaccination might contribute to long-term maintenance of immunity to disease caused by the DEN viruses. Third, it may be possible to immunize infants in the presence of maternally acquired antibodies against DEN viruses.

Immunization with two doses of an attenuated TV resulted in the induction of a broad immune response against multiple members of each DEN serotype. Since multiple DEN virus genotypes of each serotype are present in different geographic regions (37), it is necessary to examine the ability of a TV to induce a broadly reactive antibody response capable of neutralizing the vast majority of DEN virus strains. Although DEN viruses have been assigned to different genotypes, subtype members are highly related in their E protein sequence, so it was anticipated that a broad response would be achieved. The neutralizing antibody response induced after two doses of TV-2 was tested against genetically diverse members of each DEN virus serotype. The neutralization levels induced by two doses of TV-2 were similar in magnitude and breadth to those induced by a single dose of T-*wt*. However, two exceptions were noted. The antibody response induced by the rDEN3/4Δ30 component to DEN3 Sri Lanka/91 was low, and no response to DEN3 Fiji/92 was observed despite the strong immunity induced by T-*wt*. Interestingly, the E protein of Fiji/92 is the most closely related to that of the parent virus, DEN3 Sleman/78.

The difference in neutralizing activity between the serum from monkeys inoculated with T-*wt* and monkeys immunized with TV-2 is surprising and unexplained. The observed low neutralizing activity of serum from TV-2-immunized monkeys cannot simply be explained by an inherent difficulty of the in vitro neutralization of DEN3 Sri Lanka/91 and DEN3 Fiji/92 since serum from monkeys immunized with T-*wt* effectively neutralized both viruses. However, this variable neutralizing antibody response might be explained by the presence of a single amino acid change (Gln > Ser at amino acid 444) in the E protein of the rDEN3/4Δ30 virus used in this study compared to its parent, DEN3 Sleman/78 (2). Additional rDEN3/4Δ30 viruses lacking changes in the DEN3 E protein are currently being generated and may be used to test this hypothesis.

Despite our findings, the accepted dogma suggests that infection with a given DEN serotype will provide long-term protection from all subtypes within the original infecting serotype (12). Our observation that some strains escaped in vitro neutralization may not be relevant to vaccine efficacy and may

show a limitation of using an in vitro neutralization assay as a surrogate for vaccine efficacy testing. Nevertheless, the importance of developing a DEN virus vaccine which confers broad protection from identified subtypes dictates that we confirm that the accepted dogma is, in fact, true.

In summary, the TV-2 and TV-3 formulations described here have several advantages that identify them as promising vaccine candidates. First, the attenuation of each virus component in the tetravalent formulation has been established. Second, each component contains the Δ30 deletion in the 3′ UTR, which precludes the generation of nonattenuated wild-type viruses caused by recombination among tetravalent components (33, 46). Third, two immunizations separated by 4 months with TV-2 or TV-3 induced a high, balanced level of neutralizing antibodies against each serotype above levels previously shown to be protective. Fourth, a neutralizing antibody response against genetically diverse strains of each serotype was induced. Fifth, the vaccines achieve virus titers of greater than 6.5 $\log_{10}$ PFU/ml in Vero cells and can be produced economically. Finally, alternative vaccine candidates and modifying mutations exist which can be utilized to generate additional formulations if the tetravalent formulations described here fail to yield a balance of attenuation and immunogenicity in humans (3, 22).

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