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Targeted Mutagenesis as a Rational Approach to Dengue Virus Vaccine Development

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Abstract

The recombinant dengue virus type 4 (rDEN4) vaccine candidate, rDEN4Δ30, was found to be highly infectious, immunogenic and safe in human volunteers. At the highest dose (10^5 PFU) evaluated in volunteers, 25% of the vaccinees had mild elevations in liver enzymes that were rarely seen at lower doses. Here, we describe the generation and selection of additional mutations that were introduced into rDEN4Δ30 to further attenuate the virus in animal models and ultimately human vaccinees. Based on the elevated liver enzymes associated with the 10^5 PFU dose of rDEN4Δ30 and the known involvement of liver infection in dengue virus pathogenesis, a large panel of mutant viruses was screened for level of replication in the HuH-7 human hepatoma cell line, a surrogate for human liver cells and selected viruses were further analyzed for level of viremia in SCID-HuH-7 mice. It was hypothesized that rDEN4Δ30 derivatives with restricted replication in vitro and in vivo in HuH-7 human liver cells would be restricted in replication in the liver of vaccinees. Two mutations identified by this screen, NS3 4995 and NS5 200,201, were separately introduced into rDEN4Δ30 and found to further attenuate the vaccine candidate for SCID-HuH-7 mice and rhesus monkeys while retaining sufficient immunogenicity in rhesus monkeys to confer protection. In humans, the rDEN4Δ30-200,201 vaccine candidate administered at 10^5 PFU exhibited greatly reduced viremia, high infectivity and lacked liver toxicity while inducing serum neutralizing antibody at a level comparable to that observed in volunteers immunized with rDEN4Δ30. Clinical studies of rDEN4Δ30-4995 are ongoing.

1 Introduction

The four DENV serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, cause considerably more human disease than any other arbovirus. DENV is currently receiving heightened research interest because of the intense spread and increased prevalence of the disease

throughout tropical and subtropical regions of the world. In fact, dengue hemorrhagic fever (DHF) cases have now been confirmed in more than 60 countries and DENV is endemic in more than 100 countries including most of Southeast Asia, South America, Central America and the Caribbean and South Pacific regions (Gubler and Meltzer 1999; WHO 1997). Prior to the 1970s, only five Southeast Asian countries had documented DHF (Gubler 1997). Currently a licensed vaccine is not available to confer protection from dengue disease but the development of a tetravalent DENV vaccine has recently received considerable effort with substantial advancement (Whitehead et al. 2007).

Live attenuated tetravalent vaccines for DENV are currently in the most advanced stages of development. Nevertheless, several factors have complicated the development of an effective DENV vaccine including the lack of an animal model for DENV disease and the need to simultaneously protect against all four serotypes. A vaccine must confer protection from each of the DENV serotypes because all four serotypes commonly circulate in endemic regions and secondary infection with a heterologous serotype is associated with increased disease severity by a mechanism termed antibody-dependent enhancement (Halstead 2003). It is imperative that an acceptable DENV vaccine elicits a consistently strong and protective neutralizing antibody response against each of the four serotypes using a tetravalent formulation of antigens. The benefits of live attenuated vaccines for DENV are believed to outweigh the potential risks inherent with live virus vaccines. Live attenuated vaccines can induce potent humoral and cellular immune responses, mimic natural infection and can provide lifelong immunity. In addition, live attenuated vaccines are the most economical option because of the low cost of manufacture in Vero cell tissue culture and the high level of infectivity of the vaccine viruses (Blaney et al. 2006).

2 The rDEN4Δ30 Vaccine Candidate

Conventional methods of serial passage of virus in an animal host (e.g., mouse brain) or in tissue culture were used to develop the first generation live attenuated DENV vaccine candidates (Bhamarapravati and Yoksan 1997; Halstead and Marchette 2003; Sabin 1952). More recently, recombinant DNA methodology and reverse genetics has been utilized to design live attenuated recombinant DENV vaccine candidates (Durbin et al. 2001; Guirakhoo et al. 2006). For recovery of an infectious flavivirus, which contains a single-stranded, positive-sense RNA genome, RNA transcripts are produced from cloned cDNAs in vitro and then delivered into susceptible tissue culture cells by transfection or electroporation (Lai et al. 1991; Rice et al. 1989). This methodology allows recovery of well-defined, genetically homogeneous virus populations with a safe and easily characterized passage history. Site directed mutagenesis of cloned virus cDNAs has provided mutant viruses bearing engineered mutations that can be examined in vivo for their level of attenuation and stability (Blaney et al. 2002; Hanley et al. 2002; Men et al. 1996). Importantly, recombinant DNA methodology can further be used to modify and combine mutations to develop a myriad of well-defined vaccine candidates.

The first reported recombinant DENV to be evaluated in humans was the rDEN4Δ30 vaccine candidate that contains a 30 nucleotide deletion in the 3' UTR, removing nucleotides 10,478–10,507 (Durbin et al. 2001, 2005). Preclinical evaluation of rDEN4Δ30 demonstrated that the vaccine candidate had restricted replication in rhesus monkeys as indicated by a shortened duration and lower magnitude of viremia compared to wild type DENV-4 (Durbin et al. 2001; Men et al. 1996). Comparison of DENV-4 and rDEN4Δ30 virus replication in mosquitoes indicated that the Δ30 mutation restricted dissemination from the mid-gut to the head of infected mosquitoes, blocking a necessary step for virus transmission (Troyer et al. 2001). Importantly, the rDEN4Δ30 virus replicates to titers of $> 10^7$ PFU/ml in qualified Vero cells, which are suitable for vaccine production.

For the initial clinical evaluation, 20 adult volunteers were administered a single subcutaneous dose of 10^5 PFU rDEN4Δ30 (Table 1) (Durbin et al. 2001). Serious clinical responses were not observed and the vaccine was well tolerated. The most frequent clinical reactions were transient and asymptomatic including a maculopapular rash in 50% of volunteers, increased alanine aminotransferase (ALT) levels in 25% of volunteers and neutropenia in 15% of volunteers. Transient elevations in serum liver enzymes are also seen in vaccinees infected with other live attenuated DENV vaccine candidates (Eckels et al. 1984; Edelman et al. 1994; Kanesa-athan et al. 2001; Vaughn et al. 1996) and the levels observed in vaccinees are generally much lower than those observed in humans experiencing DF or DHF/DSS. Furthermore, the hepatomegaly that is observed in some dengue patients (Kalayanaroj et al. 1997; Kuo et al. 1992; Mohan et al. 2000; Wahid et al. 2000) has not been observed in vaccinees. Hepatotropism is a natural feature of DENV infection of humans and the rDEN4Δ30 vaccine candidate likely exhibited a low level of residual hepatotropism with asymptomatic hepatotoxicity at the high dose of 10^5 PFU. The mean peak titer of rDEN4Δ30 virus in serum ($10^{1.6}$ PFU/ml) of vaccinees is much lower than that observed in natural DF and DHF, which can be 10^6 infectious units/ml or greater (Endy et al. 2004; Vaughn et al. 2000). Despite the low levels of viremia observed in vaccinees, the rDEN4Δ30 vaccine candidate was strongly immunogenic. Each vaccinee (viremic and nonviremic) seroconverted to DENV-4 with a geometric mean neutralizing antibody titer (GMT) of 1:399 (seroconversion is defined as a fourfold or greater increase in neutralizing antibody level after immunization). Importantly, *Aedes* mosquitoes were fed on a subset of volunteers around the time of peak of viremia and the vaccine virus was not transmitted from the infected vaccinees to the feeding mosquitoes (Troyer et al. 2001).

While the initial evaluation of rDEN4Δ30 at 10^5 PFU indicated that the vaccine was well-tolerated and safe, two approaches were pursued to decrease the mild reactogenicity. First, a placebo-controlled dose response study was performed to determine the 50% human infectious dose (HID_{50}) of the vaccine and to examine if lowering the dose could reduce the rash or the laboratory abnormalities observed in vaccinees (Durbin et al. 2005). The HID_{50} was determined to be < 10 PFU since 95–100% of vaccinees seroconverted at doses of 10^3 , 10^2 and 10^1 PFU (Table 1). The frequency of detectable viremia in volunteers was not different among the dose cohorts but mean peak virus titers among viremic volunteers were approximately tenfold lower for the 10^3 , 10^2 and 10^1 PFU cohorts in comparison to the 10^5 PFU cohort. In addition, 95–100% of volunteers seroconverted to DENV-4 with consistently high neutralizing antibody titers regardless of vaccination dose. At a dose of 10^3 PFU, only a single volunteer had an elevated serum ALT level and elevated ALT levels were not detected in any volunteer in the 10^2 or 10^1 PFU cohorts, indicating that reduction of dose was effective in decreasing the mild hepatotoxicity of rDEN4Δ30. However, the frequency of rash and neutropenia was not related to vaccine dose and remained similar in each dose cohort. Studies performed by others have also indicated that reducing the vaccine dose may not reliably reduce clinical signs or laboratory abnormalities (Kitchener et al. 2006; Sabchareon et al. 2004, 2002).

We also pursued an additional genetic approach to reduce the mild reactogenicity observed with rDEN4Δ30 by developing further attenuated variants of rDEN4Δ30 by the introduction of additional attenuating mutations. We especially sought mutations that limited replication in vitro or in vivo in HuH-7 cells, our surrogate for human liver cells.

3 Development of Further Attenuated rDEN4Δ30 Vaccine Candidates

3.1 Generation of Mutant rDEN4 Viruses

The identification of mutations that attenuate DENV has been a major focus of our strategy for the development of live attenuated DENV vaccine candidates. The availability of a panel

of well-characterized attenuating mutations that could be introduced into wild type DENV or into incompletely attenuated vaccine candidates to decrease their reactogenicity would promote the development of safe and immunogenic vaccines. Toward this end, we have generated a large panel of mutations in rDEN4 using two approaches: paired charge-to-alanine mutagenesis and chemical mutagenesis.

Paired charge-to-alanine mutagenesis of the DENV-4 NS5 polymerase gene was conducted by mutating the eighty pairs of charged amino acids present in DENV-4 NS5 to Ala-Ala and individually incorporating them into the full length DENV-4 cDNA clone (Hanley et al. 2002). Of the eighty cDNA clones, 32 were found to yield infectious virus upon transfection of Vero and/or C6/36 mosquito cells. In a separate strategy, wild type DENV-4 was grown in Vero cells in the presence of 5-fluorouracil, a chemical mutagen, which resulted in a 100-fold reduction in virus yield (Blaney et al. 2001). Virus progeny were terminally diluted to isolate 1,248 virus clones that were then expanded into small stocks for subsequent screening. Viruses with desirable phenotypes, as described below, were then fully sequenced and the identified mutations were cloned individually into rDEN4 to generate recombinant viruses. Recombinant viruses bearing individual mutations were then studied to confirm that the mutation was responsible for the observed phenotypes. This extra step was not required for the charge-to-alanine mutant viruses because these viruses already contained genetically isolated mutations.

3.2 Selection of Mutant rDEN4 Viruses

The mutant viruses described above were initially screened for temperature sensitivity (*ts*) and small plaque (*sp*) size in Vero cells and the human hepatoma cell line, HuH-7, in an effort to identify presumptive liver-specific attenuating mutations. All mutant viruses that possessed *ts* and/or *sp* phenotypes were also screened for reduced replication in suckling mouse brain as an additional measure of overall level of attenuation. Numerous attenuating mutations conferring an array of the aforementioned attenuation phenotypes were identified and have been described (Blaney et al. 2001, 2002, 2003a; Hanley et al. 2002).

A novel small animal model, SCID-HuH-7 mice, was next developed to identify mutations that might be useful to reduce the low level of hepatotoxicity observed in the rDEN4Δ30 vaccinees. In this animal model of DENV infection, HuH-7 cells are engrafted in SCID mice and allowed to form intraperitoneal tumors into which virus can then be directly injected (Blaney et al. 2002). Subsequent viremia in the SCID-HuH-7 mouse is believed to arise primarily from the human liver cells comprising the tumor mass since DENV administered peripherally to age-matched, nonengrafted mice are highly restricted in replication. Importantly, the relative level of virus replication in SCID-HuH-7 mice correlates with that of rhesus monkeys, which serve as the most widely used animal model for evaluation of the level of attenuation of DENV vaccine candidates (Blaney et al. 2006). In addition, wild type DENV viremia in SCID-HuH-7 mice reaches high levels ($>10^6$ PFU/ml) similar to those observed in humans with severe disease (Vaughn et al. 2000).

Nineteen chemically mutagenized viruses and thirteen charge-to-alanine mutant viruses with *ts* phenotypes in HuH-7 cells were screened in SCID-HuH-7 mice and several were identified with significant reductions in replication compared to wild type DENV-4 (Blaney et al. 2002; Hanley et al. 2004). One such mutation was the 200,201 mutation named for the mutated amino acid pair in the NS5 protein. The 200,201 mutation was found to confer greater than a 100-fold reduction in replication in SCID-HuH-7 mice and importantly, each amino acid change was found to confer this attenuation when the paired mutations were studied separately (Fig. 1). Since both NS5 200 and 201 mutations independently contribute to the attenuation phenotype of rDEN4-200,201, a virus with the pair of mutations should be phenotypically stable since the two mutated codons would require two nucleotide

substitutions each to change the alanine substitution to the original charged amino acid residue in the wild type virus (Hanley et al. 2004). The 200,201 mutation and the previously described $\Delta 30$ mutation were next combined into a single virus to create the rDEN4 $\Delta 30$ -200,201 vaccine candidate.

Several other mutations that restricted replication in SCID-HuH-7 mice were identified and modified rDEN4 $\Delta 30$ derivatives were generated with these additional mutations and were shown to be further attenuated in SCID-HuH-7 mice (Hanley et al. 2004). One such virus, rDEN4 $\Delta 30$ -4995, contains the 4995 mutation that was previously found to confer a *ts* phenotype in Vero and HuH-7 cells, to enhance replication in Vero cells at permissive temperature and to cause a 1,000-fold reduction in replication in suckling mouse brain (Blaney et al. 2001; 2003b). Despite the fact that the 4995 mutation alone did not confer a significantly decreased level of replication in SCID-HuH-7 mice, it was discovered that combining the 4995 and $\Delta 30$ mutations resulted in a virus with a level of replication in SCID-HuH-7 mice similar to that of rDEN4-200,201 (Fig. 1) (Hanley et al. 2004). In rDEN4 $\Delta 30$ -4995, the mutations at genome position 4995,4996 and 4997 result in a serine to leucine change at amino acid position 158 of NS3. The mutations incorporated at codon 158 (wt. TCA $\rightarrow$ mut. CTT) in rDEN4 $\Delta 30$ -4995, would require two nucleotide changes for reversion to wild type sequence. While other modified rDEN4 $\Delta 30$ viruses have been studied (Hanley et al. 2004), we focus now on rDEN4 $\Delta 30$ -200,201 and rDEN4 $\Delta 30$ -4995, which were found to replicate efficiently in Vero cells and which were attenuated in SCID-HuH-7 mice.

3.3 Preclinical Studies of rDEN4 $\Delta 30$ -200,201 and rDEN4 $\Delta 30$ -4995

The rDEN4 $\Delta 30$ -200,201 and rDEN4 $\Delta 30$ -4995 viruses were evaluated for replication in SCID-HuH-7 mice and found to be at least 250-fold restricted compared to wild type DENV-4 (Fig. 1). These two modified rDEN4 $\Delta 30$ vaccine candidates were also significantly restricted in replication (40-50-fold) when compared to the rDEN4 $\Delta 30$ parent virus. Thus, in the SCID-HuH-7 mouse model, both rDEN4 $\Delta 30$ -200,201 and rDEN4 $\Delta 30$ -4995 had clear evidence of further attenuation. The level of attenuation achieved by the combination of the 200,201 and $\Delta 30$ mutations was not greater than the 200,201 mutation alone indicating that the effect of each mutation was not additive. In contrast, the combination of the 4995 and $\Delta 30$ mutations synergistically increased the level of attenuation in the SCID-HuH-7 mice.

Mutant viruses rDEN4 $\Delta 30$ -200,201 and rDEN4 $\Delta 30$ -4995 were more attenuated than rDEN4 $\Delta 30$ in rhesus monkeys (Table 2) and these observations were similar to those in SCID-HuH-7 mice. None of four monkeys inoculated with rDEN4 $\Delta 30$ -200,201 developed detectable viremia, while each of four rDEN4 $\Delta 30$ -vaccinated monkeys was viremic, indicating that the 200,201 mutation restricted replication of rDEN4 $\Delta 30$. Despite the lack of detectable viremia, each monkey was infected with rDEN4 $\Delta 30$ -200,201 as demonstrated by a 100% frequency of seroconversion and complete protection from challenge with wild type DENV-4 (data not shown). Importantly, the GMT was comparable to that of rDEN4 $\Delta 30$ indicating that rDEN4 $\Delta 30$ -200,201 was not over-attenuated. rDEN4 $\Delta 30$ -4995 had a replication phenotype in rhesus monkeys that appeared to be intermediate between that of rDEN4 $\Delta 30$ and rDEN4 $\Delta 30$ -200,201 (Table 2). The incidence (50%) and duration (0.8 days) of viremia was approximately half of that observed for rDEN4 $\Delta 30$, even though the peak virus titer was not different. As observed for monkeys receiving rDEN4 $\Delta 30$ -200,201, all rDEN4 $\Delta 30$ -4995 vaccinated monkeys seroconverted and were completely protected from challenge.

The further attenuation of rDEN4 $\Delta 30$ in SCID-HuH-7 mice and rhesus monkeys by the introduction of the 200,201 or 4995 mutation indicated that these two modified vaccine

candidates should be evaluated in humans. Such a study would not only allow for evaluation of clinical reactogenicity but would also be useful in determining if the differences in replication between these vaccine candidates and their rDEN4Δ30 parent virus that were seen in both SCID-HuH-7 mice and rhesus monkeys would also be seen in humans. Such a correlation would serve as a guide for the use of these preclinical phenotypes in making decisions about which vaccine candidates should be evaluated in human subjects. Because the rDEN4Δ30-200,201 vaccine candidate was the most attenuated in rhesus monkeys and has the added advantage of increased genetic stability, it was chosen first to be tested in humans as described below. Clinical evaluation of rDEN4Δ30-4995 is currently ongoing.

4 Clinical Evaluation of rDEN4Δ30-200,201

The rDEN4Δ30-200,201 vaccine candidate was evaluated at a dose of 10^5 PFU in a double-blind phase I study of 28 volunteers including 8 placebo recipients and compared to the response of volunteers in the previous study of rDEN4Δ30 given at a 10^5 PFU dose (McArthur et al. 2008). As expected, volunteers receiving the rDEN4Δ30-200,201 vaccine did not develop significant local or systemic illness. Viremia was not detectable in any vaccinee given rDEN4Δ30-200,201, which is a striking difference from the 70% incidence of viremia that was observed in the previous study of rDEN4Δ30 at the 10^5 PFU dose (Table 3). Even if a low level of viremia for rDEN4Δ30-200,201 was present below the limit of detection ($0.5 \log_{10}$ PFU/ml), it would be at least tenfold reduced compared to that of rDEN4Δ30 ($1.6 \log_{10}$ PFU/ml). Despite the lack of detectable viremia in rDEN4Δ30-200,201 vaccinees, all volunteers were infected as indicated by the 100% seroconversion rate on day 28 (data not shown). On day 42, the neutralizing antibody response against DENV-4 induced by rDEN4Δ30-200,201 (GMT of 1:84) was less than twofold different than that observed for rDEN4Δ30 (GMT of 1:156) (Table 3). This suggests that sufficient replication of rDEN4Δ30-200,201 occurred in the vaccinees to result in the induction of a moderate level of immunity. The virological and immunological results of this study correlate well with the observations seen in the rhesus monkey studies of rDEN4Δ30-200,201 (Table 2 vs. Table 3) confirming the utility of this animal model in the preclinical evaluation of DENV-4 vaccines.

The reactogenicity profile of rDEN4Δ30-200,201 was also addressed in this study (McArthur et al. 2008). The incidence of rash was lower in the rDEN4Δ30-200,201 vaccinees (20%) when compared to the rDEN4Δ30 vaccinees (50%) at the 10^5 PFU dose. However, the most conspicuous difference between the cohorts was the lack of ALT elevations in volunteers receiving rDEN4Δ30-200,201 versus the 25% incidence in the rDEN4Δ30 vaccinees. This observation indicates that the mild hepatotoxicity of rDEN4Δ30 was abrogated by the inclusion of the 200,201 mutation and suggests that the 200,201 mutation restricted replication specifically in the liver of vaccinees. It is possible that the 200,201 mutation also decreased replication at a peripheral site.

These studies indicate that the selection of mutations to further attenuate rDEN4Δ30 for SCID-HuH-7 mice was a rational approach to vaccine design and resulted in the intended decrease in the level of reactogenicity of rDEN4Δ30-200,201 for humans while maintaining sufficient immunogenicity. In the current case, a mutation that restricted replication in HuH-7 cells, a human liver cell line, also decreased the hepatotoxicity of rDEN4Δ30 for humans. This approach has also been applied to the development of attenuated mutant neurotropic flaviviruses that are selected for restricted replication in neuronal cells in culture and that were subsequently found to have decreased neurovirulence (Rumyantsev et al. 2006). The strong immunogenicity of rDEN4Δ30-200,201, despite its reduced viremia and putative restricted replication in the liver compared to rDEN4Δ30, suggests that this vaccine candidate is replicating efficiently in some tissue(s) of the host. Using reverse genetics to

develop these vaccine candidates has permitted the rapid importation of desired attenuating mutations into wild type virus or incompletely attenuated mutants. The success of this study confirms the utility of such an approach to live attenuated vaccine development in which mutations are added sequentially to a virus with clinical testing after each addition to identify a vaccine virus that has achieved an acceptable balance between attenuation and immunogenicity. Based on the highly restricted replication, lower reactogenicity and similar immunogenicity of rDEN4Δ30-200,201 compared to rDEN4Δ30, the new vaccine candidate can be strongly considered for inclusion in a tetravalent vaccine formulation. At present we favor the use of the rDEN4Δ30 at a dose of 10^3 PFU since it is safe, economical to manufacture and immunogenic at this dose, with rDEN4Δ30-200,201 serving as a backup vaccine candidate, if needed. In addition, the 200,201 mutation may be also useful for further attenuating the DENV, West Nile virus and tick-borne encephalitis virus antigenic chimeric viruses that have been created using the rDEN4Δ30 virus (Durbin et al. 2006b; Pletnev et al. 2001, 2006).

5 Future Directions

Current studies in humans focus on the analysis of safety, infectivity and immunogenicity of individual vaccine candidates, including rDEN4Δ30-200,201 and rDEN4Δ30-4995, so that a tetravalent formulation can be identified. The development and clinical evaluation of rDEN4Δ30 has led to two strategies for development of vaccine candidates for the other DENV serotypes (Blaney et al. 2006). First, reverse genetics has been used to introduce the structurally conserved attenuating Δ30 mutation into the 3'-UTR of cDNA clones of DENV-1, DENV-2 and DENV-3 (Blaney et al. 2004a, b, 2005; Durbin et al. 2006a; Whitehead et al. 2003a). Alternatively, antigenic chimeric viruses have been generated by replacement of the structural proteins of the attenuated rDEN4Δ30 vaccine candidate with those from DENV-1, DENV-2 or DENV-3 (Blaney et al. 2004a; 2007, Durbin et al. 2006b; Whitehead et al. 2003b). Targeted mutagenesis of the DENV-3 3'-UTR has also been used to identify additional attenuated vaccine candidates for this serotype (Blaney et al. 2008). In conclusion, the ability to generate and modify vaccine candidates by reverse genetics and targeted mutagenesis has increased the likelihood that four suitable vaccine candidates can be identified for a tetravalent formulation. However, additional research is required for the identification of a suitable tetravalent DENV vaccine and for the implementation of its use.

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Fig. 1.

Replication of rDEN4 viruses in SCID-HuH-7 mice. Groups of SCID mice were transplanted intraperitoneally with 10^7 HuH-7 cells. Approximately six weeks later, tumor-bearing mice received virus injections of 10^4 PFU directly into the tumor. Serum was collected on day 7 and virus titer in serum was determined by plaque assay in Vero cells. Standard error bars and animal numbers in each group are indicated. Asterisks indicate that mean virus titers were significantly lower than rDEN4Δ30 as determined by Tukey-Kramer posthoc test ($P < 0.05$)

Effect of rDEN4Δ30 vaccine dose on virus replication, immunogenicity and clinical signs in dose cohorts of 20 volunteers

Table 1

Dose (PFU)	% of volunteers with viremia	Mean peak virus titer ± SE (log ₁₀ PFU/ml serum) ^a	Geometric mean serum neutralizing antibody titer ^b	% seroconversion ^c	% of volunteers with indicated clinical sign:					Elevated ALT level ^e
					Fever	Rash	Headache	Neutropenia ^d		
10 ⁵	70	1.6 ± 0.1	399	100	5 ^f	50	35	15	25	
10 ³	35	0.5 ± 0.1	129	95	0	55	35	25	5	
10 ²	55	0.7 ± 0.1	181	95	5 ^g	80	45	20	0	
10 ¹	65	0.6 ± 0.1	355	100	0	75	45	25	0	
Placebo ^h	0	< 0.5	< 10	0	0	0	42	0	0	

^aCalculated for viremic volunteers only. The lower limit of detection is 0.5 log₁₀ PFU/ml serum

^bReciprocal plaque reduction (60%) neutralizing antibody titer on day 42

^cPercent seroconversion defined as a fourfold or greater increase in serum neutralizing antibody level to DENV-4

^dNeutropenia defined as an absolute neutrophil count of < 1,500 cells/mm³

^eElevated ALT level defined as any value above the upper limit of normal (for males, > 72 U/L⁻¹; for females, > 52 U/L⁻¹)

^fFever occurred on days 3 and 5; maximum temp was 100.5°F

^gFever occurred on day 3; maximum temp was 100.5°F

^hn = 12

Table 2

Viremia and immunogenicity in rhesus monkeys

Virus ^a	No. of monkeys	% with viremia	Mean no. days with viremia	Mean peak virus titer (log ₁₀ PFU/ml) ^b	Geometric mean serum neutralizing antibody titer ^c
rDEN4	8	100	3.0	2.2 ± 0.2	322
rDEN4Δ30	4	100	2.0	1.4 ± 0.2	154
rDEN4Δ30-4995	4	50	0.8	1.1 ± 0.1	79
rDEN4Δ30-200,201	4	0	0.0	< 1.0	139

^aGroups of rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of the indicated virus in a 1 ml dose. Serum was collected on days 0–8, 10, and 28^bVirus titer in serum was determined by plaque assay in Vero cells. The limit of detection was 1.0 log₁₀ PFU/ml^cReciprocal plaque reduction (60%) neutralizing antibody titer on day 28

Table 3

rDEN4Δ30-200,201 is more attenuated than rDEN4Δ30 when administered as a 10⁵ PFU dose in 20 volunteers

Virus	% of volunteers with viremia	Mean peak virus titer ± SE (log ₁₀ PFU/ml serum) ^a	Geometric mean serum neutralizing antibody titer ^b	% seroconversion ^c	% of volunteers with indicated clinical sign:				Elevated ALT level ^e
					Fever	Rash	Headache	Neutropenia ^d	
rDEN4Δ30 ^f	70	1.6 ± 0.1	156	100	5 ^g	50	35	15	25
rDEN4Δ300-200,201	0	< 0.5	84	100 ^h	5 ⁱ	20	30	10	0 ^j
Placebo ^k	0	n/a	<10	0	0	0	25	25	0

^aCalculated for viremic volunteers only. The lower limit of detection is 0.5 log₁₀ PFU/ml serum

^bReciprocal plaque reduction (60%) neutralizing antibody titer of day 42 serum

^cPercent seroconversion defined as a fourfold or greater increase in serum neutralizing antibody level to DENV-4 on day 28 or 42

^dNeutropenia defined as an absolute neutrophil count of 1,500 cells/mm³

^eElevated ALT level defined as any value above the upper limit of normal (for males, >72 U/L⁻¹; for females, > 52 U/L⁻¹)

^fHistorical data, except for the neutralizing antibody titer that was performed concurrently with the rDEN4Δ30-200,201 group. Although the neutralizing antibody data shown in Tables 1 and 3 are for the same sera from the rDEN4Δ30 group, the mean values differ slightly, reflecting assay to assay variability

^gFever occurred on days 3 and 5; maximum temp was 100.5°F

^hOne volunteer was excluded from serology because of pregnancy; total of 19 volunteers tested

ⁱFever occurred on day 15; maximum temp was 100.6°F

^jIncidence significantly lower than rDEN4Δ30 group (25%) determined by Fisher Exact Test (P < 0.05)

^kn = 8