A Live, Attenuated Dengue Virus Type 1 Vaccine Candidate with a 30-Nucleotide Deletion in the 3′ Untranslated Region Is Highly Attenuated and Immunogenic in Monkeys

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The Δ30 deletion mutation, which was originally created in dengue virus type 4 (DEN4) by the removal of nucleotides 172 to 143 from the 3′ untranslated region (3′ UTR), was introduced into a homologous region of wild-type (wt) dengue virus type 1 (DEN1). The resulting virus, rDEN1Δ30, was attenuated in rhesus monkeys to a level similar to that of the rDEN4Δ30 vaccine candidate. rDEN1Δ30 was more attenuated in rhesus monkeys than the previously described vaccine candidate, rDEN1mutF, which also contains mutations in the 3′ UTR, and both vaccines were highly protective against challenge with wt DEN1. Both rDEN1Δ30 and rDEN1mutF were also attenuated in HuH-7-SCID mice. However, neither rDEN1Δ30 nor rDEN1mutF showed restricted replication following intrathoracic inoculation in the mosquito Toxorhynchites splendens. The ability of the Δ30 mutation to attenuate both DEN1 and DEN4 viruses suggests that a tetravalent DEN vaccine could be generated by introduction of the Δ30 mutation into wt DEN viruses belonging to each of the four serotypes.

There are four serotypes of dengue virus (dengue virus type 1 [DEN1], DEN2, DEN3, and DEN4) that annually cause an estimated 50 to 100 million cases of dengue fever and 500,000 cases of the more severe form of dengue virus infection known as dengue hemorrhagic fever/dengue shock syndrome (6). Dengue virus is widely distributed throughout the tropical and semitropical regions of the world, and the number of dengue virus infections continues to increase due to the expanding range of its Aedes aegypti mosquito vector. A vaccine is not available for the control of dengue disease despite its importance as a reemerging disease. The goal of immunization is to protect against dengue virus disease by the induction of a long-lived neutralizing antibody response against each of the four serotypes. Simultaneous protection against all four serotypes is required, since an increase in disease severity can occur in persons with preexisting antibodies to a heterotypic dengue virus as the parent for the introduction of the Δ30 mutation (5). One such stem-loop structure, identified as TL2 in the proposed secondary structure of the 3′ UTR (14), was previously removed by deletion of 30 nucleotides from the DEN4 genome (3′ nucleotides 172 to 143) (12) and has subsequently been designated as the Δ30 mutation (5). The resulting virus, rDEN4Δ30, was shown to be attenuated in rhesus monkeys compared to parental viruses containing an intact TL2 sequence (5). In addition, the Δ30 mutation was shown to restrict the capacity for dissemination of DEN4 virus from the midgut to the head of mosquitoes (20). As a vaccine candidate, rDEN4Δ30 (also referred to as 2Δ30) was administered to 20 adult human volunteers and shown to be highly immunogenic and well tolerated without causing systemic illness (5).

Based on the success of this vaccine candidate, a strategy for the development of additional vaccine candidates representing the other three DEN virus serotypes was foreseen in which wild-type (wt) dengue viruses could be similarly attenuated for vaccine use by incorporation of mutations in the 3′ UTR. As a first step, we introduced the Δ30 mutation into the homologous region of the 3′ UTR of DEN1 virus and evaluated the level of replication of the resulting virus in rhesus monkeys and mosquitoes. Although the individual nucleotides are not well conserved in the TL2 region of each of the four DEN virus serotypes, appropriate base pairing preserves the stem-loop structure for DEN1 and DEN4 (Fig. 1A). The use of wt DEN1 virus as the parent for the introduction of the Δ30 mutation also permitted a comparison of the level of attenuation of rDEN1Δ30 with that of the previously described rDEN1mutF virus, which also contains mutations in the 3′ UTR (11). The mutF mutation consists of a pair of deleted nucleotides and a two-nucleotide substitution in the terminal 3′ stem-loop structure conserved among all flavivirus species (22).
To introduce the Δ30 mutation into a DEN virus other than DEN4, the DEN1 Western Pacific (WP) strain was engineered to contain the mutation. The DEN1 cDNA clone, pRS424DEN1WP (16), was used as the template in PCR to generate a 292-nucleotide fragment designed to remove 30 nucleotides as shown in Fig. 1B. The original pRS424DEN1WP cDNA clone was digested with ApaI (cleaves just upstream of the Δ30 mutation) and SacII (cleaves downstream of the 3′ end), mixed with the PCR product, and used to transform yeast strain YPH57 as described previously (13). DNA isolated from two independent yeast colonies was used to transform Escherichia coli strain STBL2 (Invitrogen, Carlsbad, Calif.). Plasmid DNA suitable for generating RNA transcripts was prepared, and the presence of the Δ30 mutation was verified by sequence analysis.

For transcription and generation of virus, pRS424DEN1Δ30 was linearized with SacII and used as the template in a transcription reaction with SP6 RNA polymerase as previously described (13). Transcripts were electroporated into LLC-MK2 cells, the generation of virus was confirmed by observation of cytopathic effects and immunofluorescence, and the cultures were harvested on day 14. The recovered recombinant virus was terminally diluted twice and amplified in Vero cells. The genome of the resulting virus, rDEN1mutF, was sequenced to confirm the presence of the mutF mutation. Incidental mutations arising from virus passage in tissue culture were identified in all viruses by sequence analysis and are listed in Table 1.

The replication, immunogenicity, and protective efficacy of rDEN1Δ30 were compared to those of wt parental rDEN1 virus and rDEN1mutF in juvenile rhesus monkeys. Dengue virus-seronegative monkeys were injected subcutaneously with 5.0 log10 PFU of virus. Monkeys were observed daily, and blood was collected on days 0 to 10 and 28 and serum was stored at −70°C. Titers of virus in serum samples were determined by plaque assays in Vero cells as described previously (5). Plaque reduction neutralization titers against wt rDEN1 were determined for the day 28 serum samples by using methods previously described (5). All monkeys were challenged subcutaneously on day 28 with 5.0 log10 PFU of wt rDEN1, and blood was collected for 10 days. Virus titers in postchallenge sera were determined by plaque assays in Vero cells. Monkeys inoculated with wt rDEN1 or rDEN1mutF were viremic for 2 to 3 days, with mean peak titers of 2.1 and 1.4 log10 PFU/ml, respectively (Table 2; Fig. 2). Monkeys inoculated with rDEN1Δ30 were viremic for less than 1 day, with a mean peak titer of 0.8 log10 PFU/ml, a level of replication significantly lower than that of wt rDEN1 (Tukey-Kramer test; P < 0.05), indicating that the Δ30 mutation is capable of attenuating DEN1. Although monkeys inoculated with rDEN1mutF showed a decreased level of viremia compared to those inoculated with wt rDEN1, this difference was not statistically significant. Previously published results for studies with rhesus monkeys have shown a similar level of attenuation for rDEN1mutF (11). In addition, Markoff et al. have shown that there was a difference in the mean number of days of viremia (determined by reverse transcription-PCR) between monkeys that received DEN1mutF virus and those that received wt DEN1 (11). No such difference was observed in the present study.

The neutralizing antibody titer was lower in monkeys inoculated with rDEN1Δ30 than in animals inoculated with either wt rDEN1 or rDEN1mutF (Table 2). This lower immunogenicity of rDEN1Δ30 was consistent with its greater level of attenuation. Importantly, the immunogenicity of rDEN1Δ30 cDNA clone was constructed as previously described (11), and virus was initially recovered from transfected Vero cells. Virus stocks were terminally diluted twice and amplified in Vero cells. The genome of the resulting virus, rDEN1mutF, was sequenced to confirm the presence of the mutF mutation. Incidental mutations arising from virus passage in tissue culture were identified in all viruses by sequence analysis and are listed in Table 1.

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### Table 1. Incidental mutations observed among the recombinant DEN1 viruses

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Gene</th>
<th>Nucleotideb</th>
<th>Aminoacidb</th>
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<tbody>
<tr>
<td>wt rDEN1</td>
<td>prM</td>
<td>C316U</td>
<td>A240V</td>
</tr>
<tr>
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<td>NS4B</td>
<td>U7165G</td>
<td>F2257L</td>
</tr>
<tr>
<td>wt rDEN1</td>
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<td>U7173C</td>
<td>V2360A</td>
</tr>
<tr>
<td>rDEN1mutF</td>
<td>prM</td>
<td>C612U</td>
<td>T1748U</td>
</tr>
<tr>
<td>rDEN1Δ30</td>
<td>E</td>
<td>A1748U</td>
<td>T552S</td>
</tr>
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</table>

*a GenBank accession numbers: wtDEN1, AY145121; rDEN1mutF, AY145122; rDEN1Δ30, AY145123.

*b The wt nucleotide is on the left of the nucleotide number; the mutant nucleotide is on the right.

*c Amino acid residue in DEN1 polyprotein; the predicted amino acid is on the left of residue number; the mutant amino acid residue is on the right.
and rDEN1mutF was sufficient to protect the animals against wt rDEN1 virus challenge. wt rDEN1 virus was not detected in any serum sample collected following virus challenge, and no monkey achieved an increase greater than threefold in the level of serum-neutralizing antibody following virus challenge, indicating that monkeys were completely protected following immunization with either wt rDEN1 or vaccine candidates rDEN1Δ30 and rDEN1mutF. The level of attenuation specified by the Δ30 mutation is comparable in both the DEN1 and DEN4 genetic backgrounds (Fig. 2). However, because vaccine candidate rDEN1Δ30 also contains a missense mutation in the DEN4 30 mutation from background incidental mutations by its introduction into additional vaccine candidates will be necessary to further define its phenotype.

A rodent model for the evaluation of attenuated strains of DEN virus has recently been described (1, 2) and consists of SCID mice bearing intraperitoneal tumors of the human liver cell line HuH-7. Following inoculation of DEN virus directly into the tumor, virus can be detected in the serum. As previously reported, recombinant DEN4 virus replicates to greater than 6.0 log10 PFU/ml of serum in these mice, while the replication of DEN4 virus bearing the Δ30 mutation was reduced by about 10-fold (2). The replication of rDEN1Δ30 was compared to that of wt rDEN1 virus and rDEN1mutF in HuH-7-SCID mice (Table 2). Both rDEN1Δ30 and rDEN1mutF replicated to a level approximately 100-fold less than that of their wt rDEN1 parent. These results further validate the use of the HuH-7-SCID mouse model for the evaluation of attenuated strains of DEN virus, with results correlating closely with those observed with rhesus monkeys.

The DEN4 vaccine candidate bearing the Δ30 mutation was previously shown not to be transmitted to Aedes albopictus mosquitoes fed on 10 vaccinees, all of whom were infected with the vaccine strain (20). Because this lack of transmissibility was attributed to both the low level of viremia in vaccinees and to the restricted capacity of rDEN4∆30 to disseminate from the midgut of the mosquito to its head, a phenotype previously shown to be specified by the Δ30 mutation (20), it was important to evaluate the ability of rDEN1Δ30, rDEN1mutF, and wt virus to infect and replicate in mosquitoes. This was studied by determining the mosquito 50% infectious dose (MID50) of each virus following oral feeding or intrathoracic inoculation.

Maintenance, infection, and detection of viral antigen in A. aegypti, A. albopictus, and Toxorhynchites splendens were conducted as previously described (20). Briefly, A. aegypti and A. albopictus were fed a blood meal containing serial dilutions of

![FIG. 2. (A) Viremia in monkeys inoculated with DEN1 vaccine candidates. Groups of four rhesus monkeys were inoculated subcutaneously with 5.0 log10 PFU of the indicated virus in a 1-ml dose. Serum was collected daily, and virus titers were determined by plaque assays in Vero cells. Mean virus titers are shown for monkeys in each group.](image-url)
The Δ30 and mutF mutations do not decrease injectivity of rDEN1 virus inoculated intrathoracically into T. splendens

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose* (log10 PFU)</th>
<th>No. tested</th>
<th>No. (%) infectedb</th>
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<tbody>
<tr>
<td>wt rDEN1</td>
<td>3.5</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>8</td>
<td>6 (75)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>7</td>
<td>5 (71)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5</td>
<td>3 (60)</td>
</tr>
<tr>
<td>rDEN1mutF</td>
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<td>8</td>
<td>7 (88)</td>
</tr>
<tr>
<td></td>
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<td>5</td>
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<td>0.3</td>
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<td>7 (88)</td>
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</tr>
<tr>
<td></td>
<td>0.7</td>
<td>6</td>
<td>5 (83)</td>
</tr>
</tbody>
</table>

a Virus titer administered intrathoracically in a 0.2-μl inoculum. MID90 (log10 PFU): wt rDEN1, <0.5; rDEN1mutF, <0.3; rDEN1Δ30, <0.7.
b Number of mosquitoes with IFA-detectable antigen in head tissue prepared 21 days after inoculation.

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REFERENCES