Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2

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Abstract

Background: A dengue virus type 2 (DEN-2 Tonga/74) isolated from a 1974 epidemic was characterized by mild illness and belongs to the American genotype of DEN-2 viruses. To prepare a vaccine candidate, a previously described 30 nucleotide deletion (Δ30) in the 3′ untranslated region of DEN-4 has been engineered into the DEN-2 isolate.

Methods: A full-length cDNA clone was generated from the DEN-2 virus and used to produce recombinant DEN-2 (rDEN-2) and rDEN2Δ30. Viruses were evaluated for replication in SCID mice transplanted with human hepatoma cells (SCID-HuH-7 mice), in mosquitoes, and in rhesus monkeys. Neutralizing antibody induction and protective efficacy were also assessed in rhesus monkeys.

Results: The rDEN2Δ30 virus was ten-fold reduced in replication in SCID-HuH-7 mice when compared to the parent virus. The rDEN-2 viruses were not infectious for Aedes mosquitoes, but both readily infected Toxorynchites mosquitoes. In rhesus monkeys, rDEN2Δ30 appeared to be slightly attenuated when compared to the parent virus as measured by duration and peak of viremia and neutralizing antibody induction. A derivative of rDEN2Δ30, designated rDEN2Δ30-4995, was generated by incorporation of a point mutation previously identified in the NS3 gene of DEN-4 and was found to be more attenuated than rDEN2Δ30 in SCID-HuH-7 mice.

Conclusions: The rDEN2Δ30 and rDEN2Δ30-4995 viruses can be considered for evaluation in humans and for inclusion in a tetravalent dengue vaccine.

Background

The increased prevalence of disease caused by the mosquito-borne dengue (DEN) viruses (four serotypes; DEN-1 – DEN-4) has intensified the effort to generate a vaccine that would both confer protection and be economically feasible for use in countries with limited resources for healthcare [1]. Dengue fever and dengue hemorrhagic fever and shock (DHF/DSS) are a severe disease burden for tropical and semitropical countries inhabited by more than 2.5 billion people [2]. Risk factors for the more severe disease, DHF/DSS, include the strain of virus, age and genetic background of the host, and secondary
infection by a DEN serotype different from that which caused the primary infection [2]. Increased risk associated with secondary infection by a different DEN serotype is believed to be caused both by increased virus replication resulting from antibody-dependent enhancement and by augmented immune activation induced by the secondary infection [3,4]. Typically, regions with DHF/DSS have all four DEN serotypes circulating simultaneously, and an effective DEN vaccine must contain a tetravalent formulation that confers protection against each of the four DEN serotypes.

Immunity to the DEN viruses is primarily mediated by neutralizing antibodies directed against the envelope (E) glycoprotein, and most vaccine strategies aim to induce antibody against this major protective antigen. Live attenuated tetravalent vaccines appear to be the best vaccine candidates since they are economical to manufacture and they induce long-term immunity with the live attenuated yellow fever virus vaccine serving as a successful model flavivirus vaccine [5]. Several strategies to produce live attenuated tetravalent vaccines are being pursued including attenuation of viruses by conventional passage in tissue culture or introduction of defined attenuating mutations into recombinant DEN viruses [6-9]. In addition, chimeric dengue viruses are being evaluated that contain the E protein of a DEN virus on a background of either an attenuated DEN virus from a different serotype or a more distantly related, but attenuated, flavivirus [10-12].

We have previously described attenuated and immunogenic monovalent vaccine candidates for DEN-1, DEN-2, DEN-3, and DEN-4 that were generated by two distinct recombinant methodologies. Using the first methodology, nucleotides 10478–10507 were deleted from the 3’ UTR (Δ30) of a wild type cDNA clone for DEN-4 to generate a vaccine candidate, rDEN4Δ30, which is safe, attenuated, and immunogenic in rhesus monkeys and humans [13]. Incorporation of the A30 mutation into an infectious cDNA clone of DEN-1 wild type virus at a site homologous to that in DEN-4 attenuated DEN-1 for rhesus monkeys and is currently being evaluated in humans [14]. The Δ30 mutation did not confer attenuation upon DEN-3 for reasons that have not been defined [15]. Thus, this approach has yielded live attenuated virus vaccine candidates for both DEN-1 and DEN-4. Using a second methodology, antigenic chimeric viruses have been generated by replacing the membrane protein (M) and E structural genes of rDEN4Δ30 with those from DEN-2 or DEN-3 [12,15]. These antigenic chimeric viruses were attenuated and immunogenic in rhesus monkeys and represent vaccine candidates for DEN-2 and DEN-3. We have also described a set of point mutations that can attenuate wild type rDEN-4 for SCID mice transplanted with human liver cells (SCID-HuH-7) or for rhesus monkeys [16,17]. Such mutations identified in rDEN-4 could be introduced into conserved sites of cDNA clones for other DEN serotypes to fine-tune the level of attenuation of vaccine candidates.

We have found it prudent to pursue several strategies to develop a live attenuated virus vaccine for each dengue serotype recognizing that it has been a challenge to achieve a satisfactory balance between attenuation and immunogenicity [15,18-20]. Thus, in addition to the antigenic chimeric DEN-2 vaccine candidate described above, a second approach was pursued in the present study that involved the construction of an infectious cDNA clone of a wild type DEN-2 virus isolated in Tonga [21], and the generation of DEN-2 vaccine candidates by the sequential introduction of defined attenuating mutations into the recombinant version of the DEN-2 Tonga/74 wild type virus. The rDEN2Δ30 vaccine candidate was evaluated for replication in SCID-HuH-7 mice, mosquitoes, and rhesus monkeys. In addition, an attenuating point mutation, previously described in DEN-4, was introduced into the rDEN2Δ30 virus, and this rDEN2Δ30 derivative was characterized in SCID-HuH-7 mice.

**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 (FBS), 1 mM L-glutamine and 0.05 mg/ml gentamicin (Invitrogen). C6/36 cells (Aedes albopictus mosquito cells) were maintained at 32°C in Minimal Essential Medium (MEM) containing Earle's salts and 25 mM HEPES buffer (Invitrogen) and supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (Invitrogen).

A dengue virus type 2 isolate, Tonga/74, was provided by Dr. Duane Gubler (CDC, Fort Collins, CO). The virus was isolated during a 1974 dengue outbreak in the South Pacific island of Tonga [21]. The virus was isolated by inoculation of patient sera into Aedes albopictus mosquitoes, and subsequent passage in C6/36 cells before determination of genomic sequence.

**Sequence analysis**

Viral RNA was isolated from DEN-2 Tonga/74 wild type virus using the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using random hexamer primers and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Overlapping PCR fragments of approximately 2000 base pairs were generated using DEN-2 specific primers and Advantage
cDNA polymerase (Clontech, Palo Alto, CA). Both strands of the resulting PCR fragments were sequenced directly on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using DEN-2 specific primers in BigDye terminator cycle sequencing reactions (Applied Biosystems) and the results were assembled into a consensus sequence. To determine the nucleotide sequence of the genomic 5' and 3' regions, the 5' cap nucleoside of the viral genome was removed with tobacco acid pyrophosphatase (Epicentre Technologies, Madison, WI), followed by circularization of the genome using T4 RNA ligase (Epicentre Technologies). An RT-PCR fragment spanning the ligation junction was generated and sequenced using DEN-2 primers. For the DEN-2 Tonga/74 consensus sequence, GenBank accession number AY744147 was assigned.

Genetic construction of rDEN-2 Tonga/74 cDNA clone

cDNA fragments of DEN-2 Tonga/74 were generated by reverse-transcription of the genome as indicated in Figure 1. Each fragment was subcloned into a plasmid vector and sequenced to verify that it matched the consensus sequence as determined for the virus. This yielded seven cloned cDNA fragments spanning the genome. Cloned fragments were modified as follows: Fragment X, representing the 5' end of the genome was abutted to the SP6 promoter; Fragment I, was modified to contain a SpeI restriction site at genomic nucleotide 2353; Fragment R was modified to contain a Spel restriction site also at genomic nucleotide 2353, and, to stabilize the eventual full-length clone, two additional mutations at nucleotides 2362 – 2364 and 2397 were created to ensure that translation stop codons were present in all reading frames other than that used to synthesize the virus polyprotein; Fragment A was modified at nucleotide 3582 to ablate a naturally occurring SpeI restriction site and at nucleotide 4497 to ablate a naturally occurring KpnI restriction site; Fragment C was modified at nucleotide 9374 to ablate a naturally occurring KpnI restriction site; and Fragment Y, representing the 3' end of the genome was abutted to a KpnI restriction site. All mutations introduced into the cloned cDNA fragments were translationally-silent, thereby preserving the wild-type polyprotein sequence. Each fragment was added incrementally between the Ascl and KpnI restriction sites of DEN-4 cDNA clone p4 (GenBank accession number: AY648301) to generate a full-length DEN-2 cDNA clone (p2) with the same vector background successfully used to generate rDEN-4 and rDEN4A30 virus [13]. cDNA clone p2 was sequenced to confirm that the virus genome region matched the DEN-2 Tonga/74 consensus amino acid and nucleotide sequence, with the exception of the translationally-silent modifications noted above. The ∆30 mutation which removes nucleotides 10541–10570 was introduced into Fragment Y to generate Fragment YA30. To create p2∆30, the Fragment Y region of p2 was replaced with Fragment YA30 (Figure 1). The genomic region of each full-length cDNA was sequenced as described above and GenBank accessions were assigned as follows (cDNA clone: accession numbers): p2: AY744148, p2∆30: AY744149.

Using site-directed mutagenesis, an attenuating amino acid change characterized in the NS3 gene of DEN-4 (nt 4995–7; a.a. 158, Ser→Leu) was introduced into the p2∆30 cDNA clone [17]. A mutagenic oligonucleotide was designed to change DEN-2 NS3 amino acid 158 from Ser (AGT) to Leu (CTA) and used to construct the cDNA clone, p2∆30-4995 (accession number: AY744150), which was sequenced for confirmation of nucleotide changes.

Recovery of rDEN-2 viruses

cDNA clones were linearized with Acc65I (isoschizomer of KpnI which cleaves leaving only a single 3' nucleotide) and were transcribed in vitro using the AmpliCap SP6 Message Maker kit (Epicentre Technologies, Madison, WI). Purified transcripts were then transfected into Vero or C6/36 cells. Viruses recovered in C6/36 cells were passaged 3 times in Vero cells, and all viruses were biologically cloned by terminal dilution in Vero cells. The genomes of recombinant viruses used to infect rhesus monkeys were completely sequenced as described above to identify adventitious mutations that had accumulated during transfection and biological cloning.

Replication in SCID-HuH-7 mice

Four to six week-old SCID mice (Taconic, Germantown, NY) were injected intraperitoneally with 10^7 HuH-7 cells suspended in 0.2 ml phosphate-buffered saline. Tumors were detected in the peritoneum, and mice were infected by direct inoculation of the tumor with 10^4 PFU of virus in 0.05 ml Opti-MEM (Invitrogen). On day 7 post-infection, serum was obtained from cardiac blood and stored at -70°C. Virus titer in serum samples was determined by plaque assay in Vero cells.

Replication, immunogenicity, and protection in rhesus monkeys

The DEN-2 viruses were evaluated in rhesus macaques using established methods [13]. DEN virus sero-negative monkeys were injected subcutaneously with 10^5 PFU virus diluted in L-15 medium (Invitrogen) or with a mock inoculum. Serum was collected on days 0–6, 8, 10, 12 and 28 after inoculation and stored at -70°C. Virus titer was determined for each serum sample by plaque assay in Vero cells and serum neutralizing antibody titer was determined for serum from days 0 and 28 by plaque reduction neutralization test. On day 28, monkeys were challenged with 10^5 PFU of DEN-2 Tonga/74, and serum was
collected on days 29–34, 36, and 56. Virus titer was determined for serum from days 28–34 and 36 and serum neutralizing antibody titer was determined for serum from day 56.

**Virus replication in mosquitoes**
Replication in *Aedes aegypti* and *Toxorynchites amboinensis* mosquitoes was evaluated as previously described [22]. Briefly, *A. aegypti* were fed blood meals containing serial 10-fold dilutions of virus. After 21 days, viral antigen was detected in head and midgut preparations by immunofluorescence assay using DEN-2-specific hyperimmune mouse ascitic fluid and fluorescein isothiocyanate conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD), and the mosquito infectious dose-50% (MID50) was determined. *T. amboinensis* were inoculated intrathoracically with a 0.2 ul dose containing serial ten-fold dilutions of virus and incubated for 14 days. Head preparations were made and antigen visualized as described above.

**Results**
**Generation and sequence analysis of recombinant DEN-2 Tonga/74 viruses**
A full-length cDNA clone, p2, was constructed that matched the genomic consensus sequence of the American genotype DEN-2 isolate, Tonga/74, with the exception...
of translationally-silent modifications made to facilitate cloning (Figure 1). The previously described Δ30 deletion mutation was incorporated into the p2 cDNA clone to form p2Δ30 [13]. The rDEN-2 virus was recovered in C6/36 and Vero cells, but the presence of the Δ30 mutation limited recovery to only C6/36 cells. After passage in Vero cells, adaptation mutations were identified by sequence analysis as had been described for other DEN viruses [23]. Both rDEN-2 and rDEN2Δ30 viruses accumulated a single nucleotide change in NS4B at nt 7169 encoding a Val→Ala change at amino acid position 115 as has been observed for rDEN-3 (Table 1) [15]. The same nucleotide change was previously reported to occur at the homologous site following passage of DEN-4 in Vero cells resulting in a Leu→Ser change (Table 1) [23]. Inclusion of the 7169 mutation into the p2Δ30 cDNA permitted recovery in both C6/36 and Vero cells (data not shown). The rDEN2Δ30 virus reached a virus titer of 6.6 log 10PFU/ml in Vero cells.

**Replication of rDEN-2 viruses in SCID-HuH-7 mice**

As an initial evaluation of replication of the DEN-2 Tonga/74 virus and the rDEN-2 viruses, replication in SCID mice transplanted with HuH-7 human hepatoma cells (SCID-HuH-7 mice) was tested. Wild-type viruses from each DEN serotype have been shown to replicate to approximately 6.0 log 10PFU/ml serum in SCID-HuH-7 mice, and an att phenotype in SCID-HuH-7 mice has been shown to be a predictor of reduced replication in rhesus monkeys [12,14,15,17]. The parent DEN-2 Tonga/74 virus replicated efficiently in SCID-HuH-7 mice and reached a mean titer in serum of 5.9 log 10PFU/ml (Table 2) similar to that previously observed with the DEN-2 New Guinea C (NGC) prototype strain [12]. The rDEN-2 virus replicated to the same level as the wild-type isolate, while rDEN2Δ30 was 10-fold restricted in replication. This reduction was statistically significant (Tukey-Kramer post-hoc test; P < 0.05), and was similar to that observed for the well-characterized rDEN4Δ30 virus [17].

### Table 1: Missense and UTR mutations which arose spontaneously in rDEN-2 and rDEN2Δ30 viruses during passage in Vero cells.

| Virus   | Gene     | Nucleotide position | Nucleotide substitution | Amino acid position<sup>a</sup> | Amino acid change |
|---------|----------|---------------------|-------------------------|---------------------------------|------------------|
| rDEN-2  | NS4B     | 7169<sup>b</sup>    | U > C                   | 115                             | Val > Ala        |
|         | NS5      | 9248                | A > C                   | 560                             | Glu > Ala        |
| rDEN2Δ30| NS4B     | 7169<sup>b</sup>    | U > C                   | 115                             | Val > Ala        |
| rDEN2Δ30-4995| NS3  | 4949                | A > G                   | 143                             | Lys > Arg        |
|         | NS4B     | 7169<sup>b</sup>    | U > C                   | 115                             | Val > Ala        |
|         | 3’ UTR   | 10322               | G > A                   | --                              | --               |

<sup>a</sup> Amino acid position in the individual DEN-2 protein.

<sup>b</sup> Nucleotide position 7169 in DEN-2 corresponds to nt 7162 in rDEN-4, and the wild-type amino acid is Val and Leu, respectively [23].

### Table 2: Addition of the Δ30 mutation to rDEN-2 decreases replication in SCID-HuH-7 mice by 10-fold.

| Virus         | No. of mice | Mean virus titer (log<sub>10</sub>PFU/ml ± SE) |
|---------------|-------------|-----------------------------------------------|
| DEN-2 Tonga/74| 6           | 5.9 ± 0.3                                      |
| rDEN-2        | 7           | 5.9 ± 0.2                                      |
| rDEN2Δ30      | 9           | 4.9 ± 0.2                                      |

<sup>a</sup> Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10<sup>4</sup> PFU virus. Serum was collected on day 7 and titered in Vero cells.

#### Replication of rDEN-2 viruses in mosquitoes

The DEN-2 viruses were evaluated for infectivity of *Aedes aegypti* fed on an infectious bloodmeal (oral infectivity only) and for *Toxorynchites amboinensis* inoculated intrathoracically (Table 3). At the doses tested neither DEN-2, rDEN-2, or rDEN2Δ30 were detected in the midgut or head of *A. aegypti* mosquitoes which had fed on an infectious bloodmeal 21 days earlier. The inability to infect the midgut led to a lack of infection in the head tissue. This indicates that the DEN-2 Tonga/74 viruses are poorly infectious for *A. aegypti* mosquitoes by oral infectivity, as has been demonstrated for multiple DEN-2 viruses of the American genotype [24,25]. In contrast the DEN-2 NGC prototype strain, an Asian genotype member, was highly infectious in *A. aegypti* mosquitoes when tested previously but it was not included here as a concurrent control [12].

The defect in rDEN-2 infectivity for *A. aegypti* was further investigated by directly inoculating the same virus stocks intrathoracically into *T. amboinensis* and measuring the ability of the viruses to infect the head tissues. Both rDEN-2 and rDEN2Δ30 were highly infectious by intrathoracic inoculation (Table 3). The Δ30 mutation did not alter the infectivity of rDEN-2 following intrathoracic inoculation,
a property also previously observed for DEN-1, -3 and -4 [14,15,22]. These results indicate that the lack of infectivity for A. aegypti was likely caused by the inability of the DEN-2 Tonga/74 viruses to establish a midgut infection and that the viruses retained the ability to infect head tissues.

**Replication, immunogenicity, and protective efficacy in rhesus monkeys**

The replication (viremia), immunogenicity, and protective efficacy of the DEN-2 viruses in monkeys were studied. Monkeys inoculated with the DEN-2 Tonga/74 wild-type isolate were viremic for an average of 4.5 days with a mean peak titer of 2.1 log10PFU/ml (Table 4). Inoculation with rDEN-2 resulted in detectable viremia for 4.0 days with a mean peak titer of 1.9 log10PFU/ml. While the levels of rDEN2Δ30 replication (2.8 days viremia; mean peak titer of 1.7 log10PFU/ml) were lower than DEN-2 and rDEN-2, the differences were not as dramatic as had been observed for rDEN1Δ30 and rDEN4Δ30 when compared to their parent viruses [13,14]. The level of neutralizing antibodies induced by the rDEN2Δ30 virus was also less than that induced by the wild-type DEN-2 viruses, a finding consistent with the decreased replication exhibited by this vaccine candidate. Therefore, by three quantitative measures, duration and peak titer of viremia and the level of neutralizing antibodies induced, rDEN2Δ30 appeared to be attenuated when compared to DEN-2 Tonga/74. When vaccinated monkeys were challenged with DEN-2 Tonga/74, all monkeys were protected, as indicated by the lack of viremia (Table 4).

The 4995 mutation further attenuates rDEN2Δ30 in SCID-HuH-7 mice

Based on the limited attenuation conferred upon rDEN-2 by the Δ30 mutation in rhesus monkeys, we sought to construct a further attenuated derivative of rDEN2Δ30. To further attenuate rDEN2Δ30, an att mutation that has been characterized in another DEN serotype was imported into a homologous region in DEN-2. One such mutation, the 4995 mutation in DEN-4 NS3 at amino acid 158 (Ser→Leu), was previously incorporated into the DEN-4 vaccine candidate, rDEN4Δ30, and found to further attenuate the virus for SCID-HuH-7 mice and rhesus monkeys [17]. Site directed mutagenesis was used to introduce a Ser→Leu mutation at amino acid 158 of NS3 in rDEN2Δ30-7169, and the rDEN2Δ30-4995 virus was recovered in C6/36 cells and propagated in Vero cells reaching a virus titer of 6.2 log10PFU/ml (Table 1). Importantly, the resulting Leu codon would require two nucleotide changes to revert to one of the six codons encoding a Ser residue.

Replication in the SCID-HuH-7 mouse model was used as an initial assessment of the rDEN2Δ30-4995 virus phenotype. Table 5 includes results from three separate experi-
ments (including those from Table 1) and confirms the approximate 10-fold reduction in replication conferred by the Δ30 mutation upon rDEN-2 replication in SCID-HuH-7 mice. The rDEN2Δ30-4995 virus had a mean peak virus titer of 4.6 log10 PFU/ml which was only a modest reduction from that of rDEN2Δ30, 5.2 log10 PFU/ml. However, comparison of a large number of samples indicated that the reduction in virus titer conferred by the NS3 4995 mutation upon rDEN2Δ30 was statistically significant (rDEN2Δ30-4995 versus rDEN2Δ30; Tukey-Kramer post-hoc test; P < 0.05). The virus titer of rDEN2Δ30-4995 virus in SCID-HuH-7 mice was over 60-fold reduced from that of the rDEN2 parent virus.

Discussion

Development of a live-attenuated tetravalent dengue vaccine has been complicated by two major factors. First, monovalent vaccine candidates that exhibit a satisfactory balance between attenuation and immunogenicity have been difficult to identify [15,18-20]. Second, satisfactorily attenuated tetravalent vaccine formulations that induce a broad neutralizing antibody response against each of the four DEN serotypes have been difficult to develop [6,10,20,26]. For these reasons, we have sought to develop multiple vaccine candidates for each DEN serotype to increase the likelihood that a vaccine with a satisfactory balance between attenuation and immunogenicity will be identified. To produce a live-attenuated DEN-2 vaccine candidate, we previously generated an antigenic chimeric virus, rDEN2/4Δ30, expressing the M and E structural genes of the DEN-2 NGC strain on the attenuated rDEN4Δ30 background [12]. The vaccine candidates described in the present study, rDEN2Δ30 and rDEN2Δ30-4995, could serve as alternates to this antigenic chimeric virus if evaluation of the rDEN2/4Δ30 virus in humans, either as a monovalent vaccine or as a component of a tetravalent vaccine, indicates that it lacks a balance between attenuation and immunogenicity.

It was hoped that each of the four components of a tetravalent vaccine, consisting of DEN-1, -2, -3, and -4 wild type viruses, each with the common 30 nucleotide deletion mutation in the 3’ UTR, would exhibit a similar level of attenuation in animal models [13-15]. Unfortunately, the level of attenuation conferred by the Δ30 mutation upon each of the four serotypes has proven to be variable. In rhesus monkeys, the rDEN2Δ30 virus appears to have an intermediate attenuation phenotype in between that of the attenuated rDEN1Δ30 and rDEN4Δ30 and the non-attenuated rDEN3Δ30 [13-15]. Although rDEN2Δ30 was

Table 4: Replication and immunogenicity of DEN-2 viruses in rhesus monkeys

| Virusa | % with viremia | Mean no. of viremic days per monkeyb | Mean peak virus titer (log10PFU/ml ± SE) | Geometric mean serum neutralizing antibody titer on day 28 (reciprocal dilution)c | Virus replication after challengedd |
|--------|----------------|-------------------------------------|----------------------------------------|-----------------------------------------|----------------------------------|
|        | % with viremia | Mean no. of viremic days per monkeyb | Mean peak virus titer (log10PFU/ml ± SE) | Geometric mean serum neutralizing antibody titer on day 28 (reciprocal dilution)c | % with viremia | Mean peak virus titer (log10PFU/ml ± SE) |
| Mock   | 0              | 0                                   | <0.7                                   | <10                                    | 100                             | 2.1 ± 0.1 |
| DEN-2 Tonga/74 | 100          | 4.5                                 | 2.1 ± 0.3                              | 311                                    | 0                              | <0.7     |
| rDEN-2     | 100          | 4.0                                 | 1.9 ± 0.1                              | 173                                    | 0                              | <0.7     |
| rDEN2Δ30   | 100          | 2.8                                 | 1.7 ± 0.2                              | 91                                     | 0                              | <0.7     |

| Virusa | % with viremia | Mean no. of viremic days per monkeyb | Mean peak virus titer (log10PFU/ml ± SE) | Geometric mean serum neutralizing antibody titer on day 28 (reciprocal dilution)c | Virus replication after challengedd |
|--------|----------------|-------------------------------------|----------------------------------------|-----------------------------------------|----------------------------------|
| Mock   | 0              | 0                                   | <0.7                                   | <10                                    | 100                             | 2.1 ± 0.1 |
| DEN-2 Tonga/74 | 100          | 4.5                                 | 2.1 ± 0.3                              | 311                                    | 0                              | <0.7     |
| rDEN-2     | 100          | 4.0                                 | 1.9 ± 0.1                              | 173                                    | 0                              | <0.7     |
| rDEN2Δ30   | 100          | 2.8                                 | 1.7 ± 0.2                              | 91                                     | 0                              | <0.7     |

a Groups of rhesus monkeys (mock: n = 2; viruses: n = 4) were inoculated subcutaneously with 10⁵ PFU of the indicated virus in a 1 ml dose. Serum was collected daily for day 0 to 6; day 8, 10, 12 and 28.
b Virus titer in serum was determined by plaque assay in Vero cells. The lower limit of detection was 0.7 log10 PFU/ml. Viremia was not detected in any monkey after day 6.
c Plaque reduction (60%) neutralizing antibody titers were determined using DEN-2 Tonga/74. Limit of detection was < 1:10. All monkeys inoculated with virus had a four-fold or greater increase in neutralizing antibody titer.
d Vaccinated rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of DEN-2 Tonga/74 virus in a 1 ml dose. Serum was collected daily for day 0 to 8.

Table 5: Addition of the 4995 mutation further attenuates rDEN2Δ30 in SCID-HuH-7 mice.

| Virus | No. of mice | Mean virus titer (log10PFU/ml ± SE) |
|-------|-------------|-------------------------------------|
| DEN-2 Tonga/74 | 6   | 5.9 ± 0.3 |
| rDEN-2     | 15          | 6.4 ± 0.2 |
| rDEN2Δ30   | 22          | 5.2 ± 0.1 |
| rDEN2Δ30-4995 | 28         | 4.6 ± 0.2 |

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| rDEN-2     | 15          | 6.4 ± 0.2 |
| rDEN2Δ30   | 22          | 5.2 ± 0.1 |
| rDEN2Δ30-4995 | 28         | 4.6 ± 0.2 |

a Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10⁴ PFU virus. Serum was collected on day 7 and titered in Vero cells. Data is included from three separate experiments.
slightly attenuated compared to its DEN-2 parent virus in rhesus monkeys, the reduction in replication was less than that of rDEN1A30 and rDEN4A30. While the latter two viruses had detectable viremia in only 50% of monkeys, a mean number of viremic days of less than one day, and a mean peak viremia of less than 1.0 log10PFU/ml [13,14], the rDEN2A30 virus infected 100% of the rhesus monkeys and reached a peak virus titer of 1.7 log10PFU/ml. However, the 10-fold reduction of replication of rDEN4A30 and rDEN2A30 in SCID-HuH-7 mice, compared to that of their respective wild type parents, was similar. To date, rDEN4A30 is the only A30 vaccine candidate that has been tested in humans, and it was found to be both safe and immunogenic [13]. If the level of attenuation in SCID-HuH-7 mice serves as a better guide to attenuation in humans, rDEN2A30 might be satisfactorily attenuated in humans since its level of attenuation for SCID-HuH-7 mice and that of the rDEN4A30 vaccine candidate are comparable.

To construct a further attenuated derivative of rDEN2A30, the 4995 mutation present in the NS3 gene of DEN-4 at amino acid 158 (Ser→Leu) was introduced into the homologous region of the NS3 protein of rDEN2A30 [17]. Although the 4995 mutation results in a single amino acid change and thus may be susceptible to rever- sion, the mutant leucine codon selected for insertion into rDEN2A30-4995 would require two nucleotide changes to revert to a serine codon. Introduction of the 4995 mutation into rDEN4A30 resulted in a 100-fold greater reduction of replication in SCID-HuH-7 mice [17]. In rDEN2A30, its introduction resulted in nearly a 10-fold reduction in virus titer, a smaller but still statistically significant reduction. These results provide a second example of the difficulty in predicting the precise level of attenuation following import of an attenuating mutation into a different DEN serotype. Nevertheless, the rDEN2A30-4995 vaccine candidate is more attenuated than its rDEN2A30 parent and warrants evaluation in rhesus monkeys and humans.

Epidemiologic and molecular pathogenesis studies of DEN-2 strains support the concept that the DEN-2 Tonga/74 virus, from which the vaccine candidates were derived, may naturally have a lower level of virulence than other DEN-2 viruses. If the DEN-2 Tonga/74 parent virus is naturally attenuated to some degree, only a small incremental increase in attenuation might be required to satisfactorily attenuate it for humans. Gubler et al. investigated the 1974 outbreak of DEN-2 infection in the Pacific island of Tonga [21]. In comparison to a subsequent DEN-1 outbreak, the 1974 DEN-2 outbreak was distinguished by mild disease with few hemorrhagic sequelae, low viremia, and an overall slow spread of virus infection [21]. The weak DEN-2 outbreak was proposed to be a result of the circulation of a strain with an inherently low level of virulence [21].

Since the Tonga/74 outbreak, additional evidence has emerged that supports the suggestion that there are at least two circulating lineages of DEN-2 viruses that differ in virulence [27-29]. The DEN-2 Tonga/74 virus is a member of the DEN-2 American genotype, which as a group appears to possess lower virulence than that of the Asian genotype of DEN-2 viruses [28,29]. Despite the presence of the American DEN-2 genotype viruses and limited co-circulation of DEN-1 and DEN-3 viruses in the Americas in the 1960s and 1970s, the first major epidemic of DHF/DSS in the Americas occurred only after the introduction of a DEN-2 Asian genotype virus in 1981 [27-30]. It was thought that genetic differences might have contributed to this difference in virulence and evidence to this effect has been forthcoming. Rico-Hesse and colleagues have defined genetic elements within the genome of DEN-2 American genotype viruses which distinguish them from members of the Asian genotype [31]. In addition, using chimeric rDEN-2 American/Asian viruses, introduction of three genetic elements (a point mutation in the E gene, the 5′ UTR, and the 3′ UTR) of the American genotype was found to confer reduced virus replication in dendritic cells and monocytes upon an Asian genotype rDEN-2 [32]. The Tonga/74 virus shares each of these three attenuating genetic determinants specific to the American genotype [31,32], which provides a possible explanation for its lower virulence in humans. The rDEN2A30 vaccine candidate, whose parent is the DEN-2 Tonga/74 American genotype virus, thus contains naturally occurring and experimentally introduced attenuating mutations. Thus, the small incremental increase in attenuation provided by the A30, with or without the 4995 mutation, might prove to satisfactorily attenuate the DEN-2 Tonga/74 for humans.

The American genotype DEN-2 viruses exhibit decreased infectivity for Aedes mosquitoes in comparison to Asian DEN-2 viruses [24,25]. Consistent with these observations, the wild type New Guinea C Asian DEN2 virus was highly infectious for Aedes mosquitoes in our laboratory [12] whereas the Tonga/74 American genotype virus was poorly infectious by the oral route (present study). In fact, the increased prevalence of DEN-2 viruses of the Asian genotype in the Americas has been suggested to be a result of their enhanced transmission [28]. However, the active circulation of American genotype viruses over many decades indicates that mosquito transmission does occur and large epidemics have been associated with viruses of this genotype [21,27]. At the doses tested, neither the DEN-2 Tonga/74 isolate nor the recombinant viruses were found to infect the midgut or head of Aedes aegypti mosquitoes fed an infectious blood meal. Since rDEN-2 and
rDEN2A30 viruses were infectious by intrathoracic inoculation of *Toxorhynchites* mosquitoes, the lack of infectivity for *A. aegypti* was likely caused solely by the inability of the DEN-2 Tonga/74 viruses to establish a midgut infection. Decreased infectivity for *Aedes* mosquitoes could serve to help limit transmission of the vaccine virus.

**Conclusions**

The live-attenuated DEN-2 virus candidates described here, rDEN2A30 and rDEN2A30-4995, have several properties desired in a live attenuated vaccine virus for humans. First, both viruses reached a titer over 6.0 log\(_{10}\)PFU/ml in Vero cells that would permit economical manufacture. Second, the viruses are derived from the DEN-2 Tonga/74 strain, a member of the American genotype, which has been associated with decreased virulence. Third, rDEN2A30 was attenuated for replication in SCID-HuH-7 mice and slightly attenuated for rhesus monkeys while inducing a protective neutralizing antibody response. Fourth, rDEN2A30-4995 was more attenuated in SCID-HuH-7 mice than rDEN2A30. Fifth, the DEN-2 Tonga/74 strain, like other members of the American genotype, is poorly infectious for *Aedes aegypti* mosquitoes which would help to limit uncontrolled transmission of the vaccine virus.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

J.B. recovered viruses, conducted animal studies, and drafted the manuscript. C.H. and S.W. constructed the DEN-2 cDNA clone and C.H. performed sequencing. K.H. performed mosquito studies. B.M. and S.W. supervised the study and participated in planning and design. All authors read and approved the manuscript.

**References**

1. Almond J, Clemens J, Engers H, Halstead S, Khiem H, Pablos-Mendez A, Pereskov Y, Tram T: Accelerating the development and introduction of a dengue vaccine for poor children. 5-8 December 2001, Ho Chi Minh City, VietNam. Vaccine 2002, 20:3043-3046.
2. Gubler D: Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 1998, 11:480-496.
3. Rothman AL, Ennis FA: Immunopathogenesis of dengue hemorrhagic fever. Virology 1999, 257:1-6.
4. Morens DM: Antibody-dependent enhancement of infection and the pathogenesis of viral disease. Clin Infect Dis 1994, 19:500-512.
5. Monath TP: Yellow fever. Vaccines 3rd edition. Edited by: Plotkin S and Orenstein W A. Philadelphia, W.B. Saunders Co.; 1999:815-879.
6. Sun W, Edelman R, Kanasa-Thanan N, Eckels KH, Putnak JR, King AD, Hoang HS, Tang D, Scherer JM, Hoke C H, Jr., Innis BL: Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. Am J Trop Med Hyg 2003, 69:24-31.
7. Blaney J, Jr., Johnson DH, Firestone CY, Hanson CT, Murphy BR, Whitehead SS: Chemical mutagenesis of dengue virus type 4 yields mutant viruses which are temperature sensitive in vero cells or human liver cells and attenuated in mice. J Virol 2001, 75:9731-9740.
8. Markoff L, Pang X, Hough HS, Falgout B, Olsen R, Jones E, Polo S: Derivation and characterization of a dengue type 1 host range-restricted mutant virus that is attenuated and highly immunogenic in monkeys. J Virol 2002, 76:3318-3328.
9. Bhamarapravati N, Sutee Y: Live attenuated tetravalent dengue vaccine. Vaccine 2000, 18 Suppl:34-47.
10. Guirakho F, Punugachev K, Arroyo J, Miller C, Zhang ZX, Wetzlin R, Georgkopoulos K, Catalan J, Ocran S, Draper K, Monath TP: Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 2002, 298:146-159.
11. Huang CY, Butrapet S, Tsuchiya KR, Bhamarapravati N, Gubler DJ, Kinney RM: Dengue 2 PDK-53 Virus as a Chimeric Carrier for Tetravalent Dengue Vaccine Development. J Virol 2003, 77:1436-1447.
12. Whitehead SS, Hanley KA, Blaney JE, Gilmore LE, Elkins WR, Murphy BR: Substitution of the structural genes of dengue virus type 4 with those of type 2 results in chimeric vaccine candidates which are attenuated for *Aedes* mosquitoes, mice, and rhesus monkeys. Vaccine 2003, 21:4307-4316.
13. Durbin AP, Karron RA, Sun W, Vaughn DW, Reynolds MJ, Perreault Jr, Thumar B, Men R, Lai CJ, Elkins WR, Chanock RM, Murphy BR, Whitehead SS: Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30-nucleotide deletion in its 3'-untranslated region. Am J Trop Med Hyg 2001, 65:405-413.
14. Blaney J, Blaney KA, Hanley J, Markoff L, Murphy BR: 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. J Virol 2003, 77:1653-1657.
15. Blaney J, Jr., Hanson CT, Firestone CY, Hanley KA, Murphy BR, Whitehead SS: Genetically modified, live attenuated dengue virus type 3 vaccine candidates. Am J Trop Med Hyg 2004, in press.
16. Blaney J, Jr., Johnson DH, Maniopon GG, Firestone CY, Hanson CT, Murphy BR, Whitehead SS: Genetic basis of attenuation of dengue virus type 3 small plaque mutants with restricted replication in suckling mice and in SCID mice transplanted with human liver cells. Virology 2002, 300:125-139.
17. Hanley KA, Manlucu LR, Maniopon GG, Hanson CT, Whitehead SS, Blaney J, Jr.: Introduction of mutations into the nonstructural genes or 3' untranslated region of an attenuated dengue virus type 4 vaccine candidate further decreases replication in rhesus monkeys while retaining protective immunity. Vaccine 2004, 22:3440-3448.
18. Eckels KH, Dubois DR, Putnak R, Vaughn DW, Innis BL, Henchal EA, Hoke C H, Jr.: Modification of dengue virus strains by passage in primary dog kidney cells: preparation of candidate vaccine candidates and immunization of monkeys. Am J Trop Med Hyg 2003, 69:12-16.
19. Kanasa-Thanan N, Edelman R, Tacket CO, Wasserman SS, Vaughn DW, Coster TS, Kim-Ahn GJ, Dubois DR, Putnak JR, King A, Summers PL, Innis BL, Eckels KH, Hoke C H, Jr.: Phase 1 studies of Walter Reed Army Institute of Research candidate attenuated dengue virus vaccines: selection of safe and immunogenic monovalent vaccines. Am J Trop Med Hyg 2003, 69:17-23.
20. Kanasa-Thanan N, Sun W, Gubler DJ, Van Albert S, Putnak JR, King AD, Raengsakulsrach B, Christ-Schmidt H, Giltos K, Zahradnik JM, Vaughn DW, Innis BL, Saluzzo JF, Hoke Jr CH: Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. Vaccine 2001, 19:3179-3188.
21. Gubler DJ, Reed D, Rosen L, Hitchcock J, Jr.: Epidemiologic, clinical, and virologic observations on dengue in the Kingdom of Tonga. Am J Trop Med Hyg 1978, 27:581-589.
22. Troyer JM, Hanley KA, Whitehead SS, Strickman D, Karron RA, Durbin AP, Murphy BR: A live attenuated recombinant dengue 4 virus vaccine candidate with restricted capacity for dissemination in mosquitoes and lack of transmission from vaccinees to mosquitoes. Am J Trop Med Hyg 2001, 65:414-419.
23. Blaney JE, Maniopon GG, Firestone CY, Johnson DH, Hanson CT, Murphy BR, Whitehead SS: Mutations which enhance the replica-
tion of dengue virus type 4 and an antigenic chimeric dengue virus type 2/4 vaccine candidate in Vero cells. Vaccine 2003, 21:4317-4327.

24. Armstrong PM, Rico-Hesse R: Differential susceptibility of Aedes aegypti to infection by the American and Southeast Asian genotypes of dengue type 2 virus. Vector Borne Zoonotic Dis 2001, 1:159-168.

25. Armstrong PM, Rico-Hesse R: Efficiency of dengue serotype 2 virus strains to infect and disseminate in Aedes aegypti. Am J Trop Med Hyg 2003, 68:539-544.

26. Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, Sirivichayakul C, Pongsaa K, Poijaroen-Anant C, Chokejindachai W, Jagudee A, Saluzzo JF, Bhamarapravati N: Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. Am J Trop Med Hyg 2002, 66:264-272.

27. Watts DM, Porter KR, Puvatana P, Vasquez B, Calampa C, Hayes CG, Halstead SB: Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever [see comments]. Lancet 1999, 354:1431-1434.

28. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RM, da Rosa AT: Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 1997, 230:244-251.

29. Rico-Hesse R: Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology 1990, 174:479-493.

30. Gubler DJ, Clark GG: Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis 1995, 1:55-57.

31. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, de Chacon, Ramos C, Rico-Hesse R: Dengue virus structural differences that correlate with pathogenesis. J Virol 1999, 73:4738-4747.

32. Cologna R, Rico-Hesse R: American genotype structures decrease dengue virus output from human monocytes and dendritic cells. J Virol 2003, 77:3929-3938.

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