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Preclinical development of a dengue tetravalent recombinant subunit vaccine: Immunogenicity and protective efficacy in nonhuman primates

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ABSTRACT

We describe here the preclinical development of a dengue vaccine composed of recombinant subunit carboxy-truncated envelope (E) proteins (DEN-80E) for each of the four dengue serotypes. Immunogenicity and protective efficacy studies in Rhesus monkeys were conducted to evaluate monovalent and tetravalent DEN-80E vaccines formulated with ISCOMATRIX™ adjuvant. Three different doses and two dosing regimens (0, 1, 2 months and 0, 1, 2, and 6 months) were evaluated in these studies. We first evaluated monomeric (DEN4-80E) and dimeric (DEN4-80EZip) versions of DEN4-80E, the latter generated in an attempt to improve immunogenicity. The two antigens, evaluated at 6, 20 and 100 μg/dose formulated with ISCOMATRIX™ adjuvant, were equally immunogenic. A group immunized with 20 μg DEN4-80E and Alhydrogel™ induced much weaker responses. When challenged with wild-type dengue type 4 virus, all animals in the 6 and 20 μg groups and all but one in the DEN4-80EZip 100 μg group were protected from viremia. Two out of three monkeys in the Alhydrogel™ group had breakthrough viremia. A similar study was conducted to evaluate tetravalent formulations at low (3, 3, 3, 6 μg of DEN1-80E, DEN2-80E, DEN3-80E and DEN4-80E respectively), medium (10, 10, 10, 20 μg) and high (50, 50, 50, 100 μg) doses. All doses were comparably immunogenic and induced high titer, balanced neutralizing antibodies against all four DENV. Upon challenge with the four wild-type DENV, all animals in the low and medium dose groups were protected against viremia while two animals in the high-dose group exhibited breakthrough viremia. Our studies also indicated that a 0, 1, 2 and 6 month vaccination schedule is superior to the 0, 1, and 2 month schedule in terms of durability. Overall, the subunit vaccine was demonstrated to induce strong neutralization titers resulting in protection against viremia following challenge even 8-12 months after the last vaccine dose.

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1. Introduction

Dengue is the most important mosquito-borne viral illnesses of the tropics and the subtropics, with significant morbidity and mortality [1–4]. Recent studies suggest that an estimated 4 billion people around the globe are at risk of infection with approximately 390 million infections occurring worldwide annually, of which there are about 96 million symptomatic dengue cases [5,6]. Though mainly a disease of the tropics and subtropics, dengue is rapidly becoming a global public health concern due to the spread of the mosquito vector, increased urbanization, international commerce and global air travel.

Dengue virus belongs to the *Flavivirus* genus in the family *Flaviviridae* [7]. There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4), which are transmitted by *Aedes* sp. of mosquitoes. Infection with any of the virus serotypes ranges from asymptomatic infection to flu-like illness (dengue fever [DF]), and rarely to the severe life-threatening forms of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [8,9]. Generally, it is believed that infection with a single serotype of dengue leads to life-long protection against that particular serotype, but not the other serotypes. However, when an individual previously exposed to one serotype is infected at a later date with a different serotype, they are

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at increased risk of developing the severe forms of illness, DHF and DSS. Due to the existence of four serotypes and the complex nature of the illness, it is believed that a dengue vaccine should be tetravalent in nature so that it will provide robust protective immunity against all four serotypes simultaneously and not sensitize people for DHF/DSS [10,11].

Currently, there are no vaccines or antiviral therapies available for dengue and the only line of treatment is supportive therapy [12]. Given the expanding global burden of disease, there is an increased need for a dengue vaccine. Several efforts have been underway over the past 70 years toward the development of a dengue vaccine. Vaccine candidates at various stages of development include live attenuated viruses (LAV), inactivated viruses, protein subunit vaccines, vectored vaccines, and DNA vaccines [13–15]. Moreover, each of the vaccine concepts is associated with its own advantages and disadvantages in terms of safety, immunogenicity, and feasibility of developing into a product. The most advanced clinical candidate, a tetravalent vaccine consisting of recombinant chimeric yellow fever LAV expressing the prM and E genes of each of the four DENV serotypes (CYD), has recently completed Phase 3 clinical trials [16–18]. Tetravalent LAV formulations have a history of and the potential for viral interaction/interference resulting in the failure to generate balanced tetravalent immunity/balanced protection. The modest efficacy reported for the CYD vaccine in Phase II and III trials (especially for flavivirus naïve subjects and against DENV2, Sanofi trial) further emphasizes that there are practical challenges for attaining a balanced protective responses with LAV vaccines [10,16–19]. Furthermore, the prolonged three-dose regimen (0, 6 and 12 months) used for the CYD LAV presents challenges for implementation and compliance.

Recombinant subunit vaccines offer several potential advantages compared to LAV approaches. These include, (1) improved safety in populations where LAV's are often not used (e.g. the very young, very old and immune compromised); (2) they are not subject to the issue of viral interference and the level of each component can be easily adjusted to target balanced tetravalent immune responses; (3) they offer the potential for an accelerated dosage regimen which can lead to greater vaccine compliance and suitability for travelers, military personnel and use during outbreaks [20]. However, non-replicating vaccines such as recombinant subunits may have some limitations. These include a higher cost of goods, immune responses that may encompass more limited epitope coverage or be of shorter duration, and a reduced ability to induce cell mediated immune responses compared to live vaccines. However, these limitations may be overcome at least in part by the addition of adjuvants. Therefore in order to capitalize on the advantages of non-replicating vaccines while limiting the disadvantages we are currently developing a tetravalent recombinant subunit based dengue vaccine in combination with ISCOMATRIX™ adjuvant. In this paper, we describe the preclinical development of a tetravalent formulation of a recombinant subunit vaccine containing a carboxy-truncated envelope (E) protein (DEN-80E) of each of the four dengue serotypes.

2. Materials and methods

2.1. Cells and viruses

Vero cells (ATCC CCL-81) were maintained in Medium 199 supplemented with 10% heat-inactivated fetal bovine serum (HyClone), glutamine (Mediatech) and penicillin-streptomycin (Mediatech). These cells were used for virus growth, virus titration and virus neutralization assays. Stably transformed *Drosophila* S2 cells that were used to express the DEN-80E subunit proteins of the four dengue viruses have been described elsewhere in detail [21].

The viruses used in the assays described here were DENV-1 (strain WestPac-74), DENV-2 (strain S18603), DENV-3 (strain CH53489), and DENV-4 (strain TVP-360). All the viruses were kindly provided by Alan Barrett (University of Texas Medical Branch, Galveston, TX). Viruses were amplified in Vero cells cultured at 37 °C at a low multiplicity of infection. Briefly, Vero cells grown in 225 cm^2 flasks were infected with 0.01 multiplicity of infection of each of the DENV subtypes. Supernatant from each of the infected cultures was harvested at 5 days post-infection, clarified at 1000 × *g* for 10 min, divided into 0.2–0.5 mL aliquots, flash-frozen on dry ice, and stored at −70 °C. All virus aliquots were thawed on ice immediately prior to use in all assays.

2.2. Dengue envelope (E) proteins

The DEN-80E proteins belonging to all four DENV serotypes (DENV-1 strain 258848, DENV-2 strain PR159 S1, DENV-3 strain CH53489, and DENV-4 strain H241) were expressed in *Drosophila* S2 cells [21]. Briefly, dengue sequences encoding the full-length prM protein and 80% of the E protein (truncation at amino acid 395 for DENV-1, DENV-2 and DENV-4, 393 for DENV-3) were individually cloned into the *Drosophila* expression vector pMttΔXho and then transformed into *Drosophila* S2 cells. The carboxy terminal truncations remove the carboxy stem and transmembrane region of the E protein and result in secretion of DEN-80E with native-like biological structure into the cell culture medium. The secreted recombinant DEN-80E subunits were purified from clarified cell culture medium by immunoaffinity chromatography using a conformationally sensitive monoclonal antibody (4G2) [22]. The purified bulk biologic substances were formulated in phosphate buffered saline at pH 7.2 and filtered into single-use bags and stored at −20 °C.

DEN4-80EZip is a dimeric version of the DENV-4 envelope protein (derived from H241 stain). It consists of the DEN4-80E protein joined to a C-terminal flexible linker and leucine zipper domain to facilitate dimerization. The dimeric version of DEN4-80E was generated as a potential mechanism to improve the immunogenicity of the monomeric DEN4-80E which had been noted to induce slightly lower virus neutralizing antibody titers in previous preclinical studies [21]. To generate the DEN4-80EZip expression plasmid, the DEN4-80E monomer-expressing plasmid was modified to include a leucine zipper region fused in frame at the carboxy terminus of the DEN4-80E sequence to allow for the expression of a DEN4-80EZip dimer product. The third to last codon in the leucine zipper sequence was designed as a cysteine to allow for the two monomers which comprise the dimer to be linked via a disulfide bond. The disulfide linked DEN4-80E leucine zipper dimer is referred to as DEN4-80EZip. The method of production of DEN4-80EZip is essentially the same as that of the other DEN-80E proteins (see above) with the exception that the purified biological substance is formulated in 20 mM glycine pH 9.0. Since DEN4-80EZip and DEN4-80E are based on the same amino acid sequence expressed in the same expression system and react with conformationally sensitive monoclonal antibodies in the same way it is presumed that the structures are similar. However, neither the monomeric nor the dimeric form of DEN4-80E have been analyzed by X-ray crystallography so the exact structures have not been confirmed.

2.3. Rhesus monkey studies

All animal studies were performed at the New Iberia Primate Research Center (New Iberia, LA) according to the Institutional Animal Care and Use Committee-approved protocols. Healthy adult,

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