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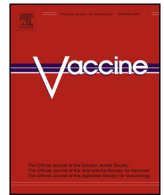

Generation and preclinical evaluation of a DENV-1/2 prM + E chimeric live attenuated vaccine candidate with enhanced prM cleavage

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ABSTRACT

In the absence of a vaccine or sustainable vector control measures, illnesses caused by dengue virus infection remain an important public health problem in many tropical countries. During the export of dengue virus particles, furin-mediated cleavage of the prM envelope protein is usually incomplete, thus generating a mixture of immature, partially mature and mature extracellular particles. Variations in the arrangement and conformation of the envelope proteins among these particles may be associated with their different roles in shaping the antibody response. In an attempt to improve upon live, attenuated dengue vaccine approaches, a mutant chimeric virus, with enhanced prM cleavage, was generated by introducing a cleavage-enhancing substitution into a chimeric DENV-1/2 virus genome, encoding the prM + E sequence of a recent DENV-1 isolate under an attenuated DENV-2 genetic background. A modest increase in virus specific infectivity observed in the mutant chimeric virus affected neither the attenuation phenotype, when assessed in the suckling mouse neurovirulence model, nor multiplication in mosquitoes. The two chimeric viruses induced similar levels of anti-DENV-1 neutralizing antibody response in mice and rhesus macaques, but more efficient control of viremia during viral challenge was observed in macaques immunized with the mutant chimeric virus. These results indicate that the DENV-1/2 chimeric virus, with enhanced prM cleavage, could be useful as an alternative live, attenuated vaccine candidate for further tests in humans.

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1. Introduction

Illnesses resulting from dengue virus (DENV) infection continue to pose a major health problem in many tropical countries [1]. It is estimated that global dengue incidence has increased 30 folds over the last 50 years [2]. The economic burden due to dengue is enormous [3–5], reaching an annual average of US$ 2.1 billion in the Americas alone [6]. Infection by any one of the four dengue serotypes causes a spectrum of clinical outcomes and induces a brief period of cross-protective immunity [7]. In endemic areas, secondary infection with heterologous serotypes leads to an increased risk of developing severe outcomes [8–10]. A lack of association between serotype and severe illness [11] suggests that all four serotypes should be included in a dengue vaccine.

Development of a dengue vaccine suitable for general use remains a challenge. Several vaccine candidates have been generated and evaluated at various phases of testing [12–14], but none has yet met all desirable properties of an ideal dengue vaccine [15]. Incorporation of recent knowledge on virus structure and relevant immune responses in the design of next generation vaccine candidates should facilitate vaccine development effort. Tests on candidates containing different structural elements may help in identifying features that should be included in, or excluded from, an efficacious dengue vaccine.

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DENV particles are assembled intracellularly as immature ones containing an envelope layer embedded with prM + E heterodimeric spikes [16]. The prM protein in the immature particles forms a cap-like structure covering the E fusion loop, resulting in inhibition of E-mediated fusion [17] and reduction of cell binding [18]. During export, furin-mediated internal cleavage of prM generates mature particles, composed of E homodimers arranged in a flattened configuration on the envelope [19]. Reorganization of E in the mature particles results in altered accessibility of certain epitopes for antibody recognition [20]. Cleavage of prM is, however, often inefficient [21,22]. Among the extracellular particles, mature, immature and partially mature ones coexist [23–26]. Antibody-bound prM-containing particles may enter FcγR-bearing cells and become infectious upon further cleavage of prM [27,28]. Whether prM-containing particles are present extracellularly *in vivo*, and how they influence the immune responses, remain unclear.

Following DENV infection, antibodies that recognized serotype-specific epitopes on the receptor-binding domain III (EDIII) of the E protein constitute a small fraction of virus-binding antibodies in immune sera, and play a minor role in neutralization [29,30]. B lymphocytes capable of producing cross reactive anti-prM and anti-E antibodies are commonly present [31–34]. Anti-prM antibodies do not, or weakly, neutralize virus infectivity [35–38], but may augment entry of prM-containing particles and contribute to antibody-dependent enhancement of infection [18,33,35,39]. Vaccine that induces minimal cross-reactive anti-prM antibody response may be desirable [33].

This study generated a live, attenuated vaccine candidate, with reduced prM content, from a chimeric virus that contained the prM + E sequence of a recent DENV-1 isolate and an attenuated DENV-2 background [40]. Preclinical evaluation of the two chimeric viruses was performed in mice, mosquitoes and monkeys.

2. Materials and methods

2.1. Viruses

DENV-1 strains 03-0398 and 03/135, and DENV-2 strain 03-0420 were isolated from Thai pediatric patients in 2003. Four DENV strains (DENV-1 strain 16007, DENV-2 strain 16681, DENV-3 strain 16562 and DENV-4 strain 1036) were provided for use in the plaque reduction neutralization test (PRNT) by Dr. Ananda Nisalak of the Armed Forces Research Institute for Medical Sciences (AFRIMS), Bangkok. Viruses were propagated in Vero cells and stored in 20% fetal bovine serum at −70 °C. Infectious viruses were quantitated by focus immunoassay titration in Vero cells [22] or plaque titration in LLC-MK_2 cells [41].

2.2. Generation of chimeric viruses

The cDNA clone of the DENV-2 attenuated parent virus, 16681-3pm, was generated by introducing attenuating mutations of strain 16681-PDK53 [40]—5′UTR C57T, NS1 Gly53Asp and NS3 Glu250Val—into a cDNA clone of strain 16681 [42]. To construct the DENV-1/2 chimeric cDNA clone, the prM + E region of strain 03-0398 was amplified from patient plasma and exchanged with a corresponding region in the 16681-3pm cDNA clone. Substitution of the P3 acidic residue at the polyprotein position 203 with alanine was performed by site directed mutagenesis. Details of the construction and primer sequences are available upon request.

Chimeric viruses were generated by Lipofectamine 2000-mediated transfection of Vero cells with capped RNA transcripts [22]. They were propagated for 4 additional passages at 37 °C (passage 2) and 32 °C (passages 3–5). Recombinant DENV-2 containing the attenuating mutations and prGlu203Ala substitution were designated as 16681-4pm. cD1-3pm and cD1-4pm denoted chimeric viruses containing the prM + E region from DENV-1 and aspartic acid or alanine at residue 203, respectively. Sequence analyses revealed an unintended Lys204Arg substitution in the E gene of both chimeric viruses, and a silent T1582C substitution in cD1-3pm.

2.3. Determination of plaque size, virus titer and specific infectivity

Ten-fold serially diluted virus suspensions were inoculated onto confluent LLC-MK_2 monolayers. After adsorption at 37 °C in 5% CO_2 for 90 min, an overlay medium containing 1% Noble agar was added. Plaque sizes were recorded on day 7 of incubation.

Genome-containing particles of DENV-2 were quantitated by a real-time RT-PCR method [21]. The sensitivity of detection was 1.25 FFU/ml. DENV-1 was quantitated by the method of Houng et al. [43]. Specific infectivity was calculated by dividing infectious virus titer with genomic RNA concentration, and expressed as FFU/1000 RNA copies.

2.4. Suckling mouse neurovirulence study

BALB/c mice aged 1–2-days old were injected intracranially with 100 PFU of virus per mouse. Neurological abnormalities and death were observed daily for 3 weeks.

2.5. Immunogenicity study in mice

BALB/c mice aged 4–6-weeks old were injected subcutaneously with viruses at 10, 100, or 1000 PFU/mouse. Levels of neutralizing antibody in sera collected on days 14 and 28 post-injection were determined on LLC-MK_2 cells using the PRNT50 end point.

2.6. Virus replication in mosquitoes

Laboratory-reared *Aedes aegypti* mosquitoes aged 3–5-days old were intrathoracically inoculated with 10 PFU/mosquito and subsequently maintained at 28 °C on a 10% sucrose solution. On days 7 and 14 post-injection, five mosquitoes from each group were chilled, triturated and suspended, and infectious viruses were quantitated by plaque titration.

2.7. Immunization of monkey and virus challenge

Fourteen 5- to 10-year-old healthy rhesus macaques (*Macaca mulatta*), with undetectable neutralizing antibody against DENV, were injected subcutaneously in the upper arm with 1 ml (1×10^5 PFU) of strain 03-0398 ($n = 2$) or chimeric viruses ($n = 6$ per virus) under ketamine hydrochloride-induced anesthesia. Two animals received 1 ml of PBS containing 20% fetal bovine serum.

On day 90 post immunization, PBS- and DENV-1strain 03-0398-injected groups were challenged with DENV-2 strain 03-0420 at 1×10^5 PFU per animal. Half ($n = 3$) of the cD1-3pm- and cD1-4pm-immunized groups were challenged with DENV-1 strain 03-0398 at 1×10^5 PFU per animal, while the other half received DENV-2 strain 03-0420 at 1×10^5 PFU per animal. Femoral vein blood samples were taken at various time points, processed, encoded and stored at −70 °C; and subsequently blind tested for the presence of virus and neutralizing antibody. The animals were kept in separate cages equipped with mosquito screens, and provided with the usual water and food allowance. Body temperature was taken twice daily and body weight measured once daily.

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