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Generation and preclinical immunogenicity study of dengue type 2 virus-like particles derived from stably transfected mosquito cells

Amporn Suphatrakul[a], Thippawan Yasanga[b], Poonsook Keelapang[c], Rungtawan Sriburi[c], Thaneeya Roytrakul[a,d], Rojjanaporn Pulmanausahakul[e], Utaiwan Utaipat[f], Yanee Kawilapan[c], Chunya Puttikhunt[a,d], Watchara Kasinrerk[g,h], Sutee Yoksan[e], Prasert Auewarakul[e], Prida Malasit[a,d], Nicha Charoensri[i,*], Nopporn Sittisombut[a,c,**]

[a] *Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok 10700, Thailand*
[b] *Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*
[c] *Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*
[d] *Dengue Hemorrhagic Fever Research Unit, Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*
[e] *Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand*
[f] *Research Institute for Health Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[g] *Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[h] *Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[i] *Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

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ABSTRACT

Recent phase IIb/III trials of a tetravalent live attenuated vaccine candidate revealed a need for improvement in the stimulation of protective immunity against diseases caused by dengue type 2 virus (DENV-2). Our attempts to develop particulate antigens for possibly supplementing live attenuated virus preparation involve generation and purification of recombinant DENV-2 virus-like particles (VLPs) derived from stably (prM+E)-expressing mosquito cells. Two VLP preparations generated with either negligible or enhanced prM cleavage exhibited different proportions of spherical particles and tubular particles of variable lengths. In BALB/c mice, VLPs were moderately immunogenic, requiring adjuvants for the induction of strong virus neutralizing antibody responses. VLPs with enhanced prM cleavage induced higher levels of neutralizing antibody than those without, but the stimulatory activity of both VLPs was similar in the presence of adjuvants. Comparison of EDIII-binding antibodies in mice following two adjuvanted doses of these VLPs revealed subtle differences in the stimulation of anti-EDIII binding antibodies. In cynomolgus macaques, VLPs with enhanced prM cleavage augmented strongly neutralizing antibody and EDIII-binding antibody responses in live attenuated virus-primed recipients, suggesting that these DENV-2 VLPs may be useful as the boosting antigen in prime-boost immunization. As the levels of neutralizing antibody induced in macaques with the prime-boost immunization were comparable to those infected with wild type virus, this virus-prime VLP-boost regimen may provide an immunization platform in which a need for robust neutralizing antibody response in the protection against DENV-2-associated illnesses could be tested.

1. Introduction

A number of candidates have been developed in the search for an effective vaccine against dengue [1]. Recent phase IIb/III trials of a tetravalent dengue-yellow fever chimeric live attenuated virus preparation revealed an inefficient protection against dengue caused by DENV-2 in children immunized on a year-long

* Corresponding author. Tel.: +66 053935332; fax: +66 053217144.
** Corresponding author at: Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail addresses: nicha@kku.ac.th (N. Charoensri), nopporn.sittiso@cmu.ac.th (N. Sittisombut).

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three-dose schedule [2–4]. This result was unexpected, as a high proportion of volunteers had been infected with DENV prior to enrollment, and the level of DENV-2 neutralizing antibodies in the vaccinated group was comparable to those directed against other serotypes. An alternative vaccine candidate may be needed for successful immunization against diseases caused by DENV-2 infection.

Virus-like particles (VLPs) offer a number of advantages as a dengue vaccine candidate over soluble E protein or live attenuated candidates. Flaviviral VLPs are assembled from two glycoproteins, prM and E, forming enveloped particles with icosahedral symmetry [5]. Similarities between VLPs and virions in the conformation and arrangement of receptor-binding E protein may help focus antibody responses following VLP immunization toward epitopes that are exposed on virions [6]. In structure-based design of particulate vaccines, alterations of critical components of prM and E, such as the pr-M junction [7] and the E fusion loop [8,9], to improve yield allow the generation of particles that may not be infectious, and thus can be applied only to VLPs.

Extracellular particles of dengue virus are heterogeneous in size and maturation state, ranging from immature and partially mature particles to fully mature particles [reviewed in 10]. Conserved sequence of charged amino acids nearby the pr-M junction influences the extent of furin-mediated cleavage of prM during export [11,12]. Selected substitutions of these residues modify the proportion of particles at different maturation levels [13], which might be useful in the preparation of particulate vaccine candidates of desired properties. While it is unclear how and to what extent variations in the maturation state of DENV particles affect immune response in naturally infected persons, a comparison of DENV-1/2 chimeric live attenuated vaccine candidates (LAV) in monkeys revealed that a candidate with enhanced prM cleavage resulted in more efficient protection against viremia during challenge with wild-type viruses [14]. In this study, the influence of DENV particle maturation state in the stimulation of antibody responses was explored further by generating DENV-2 VLPs with different prM cleavage levels and comparing their immunogenicity in mice. Subsequently, the possibility for use of VLPs as a boosting antigen in a prime-boost regimen was examined in monkeys.

2. Materials and methods

2.1. Cells and viruses

C6/36 cells [15] were maintained at 29 °C in Leibowitz's L-15 medium supplemented with 0.29% tryptose phosphate broth, 10% fetal bovine serum (FBS), and antibiotic/glutamine solution. Vero cells (ATCC CCL-81) were propagated at 37 °C employing minimal essential medium containing 10% FBS, antibiotic/glutamine solution, and 0.22% sodium bicarbonate.

DENV-2 strains 03-0420 and NS1-123, and DENV-1 strain 03-0398 were isolated from Thai pediatric patients in 2003. As described previously [14], a chimeric DENV-1 LAV was generated by exchanging the prM+E coding region in a cDNA clone of strain 16681-3pm [14], which contained attenuation-associated substitutions of strain 16681 PDK-53 [16], with that of strain 03-0398. In the same manner, chimeric DENV-2 LAVs were generated by substituting with the envelope protein genes from strains 03-0420 or NS1-123. A comparison in cynomolgus macaques revealed that NS1-123-derived LAV induced lower neutralizing antibody response than 03-0420-derived LAV; macaques primed with NS1-123-derived LAV were employed in the prime-boost study.

2.2. Generation of (prM+E)-expressing C6/36 clones

prM cleavage-modifying substitutions, with or without an E fusion loop mutation, were introduced into pIE1-SP-prME, which encoded codon-optimized sequences for a defensin A-derived prM signal peptide, the prM and EDI-III domains of strain 03-0420, and the E stem-anchor region of Japanese encephalitis virus (JEV) [17]. C6/36 cells were transfected with plasmids and selected with 25 μg/ml of blasticidin. Following limiting dilution, clones were screened for high extracellular E levels by dot enzyme immunoassay. Selected clones had been propagated without blasticidin for up to 50 passages while maintaining expression of transfected genes.

2.3. Purification of particles and negative staining

VLPs and native viral particles were purified by centrifugation in a potassium tartrate/glycerol gradient as described previously [17] and stored in 20% glycerol at −20 °C. For electron microscope visualization, 1 μg was applied onto a Formvar carbon-coated copper grid (Electron Microscopy Sciences, Hatfield, PA, USA), fixed with 1% glutaraldehyde and stained with 1% uranyl acetate. Grids were air-dried and examined at 25,000–50,000×.

2.4. Adjuvants

Saponin-based adjuvant (AbISCO-100) was obtained from Novavax AB (formerly Isconova AB, Uppsala, Sweden). Aluminum hydroxide gel (Alhydrogel, 2%), squalene-based oil-in-water adjuvant (Addavax), cyclic diguanylate monophosphate (c-di-GMP VacciGrade) were procured from Invivogen (San Diego, CA, USA). The Sigma adjuvant system (2% squalene oil-in-water emulsion containing monophosphoryl lipid A [*Salmonella minnesota*] and synthetic trehalose dicoryno-mycolate) was from Sigma (St. Louis, MO, USA). The amount of each adjuvant employed was determined from the recommended dose or dose range provided by the suppliers. Adjuvants were employed, as follows: AbISCO-100, 12 μg/mouse and 50 μg/monkey; Alhydrogel, 50 μl/mouse; Addavax, 50 μl/mouse; Sigma adjuvant system, 100 μl/mouse; and c-di-GMP, 25 μg/mouse.

2.5. Immunization

Female, 8-week-old, BALB/c mice were injected with VLPs either intraperitoneally in the preliminary experiments or subcutaneously at the tail base in subsequent experiments. VLPs plus the Sigma adjuvant system were administered intraperitoneally and VLPs plus other adjuvants subcutaneously according to the manufacturers' recommendations. Blood samples were obtained from retro-orbital plexus or heart under general anesthesia.

Two- to four-year-old healthy cynomolgus macaques (*Macaca fascicularis*; five males, BW 2.4–3.2 kg; four females, BW 2.2–2.8 kg) with undetectable neutralizing antibody against dengue viruses were injected subcutaneously in the upper arm with phosphate buffered saline (PBS), 1×10^5 PFU of strain 03-0420, or 2×10^5 PFU of the prM+E chimeric LAV under ketamine hydrochloride-induced anesthesia. Blood samples were taken from the femoral vein, processed, and stored at −70 °C. All monkey blood samples were blind-tested for the presence of neutralizing antibody. Animals were housed in separate cages with mosquito screens and provided with usual water and food allowances.

2.6. Plaque reduction neutralization test (PRNT)

PRNT was performed using Vero cells and the 50% end point (PRNT50) according to WHO guidelines [18]. Mouse and monkey sera were diluted serially in 2-fold and 10-fold increments, respectively, beginning with 1:10. Target viruses were: DENV-1, strain 16007; DENV-2, strain 16681; DENV-3, strain 16562; and DENV-4, strains 1036 or C0036/06. In the calculation of geometric mean titer

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