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# Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen

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**Abstract**

We employed DNA shuffling and screening technologies to develop a single recombinant dengue envelope (E) antigen capable of inducing neutralizing antibodies against all four antigenically distinct dengue serotypes. By DNA shuffling of codon-optimized dengue 1–4 E genes, we created a panel of novel chimeric clones expressing C-terminal truncated E antigens that combined epitopes from all four dengue serotypes. DNA vaccines encoding these novel chimeras induced multivalent T cell and neutralizing antibody responses against all four dengue serotypes in mice. By contrast, a mixture of four unshuffled, parental DNA vaccines failed to produce tetravalent neutralizing antibodies in mice. The neutralizing antibody titers for some of these antigens could be further improved by extending the sequences to express full-length pre-membrane and envelope proteins. The chimeric antigens also protected mice against a lethal dengue-2 virus challenge. These data demonstrate that DNA shuffling and associated screening can lead to the selection of multi-epitope antigens against closely related dengue virus serotypes and suggest a broad utility for these technologies in optimizing vaccine antigens.
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## 1. Introduction

Dengue viruses are transmitted through bites of *Aedes* mosquitoes. Four virus serotypes (dengue-1 to dengue-4) of the genus *Flavivirus* can produce a spectrum of clinical illness, ranging from an uncomplicated but debilitating dengue fever (DF) to sometimes severe and fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Infection with one serotype results in a long-lasting protective immunity against the homotypic virus, but only confers short lived cross-protective immunity to heterologous serotypes [1]. Therefore, people living in endemic areas can have multiple infections, with secondary infections posing a risk for the most severe manifestation of the disease, DHF/DSS. With an estimate of up to 100 million human infections each year and several hundred thousand cases of DHF/DSS [2,3], dengue viruses have become one of the most important arthropod-borne viruses from a medical and public health perspective.

There is currently no specific treatment for dengue diseases, but modern supportive intensive care can reduce the fatality rate of DHF/DSS patients from approximately 20% in an untreated group to 0.1%. Vaccination could provide a promising approach for controlling dengue virus infections. Although the development of dengue vaccines has been ongoing for the past 50 years [4], no licensed dengue vaccine is available yet. The major challenge is to generate a vaccine that induces protective immunity against all four serotypes of dengue viruses. Several dengue

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vaccine programs have focused on manufacturing four live attenuated viruses, which are administered as a tetravalent mixture. Promising preclinical results have been achieved using this approach, but formulation complexities and competition among serotypes leading to non-uniform immune responses hamper the speed of clinical development [5–7]. Protective immunity was also achieved in animal models using inactivated dengue-2 viruses or recombinant dengue-2 E proteins as subunit vaccines against homotypic dengue-2 viruses [8,9]. Chimeric dengue-2/1, -2/3 and -2/4 virus constructs using the backbone of attenuated dengue-2 virus have been shown to retain the attenuation and elicit specific immune responses to dengue-1, -3 and -4, respectively [10]. Considerable advances have also been made in developing chimeric dengue-yellow fever viruses [11]. These chimeric dengue/dengue or dengue/yellow fever viruses could provide a basis for live attenuated tetravalent vaccine. However, manufacturing four vaccine candidates and formulation complexities pose a significant challenge to this approach as well.

DNA vaccines may offer an attractive alternative or additional platform. Feasibility of DNA vaccines is supported by induction of both humoral and cellular immune responses in several human clinical trials [12–14] and by a recent report that a West Nile virus DNA vaccine is under consideration for USDA approval for use in horses [15]. A dengue-2 DNA vaccine expressing the viral pre-membrane (prM) protein and the amino terminal 92% of envelope protein was shown to elicit neutralizing and protective antibody responses in mice challenged with live dengue-2 virus [16]. A dengue-1 construct expressing prM and full-length envelope was shown to produce virus-like particles in transfected cells, induce neutralizing antibodies in mice [17], and protect rhesus macaques and *Aotus* monkeys when challenged with the corresponding live dengue-1 virus [18,19]. However, progress in this area has been slow, and is attributed to the need for developing 4 individual vaccines, which then must be formulated optimally to produce the tetravalent vaccine. It would be highly beneficial to develop a single vaccine that combines attributes from all four dengue serotypes and elicits protective immune responses to each of them.

We have addressed these challenges by using directed molecular evolution by DNA shuffling and screening to generate chimeric dengue antigens, which combine neutralizing epitopes from all four dengue virus E proteins. Several studies have been conducted to identify the location and functional importance of neutralizing antibody epitopes for the dengue-2 E antigen [20–23], but currently available information regarding the structure, localization and sequences of the antigenic epitopes exposed on the virion surfaces of all four dengue serotypes is insufficient to enable rational design of broadly protective, multivalent recombinant dengue antigens. DNA shuffling and screening was therefore a practical tool to search for tetravalent dengue vaccine candidates, since it does not require an understanding of the number and location of specific neutralizing epitopes but simply relies on a functional screen for desired improvements. Shuffling technologies have previously been used for a wide-range of applications and have been successful, for example, in evolving interferon-α, interleukin-12, co-stimulatory molecules and viruses [24–28]. In the present study, we describe directed molecular evolution of dengue envelope genes to generate a single chimeric antigen that induces neutralizing antibodies against all four dengue serotypes in mice when administered as a DNA vaccine.

## 2. Materials and methods

### 2.1. Cells, antigens, and antibodies

HEK293 cells (ATCC) were grown in DMEM medium, supplemented with 10% fetal bovine serum (FBS). Dengue antigens used for ELISA and ELISpot assays included recombinant dengue-3 and dengue-4 antigens, and dengue-1, -2, -3, and -4 virus infected vero cell lysates, dengue virus infected insect cell lysates (Immunology Consultants Laboratory, Oregon), and vero cell derived purified dengue-2 virus (Microbix Biosystems, Canada.). The recombinant dengue-3 and dengue-4 antigens, comprising the viral prM and E proteins, were expressed in HEK293 cells. The high molecular weight particles formed by these proteins were purified from the cell culture medium by sucrose gradient centrifugation and Sephadex$^{TM}$ G-25 column purification (PD-10, Amersham Biosciences, Uppsala, Sweden). Protein concentration was determined by BCA Protein Assay kit (Pierce, Rockford, IL). Endotoxin level was measured using Limulus Amebocyte Lysate kit (QCL-1000, BioWhittaker, Walkersville, MD). Vero cell propagated Dengue-1 (strain Western Pacific 74), dengue-2 (strain 16803), dengue-3 (strain CH53489), and dengue–4 (strain 341750) viruses used for virus neutralization assays (PRNT) were obtained from the Walter Reed Army Research Institute.

Hyperimmune mouse ascetic fluids (HMAF) for dengue-2 and -3 (ATCC) and dengue-1 and -4 (WHO collection, gift from R. Shope) were used for flow cytometry and immunoblot analyses. Anti-dengue human plasma IgG was obtained from Immunology Consultants Laboratory, Oregon. Monoclonal dengue-2 antibody 3H5 was received from Chemicon.

### 2.2. Construction of shuffled libraries and plasmid preparations

Sequence information for dengue virus prM and E proteins was derived from GenBank entries. The prM and E sequences of dengue-1, -2 and -4 were derived from sequence accession numbers B32401, P30026, and AAB706903, respectively. For dengue-3, prM and E sequences were derived from sequence accession numbers P27915 and AAD37781, respectively. The hydrophobic membrane anchor and the hydrophilic cytoplasmic sequences of the E proteins were

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