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A novel dengue virus serotype-2 nanovaccine induces robust humoral and cell-mediated immunity in mice

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ABSTRACT

Dengue virus (DENV), a member of the *Flaviviridae* family, can be transmitted to humans through the bite of infected *Aedes* mosquitoes. The incidence of dengue has increased worldwide over the past few decades. Inadequate vector control, changing global ecology, increased urbanization, and faster global travel are factors enhancing the rapid spread of the virus and its vector. In the absence of specific antiviral treatments, the search for a safe and effective vaccine grows more imperative. Many strategies have been utilized to develop dengue vaccines. Here, we demonstrate the immunogenic properties of a novel dengue nanovaccine (DNV), composed of ultraviolet radiation (UV)-inactivated DENV-2, which has been loaded into the nanoparticles containing chitosan/*Mycobacterium bovis* Bacillus Calmette-Guerin cell wall components (CS/BCG-NPs). We investigated the immunogenicity of DNV in a Swiss albino mouse model. Inoculation with various concentrations of vaccine (0.3, 1, 3 and 10 μg/dose) with three doses, 15-day apart, induced strong anti-dengue IgM and IgG antibodies in the mouse serum along with neutralizing antibody against DENV-2 reference strain (16681), a clinical-isolate strain (00745/10) and the mouse-adapted New Guinea-C (NGC) strain. Cytokine and chemokine secretion in the serum of DNV-immunized mice showed elevated levels of IFN-γ, IL-2, IL-5, IL-12p40, IL-12p70, IL-17, eotaxin and RANTES, all of which have varying immune functions. Furthermore, we observed a DNV dose-dependent increase in the frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells after *in vitro* stimulation of nucleated cells. Based on these findings, DNV has the potential to become a candidate dengue vaccine.

1. Introduction

Dengue virus (DENV), of the genus *Flavivirus*, family *Flaviviridae*, can be grouped into four antigenically distinct serotypes (DENV 1–4). DENV is transmitted to humans through the bite of DENV-infected *Aedes aegypti* and *Aedes albopictus*. Natural DENV infections are thought to induce lifelong protection against the infecting homologous serotype and short-term cross protection against heterologous serotypes. Recent estimates indicate that up to 390 million infections occur annually worldwide, with approximately 96 million symptomatic cases [1].

DENV infections generally present with a wide range of clinical symptoms, varying from asymptomatic infection, to undifferentiated dengue fever (DF), to dengue hemorrhagic fever (DHF), dengue shock syndrome [2], or other severe forms of dengue [2]. The mechanisms underlying severe dengue disease are believed to be related to pathogenic manifestations of the immune response, including antibody cross-reactivity to the vascular endothelium, disease-enhancing antibodies, complement proteins and byproducts, and soluble mediators such as cytokines and chemokines [3]. How each of these elements contributes to disease pathology is not clear.

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DHF and DSS occur in secondary heterologous DENV infection at a 15–80 fold higher frequency than in primary infection. These levels underscore the significance of preexisting anti-DENV antibodies, which may enhance infections by heterologous DENV serotypes. There is no DENV-specific treatment available, increasing the need for an effective vaccine that can provide simultaneous protection against all four serotypes with minimal reactogenicity. Currently, there are no licensed dengue vaccines. However, there are several vaccine candidates under development, all using different strategies to provide protection. These include live-attenuated vaccines [4–6], purified inactivated vaccines [7,8], recombinant vaccines [9], chimeric vaccines [10], virus-like particles vaccines [11,12] and subunit vaccines [13,14]. Many of these are in preclinical and clinical trials. Vaccine development is hampered by the complexity of the immune responses, the potential for interference when all four DENV serotypes are delivered simultaneously, and the theoretical safety risk of vaccine-related immune enhancement.

Subunit and recombinant protein vaccines are thought to be safer than live-attenuated vaccines since their composition is devoid of replicative forms of the virus, usually associated with reactogenicity. However, subunit and recombinant vaccines can fail to elicit robust cell-mediated immune responses and are also easily degraded by lysosomal enzymes [15,16]. The dengue/yellow fever chimeric vaccine (CYD-TDV) is the most advanced candidate dengue vaccine, currently in phase III clinical trials [17]. It is unknown how the lack of DENV non-structural (NS) proteins, a source of T cell epitopes, in the CYD-TDV will affect long-term immunogenicity and protection. Purified inactivated vaccines can be done by several methods including heat-inactivation, chemical inactivation and UV irradiation. Formalin inactivation is widely used in many vaccines such as polio virus, JEV, dengue and influenza virus. It is known that formalin treatment destroys important viral epitopes [18,19]. A more recent approach in vaccine development links DENV to the phosphoreactive psoralen, 4′-aminomethyltrioxalen hydrochloride [20], which cross-links pyrimidine residues when exposed to UV-A radiation [21]. The AMT-UV inactivated virus freely penetrates the cell phospholipid bilayer, while retaining intact structural and non-structural epitopes. This approach may increase the vaccine-induced cytotoxic T cell responses, similar to what has been reported for NS3[22].

Improving vaccine immunostimulatory properties may require the use of adjuvants and improved delivery systems. Nanotechnology is fast becoming a widely used approach for drug and vaccine delivery. Several methods are used to generate nanoparticles (NPs) including coacervation [23], emulsion [24] and polymerization [25]. Because of their small size, NPs easily cross the blood brain barrier and access the interior of cells and various intracellular compartments [26]. NPs stimulate antigen uptake by antigen-presenting cells (APCs) [27], while reducing toxicity and other side effects associated with drugs [28,29]. In animal models, NPs have been used to improve delivery and efficacy of influenza [30–32] and hepatitis B vaccines [33,34], underscoring NPs applicability to other experimental vaccines. The cell wall components of *Mycobacterium bovis* Bacillus Calmette-Gurin (BCG-CWCs) are widely used as adjuvant for several vaccine developments. BCG-CWCs serve as a ligand for TLR2/4 that pasty share their signaling pathway through MyD88, an adaptor that is essential for effective cytotoxic T lymphocytes induction, and TIRAP, lead to the activation of NF-κB and productions of cytokine such as TNFα, IL-1β, IL-6, IL-8 and chemokine like MIP and [35]. It has been It has been reported that mice treated i.p. with BCG showed robust antigen presentation and increased IL-1, IL-6 and TNFα production in peritoneal macrophages [36,37]. Since BCG-CWCs have the capability in modulating of T cell responses, CS/BCG-NPs were used as an adjuvant and delivery system for DNV in this study.

Table 1
Composition of DNV. A constant amount of the BCG-CWCs adjuvant was loaded into CS-NPs by adsorption–mixing method. Varying amounts of UVI-DENV were loaded by adsorption–mixing method to form DNV.

Groups	CS-NPs (μg/ml)	BCG-CWCs (μg/ml)	UVI-DENV antigen (μg/ml)
Saline control	–	–	–
Adjuvant	45	20	–
0.3 μg of DNV	45	20	3
1 μg of DNV	45	20	10
3 μg of DNV	45	20	30
10 μg of DNV	45	20	100

Here, we report the ability of DNV, composed of AMT-UV-inactivated whole DENV2 loaded chitosan (CS)/*M. bovis* Bacillus Calmette-Guerin (BCG) cell wall components nanoparticles (CS/BCG-NPs), to stimulate humoral and cellular immunogenicity in a Swiss albino mouse model.

2. Materials and methods

2.1. Adjuvant preparation and vaccine formulation

CS/BCG-NPs were used as the adjuvant delivery carriers for dengue nanovaccine (DNV). Preparation of the adjuvant was started from chitosan core-shell nanoparticles synthesis by an emulsifier-free emulsion-polymerization method [38]. Cell wall components of *M. bovis* BCG were isolated from heat-inactivated *M. bovis* BCG Tokyo 172 by French pressure cell press [39]. No toxicity was found when the adjuvant was tested in primary human DCs, THP-1 cells and LLC-MK2 cells (data not shown). The integrity of the component was checked using an anti-lipoarabinomannan (LAM) antibody. The antigenicity of the sucrose gradient-purified AMT-UV-inactivated DENV-2 strain 16681 (UVI-DENV) [7,21,40] was tested by typing ELISA using 4G2, 3H5 and 2H2 monoclonal antibodies (data not shown). Protein concentration was determined by bicinchoninic acid (BCA) assay using bovine serum albumin as a standard (Bio-Rad Laboratories, USA). The complete inactivation of the UVI-DENV-2 antigen was verified by inoculating the antigen in LLC-MK2 cells and incubating the cells 6 days, after which we were unable to detect any plaque-forming units in the undiluted preparation (data not shown).

To generate DNV, BCG and UVI-DENV were sequentially loaded onto CS-NPs by an absorption-mixing method at 250 rpm for 16 h [41]. The constant amount of adjuvant was used in adjuvant or various concentrations of DNV immunized mice (Table 1). DNV was cleaned by centrifugation at 10,000 rpm for 1 h to remove unbound components. Vaccine particle size and surface charge were determined by zetasizer (NanoZS 4700, Malvern Instruments, UK). Loading efficacy of UVI-DENV was determined by staining the DNV with FITC-conjugated anti-flavivirus antibodies. The frequency of UVI-DENV present on NPs was observed by BD LSRFortessa™ Cell Analyzer (BD Biosciences, data not shown).

2.2. Animals

Adult female Swiss albino mice, *Mus musculus* ICR outbred strain aged 6 to 8 weeks and of 25–30 g of body weight were maintained at the Department of Veterinary Medicine, United States Army Medical Component-Armed Forces Research Institute of Medical Sciences (USAMC-AFRIMS) (Bangkok, Thailand) under good animal welfare conditions. All procedures involving mice were performed in compliance with national laws and institutional policy and with the permission of the AFRIMS Institutional Animal Care and Use Committee.

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