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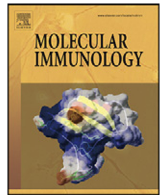

Preliminary evaluation of DNA vaccine candidates encoding dengue-2 prM/E and NS1: Their immunity and protective efficacy in mice

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ABSTRACT

Public health is still seriously threatened by dengue virus (DENV) and no vaccine against DENV is yet available for clinical use till now. In this study, DNA vaccine candidates encoding DENV serotype 2 (DENV-2) prM/E (premembrane and envelope proteins) and NS1 (non-structural 1 protein) with or without a gene adjuvant, granulocyte-macrophage colony-stimulating factor (GM-CSF), were evaluated in the aspects of immunity and protective efficacy in mice. We constructed three plasmids, pCAG-prM/E (which only expressed DENV2 prM/E), pCAG-prM/E/NS1 (which only expressed DENV2 prM/E/NS1) and pCAG-DG (which co-expressed DENV2 prM/E/NS1 and GM-CSF). The expressions of the recombined plasmids were analyzed by immuno-staining in Vero cells. Antibody responses and neutralization activity of the sera from the mice were assayed by ELISA and plaque reduction neutralization test after immunization with the plasmids. Immunized BALB/c mice were intracerebrally challenged with DENV2 to evaluate protective efficacy of the plasmids. The recombinant plasmids could be efficiently expressed in Vero cells and induced different levels of specific anti-DENV2 immune responses. The immunized mice were partially protected. The highest survival rate was observed in the pCAG-DG group although the anti-DENV2 titer and neutralization antibody titer were not the highest among the three groups. Our data suggested that pCAG-DG offered better protection against DENV2 infection.

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1. Introduction

Dengue viruses (DENV), belonging to the *Flaviviridae* family, are composed of four distinct serotypes (DENV1–4). The genome of the viruses is a single-stranded positive-sense RNA of approximately 11 kb that encodes three structural proteins, capsid (C), premembrane (prM) and envelope (E) proteins, and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). DENV are transmitted to humans by the mosquitoes, *Aedes aegypti*, and can cause the self-limiting dengue fever (DF), the more severe dengue hemorrhagic fever (DHF) or fatal dengue shock syndrome (DSS) throughout tropical and subtropical regions of the world. It is estimated that up to 100 million infections of dengue occur annually, resulting in about 500,000 cases of DHF and 24,000 deaths each year (Rigau-Pérez et al., 1998). Dengue infection is still a severe public health problem.

Considerable research had been done toward the development of DENV vaccines (Chambers et al., 1997). The most exciting among these was the chimeric Yellow fever dengue tetravalent vaccine (CYD, ChimeriVax), which has already entered clinical trial (Guy et al., 2008). Although CYD, ChimeriVax was very promising, potential complications remain, and studies have shown that the tetravalent vaccine formulations elicit unbalanced immune responses due to viral interference (Guirakhoo et al., 2002). Such interference is particularly an important concern for dengue vaccines because the unbalanced immune responses may lead to increased disease severity when the vaccinated host acquires an infection with one of the four serotypes for which the induction of immunity is insufficient (Imoto and Konishi, 2007). Therefore, none of the traditional vaccine against DENV is yet available for clinical use till now.

DNA vaccines are able to induce long-lasting cellular and humoral immunity against some pathogens including *flaviviruses* (Donnelly et al., 1997). One of the potential advantages was the expression of viral proteins in situ after DNA immunization, leading to proper folding and posttranslational modifications, which originally occur during the course of natural viral infections (Schlesinger et al., 1987). In studies of DENV DNA vaccines, prM/M, E, NS1 and NS3 proteins were usually used as target antigens because they could elicit protective immune responses. The E protein, the viral major surface protein, contains important neutralizing epitopes. The prM protein is necessary for the proper processing and expression of the E protein. The NS1 protein associates with the E protein in the ER (endoplasmic reticulum) lumen and is involved in virion

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maturation. It can induce a strong antibody response and protect hosts through an Fc-dependent complement-mediated manner (Schlesinger et al., 1993). In fact, several investigations reported inoculation of plasmids containing *flaviviruses* prM, E, or NS1 gene to elicit specific immune responses in mice (Schlesinger et al., 1987; Ulmer et al., 1993; Donnelly et al., 1997; Colombage et al., 1998). But the efficiency of these DNA vaccines was poor. In our previous studies, we found that granulocyte-macrophage colony-stimulating factor (GM-CSF) was an effective gene adjuvant, which enhanced the immune and protective effects against Japanese encephalitis virus (JEV) and DENV1 when co-expressed with their antigens (Gao et al., 2010; Zheng et al., 2011). In this report, we constructed three recombinant plasmids containing either prM/E of DENV2 (pCAG-prM/E), prM/E/NS1 of DENV2 (pCAG-prM/E/NS1) or prM/E/NS1 fused to GM-CSF (pCAG-DG) and analyzed their immunogenicity and protective role in mice. This will allow us to evaluate whether this strategy of DNA vaccine construction could be more widely applied in other DENV serotypes and viruses.

2. Materials and methods

2.1. Cells lines, virus, and mice

Aedes albopictus mosquito cells (C6/36) were grown at 28 °C in RPMI (Roswell Park Memorial Institute) 1640 containing 10% fetal bovine serum (FBS, Gibco, Auckland, New Zealand) and were used to propagate DENV-2 (strain TR1751) which was isolated from a patient with DF and was kindly provided by Dr. A. Oya (National Institute of Infectious Disease, Japan). Vero cells were cultivated at 37 °C in Eagle's minimal essential medium (MEM, Gibco, Buffalo, America) supplemented with 5% FBS.

Female inbred BALB/c mice were purchased from the Academy of Military Medical Sciences (Beijing, China). All of the mice were maintained in specific pathogen-free environments.

2.2. Construction of plasmids

We used pCAG and pIRES (Qiagen) to construct the DNA vaccines. First, viral RNA was isolated from DENV2-infected C6/36 cells with the Biozol reagent (Bioflux, Tokyo, Japan), according to the manufacturer's protocol, and reversely transcribed to cDNA using primer DENV (−): CTC CCG CTC ATC AAG AAT AA by reverse transcriptase M-MLV (Invitrogen, Grand Island, America). Different segments of DENV2 were then amplified by PCR using the primers shown in Table 1. Then, the amplified fragments were cloned into the pCAG vector using the multiple cloning sites. Furthermore, we constructed a bicistronic plasmid expressing simultaneously DENV2 prM-E-NS1 and GM-CSF under the control of a single promoter. Briefly, DENV-2 prM-E-NS1 and GM-CSF fragments were inserted into the multiple cloning sites A and B of the pIRES, respectively. Then, the total prM-E-NS1-IRES-GM-CSF fragment was digested with the XhoI and NotI restriction enzymes (MBI, Glen Burnie, America) and subcloned into pCAG. DNA sequencing was used to verify the cloned fragments and they were proper subcloned into the vectors. The expression of these plasmids was further confirmed by indirect immuno-fluorescence in Vero cells.

For immunization, the plasmids were extracted and purified with an endotoxin-free plasmid extraction kit (Omega, Norcross, America) from transformed *Escherichia coli* JM109. Then, the purified plasmids were dissolved in sterile saline and adjusted to 1.0 mg/ml before use.

2.3. Mouse experiments

All of the experiments with mice were conducted in compliance with Ethical Principles in Animal Experimentation.

For DNA immunization, 6-week-old female BALB/c mice were used. To enhance the uptake of plasmid DNA, mice were prior injected with 50 μl 0.25% lidocaine hydrochloride in each quadriceps muscle the day before immunization. Each mouse was inoculated intramuscularly with 100 μg of the plasmids at 3-week intervals for three times. Mice injected with pCAG served as negative controls. The mice were finally euthanized.

For the protection test, three weeks after the last immunization, the mice were challenged intracerebrally with a lethal dose (50× LD50) of DENV2. The mice were monitored daily for the morbidity and mortality for 3 weeks.

2.4. Antibody assay

Mice sera samples were collected by tail bleeding at three weeks after the last immunization and analyzed for the presence of DENV2 antibodies by an enzyme-linked immunosorbent assay (ELISA). Briefly, Two-fold serial dilutions of the serum samples were added to wells coated with concentrated virus. After incubation and washing with TPBS, each well was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL, Houston, America). Afterward, the plates were washed five times with TPBS, and the color was generated by using the HRP substrate, orthophenylene diamine (OPD), and the action was stopped by addition of H_2SO_4. After measurement of the absorbance at 490 nm, the end-point titers of anti-DENV2 antibodies were determined as the reciprocal of the highest dilution, giving an optical density (OD) twice that of the non-immune serum. To determine the IgG subclass, antibody isotype ELISAs were similarly performed using anti-mouse IgG1-HRP or anti-mouse IgG2a-HRP (SBA, Oregon, America) replacing the former anti-mouse IgG-HRP.

2.5. Plaque reduction neutralization test (PRNT)

To determine the presence of the neutralizing antibodies (NAbs) in the sera of inoculated mice, sera were diluted twofold in MEM containing 2% FBS. Sera were heated at 56 °C for 30 min to inactivate complement. Serial dilutions were incubated with equal volumes of DENV2 virus at 37 °C for 1 h. Then the mixtures containing 100 PFU/well of the virion were added in duplicate to Vero cell monolayers seeded in 24-well plates. After adsorption for 1 h, Vero cells were then washed and incubated in MEM containing 5% FBS and 1.3% methyl-cellulose. After 1 week of incubation at 37 °C in 5%

Table 1
Constructions of plasmids including the components of DENV-2.

Recombinant plasmids	Amplified regions	Primers	Restriction sites
pCAG-prM/E	prM/E (367-2421)	5′-ggcc ctcgag ATG CTG AAC ATC TTG AAC A-3′	*Xho*I
		5′-cgta gcggccgc CTA GGC CTG CAC CAT AAC TCC-3′	*Not*I
pCAG-prM/E/NS1	prM/E/NS1 (367-3477)	5‘-ggcc ctcgag ATG CTG AAC ATC TTG AAC A-3′	*Xho*I
		5′-cgta gcggccgc CTA GGC TGT GAC CAA GGA GTT-3′	*Not*I
pIRES-prM/E/NS1	prM/E/NS1 (367-3477)	5′-ggcc ctcgag ATG CTG AAC ATC TTG AAC A-3′	*Xho*I
		5′-cggc acgcgt CTA GGC TGT GAC CAA GGA GTT-3′	*Mlu*I

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