

The immunogenicity of tetravalent dengue DNA vaccine in mice pre-exposed to Japanese encephalitis or Dengue virus antigens

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Summary

Background: Asian countries are an endemic area for both dengue (DENV) and Japanese encephalitis viruses (JEV). While JEV vaccines have been used extensively in this region, DENV vaccines remains under development. Whether preexisting naturally acquired or vaccination-induced immunity against JEV may affect the immune response to dengue vaccine candidate is unclear. In this study we used mice previously immunized with JEV vaccines to evaluate the impact on dengue-specific neutralizing antibody responses to a tetravalent dengue DNA vaccine candidate (TDNA).

Methods: A tetravalent cocktail of plasmids encoding pre-membrane and envelope proteins from each dengue serotype was administered into mice which had been previously primed with inactivated or live-attenuated JEV vaccines, or dengue serotype2 virus (DENV-2). Neutralizing antibody response was measured employing a plaque reduction neutralization test at two weeks

after the priming and at four weeks after the second dose of the dengue tetravalent plasmids.

Results: Inactivated or live-attenuated JEV vaccines, or DENV-2 induced low levels of neutralizing antibodies against the homologous viruses (JE and dengue virus, respectively). DENV-2 injection induced also low levels of cross-reactive antibodies against DENV-1, -3 and -4. JEV vaccines have no effect on the dengue-specific neutralizing antibody responses to the subsequent TDNA immunization. Pre-exposure to DENV-2 infection increased DENV-2 specific response neutralizing antibody to two doses of TDNA plasmids by six folds, but did not affect antibody response to other serotypes.

Conclusions: Priming with JEV vaccines did not impact on dengue virus-specific neutralizing antibody response to a dengue TDNA vaccine candidate in mice. (*Asian Pac J Allergy Immunol 2015;33:182-8*)

Keywords: dengue virus, Japanese encephalitis virus, preexisting immunity, tetravalent dengue DNA vaccine, neutralizing antibody

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Introduction

Several flaviviruses cause human diseases, including dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus and tick-borne encephalitis virus. Infection by DENV may result in undifferentiated fever, dengue fever or dengue hemorrhagic fever (DHF). Infection with any of the four serotypes of DENV induces long-term immunity against the homologous serotype. DHF, an immunopathological disease, occurs with an increased risk during secondary infections.^{1,2} Although there have been several decades of investigation, vaccines targeting all four serotypes of DENV currently remain under development.

In some DENV endemic areas, particularly the Indian subcontinent, East and South-East Asia, JEV



co-circulates with DENV.^{3,4} While JEV vaccines have been licensed in many countries and introduced to the residents in endemic areas as well as travelers, the vaccine effectiveness was initially tested in flavivirus-seronegative children or adults to avoid the influence of flavivirus preexisting immunity.^{5,6} As dengue virus may be contracted in areas co-endemic with JEV and other flaviviruses, it is important to develop dengue vaccines in conditions where flaviviruses preexisting immunity effects are taken into account in order to improve vaccine efficiency.

Previously, we have developed a tetravalent dengue DNA vaccine candidate (TDNA) expressing the premembrane and envelope proteins of all four dengue serotypes.⁷ In this study we investigated the potential impact of previous JEV immunization on the induction of dengue virus-specific antibody responses during subsequent injection with a tetravalent DENV DNA vaccine in mice.

Methods

Viruses, Cells, and JEV vaccines

DENV-1 (strain 16007), DENV-2 (strain 16681), DENV-3 (strain 16562) and DENV-4 (strain C0036) were kindly provided by Drs. Ananda Nisalak and Robert Gibbons, Armed Forces Research Institute of Medical Sciences, Thailand. JEV (Strain Beijing-1) was obtained from Dr. Thananya Thongtan, Faculty of Medicine, Chulalongkorn University. Vero, an African green monkey kidney epithelium cell line, and LLC-MK₂, a rhesus monkey kidney epithelium cell line, were propagated in minimum essential medium (MEM with Earle salts) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA) and Medium 199 supplemented with 20% FBS, respectively. DENV1-4 viral stocks were propagated in Vero cells. C6/36, an *Aedes albopictus* cell line, was propagated in 10% FBS MEM plus non-essential amino acid and used for JEV propagation. Virus titers were determined by a plaque assay on LLC-MK₂ cell monolayer.⁷ Inactivated JEV (JE-Vax, lot no. JJ5401-1) and live-attenuated (CD.JEVAX, lot no. 201003A026-2) were manufactured by Thai Government Pharmaceutical Organization, and *Chengdu* Institute of Biological Products, P.R. China, respectively.

DNA vaccine candidates

The tetravalent dengue DNA vaccine candidate was described previously.⁷ Briefly, humanized codon of consensus *prM/E* were generated from the isolates (deposited in Genbank since 1962-2003)

Table 1. Study design and immunization schedule

Study group	Baseline exposure Week 0	First TDNA Week 2	Second TDNA Week 4
Group 1	PBS ^a	TDNA ^b 100 µg	TDNA 100 µg
	Inactivated JEV ^a vaccine (1/10 of human dose)	TDNA 100 µg	TDNA 100 µg
Group 2	Live-attenuated JEV ^a vaccine (5000 PFU)	TDNA 100 µg	TDNA 100 µg
	DENV-2 ^c 10 ⁴ PFU	TDNA 100 µg	TDNA 100 µg
Group 3	TDNA 100 µg	TDNA 100 µg	TDNA 100 µg
	Negative control PBS	Empty vector ^b 66 µg	Empty vector 66 µg

^asubcutaneous injection, ^bIM-EP injection, ^cintraperitoneal injection, PBS: phosphate buffer saline, TDNA: tetravalent dengue DNA vaccine, JEV: Japanese Encephalitis Virus, DENV: dengue virus, PFU: plaque forming unit

then commercially synthesized by GeneArt, Inc., (Germany), The expression cassettes were sub-cloned into pCMVkan expression vector⁸ (National Cancer Institute, Frederick, MD, USA) designated as pCMVkanD1prME, pCMVkanD2prME, pCMVkanD3prME and pCMVkanD4prME. All recombinant plasmid constructs were confirmed by nucleotide sequence analysis. Following lipofectamine 2000 (Invitrogen, CA, USA) -mediated transfection of plasmids into Vero cells, both intracellular and secreted E protein expression were detected for each construct with an indirect immunofluorescence staining and western blot analysis, respectively.

Mice experiment

ICR mice at 4-6 weeks of age were obtained from the National Laboratory Animal Center, Mahidol University, Thailand and all experimental procedures were approved by the Committee of Animal Care and Use of Chulalongkorn University. Five-six mice/group were injected at week 0 with PBS, DENV-2 (intraperitoneally), TDNA (intramuscular

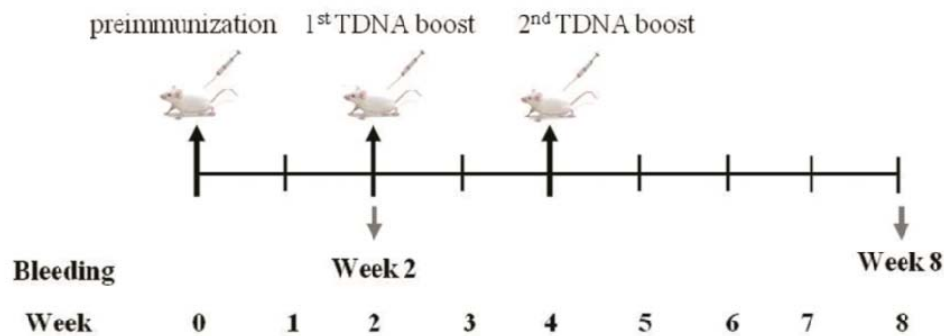


Figure 1. Mice immunization schedule. Mice were pre-exposed to JEV or DENV antigens as indicated in Table 1 at week 0. Then 2 doses of 100 μ g of TDNA were administered at 2-week interval by intramuscular electroporation. Blood was collected as indicated by grey arrows.

electroporation), inactivated, or live-attenuated JEV vaccines (subcutaneously) (Table 1). Two weeks later, mice were boosted with 100 μ g of TDNA (25 μ g each of monovalent preparation) by an IM-EP route on week 2 and week 4. IM-EP immunization procedures were performed as described previously.⁹ Blood was collected on week 2 after the first injection, and subsequently on week 4 after the second boost. Empty pCMVkan vector (66 μ g) was used as negative control for TDNA immunization. Injection and bleeding of mice were performed under isoflurane anesthesia (Baxter, IL, USA). Immunization schedule is shown in Figure 1.

Plaque reduction neutralization test (PRNT)

Virus Neutralizing Antibody (Nab) titer was determined by PRNT as described previously.^{7,10,11} Briefly, heated inactivated mice sera were 2-fold serially diluted and mixed with 30-50 PFU/well of target virus. The virus-serum mixtures were incubated at 37 °C for 1 hr and then inoculated onto LLC-MK2 monolayer for 1 hr. Cells were then overlaid with the first overlayer containing Hank's balanced salt solution supplemented with FBS, non-essential amino acid solution, vitamin solution, L-glutamine (Gibco, CA, USA) and 0.9% low-melting point agarose (Invitrogen, CA, USA). After 4 days (DENV-1; strain 16007, DENV-3; strain 16562 and DENV-4; strain C0036) or 5 days (DENV-2; strain 16681) of incubation (37 °C, 5% CO₂), the second overlayer containing 4% v/v neutral red (Sigma-Aldrich, MO, USA) was added and plaques were counted after 16-20 hr. The highest serum dilution that resulted in 50% or more reduction of the number of plaques as compared with the virus control wells was considered as the neutralizing titer (PRNT50).

Statistical analysis

Comparisons of the neutralizing antibody titers (PRNT50) at baseline and after immunization, and between the experimental groups were analyzed by 2-tailed nonparametric Mann-Whitney test and one-way ANOVA. Values were considered significant at $p < 0.05$.

Results

Induction of neutralizing antibody after priming with JEV and DENV

Low levels of DENV or JEV neutralizing antibody were detected at week 2 after injection of the corresponding priming antigens (Figure 2). Mice injected with DENV-2 generated higher antibody titer against DENV-2 than DENV-1, DENV-3 and DENV-4. The two types of JEV vaccine induced neutralizing antibodies only against JEV, but not DENV. The median PRNT50 titers were 40 and 80 for inactivated and live-attenuated JEV vaccines, respectively (Figure 2). Although the priming effect of TDNA wasn't analyzed at this time-point, our previous observation demonstrated that a single immunization of dengue serotype 2 DNA vaccine induced neutralizing antibody at PRNT50 = 10-20 (unpublished data) thus, it suggests that one dose of dengue DNA vaccine is able to be used as priming antigen as it is sufficient to induce dengue-specific neutralizing antibodies.

Neutralizing antibody responses following dengue tetravalent DNA boost

On week 4 following the immunization with two 100- μ g doses of dengue TDNA preparations, mice that were injected previously with phosphate buffered saline generated neutralizing antibodies against all four serotypes of dengue virus. The median PRNT50 titers against DENV-1, DENV-2,

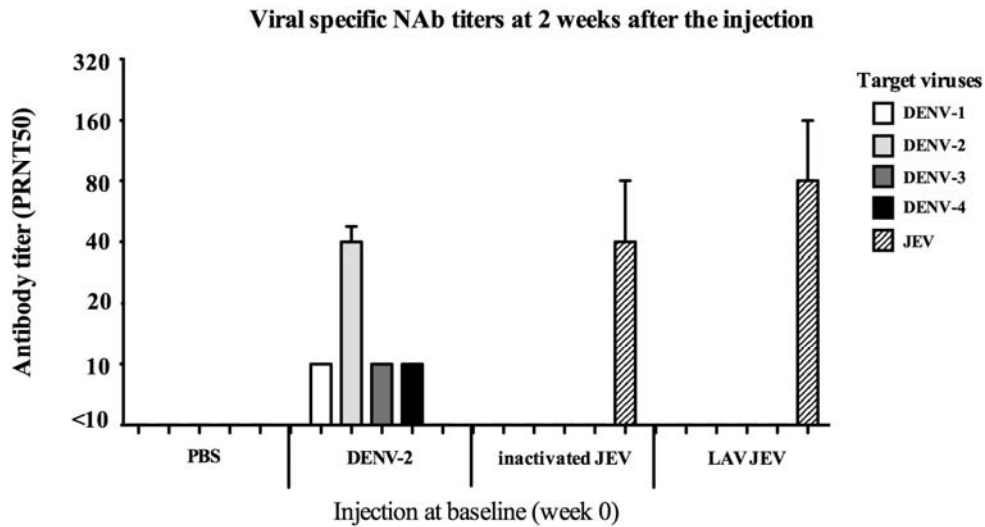


Figure 2. NAb results at 2-week post-exposure to either JEV or dengue antigens, against DENV-1 (opened bar), DENV-2 (light grey bar), DENV-3 (dark grey bar), DENV-4 (black bar) and JEV (slashed bar). Individual or pooled mice sera were tested for NAb responses to the homologous or heterologous antigens used for injection, respectively. Each bar represents median PRNT50 end-point titer with interquartile range or PRNT50 end-point titer of pooled mice sera.

DENV-3 and DENV-4 were 1:30, 1:80, 1:40 and 1:160, respectively (Figure 3A and 3B). This group of mice was used for comparison with JEV or dengue antigens-primed groups.

The role of JEV preexisting immunity on TDNA vaccine immunogenicity was investigated. We found similar levels of dengue neutralizing antibody between mice that received inactivated or live-attenuated JEV vaccines and the PBS-prime group (Figure 3A).

In contrast, in the DENV-2 pre-infected group, the median PRNT50 titer was 6-fold higher than that of the PBS-prime group (median PRNT50 = 480 vs 80) when sera were tested against DENV-2 ($p = ns$). The effect of DENV-2 infection on subsequent stimulation of DENV-neutralizing antibodies by TDNA was not detected when the test was performed using other DENV serotypes. Additionally, mice that were primed with TDNA and then boosted with two additional doses of TDNA displayed higher levels of neutralizing antibodies against all four dengue serotypes (median PRNT50 titers against DENV-1, DENV-2, DENV-3 and DENV-4 are 240, 320, 240 and 640, respectively). Significant higher levels of neutralizing antibody in TDNA-prime group when compared with PBS-prime group were detected against DENV-1 and DENV-3 and also found to be 4-fold

higher in DENV-2 (median PRNT50 = 320 vs 80) and DENV-4 (median PRNT50 = 640 vs 160), Figure 3B.

Discussion

In this study, preexisting immune response against JEV has been found to have no effect on subsequent dengue-specific neutralizing antibody responses induced by TDNA immunization in mice. After receiving 2 doses of TDNA, mice primed with a JEV vaccine either killed or live-attenuated forms, generated Neutralizing Antibodies to all DENV at the levels comparable to those that received PBS. This supports the current status of DENV and JEV as being categorized in different antigenic complexes.^{12,13} In addition, a comparison study between DENV and JEV showed only 31-38% and 45.5-51% amino acid homology within prM and E, respectively. Similarly, Konishi and colleague¹⁴ have observed that the balance of NAb titers induced by their dengue tetravalent DNA vaccine candidate was not affected by preexisting immunity against JEV and Yellow fever virus (YFV). Moreover, mice pre-infected with DENV-2 rapidly increased DENV-2 specific-NAb after the first tetravalent DNA vaccine injection but NAb to all serotype was balanced by giving the subsequent dose. This observation slightly differs from the

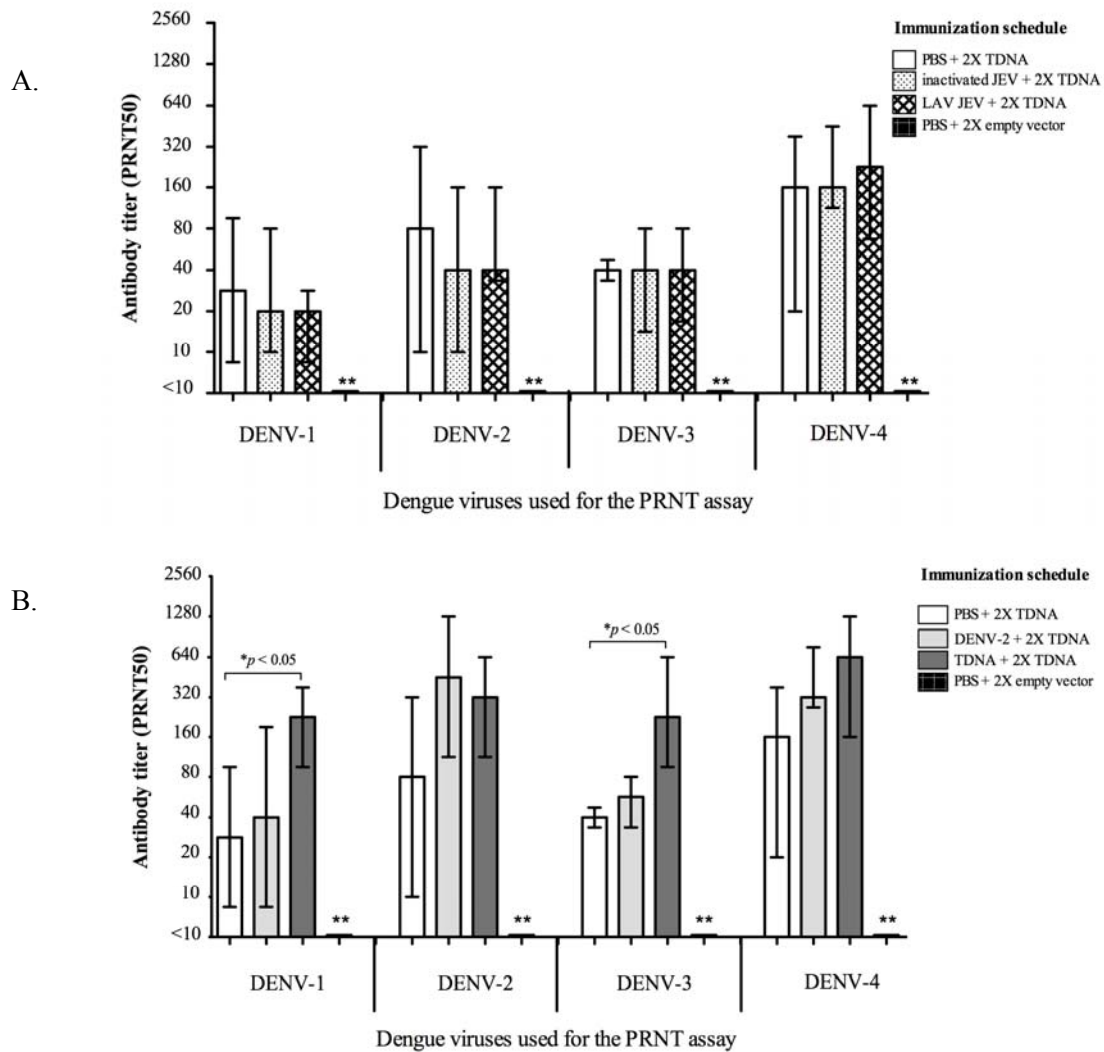


Figure 3. NAb of JEV or dengue antigens pre-exposed mice at 4-week after 2nd TDNA boost, at baseline, mice were injected either with JEV vaccines (A) or dengue serotype 2 virus (B). NAb of individual mice ($n = 5-6$ each group) were analyzed against DENV-1, DENV-2, DENV-3 and DENV-4. Each bar represents median PRNT50 end-point titer to DENV with interquartile range, * and ** represent $p < 0.05$ and $p < 0.01$, respectively.

present findings where additional TDNA injections did not generate a balance in NAb response to all 4 serotypes. A possible explanation for this discrepancy is the relatively smaller amount of DENV-2 (50-5000-fold lower) used in our study which may have resulted in lower cross-reactive priming effects to the other serotypes. This argument is further substantiated by the observation that the NAb titers against all DENV serotype exhibit a dose-dependent pattern associated with the amount of DENV-2 used for preimmunization.¹⁴ Interestingly, in our study in autologous TDNA-primed mice where the NAb against all DENV serotypes is supposed to develop, the induced NAb

titers were not only 4-8 fold higher than those of the PBS-controls, but a more balanced NAb across serotypes was observed. This suggests that tetravalent priming, in this case with TDNA, was better both in term of the breadth and magnitude of NAb responses.

Consistent with our previous report, the negative control group, pCMVkan empty vector injection never induced any responses to the vector.⁷ Moreover, as demonstrated in Figure 2, no dengue-specific NAb responses were detected in JEV vaccines-primed mice. Thus, JEV vaccines-primed/empty vector-boost group was not included in the study.

Impacts on clinical patterns of dengue infection and subsequent dengue vaccine responses in human by prior JEV or YFV immunity have also been reported. A cohort of Thai children who had prior detectable JEV antibody had an increased occurrence and duration of dengue infection illness.¹⁵ The possible role of ADE phenomenon caused by related-flaviviruses has been proposed, however further investigation is necessary.¹⁶ In contrast, several reports of chimeric YFV-DENV (CYD) live attenuated vaccine showed that YFV-immune volunteers increased the immunogenicity to DENV compared to naïve volunteers.¹⁷⁻¹⁹ This enhancement might be due to a common immunogenic epitope among flaviviruses such as the epitope within the fusion loop.²⁰ Other regions such as prM/E (30-50% amino acid homology between YFV and DENV) or non-structural (NS) proteins within the YFV backbone are believed to also be involved in immune enhancement but their specific role remain uncertain, especially in the case of NS3 and NS5. These two YFV-proteins showed 50-60% amino acid homology with DENV.²¹

Interestingly, although pre-immunity to YFV enhanced immune responses to all dengue serotypes after ChimeriVax™-DEN2 immunization, a trend towards increased viremia was observed in the YFV-immune group.¹⁸

The incongruent observations among studies might be resulting from a combination of factors. First, the DNA vaccine used in this study contains only prM/E whereas CYD uses prM/E from DENV and other non-structural proteins from YFV as a vaccine backbone.²² Second, the species-specific responses to mice and humans could represent a confounding or modifying influence on observed immune responses. As our study was conducted on mice the observed patterns may not totally reflect response patterns in human, possibly because of differential patterns immune expression.²³⁻²⁶ Further investigations using other biological models, such as humanized mice, should provide complementary evidence and immune responses that more closely resembles human responses and consequently support stronger inferences.²⁷ Third, the responses to natural dengue infection or live-attenuated vaccine may be different from TDNA.

Although the median titers against DENV-4 in mice that received 2 doses of TDNA was higher than for other serotypes (Figure 3), the DENV-4 NAb titer was more balanced when the third TDNA was administered (group 5) with no statistically

significant difference in NAb titers among DENV serotypes. Further investigations in non-human primates are currently performed to determine whether 3 doses of TDNA are required to achieve good NAb responses both in terms of magnitude and breadth. Non-live vaccine platforms such as tetravalent dengue DNA vaccines are thought to present several advantages over LAV because they do not show interference among vaccine components and consequently have the potential to be more efficient at protecting against subsequent viral challenges.²⁸

In summary, we provided important information for further study or usage of a dengue DNA vaccine candidate in prior-vaccinated or naturally infected people in DENV and JEV endemic areas. There was no significant effect of pre-exposure to JEV on TDNA immunogenicity in mice.

Conflict of interests

The authors have declared that no competing interests exist.

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