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Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice

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Abstract

We developed a dengue tetravalent DNA vaccine consisting of plasmids expressing premembrane and envelope genes of each of four serotypes of dengue viruses. BALB/c mice immunized twice with the tetravalent vaccine at a dose of 100 μg (25 μg for each serotype) using a needle-free jet injector developed neutralizing antibodies against all serotypes. There was no interference among the four components included in this combination vaccine. Tetravalent vaccine-immunized mice showed anamnestic neutralizing antibody responses following challenge with each dengue serotype: responses to challenges from serotypes different to those used for neutralization tests were also induced.

Keywords: Dengue; Vaccines; Combined; DNA Vaccines

1. Introduction

Dengue fever and its severe form, dengue hemorrhagic fever (DHF), are mosquito-borne viral diseases distributed in most tropical and subtropical regions with an estimated 50–100 million human cases annually [1–4]. Four serotypes of dengue viruses (DENV1, DENV2, DENV3, and DENV4) are the causative agents. Currently, no approved vaccines are available, and various strategies have been used to develop dengue vaccine candidates [5–7].

A mechanism causing DHF upon infection with one particular dengue serotype has been considered to be the presence of non-neutralizing crossreactive antibodies and cross-reactive memory T lymphocytes induced by an earlier infection with other serotypes [7–9]. Epidemiologically, people once infected with a serotype are usually protected from the disease from a subsequent homotypic infection [10]. Therefore, for developing a safe and effective dengue vaccine, a combination of vaccines that can induce immune responses against all four serotypes is required, i.e. a tetravalent vaccine. In clinical trials, attenuated tetravalent vaccines have produced high seroconversion rates to all four serotypes after two or three doses [11–13], but concerns have been raised about interference in virus replication among serotypes, which may cause imbalanced immune responses and enhanced disease severity [7]. A chimeric tetravalent vaccine produced high levels of neutralizing antibody and viremia protection against all serotypes after a single dose in preclinical evaluation using non-human primates [14], and clinical trials are in progress [6–7]; however, the possibility of genetic recombination with virulent viruses remains a concern [7,15].

DNA vaccines are a promising gene-based vaccine strategy and their effectiveness has been demonstrated in various virus systems [16–19]. In light of the advantages of DNA vaccines over other types, the strategy is considered suitable for developing a dengue tetravalent vaccine. DNA vaccines do not seem to cause interference upon combined immunization. Although immune interference may occur, combined immunizations with two DNA vaccines against closely related flaviviruses, tick-borne encephalitis viruses, did not show detectable interference [20]. The use of DNA vaccines in combination vaccines is considered a promising area [21]. In addition, DNA vaccines are durable and inexpensive for production and transportation, facilitating their introduction into areas with endemic dengue. Several flavivirus DNA vaccines,

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including those against dengue, have already been developed (reviewed in [21–23]).

Dengue viruses belong to the genus flavivirus [24]. The envelope of flavivirus virions contains two protein species: envelope (E), the major surface protein on which most of the neutralizing epitopes are located, and membrane (M) which is produced in infected cells as a precursor, prM. Coexpression of flavivirus prM and E genes in mammalian cells has been demonstrated to produce immunogenic, extracellular subviral particles (EPs) containing prM/M and E proteins [21–22,24]. Using the prM/E gene strategy, we have developed pcDNA3-based DNA vaccines for JEV [25], DENV1 [26] and DENV2 [27], all of which are able to induce neutralizing antibodies in mice.

In the present study, we newly constructed DNA vaccines against DENV3 and DENV4 based on the prM/E strategy. These vaccines were combined with two previously constructed DNA vaccines against DENV1 [26] and DENV2 [27], to produce a dengue tetravalent DNA vaccine. For the first evaluation of the dengue tetravalent DNA vaccine based on the prM/E strategy, we used a murine model and prototypes as vaccine strains.

2. Materials and methods

2.1. Viruses

The Mochizuki strain of DENV1 [26] and the New Guinea C (NGC) strain of DENV2 [27] have been described previously. The H87 strain of DENV3 and the H241 strain of DENV4, which had been passaged through suckling mouse brains (SMBs) for 30 and 18 times, respectively, were provided by Dr. Nobuya Fujita of the Department of Microbiology, Kobe University School of Medicine, Japan, who had originally received the viruses from Dr. Nick Karabatsos of the Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, CT. These viruses were passaged two times through SMBs, and 10% homogenate was used to immunize mice for preparation of polyclonal antibodies and to challenge mice for evaluation of anamnestic immune responses. These viruses passaged two times through SMBs in our laboratory were further passaged two times through a mosquito cell line, C6/36 [28]; the infected culture fluid was used for sedimentation analyses, and virion fractions purified using sucrose density gradients (see below) from infected culture fluids were used to extract RNA for vaccine construction and to immunize rabbits for preparation of polyclonal antibodies. These viruses were further passaged one or two times through a mammalian cell line, Vero [29], and the infected culture fluid was used for neutralization tests and antigens for ELISA. For comparison with the H241 strain maintained in our laboratory, another H241 strain was provided by Dr. Tomohiko Takasaki of the National Institute of Infectious Diseases (NIID), Japan. Virus titers were determined on Vero cell monolayers and plaques were visualized by the immunochemical staining method using a monoclonal 4G2 and an avidin-biotin system as previously described [26].

2.2. Plasmids

The pcDNA3-based plasmids encoding prM and E of DENV3 (designated pcD3ME) and DENV4 (pcD4ME) were constructed essentially by the method previously described for construction of DNA vaccines against DENV1 (pcD1ME [26]) and DENV2 (pcD2ME [27]). Briefly, viral RNA extracted from purified virion fraction was used for production of the cDNA containing prM/E genes of each virus by reverse transcriptase-polymerase chain reaction (RT-PCR). For construction of pcD3ME, the RT reaction was performed with an antisense primer corresponding to the carboxy-terminal 6 codons of E based on a reported sequence for the H87 strain [30], which was adjacent to a termination codon (TAA) and an *Xba*I site (TGCTCTAGATTAAGCTTG-CACCACGACCCCCAG). PCR was performed with the above antisense primer and a sense primer including an *Eco*RV site, an efficient eukaryotic initiation site (ACC [31]) and a start codon (ATG), followed by the codons encoding Ile-Asn-Lys-Arg-Lys-Lys-Thr-Ser-Leu of the prM signal sequence (CGCGATATCACCATGATCAACAAACG-GAAAAAGACATCGCTC). The amplified cDNA containing the prM/E gene cassette was inserted into the pcDNA3 vector (Invitrogen) at the *Eco*RV/*Xba*I site. For construction of pcD4ME, a reported sequence for the H241 strain [32] was used for designing the antisense primer including the *Xba*I site (TGCTCTAGATTATGCTTGAACTGTGAAGC-CCAG) and the sense primer including the *Eco*RI site and the codons encoding Leu-Asn-Gly-Arg-Lys-Arg-Ser-Thr-Met of the prM signal sequence (CCGGAATTCACCAT-GTTGAATGGGAGAAAAAGGTCAACAATG). All plasmid DNAs were purified using a Qiagen Plasmid Kit.

Nucleotide sequences of the prM/E region of the newly constructed plasmids, pcD3ME and pcD4ME, differed from those of the corresponding virus strains registered in GenBank [30,32] at several points with amino acid alterations, as listed in Table 1. The prM/E region of pcD3ME differed at two amino acids, probably due to the difference in passage history of the H87 strain between the two laboratories. Unexpectedly, a significant number of amino acids differed between pcD4ME and the sequence reported for H241 [32], and were accompanied by an addition of one amino acid. To confirm the prM/E sequence of the H241 strain from our laboratory, the same strain but supplied by a different laboratory (NIID) was sequenced, and we determined that the two strains were identical.

2.3. Antibodies

Monoclonal antibodies were 2H2 (prM-specific, dengue complex-crossreactive [33]) and 4G2 (E-specific, flavivirus group-crossreactive [34]), provided by Dr. Takasaki of NIID). Mouse or rabbit polyclonal antibodies to DENV1, DENV3 or

Table 1
Differences of nucleotide sequences accompanied by alterations of deduced amino acid sequences in the prM and E regions of pcD3ME and pcD4ME from those reported for DENV3 (H87 strain) and DENV4 (H241 strain), respectively

Vaccine plasmid	Coding region	Nucleotide number	Nucleotide		Amino acid	
			Report[a]	Plasmid	Report[a]	Plasmid
pcD3ME	E	1362[b]	T	C	Val	Ala
	E	1805	A	G	Lys	Glu
pcD4ME	prM	449[c]	T	C	Ile	Thr
	E	905	C	T	Ser	Leu
	E	1255	A	G	Ile	Val
	E	1301	C	T	Thr	Ile
	E	1589	T	C	Ile	Thr
	E	1669	A	G	Met	Val
	E	1673	T	A	Phe	Try
	E	1897	C	T	Pro	Ser
	E	1915	A	T	Asn	Try
	E	1949	G	C	Arg	Pro
	E	1954–1956	TTG	TTTGGG	Leu	Phe-Gly

[a] Sequences were obtained from [30] (for H87) and [32] (for H241) with GenBank accession numbers of M93130 (for H87) and S66064 (forH241).
[b] Nucleotide number was described by defining 1 as the first nucleotide of the sequence in the 5′-untranslated region for the H87 strain, according to the style registered in GenBank.
[c] Nucleotide number was described by defining 1 as the first nucleotide of the C protein initiation codon for the H241 strain, according to the style registered in GenBank.

DENV4 were obtained essentially as previously described for production of antibodies to DENV2 [35]. Briefly, adult ICR mice were repeatedly immunized with DENV1 (Mochizuki), DENV3 (H87) or DENV4 (H241) in a form of 10% SMB homogenate, followed by the inoculation of sarcoma 180 cells to produce hyperimmune mouse ascitic fluid (HMAF). Also, a rabbit was repeatedly immunized with a purified virion fraction that was dissociated with 0.05% Triton X-100.

2.4. Cell transfection and immunochemical staining

A mammalian cell line, CHO-K1, was used for expression studies essentially as previously described [27]. Briefly, cells were transfected with 2 μg of pcD3ME or pcD4ME and maintained in MEM containing 1% BSA for 24 h at 37 °C. Cells were fixed and then stained with monoclonal antibodies specific for dengue viral proteins and an avidin-biotin system.

2.5. Sedimentation analysis

Extracellular antigens produced by CHO cells transfected with pcD3ME or pcD4ME were analyzed on sucrose density gradients as previously described for analysis of JEV EPs [29]. Briefly, culture fluids harvested 48 h after transfection were precipitated with polyethylene glycol (PEG) and analyzed on a 10–40% (w/w) continuous sucrose gradient. As controls, culture fluids harvested from C6/36 cells 72 h after infection with DENV3 or DENV4 were used.

2.6. ELISA for detection of E antigen

DENV3 and DENV4 E antigens were detected using a sandwich ELISA as previously described [29]. Briefly, microplates were sensitized with polyclonal rabbit antibody against DENV3 or DENV4. The plates were then incubated serially with test samples, an anti-E monoclonal 4G2, alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate, followed by spectrophotometry at 410 nm.

2.7. Mouse experiments

Four-week-old male BALB/c mice (CLEA Japan, Tokyo, Japan) were used for all experiments. For evaluating immunogenicity of DNA vaccines, groups of 5–10 mice were inoculated twice at an interval of 3 or 7 weeks with plasmid DNAs using a spring-powered needle-free jet injector (ShimaJET; Shimadzu Corp., Kyoto, Japan [26]). The amount of vaccine DNA was 100 μg for the tetravalent vaccine (a mixture of 25 μg of each of pcD1ME, pcD2ME, pcD3ME and pcD4ME) and 25 μg for each monovalent vaccine. To adjust the molarity of the vector plasmid DNA, the monovalent vaccine (25 μg) was mixed with 55 μg of pcDNA3, which corresponded to 75 μg of monovalent vaccines based on molecular weights of pcDNA3 (5446 base pairs) and monovalent vaccines (7459–7491 base pairs). For a non-vaccine control, 73 μg of pcDNA3 was used, which contained the same molarity of the vector DNA as 100 μg of vaccine DNA. Plasmid DNAs were diluted in PBS to adjust the volume of DNA solution to 100 μl per mouse, with 50 μl being used for inoculation into each thigh. Retroorbital blood was collected from immunized mice. Individual sera were examined for ELISA antibody and pooled sera were examined for neutralizing antibody.

For evaluating anamnestic neutralizing antibody responses, groups of six mice immunized twice at a 3-week interval with 100 μg of the tetravalent vaccine or 73 μg of pcDNA3 were challenged intraperitoneally (i.p.) with each serotype of dengue viruses in the form of 10% SMB

homogenate (10^8 FFU per mouse) 3 weeks after the second immunization. Challenge with this dose of virus through an i.p. route does not produce death in BALB/c mice at 10 weeks of age, but enables the assessment of the level of memory B cell activity. These mice were bled 4, 7 and 14 days after challenge, and sera were isolated, pooled and examined for neutralizing antibody.

All the animal experiments in the present study were approved by the Committee for Animal Experiments, the Kobe University School of Medicine.

2.8. Neutralization tests

Neutralizing antibodies elicited in immunized mice were determined essentially by our previously described method using plaque reduction assays performed with DENV1 in the presence of rabbit complement [26]. Each of four virus serotypes harvested from infected Vero cell cultures was used as an antigen. For all serotypes, plaques were visualized by the immunostaining method. The neutralizing antibody titer was expressed as the maximum serum dilution yielding a 70% reduction in plaque number.

2.9. ELISA for quantifying antibodies to dengue viruses in mouse sera

A conventional ELISA using captured antigen was performed for quantifying antibodies to dengue viruses in mouse sera essentially by a method previously described for quantification of antibodies to JEV [29]. Microplates were sensitized with rabbit hyperimmune sera against each dengue serotype and incubated with culture fluids harvested from Vero cells infected with the corresponding serotypes. Sensitized plates were incubated serially with test sera, alkaline phosphatase-conjugated anti-mouse IgG and *p*-nitrophenyl phosphate. Tests were done in duplicate. To minimize interplate variations, a constant positive control serum prepared from HMAF was included in every plate, and absorbances obtained with test samples were adjusted with the value for the positive control as 1.0. The adjusted absorbances were expressed as ELISA values.

3. Results

3.1. Evaluation of pcD3ME and pcD4ME

To evaluate pcD3ME and pcD4ME for in vitro expression, CHO-K1 cells transfected with either of these plasmids and incubated at 37 °C for 24 h were examined for the presence of viral antigens by immunostaining with HMAF against the corresponding virus (DENV3 or DENV4) and monoclonals directed to prM (2H2) or E (4G2). Cells were stained with both polyclonal and monoclonal antibodies (data not shown), indicating that pcD3ME and pcD4ME have the ability to express prM and E antigens of the corresponding dengue viruses in transfected cells.

Our previous studies have demonstrated that prM/E genes are able to produce EPs in several systems of flaviviruses including DENV1 [26], DENV2 [35] and a closely-related JEV [29]. We therefore examined our newly constructed plasmids, pcD3ME and pcD4ME, for the ability to produce EPs simply by analyzing PEG-concentrated culture fluids of transfected CHO cells on sucrose density gradients. The amount of E antigens contained in each fraction was compared with fractions harvested from identical gradients prepared with the culture fluids of C6/36 cells infected with DENV3 or DENV4. Although a slowly-sedimenting hemagglutinin peak was not clearly resolved in both the DENV3 and DENV4 gradients, the peak of E antigen obtained with pcD3ME and pcD4ME samples migrated in the predicted position relative to the virion peak obtained in the DENV3 and DENV4 gradients (data not shown). These results indicate that E antigens expressed by cells transfected with pcD3ME or pcD4ME are released from cells and these extracellular antigens are probably produced in a particulate form.

3.2. Immunogenicity of tetravalent DNA vaccine

To evaluate the tetravalent DNA vaccine for its ability to induce neutralizing antibody to all serotypes, three separate experiments were performed. Mice were immunized twice with 100 μg of the tetravalent vaccine using two protocols: (i) immunization at a 3-week interval and bleeding 3 weeks after the second immunization (Experiments 1 and 2); (ii) immunization at a 7-week interval and bleeding 5 weeks after the second immunization (Experiment 3). As shown in Table 2, two immunizations using either protocol induced detectable neutralizing antibody against any of the four serotypes. Comparison of Experiment 3 with Experiments 1 and 2 indicates that longer intervals between two immunizations and/or between bleeding and the last immunization provide higher neutralizing antibody levels. Although not shown in this table, mice inoculated twice with 73 μg of the vector pcDNA3 did not develop detectable levels (<1:10) of neutralizing antibody to any of the serotypes at the corresponding time points in all experiments.

3.3. No detectable interference in immunogenicity among 4 vaccine components

To examine if there would be any interference in immunogenicity among the four components included in the tetravalent vaccine, levels of neutralizing antibodies induced by a single component of the tetravalent vaccine (monovalent vaccine) were compared with those induced by the tetravalent vaccine against the corresponding serotypes. Groups of six mice were immunized twice with 100 μg of the tetravalent vaccine or 25 μg of monovalent vaccines at a 3-week interval (Fig. 1).

Table 2
Induction of neutralizing antibody against four dengue serotypes in mice immunized with a tetravalent DNA vaccine

Experiment	Number of mice	Interval (weeks) between first immunization[a] and		Neutralizing antibody titer[b] against			
		Second immunization[a]	Bleeding	DENV1	DENV2	DENV3	DENV4
1	10	3	6	1:10	1:10	1:40	1:10
2	8	3	6	1:10	1:40	1:10	1:10
3	5	7	12	1:640	1:640	1:1280	1:160

[a] Groups of 4-week-old BALB/c mice were immunized with 100 μg of tetravalent vaccine twice at indicated intervals.
[b] Represented as the maximum serum dilution yielding a 70% reduction in plaque number.

Neutralizing antibody levels against DENV1, DENV2 and DENV3 induced by the tetravalent vaccine were equivalent to those induced by the corresponding monovalent vaccines, whereas higher antibody levels against DENV4 were shown in tetravalent vaccine-immunized compared to monovalent vaccine-immunized mice (compare open triangles in Panels A and B, 9 and 12 weeks after the first immunization). The results were consistent with those shown by ELISA antibodies. In ELISA, antibody levels against DENV1, DENV2 and DENV3 induced by the tetravalent vaccine were not significantly different from those induced by the corresponding monovalent vaccines ($P > 0.05$ by the Student's *t*-test), whereas significantly higher antibody levels against DENV4 were shown in tetravalent vaccine-immunized than in monovalent vaccine-immunized mice at 6 weeks ($P < 0.05$), and 9 and 12 weeks ($P < 0.01$) after the first immunization. These results indicate that the tetravalent vaccine does not cause any detectable interference among the four components in terms of antibody induction in mice. Further, the difference in antibody levels against DENV4 between tetravalent and monovalent vaccines suggests that on some occasions the combined immunization may increase immunogenicity of a single component.

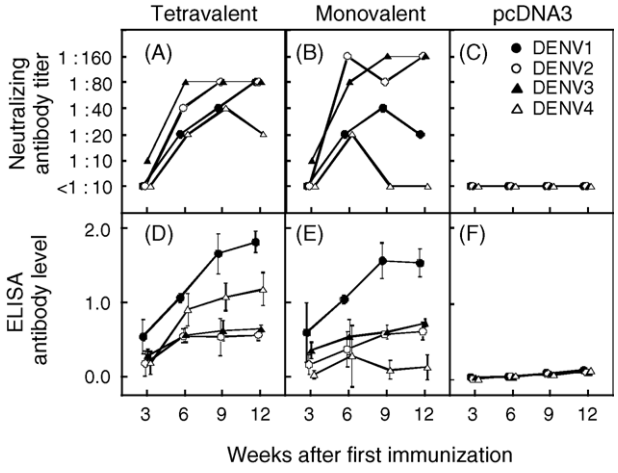

Fig. 1. Time course of neutralizing antibody titers (A–C) and ELISA antibody levels (D–F) induced in mice immunized with tetravalent or monovalent vaccines. Groups of six 4-week-old male BALB/c mice were immunized twice at an interval of 3 weeks with 100 μg of the tetravalent vaccine (mixture of 25 μg of each of pcD1ME, pcD2ME, pcD3ME and pcD4ME; A and D), 25 μg of each of monovalent vaccines mixed with 55 μg of pcDNA3 (B and E), or 73 μg of pcDNA3 (C and F; see Section 2 for details concerning DNA amounts). Neutralizing antibody titers (70% plaque reduction assay) were obtained with pooled sera, whereas ELISA antibody levels were obtained with individual sera and expressed as the mean and standard deviation (indicated by bars). Antibody titers/levels were obtained with antigens of DENV1 (closed circles), DENV2 (open circles), DENV3 (closed triangles) and DENV4 (open triangles). For mice immunized with monovalent vaccines, antibody titers/levels against the serotype corresponding to the vaccine are shown.

3.4. Duration of immunity induced by the tetravalent vaccine

To study the duration of the immune response in tetravalent vaccine-immunized mice, mice used in the experiment shown in Fig. 1 were kept until 30 weeks after the first immunization. The neutralizing antibody titers were maintained at 1:80 against DENV1 or further increased to 1:320 against DENV2, 1:160 against DENV3 and 1:40 against DENV4 (data not shown), indicating long-term immune responses induced by the tetravalent DNA vaccine.

3.5. Anamnestic neutralizing antibody responses

To estimate if the tetravalent DNA vaccine could induce memory B cells, mice immunized with the tetravalent vaccine were examined for the ability to induce an anamnestic antibody response to challenge with any of four serotypes. Groups of 6 mice were immunized with 100 μg of the tetravalent vaccine or inoculated with 73 μg of pcDNA3 twice at a 3-week interval and challenged i.p. with 10^8 FFU of each serotype 3 weeks after the second immunization. Prechallenge neutralizing antibody titers were 1:10 against DENV1, DENV2 and DENV4 and 1:40 against DENV3 in tetravalent vaccine-immunized mice, and <1:10 against all serotypes in pcDNA3-inoculated mice (data not shown). Mice were bled 4, 7 and 14 days after challenge and examined for neutralizing antibody (Fig. 2).

In pcDNA3-inoculated mice, neutralizing antibody levels were undetectable 4 days after challenge and became detectable 7 or 14 days after challenge, against any serotype. On the other hand, higher levels of neutralizing antibodies than those shown before challenge were developed in tetravalent vaccine-immunized mice 4 days after challenge. Although antibody titers induced in vaccine-immunized mice challenged with DENV4 were lower than those in vaccine-

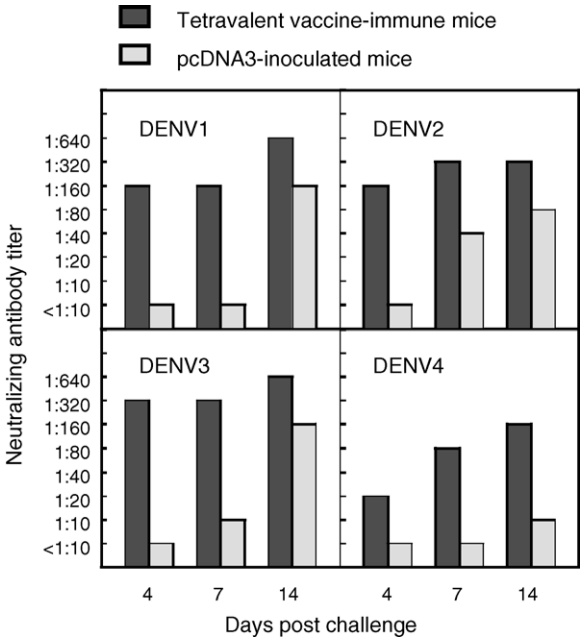

Fig. 2. Postchallenge neutralizing antibody titers in tetravalent vaccine-immunized mice (closed bars) compared with those in pcDNA3-inoculated mice (open bars). Groups of six mice immunized twice with 100 μg of the tetravalent vaccine were challenged i.p. with 10^8 FFU of each serotype (indicated in panels) 6 weeks after the first immunization (3 weeks after the second immunization), and were bled 4, 7 and 14 days after challenge. Pooled sera were examined for neutralizing antibody against each serotype used for the challenge. As a control, mice inoculated twice with 73 μg of pcDNA3 were challenged in the same protocol.

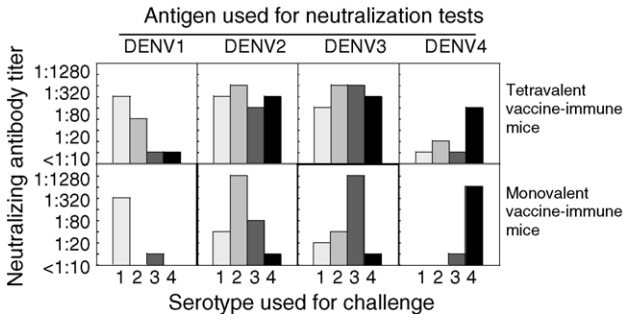

Fig. 3. Neutralizing antibody titers against four dengue serotypes in postchallenge sera obtained from mice immunized with the tetravalent vaccine or each of the four monovalent vaccines. For tetravalent vaccine-immunized mice (upper panels), sera collected 14 days after challenge, which were obtained in experiments shown in Fig. 2, were used. For monovalent vaccine-immunized mice (lower panels), mice immunized twice with each monovalent vaccine and then challenged with 10^8 FFU of the corresponding virus serotype were used. Postchallenge titers were obtained from mice challenged with DENV1 (lightest bars), DENV2 (second lightest bars), DENV3 (second darkest bars) and DENV4 (darkest bars).

immunized mice challenged with other serotypes, unimmunized mice challenged with DENV4 also developed lower titers than those challenged with other serotypes. Thus, in all serotypes, the levels of neutralizing antibody induced 4 days after challenge in vaccine-immunized mice were equivalent to those induced by unimmunized mice 14 days after challenge. These results indicate that the tetravalent vaccine can induce anamnestic antibody responses to challenge with any serotype.

Next, to assess levels of anamnestic responses to heterologous serotypes, pooled sera obtained from tetravalent vaccine-immunized mice 14 days after challenge were examined for neutralizing antibody against all serotypes. Levels of neutralizing antibodies against serotypes homologous or heterologous to those used for the challenge were compared with those obtained in mice immunized with one of four monovalent vaccines and then challenged with the corresponding serotype (Fig. 3). In monovalent vaccine-immunized mice, neutralizing antibody levels induced by homologous serotypes were ≥16-fold higher than those induced by heterologous serotypes, indicating a "specific" feature of the neutralization test. On the other hand, higher neutralizing antibody levels relative to those obtained in homologous combinations were induced after challenge with heterologous serotypes in tetravalent vaccine-immunized mice. In particular, in neutralizing antibody against DENV2 or DENV3, antibody titers induced after challenge with any serotype were equivalent (within four-fold) in tetravalent vaccine-immunized mice. These results indicate that tetravalent vaccine-immunized mice can also develop strong anamnestic neutralizing antibody responses to serotypes different from those used for the challenge.

4. Discussion

The present study demonstrated that our dengue tetravalent DNA vaccine can induce neutralizing antibodies and anamnestic neutralizing antibody responses to all serotypes in mice. The vaccine consisted of four plasmids containing the prM/E gene of each serotype. It is generally accepted that coexpression of the complete prM and E genes provides the best immunogenicity for flavivirus DNA vaccines [22]. On the other hand, the only dengue tetravalent DNA vaccine previously reported used a strategy of expressing domain III of E [36]: this vaccine induced ELISA antibodies to all serotypes and neutralizing antibody to DENV2 in mice.

Immunity to dengue virus infections has been extensively studied [8,37]. Although disease pathogenesis and protection mechanisms have not been fully clarified, neutralizing antibody has generally been used as a marker of vaccine effectiveness [4–7]. For DNA vaccines, induction of neutralizing antibody has been demonstrated using candidate monovalent vaccines against dengue type 1 and type 2 developed in other laboratories (reviewed in [21–23]). In general, disease severity is correlated with viremia levels: high levels of preexisting homotypic neutralizing antibody and low levels of viremia are associated with mild disease [5,7].

Murine models for evaluating the protective efficacy of dengue vaccine candidates have usually adopted intracranial challenge, which can cause lethal encephalitis. However, in

this case, both the disease (lethal encephalitis) and the challenge route (direct deposition into the target tissue) do not mimic the situation of man exposed to infective mosquito bites. In the present study, the ability to induce anamnestic responses to peripheral challenge was used to evaluate our tetravalent vaccine in a mouse model, as an indicator of the presence of vaccination-induced memory B cells. The production of immune responses to a peripheral amplification of virus would be expected in humans who become infected with dengue virus. From epidemiological evidence, pre-exposure to one serotype of dengue virus, which produces both B and T cell responses, is thought to prevent the development of disease on the subsequent homotypic infection [10]. Further studies using a monkey model will be needed to evaluate a protective capacity of the tetravalent DNA vaccine.

The dose of 100 μg was used for evaluation of our dengue tetravalent DNA vaccine in the present study, since this dose has been generally used for the first evaluation of a new DNA vaccine candidate in mice. For future use in humans, however, reduction of the vaccine dose would be desired. Currently, we are attempting an combined immunization of the tetravalent DNA vaccine and a protein-based vaccine, such as DENV2 EPs or an inactivated JE vaccine, to increase the vaccine effectiveness.

Long-lived antigen production is one advantage of DNA vaccines: the gene delivered into muscle cells can persist and continue to express gene products for weeks to months [38]. In the present study, long-term duration of immune responses was shown with our tetravalent vaccine for all serotypes. Also, this advantage should help overcoming difficulties in dose adjustment upon combined immunization: as shown in our previous study [26], similar levels of neutralizing antibody were induced in mice 14 weeks after immunization with the same amount of DNAs with varying abilities to produce EPs ranging over 1000-fold in vitro. This feature is particularly important for dengue vaccine, since incomplete immunization may result in enhanced disease.

Further, the tetravalent vaccine-immunized mice induced anamnestic neutralizing antibody responses to challenges with serotypes homologous and heterologous to those used for neutralization tests. Anamnestic responses to challenges with heterologous serotypes suggest that a relatively strong crossreactive neutralizing antibodies could be induced in DNA vaccine-primed mice upon infection. Considering the situation in endemic areas, this ability of our tetravalent vaccine can also contribute to long-term immune responses against all four serotypes.

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