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Evidence for antigen production in muscles by dengue and Japanese encephalitis DNA vaccines and a relation to their immunogenicity in mice

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Abstract

This study demonstrated viral antigen production in muscle tissues following inoculation with DNA vaccines and examined its relation to antibody induction in mice using the flavivirus system. To achieve detectable levels of antigen production, we used a needle-free jet injector and examined 10% homogenate of quadriceps muscle for viral antigens in a sandwich enzyme-linked immunosorbent assay. We compared DNA vaccines against dengue type 1 (designated pcD1ME), dengue type 2 (pcD2ME) and Japanese encephalitis (pcJEME). The amounts of viral envelope (E) antigen contained in muscle homogenate 1, 2, 3 and 4 days following inoculation with 50 μg of pcJEME were 1.1, 1.0, 0.3 and <0.1 ng/ml, respectively. Muscles from pcD2ME- and pcD1ME-inoculated mice did not contain detectable levels of E antigen (<0.1 ng/ml) during 4 days following inoculation. The E amounts released from Vero cells transfected with DNAs were in the order pcJEME > pcD2ME > pcD1ME. Levels of neutralizing antibody induced by two immunizations with 100 μg of each DNA vaccine using needle-free or normal needle/syringe injection systems also were in the order pcJEME > pcD2ME > pcD1ME, 2–11 weeks after the first immunization. However, the difference in antibody levels among three DNA vaccines 14–18 weeks after immunization was smaller than that in the early phase of immunization. These results provide fundamental information useful for developing combination DNA vaccines, such as a dengue tetravalent DNA vaccine, which require adjustment of immunogenicity of each component.

Keywords: Dengue; Japanese encephalitis; DNA vaccine; Needle-free injection

1. Introduction

Dengue fever and its severe form, dengue hemorrhagic fever, are diseases of world threat, which are caused by dengue viruses consisting of four serotypes [1,2]. No approved vaccines are currently available against these diseases. Since one of the possible mechanisms to cause dengue hemorrhagic fever upon infection with one serotype is the presence of non-neutralizing cross-reactive antibodies against other serotypes, combination of vaccines against each of four serotypes (tetravalent vaccine) is desired for developing an effective dengue vaccine. Clinical trials of attenuated tetravalent dengue vaccines [3–6] and preclinical evaluations of a chimeric tetravalent dengue vaccine [7,8] have shown the importance of dosage formulation to adjust immunogenicity of four different live vaccine components.

DNA vaccines are one promising gene-based vaccine, called the "third generation vaccine" [9–13]. Based on their advantages over other vaccines, the strategy is considered suitable for developing a dengue tetravalent vaccine. DNA vaccines do not cause interference due to combined immunization, different from infectious vaccines. In addition, DNA vaccines are durable, since they can persist in transfected cells and produce viral antigens for a relatively long period. Furthermore, DNA vaccines are inexpensive for production and do not require the cold chain, facilitating introduction of vaccines into dengue endemic areas. Dengue DNA vaccine candidates have been developed against dengue type 2 (DEN2) [14–16] and dengue type 1 (DEN1) [17–19].

Four serotypes of dengue viruses are members of the genus flavivirus. The flavivirus genome contains genes coding for three structural proteins including capsid (C), premembrane (prM) and envelope (E), and seven non-structural proteins [20]. Among these flavivirus genes, the co-expression of prM and E has demonstrated to induce the highest level of neutralizing antibody and protection in mice, using a system of a closely related flavivirus, Japanese encephalitis (JE) virus [21,22]. We found that extracellular

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subviral particles (EPs) produced in prM/E-expressing mammalian cells were able to induce neutralizing antibody and protection in mice [23]. Based on the prM/E gene strategy, we have developed pcDNA3-based DNA vaccines for JE [24] and DEN2 [25], both of which are able to induce neutralizing antibody in mice.

The mechanism of DNA vaccines to initiate immune responses in inoculated host animals has not yet fully elucidated [9–13]. Expression of viral antigens in dermal cells has been demonstrated by histochemical immunostaining [26], whereas delivery of foreign genes following intramuscular (i.m.) inoculation has been demonstrated using plasmids containing reporter genes [27,28]. However, levels of viral antigen produced in injected muscles have not been reported, to our knowledge, although it has been speculated that antigens are produced in the range of picograms to nanograms [13]. Quantification of in vivo antigen production provides information useful for elucidation of mechanisms of DNA vaccines to induce immune responses and for adjustment of immunogenicity of DNA vaccines for combined immunization, particularly for dengue tetravalent vaccines.

In the present study, we attempted to increase levels of viral antigen produced in muscles by using a needle-free jet injection device which has been reported to increase the uptake of [29] and immune responses to DNA vaccines [30,31]. We then compared the level of antigen production with the level of neutralizing antibody induced in mice. For this purpose, we constructed a DEN1 DNA vaccine based on the prM/E strategy and compared it with previously constructed DNA vaccines against DEN2 and JE.

2. Materials and methods

2.1. Virus

The Mochizuki strain of DEN1 virus, which had been passaged through suckling mouse brains for more than 100 times [32], was supplied from Dr. Susumu Hotta of Japan WHO Association. The virus was once passaged through C6/36 cells and used for vaccine construction, sedimentation analyses, mouse experiments and neutralization tests. The New Guinea C (NGC) strain of DEN2 virus [25] and the Nakayama strain of JE virus [24] have been described previously.

2.2. Construction of plasmids

The construction of pcDNA3-based plasmids encoding prM and E of JE virus (designated pcJEME [24]) and DEN2 virus (pcD2ME [25]), has been described previously. For construction of a DEN1 DNA vaccine plasmid, the cDNA containing prM/E genes of the Mochizuki strain was produced from purified viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) using Superscript First Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA). 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 Mochizuki strain [33], which was adjacent to a termination codon (TAA) and an *Eco*RV site (GCCGATATCTTACGCCTGAACCGTGACTCC). PCR was performed with the above antisense primer and a sense primer including an *Eco*RI site, an efficient eukaryotic initiation site (ACC) [34] and a start codon (ATG), followed by the codons encoding Asn-Arg-Arg-Lys-Arg-Ser-Val-Thr of the prM signal sequence (CCGGAATTCACCATGAATAGAAGAAAAAGATCCGTGACC). The amplified cDNA containing the prM/E gene cassette was inserted into the pcDNA3 vector (Invitrogen) at the *Eco*RI/*Eco*RV site between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from the bovine growth hormone. The construct was designated pcD1ME. The sequence of the prM/E region of pcD1ME differed at three nucleotides from the Mochizuki sequence reported from a different laboratory [33], from G to A at nucleotide number (nt#) 702 with an amino acid alteration from Gly to Asp at prM-89, from A to G at nt#1059 with an alteration from Asp to Gly at E-42 and from T to C at nt#1379 with an alteration from Tyr to His at E-149.

The DEN1 virus prM/E genes were cloned also into a vector, pCAGn-mcs-poly(A) [35,36], which was supplied by the Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan. This vector contains the human cytomegalovirus immediate early enhancer, chicken β-actin gene promoter, the polyadenylation signal derived from the bovine growth hormone, neomycin-resistance gene, and ampicillin-resistance gene. The cDNA contained in pcD1ME was amplified by PCR using sense and antisense primers containing the same nucleotide sequences as the corresponding primers described above, except for substitution of a *Xho*I for *Eco*RI site and of a *Bam*HI for *Eco*RV site. Amplified DNAs were inserted using the *Xho*I/*Bam*HI site in the polylinker between the promoter and the polyadenylation signal. The construct was designated pkD1ME. The sequence of the prM/E region of pkD1ME differed at two nucleotides from the sequence of the corresponding region of pcD1ME from G to A at nt#1059 with an amino acid alteration from Gly to Asp at E-42 and from T to C at nt#1815 with an alteration from Leu to Ser at E-294.

All plasmid DNAs (pcD1ME, pcD2ME, pcJEME, pcDNA3, pkD1ME) were purified using a Qiagen Plasmid Kit (Funakoshi Co. Ltd., Tokyo, Japan) following the manufacturer's instruction.

2.3. Generation of a stably transfected cell line

CHO-K1 cells [25] grown in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, and kanamycin at 60 μg/ml, at 37 °C in a humidified atmosphere of 5% CO_2–95% air, were transfected with 1 μg of pkD1ME

by using Transfectamine (Life Technologies, Gaithersburg, MD) according to the instructions supplied by the company. Following selection using medium supplemented with G418 (Life Technologies) at 400 μg/ml, transfected cells displaying high-level E protein expression were selected by limiting dilution cloning following the method we previously adopted for generating a cell line continuously expressing JE virus E antigen [37].

2.4. Sedimentation analysis

DEN1 virus EPs and virions were purified from culture fluids of a cell line stably transfected with pkD1ME (designated 5B cells) and Mochizuki-infected Vero cells, respectively, based on the method previously described for purification of JE virus EPs and virions [37]. Briefly, culture fluids were clarified by centrifugation and precipitated with polyethylene glycol (PEG). The precipitate was collected by centrifugation and applied on a 10–40% (w/w) continuous sucrose gradient prepared in TN buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl). Following centrifugation at 4 °C at 55,000 rpm for 60 min in a S55S rotor of a himac CS100GX micro-ultracentrifuge (Hitachi Koki, Ibaraki, Japan), fractions were collected from the bottom. Each fraction was tested for E antigen level by enzyme-linked immunosorbent assay (ELISA; see Section 2.5), infectivity by plaque assay on Vero cell monolayers (see Section 2.7), and hemagglutinating (HA) activity by a modification of the method described by Clarke and Casals [38].

2.5. ELISA for detection of E antigen

The JE [37] and DEN2 [39] virus E antigens were quantified using a sandwich ELISA as previously described. For estimation of DEN1 virus E antigen, we basically followed the ELISA method used for quantifying JE and DEN2 virus E antigens [37,39]. Briefly, 96-well plates were sensitized with an immunoglobulin fraction of polyclonal rabbit antibody against the Mochizuki strain (supplied by Dr. Hak Hotta of Kobe University School of Medicine, Japan). The plates were then incubated serially with test samples, a monoclonal anti-E antibody (4G2; provided by Dr. Tomohiko Takasaki of Department of Virology 1, National Institute of Infectious Diseases, Japan), alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. Antigen levels were calculated from absorbance values obtained with the sample and a reference standard. The homologous reference standards were used for quantification of JE and DEN2 antigens, whereas ELISA for estimation of DEN1 antigen used DEN2 virus EPs as a reference standard, due to difficulty in preparation of DEN1 virus EPs of an amount required for biochemical detection. We considered that DEN2 and DEN1 viral antigens are cross-reactive enough for estimation of DEN1 viral antigen, since DEN2 viral antigen provided similar dose response curves in ELISA systems for quantification of DEN2 or JE viral antigens.

2.6. Mouse experiment

ICR and ddY mice were purchased from Japan SLC (Shizuoka, Japan). For evaluating immunogenicity of DNA vaccines, groups of six 4-week-old male or female mice were inoculated twice at an interval of 3 weeks by i.m. injections with 100 μg of plasmid DNAs diluted in phosphate-buffered saline (PBS). In most cases, mice were inoculated using a spring-powered needle-free jet injector (ShimaJET; Shimadzu Corp., Kyoto, Japan) as instructed by the manufacturer. This injector forces DNA solution through a 0.16 mm-diameter orifice to create a high-pressure stream that penetrates the skin, depositing the DNA into the tissue beneath. We confirmed that the needle-free injector delivered Indian ink through skin into muscle tissue. For a control, a normal needle/syringe injection was used for inoculation of mice. In both injection systems, the volume of DNA solution was 50 μl for each thigh. Retroorbital blood was collected from mice mainly at 3-week intervals 2–18 weeks after the first immunization, and sera were isolated, pooled and examined for neutralizing antibody.

For evaluating antigen production in muscles, groups of six to twelve 4-week-old male ICR mice were inoculated with 50 μg of plasmid DNAs (in 50 μl) only at the right thigh. At daily intervals until the fourth day of inoculation, quadriceps muscles were collected from both thighs, weighed and homogenized in 1% bovine serum albumin (BSA) and 0.5% Triton X-100 in PBS to make a final 10% emulsion. After clarification, the homogenate was examined for antigen levels in ELISA (see Section 2.5). To eliminate non-specific ELISA reactions derived from muscle components, the ELISA absorbance obtained with muscles from the left thigh was subtracted from that obtained from the corresponding right thigh, and the E antigen amounts were estimated from the difference and the calibration curve obtained with the reference standard.

2.7. Neutralization tests

Neutralizing antibodies elicited in pcJEME- or pcD2ME-immunized mice were determined essentially as previously described, using plaque reduction assays performed with the Nakayama strain of JE virus [22] or the NGC strain of DEN2 virus [39] in the presence of rabbit complement. For pcD1ME-immunized mice, neutralizing antibodies were titrated following the method adopted for pcJEME- or pcD2ME-immunized mice [22,39], except that the Mochizuki strain of DEN1 virus was used and plaques were visualized by the immunochemical staining method. For immunochemical staining, cells were fixed, blocked with PBS containing normal horse serum at 1%, and then incubated serially with a monoclonal antibody to dengue virus E (4G2), biotinylated anti-mouse IgG, the ABC reagents, and

the VIP substrate (Vector Laboratories, Burlingame, CA). The neutralizing antibody titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque number. Both of the plaque method (adopted for testing antibody to JE or DEN2 virus) and the immunochemical staining method (adopted for testing antibody to DEN1 virus) provided equivalent neutralizing antibody titers in pilot experiments using JE and DEN2 virus.

3. Results

3.1. Evaluation of pcD1ME

3.1.1. Immunogenicity in ICR and ddY mice

To evaluate pcD1ME for immunogenicity, male and female mice of the ICR and ddY strains were immunized twice at intervals of 3 weeks with 100 μg of pcD1ME using a needle-free injector and sera were collected 2–11 weeks after the first immunization. For controls, mice were inoculated with 100 μg of pcDNA3 or vaccinated with 1×10^5 PFU of the Mochizuki strain of DEN1 virus (Table 1). All groups of pcD1ME-immunized mice developed moderate to high levels of neutralizing antibody 5–11 weeks after the first immunization. Mice inoculated with pcDNA3 did not develop detectable levels of neutralizing antibody, whereas infection with the Mochizuki strain induced high levels of neutralizing antibody. These results indicate that pcD1ME has an ability to induce neutralizing antibody in mice.

3.1.2. Production of EPs

Several attempts to generate stable cell lines producing DEN1 viral antigens using pcD1ME failed. Thus, we used pkD1ME for generating cells continuously producing DEN1 viral antigens. CHO-K1 cells were transfected with pkD1ME, selected in G418-containing medium and then cloned by limiting dilution. Although only 60–70% of E-expressing cells were observed following four passages in G418-containing medium, two cloning steps of these cells increased the percentage of E-expressing cells to more than 90%. Among several clones containing E-expressing cells, the highest yield of E antigen was shown with the clone 5B (designated 5B cells), which was used for preparing E antigen for sedimentation analysis.

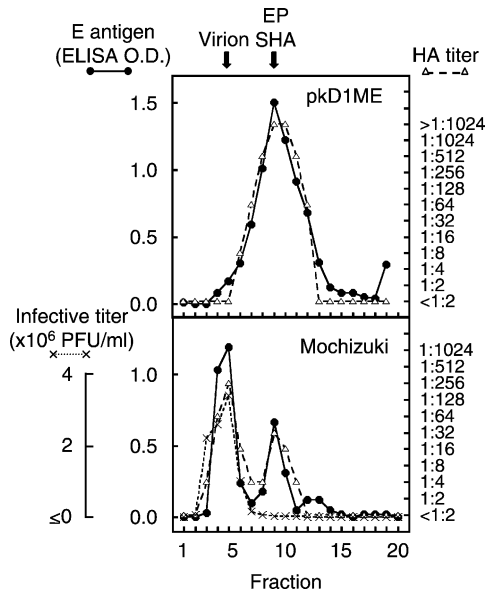

Fig. 1. Sucrose density gradient analyses of DEN1 virus E antigen released from CHO cells stably transfected with pkD1ME (5B cells). Culture fluids harvested from 5B cells (upper panel) and Mochizuki-infected Vero cells (lower panel) were precipitated with PEG and fractionated on 10–40% sucrose gradients. Fractions were examined for E antigen levels (●), HA activity (△) and infectivity (×). E antigen levels were measured in a sandwich ELISA using DEN1 virus-specific antibodies and represented as absorbance values. The arrows at the top of the figure identify peaks of virion and SHA/EP activity; fraction 1 was harvested from the bottom of the gradient (see Section 2.4 for details).

Culture fluids of 5B cells was PEG-precipitated and resolved on a 10–40% sucrose density gradient, and the amount of E antigens and HA activity contained in each fraction was compared with fractions harvested from identical gradients prepared with the culture fluids of Mochizuki-infected Vero cells. As shown in Fig. 1, these analyses identified a peak of E antigen in the 5B cell sample that co-migrated with Mochizuki-derived SHA particles. This result indicates that E antigen expressed by cells

Table 1
Induction of neutralizing antibody in ICR and ddY mice immunized with pcD1ME

Strain	Gender	Immunogen[a]	Neutralizing antibody titer[b]			
			2 weeks[c]	5 weeks	8 weeks	11 weeks
ICR	Male	pcD1ME	<1:10	1:40	1:160	1:320
ICR	Female	pcD1ME	<1:10	1:20	1:320	1:320
ddY	Male	pcD1ME	<1:10	1:20	1:160	1:320
ddY	Female	pcD1ME	<1:10	1:40	1:160	1:320
ddY	Male	pcDNA3	<1:10	<1:10	<1:10	<1:10
ddY	Male	Mochizuki	1:20	≥1:1280	≥1:1280	≥1:1280

[a] Groups of six 4-week-old mice of indicated strains were immunized twice at an interval of 3 weeks with 100 μg of DNAs using a needle-free injector or with 1×10^5 PFU of the Mochizuki strain using a normal needle/syringe by the i.p. route.
[b] Represented as the maximum serum dilution yielding a 90% reduction in plaque number.
[c] Weeks after the first immunization.

transfected with the prM/E genes of the Mochizuki strain of DEN1 virus was released from cells in a particulate form.

3.2. Comparison of pcJEME, pcD2ME and pcD1ME in levels of antigen production in muscles

Three plasmids encoding prM/E genes of each of JE, DEN2 and DEN1 viruses were compared in their abilities to produce viral antigens in muscles using male ICR mice. Since no detectable levels of E antigen was shown in mice immunized with pcJEME using a normal needle/syringe in a pilot experiment (data not shown), a needle-free injector was used to try to increase levels of antigen production in muscles. Mice were inoculated with 50 μg of DNAs at the right thigh and quadriceps muscles at both thighs were collected at daily intervals during 4 days after inoculation. The muscles were homogenized and examined for E antigen amounts in ELISA. The difference between ELISA absorbances obtained with samples from the DNA-injected (right) and non-injected (left) thighs of the same individual was considered as specific reactions due to the presence of E antigen. The ELISA absorbances obtained with muscles from the left thigh were equivalent to those obtained with muscles from mice inoculated with pcDNA3 (data not shown). As shown in Fig. 2, E antigen was detected in muscles from pcJEME-inoculated mice. Antigen levels were relatively high (approximately 1 ng/ml) 1 and 2 days following inoculation, and were decreased to 0.3 and <0.1 ng/ml 3 and 4 days following inoculation, respectively. The muscles from mice inoculated with pcD2ME or pcD1ME did not contain detectable levels of E antigen. These results indicate that needle-free injection increased antigen production in muscles of pcJEME-inoculated mice

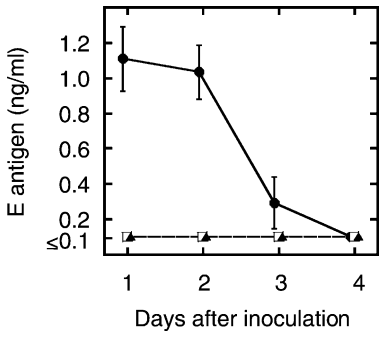

Fig. 2. E antigen production in muscles from mice inoculated with pcJEME (●), pcD2ME (□) or pcD1ME (▲). Quadriceps muscles of inoculated mice were homogenized and examined for E antigen levels in ELISA. For ELISA, the homologous reference standards were used for quantifying JE and DEN2 antigens, whereas DEN2 virus EPs were used as a reference standard in ELISA for estimation of DEN1 antigen (see Section 2.5 for details). Three individuals were used per day in the pcJEME-inoculated group, whereas in other groups three individuals were used only 1 day after inoculation and a single individual was used for each of other days, since a pilot experiment indicated that E antigen was detected in muscles only from pcJEME-inoculated mice. Vertical bars represent standard errors of data obtained from three mice.

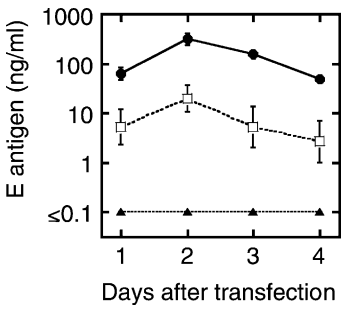

Fig. 3. E antigen levels released from Vero cells transfected with pcJEME (●), pcD2ME (□) or pcD1ME (▲). Cells grown in 35 mm-diameter dishes were transfected with 2 μg of each DNA in triplicate and maintained in 2 ml of MEM containing 10% FBS. At daily intervals, 100 μl of culture fluids were sampled and examined for E antigen levels in ELISA, during 4 days after transfection. For ELISA, the homologous reference standards were used for quantifying JE and DEN2 antigens, whereas DEN2 virus EPs were used as a reference standard in ELISA for estimation of DEN1 antigen (see Section 2.5 for details). Vertical bars represent standard errors of data obtained in triplicate.

to detectable levels and that pcJEME induced higher levels of antigen production in muscles than pcD2ME or pcD1ME.

3.3. Comparison of pcJEME, pcD2ME and pcD1ME in levels of EP production in vitro

To examine whether levels of in vivo antigen production by plasmid DNAs would be related to those of in vitro antigen production, Vero cells were transfected with pcJEME, pcD2ME or pcD1ME and cultivated in MEM containing 10% FBS for 4 days. Culture fluids were sampled at daily intervals and examined for E antigen amounts in ELISA (Fig. 3). The highest antigen yields were shown by pcJEME, whereas pcD1ME did not release detectable levels of antigens (<0.1 ng/ml). In cells transfected with pcJEME or pcD2ME, the maximum E antigen release was shown 2 days following transfection. These results indicate that the abilities of three plasmids to produce viral antigens in vitro were in the order pcJEME > pcD2ME > pcD1ME.

3.4. Comparison of pcJEME, pcD2ME and pcD1ME in immunogenicity

To directly compare abilities of three plasmids to induce neutralizing antibody, mice were immunized with 100 μg of DNAs twice at a 3-week interval using the needle-free or normal needle/syringe injection systems and sera were collected at 2, 5, 8, 11, 14 and 18 weeks following the first immunization (Fig. 4). Overall, neutralizing antibody titers induced by DNAs were in the order pcJEME > pcD2ME > pcD1ME in the early phase of immunization (2–11 weeks after the first immunization), irrespective of injection systems. In both injection systems, pcJEME-immunized mice

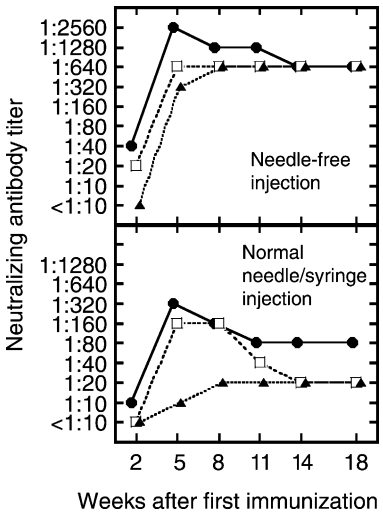

Fig. 4. Time course of neutralizing antibody titers induced in mice immunized with 100 μg of pcJEME (●), pcD2ME (□) or pcD1ME (▲) using a needle-free injector (upper panel) or a normal needle/syringe (lower panel). Groups of six 4-week-old male ICR mice were immunized twice at an interval of 3 weeks and bled 2–18 weeks after the first immunization. Neutralizing antibody titers were represented as the maximum serum dilution yielding a 90% reduction in plaque number.

developed the highest titer 5 weeks after the first immunization (2 weeks after the second immunization) and the titer was decreased to one-fourth of the highest titer 14 weeks after the first immunization. In contrast, the titers were increasing in pcD1ME-immunized mice during 8 weeks after the first immunization, and were maintained until the end of the experimental period. The time course of neutralizing antibody titers developed by pcD2ME-immunized mice showed intermediate patterns in both injection systems. Thus, the difference between pcJEME- and pcD1ME-immunized mice in the normal needle/syringe injection system was decreased from 32-fold (5 weeks after the first immunization) to 4-fold (11 weeks after the first immunization). In the needle-free injection system, all groups of mice showed equivalent antibody titers 14 and 18 weeks after the first immunization. These comparative studies demonstrate that the abilities of DNA vaccines to induce neutralizing antibody in mice were different in the early phase of immunization, but the difference in antibody levels among three DNA vaccines several weeks later was smaller than that in the early phase of immunization.

The needle-free injection provided higher levels of neutralizing antibody than the normal needle/syringe injection in all plasmids throughout the experimental period (Fig. 4). In particular, pcD1ME induced 32-fold higher neutralizing antibody titers when injected using the needle-free than normal needle/syringe system. Importantly, mice immunized with pcD1ME using the needle-free injection developed higher titers than mice immunized with pcJEME using the normal needle/syringe injection 8–18 weeks after the first immunization.

4. Discussion

In this study, antigen production in muscles following inoculation with DNA vaccines, which has not been readily detected so far, was successfully detected by using a needle-free injection device. Different from a normal needle/syringe injection, the needle-free injection system is considered to provide a more effective means to deliver genes to muscle cells. The effective delivery of vaccine DNAs to muscle cells by the needle-free system is consistent with higher immune responses induced in mice as shown in previous [30,31] and the present studies. It is possible that plasmid DNAs can be circulated following inoculation [29], but transfection of cells in tissues other than the inoculation site is not considered to frequently occur, since intravenous administration of naked DNAs do not effectively induce immune responses in host animals [40].

DNA vaccines used in the present study can produce antigen in a secreted form. Specifically, EPs are produced by the flavivirus prM/E genes contained in pcJEME, pcD2ME and pcD1ME. Therefore, the mechanism of these DNA vaccines to induce humoral immune responses seems to be similar to that of EP vaccines which have been demonstrated to induce high levels of neutralizing antibody [23,37,39]. Although it was considered that the antigen secreted from expressing muscle cells might be circulated, virus-specific antigens could not be detected in plasma following the needle-free injection with pcJEME (data not shown).

Comparison of three plasmids (pcJEME, pcD2ME, pcD1ME) indicated that levels of in vitro EP production by the prM/E genes were related to the in vitro replication level of the virus from which the prM/E genes were derived. Specifically, virus titers generally contained in culture fluid of infected cells and E antigen levels released from transfected cells (ml^{-1}) were roughly 10^8 PFU and 100 ng for JE virus, 10^7 PFU and 10 ng for DEN2 virus and 10^5–10^6 PFU and <0.1 ng for DEN1 virus, respectively (Fig. 3). Consistently, levels of E antigen released from stably transfected cells were approximately 100 ng/ml for JE virus [37] and 10 ng/ml for DEN2 virus [39]. These results are also consistent with previous poxvirus-based recombinant virus studies using JE [23] and dengue [41] virus genes expressed under the same promoter, which indicated higher levels of EP production by JE than dengue viral genes. This correlation may be attributed to the fact that the biosynthetic process of EPs mimics the virion morphogenesis [42].

Neutralizing antibody is considered the most important immune component for protection from dengue or JE [2,4]. The correlation between the ability of DNA vaccine plasmids to produce EPs in vitro and the ability of DNA vaccines to induce neutralizing antibody in mice has been shown using tick-borne encephalitis (TBE) or JE virus systems. Studies using TBE virus have demonstrated that when truncated or full-length E is co-expressed with prM, only the full-length of E induces secretion of EPs from cells [43] and neutralizing antibody and protection in mice [44]. Our previous

comparison between poxvirus-based recombinants encoding JE virus prM/E with or without NS1 indicated that the prM/E construct induced four to eight-fold higher levels of EP production in vitro and neutralizing antibody in mice than the prM/E/NS1 construct [22]. Also, our previous studies using DNA vaccines for JE [24,37] and DEN2 [25,39] suggested a similar correlation between levels of EP production in vitro [37,39] and neutralizing antibody induction in mice [24,25].

Although a relatively large difference in neutralizing antibody titers was shown in the early phase of immunization among mice immunized with plasmids producing different levels of E antigens, levels of neutralizing antibody 14 weeks after the first immunization were equivalent in the needle-free injection system or within four-fold in the normal needle/syringe injection system (Fig. 4). The decrease in neutralizing antibody titers shown in pcJEME-immunized mice from 5 to 14 weeks following the first immunization (Fig. 4) may be attributed to the fact that the level of antigen production in muscles was decreased to undetectable levels 4 days after inoculation with pcJEME (Fig. 2), since relatively high levels of antigen production is considered the most critical factor to induce neutralizing antibody in the early phase of immunization. However, following the decrease, the level of neutralizing antibody was maintained until the end of the experimental period (Fig. 4). Furthermore, levels of neutralizing antibody in pcD1ME-immunized mice increased until 8 weeks after the first immunization and were maintained until the end of the experimental period. Although the level of antigen production in the muscle were undetectable, it is speculated that very low levels of JE and dengue viral antigens were continuously produced in muscle cells, since it has been demonstrated using a reporter gene that the gene delivered into muscle cells can persist and continue to express gene products for weeks to months [27,45]. Thus, the similar levels of neutralizing antibody at the end of the experimental period are probably attributed to the long-lived antigen production at undetectably low levels by the DNA vaccine. Moreover, the neutralizing antibody titers shown at this period depended on the injection system rather than the ability of plasmid DNAs to produce antigens. The number of continuously expressing cells might be more important than the expression level of vaccine plasmids for the host to induce higher neutralizing antibody levels.

Immunogenicity of DNA vaccines depends on animals used for evaluation. Although our previous studies using ICR mice purchased from CLEA Japan have shown that i.m. or intradermal (i.d.) inoculation with 100 μg of pcJEME or pcD2ME induced only low levels of neutralizing antibody (1:10 to 1:20 in a 90% plaque reduction neutralization test [24,25]), evaluation in swine indicated that i.m. or i.d. inoculation with 100 or 450 μg of pcJEME induced higher levels of neutralizing antibody than the commercial inactivated JE vaccine [46]. Furthermore, evaluation of pNJEME (that contains the JE virus prM/E genes identical to those contained in pcJEME but is based on a vector designed to address some of the safety concerns of DNA vaccine use) in monkeys indicated that i.m. inoculation with 300 μg of pNJEME or gene gun administration with 3 μg of pNJEME induced similar levels of neutralizing antibody to those induced by the inactivated JE vaccine in most animals [47]. In the present study, ICR mice from a different breeder (Japan SLC) and ddY mice developed considerably higher levels of neutralizing antibody than the ICR strain from CLEA Japan which we had consistently used for evaluation of our DNA vaccines, indicating a relatively wide variation among mouse strains in responses to DNA vaccines.

In conclusion, the relation between levels of E antigen production in vitro and neutralizing antibody induction in mice would be a key factor for dosage formulation in combined DNA vaccines, such as dengue tetravalent DNA vaccines. However, adjustment of immunogenicity for DNA vaccines may not be as strict as considered for attenuated or chimeric tetravalent vaccines [3–8], since similar levels of neutralizing antibody were induced in mice by three DNAs with varying abilities to produce EPs ranging over 1000-fold in vitro, except for the early phase of immunization. The increasing effect of the needle-free injection system on immunogenicity of dengue DNA vaccines and the long-lived duration of antibody responses indicate the potential for developing an effective dengue tetravalent DNA vaccine.

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