表１．イヌ街上毒株 QS-05のハムスターでの病原性の変化

QS-05 (30% brain susp.)	QS-05 SMB-P1 (30% brain susp.)	QS-05 SMB-P2 (30% brain susp.)	QS-05 BHK-P10 (medium susp.)
Undiluted (50μl) 40% Lethal	Undiluted (50μl) 10% Lethal Undiluted (100μl) 33% Lethal	$IMLD_{50}=10^{-1.7}$	$IMLD_{50}=10^{-2.5}$

イヌ狂犬病ウイルス QS-05 株のハムスターでの病原性の変化。QS-05 SMB-P1 株は QS-05 株を乳のみマウス脳(SMB; suckling mouse brain)で１代継代した株、QS-05 SMB-P2 株は QS-05 株を乳のみマウス脳で２代継代した株、QS-05 BHK-P10 株は BHK-21 細胞で 10 代継代した株。QS-05 株、QS-05 SMB-P1 株、QS-05 SMB-P1 株は 30％脳懸濁液として、QS-05 BHK-P10 株はその培養液上清をハムスター接種の原液として用い、$IMLD_{50}$ を計算した。

Ⅲ. 研究成果の刊行に関する一覧表

III 研究成果の刊行に関する一覧表

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Development of an enzyme-linked immunosorbent assay for serological diagnosis of tick-borne encephalitis using subviral particles

Mayumi Obara, Kentaro Yoshii, Tomoko Kawata, Daisuke Hayasaka, Akiko Goto, Tetsuya Mizutani, Hiroaki Kariwa, Ikuo Takashima *

Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

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Abstract

The similarity of symptoms produced by tick-borne encephalitis (TBE) and Japanese encephalitis (JE) and the high degree of cross-reactivity between TBE and JE viruses by serological tests make the development of a differential diagnostic test a priority. In this study, recombinant prM/E proteins of TBE virus strain Oshima 5–10 expressed in mammalian cells resulted in the release of subviral particles (SPs) into the culture medium. Using the SPs as antigens, enzyme-linked immunosorbent assay (ELISA) systems were developed to detect TBE virus-specific IgM and IgG antibodies, designated SP-IgG and SP-IgM ELISAs, respectively. Of 83 serum samples from encephalitis patients in Khabarovsk, Russia, which were positive with the neutralization test (NT), 82 were positive by the SP-IgG ELISA, for a sensitivity of 98.8%, which was higher than that of a commercial ELISA kit. All 12 NT-negative samples were also negative by the SP-IgG ELISA (specificity, 100%). Of 17 patient samples that were NT-positive, 16 (94.1%) were positive by the SP-IgM ELISA. Of 15 paired serum samples that yielded equivocal results by NT, 11 had positive results with the SP-IgM ELISA, indicating a diagnosis of TBE infection. The SP-IgG and SP-IgM ELISAs showed no cross-reactivity with antibodies to the JE virus. The results indicate that these ELISAs will be useful for the detection of TBE-specific antibodies.

Keywords: Tick-borne encephalitis virus; ELISA; Subviral particles

1. Introduction

Tick-borne encephalitis (TBE) virus belongs to the family Flaviviridae, genus Flavivirus, and causes fatal encephalitis in humans. There are three main genetic lineages of TBE virus; the European, Siberian, and Far Eastern subtypes. They cross-react each other (Hayasaka et al., 2001). Although there are various tests for detection antibodies for the serological diagnosis of TBE, neutralization tests (NTs) are used in areas where two or more flaviviruses are endemic because of their high degree of specificity for each virus. However, neutralization tests have some disadvantages, including the requirement for a high-level biocontainment facility to handle the live viruses, the need for advanced and skilled techniques, limitations on the number of samples that can be tested, and the time-consuming nature of the neutralization reaction. On the other hand, enzyme-linked immunosorbent assays (ELISA) based on inactivated TBE virus antigens are also used widely. Since many samples can be tested in a short time under ordinary laboratory conditions with ELISA, ELISA is a useful diagnostic method. However, the production process for the ELISA antigen requires purification and inactivation of the virus using skilled techniques in laboratories with biosafety facilities. It has also been reported that the standard TBE ELISAs can cross-react with antibodies to other flaviviruses (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Therefore, serological diagnostic methods which are safe, simple, and specific to TBE virus need to be developed.

The positive single-stranded RNA genome of the genus Flavivirus consists of about 11,000 nucleotides. It encodes three structural proteins, i.e., the core (C), precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, i.e.,

* Corresponding author at: Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan. Tel.: +81 11 706 5211; fax: +81 11 706 5211.
E-mail address: takasima@vetmed.hokudai.ac.jp (I. Takashima).

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NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990). When recombinant prM and E proteins are co-expressed in mammalian cells, subviral particles (SPs) that do not contain nucleocapsids are released into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). The recombinant prM and E proteins of the Oshima 5–10 strain of TBE virus can be used to produce SPs in such systems, as described previously (Yoshii et al., 2003). SPs are expected to be useful as antigens for the serological diagnosis of Flavivirus infections because they maintain the authentic characteristics of the viral antigens. The SPs of the European subtype TBE virus, expressed in insect cells using a recombinant baculovirus system, have been used to develop an IgM ELISA (Jaaskelainen et al., 2003).

In this study, ELISAs for the detection of anti-TBE IgG and IgM were developed using SPs of the Far Eastern subtype TBE virus expressed in mammalian cells as antigens. The ELISAs were evaluated using serum samples from patients with suspected TBE from Khabarovsk, Russia, and the results were compared with those obtained using the neutralization test and two commercial ELISA kits.

2. Materials and methods

2.1. Cells and virus strains

BHK-21 cells were cultured in Eagle's minimum essential medium containing 8% fetal bovine serum (FBS) and were used for the neutralization tests. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and were used for the expression of recombinant proteins.

The Oshima 5–10 strain of TBE virus and the JaGAr-01 strain of Japanese encephalitis (JE) virus were used. The Oshima 5–10 strain was isolated from dogs in 1995 and was the Far Eastern subtype (Takashima et al., 1997). The JaGAr-01 strain was isolated from mosquitoes in Japan in 1959 (Matsuyama et al., 1960). The viruses were propagated by intracerebral inoculation of suckling mice.

2.2. Serum samples

All serum samples were heat-inactivated at 56 °C for 30 min and were stored at −40 °C. A total of 95 serum samples were collected from 43 patients in Khabarovsk, Russia, who were suspected of having TBE on the basis of clinical signs. Nine serum samples were single serum samples and 86 were paired samples from 34 patients. Acute samples were collected on from 1 to 17 days after onset of illness and convalescent samples were collected on from 5 to 72 days after onset of illness. The interval between collection of acute and convalescent samples were from 1 to 61 days. Ten serum samples were collected from patients with JE in Nepal and had been shown to have JE-specific IgM antibodies by using IgM ELISA (Akiba et al., 2001). They were all negative for TBE on the NT. Twenty-one negative control serum samples were obtained from individuals who were negative for TBE by the neutralization test.

2.3. Neutralization tests

These tests were carried out as described previously (Kariwa et al., 1995). Serum samples that produced a 50% reduction in focus formation of the Oshima 5–10 strain of TBE virus on BHK cells in 96-well plates as determined by immunohistochemical staining at a dilution of 1:20 or greater were judged to be neutralization test-positive. The patients who have greater than four-fold increase in the neutralizing titer in the convalescent phase compared with the acute phase of infection were diagnosed with TBE infection.

2.4. Plasmids and antigens

A plasmid encoding the prM and E proteins of the Oshima 5–10 strain (pCAGprME, Yoshii et al., 2003) was transfected into 293T cells as described previously (Yoshii et al., 2003). After a 48-h incubation at 37 °C, the culture medium was harvested and centrifuged at 12,000 rpm for 30 min. A one-third volume of polyethylene glycol (PEG) solution (40% w/v PEG8000, 7.6% w/v NaCl) was added to the collected culture medium supernatants. After gentle shaking at 4 °C for 2 h, the mixture was centrifuged at 10,000 rpm for 20 min. The pellets were dissolved in carbonate–bicarbonate buffer (Sigma Chemical Co., St. Louis, MO) to yield 1% of the original culture medium volume, and this was used as the positive antigen for the ELISAs. The negative antigen was prepared from the culture medium of nontransfected 293T cells.

2.5. SP-IgG ELISA

The monoclonal antibody (mAb) 1H4, which recognizes the E protein of the Oshima 5–10 strain of TBE virus (Komoro et al., 2000), was coated onto 96-well microplates (50 μl/well, 2 μg/ml in carbonate buffer). After overnight incubation at 4 °C, the plates were washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). A blocking solution (Block Ace diluted 1:4 in ddH_2O; Dai-Nippon, Osaka, Japan) was applied (200 μl/well), and the plates were incubated at 37 °C for 1 h. The plates were washed before adding the subviral particle antigen (50 μl/well, 1:10 dilution in PBST containing 0.3% bovine serum albumin) and incubating at 37 °C for 1 h. After washing, the serum samples were added (50 μl/well, 1:800 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h. Bound IgG antibodies were detected by adding 50 μl/well of alkaline-phosphatase-conjugated anti-human IgG goat IgG (1:2000 in PBST containing 0.3% bovine serum albumin; Sigma) and incubating at 37 °C for 1 h. The color reaction was developed by adding 100 μl/well of *p*-nitrophenyl phosphate and incubating at 37 °C for 90 min, and the absorbance at 405–620 nm was measured. The results for each serum sample were reported as the positive:negative ratio (P/N), that is, the ratio of the optical density (OD) with the positive antigen to the OD with the negative antigen.

2.6. SP-IgM Elisa

Anti-human IgM goat IgG (50 μl/well, 1:400 in carbonate buffer; ICN Biomedicals, Aurora, OH) was added to 96-well microplates. After overnight incubation at 4 °C, the plates were washed five times with PBST before adding 200 μl/well of blocking solution containing 3% bovine serum albumin in PBS and incubating at 37 °C for 1 h. After washing, the serum samples were added (50 μl/well, 1:100 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h before washing again. The subviral particle antigen was added (50 μl/well, 1:20 dilution in PBST containing 1% skim milk), and the plates were incubated at 37 °C for 1 h and washed again. The subviral particle antigen bound by the IgM antibodies was detected by the addition of biotinylated mAb 1H4 (50 μl/well, 0.25 μg/ml in PBST containing 0.3% bovine serum albumin) and incubation at 37 °C for 1 h, followed by washing, the addition of horseradish peroxidase–streptavidin (50 μl/well, 1:3000 in PBST containing 1% skim milk; Zymed, South San Francisco, CA), and incubation at 37 °C for 1 h. The color reaction was developed by the addition of 3,3′,5,5′-tetramethylbenzidine (100 μl/well; Sigma). The reaction was stopped after 15 min at room temperature by the addition of 100 μl of 0.5N H_2SO_4. The absorbance at 450 and 620 nm was measured using a plate reader. The P/N ratios were determined as described above for the SP-IgG ELISA.

2.7. Commercial ELISA

The commercial Immunozym FSME IgG and IgM kits (Progen Biotechnik, Heidelberg, Germany) were used for comparison with the SP-ELISA. In this commercial ELISA, the European subtype virus was used as antigens.

3. Results

3.1. SP-IgG ELISA

Ninety-five serum samples from patients with suspected TBE and 21 negative control samples were tested for the presence of anti-TBE IgG antibodies using the SP-IgG ELISA, and the sensitivity and specificity of the test were determined by comparison with the results of the neutralization test, using the corresponding cut-off values (Fig. 1). The sensitivity of the SP-IgG ELISA decreased with increasing cut-off values, while the specificity increased. The difference between the sensitivity and specificity was minimal when a cut-off value of 1.155 was used. At a cut-off value of 1.155, the sensitivity of the SP-IgG ELISA about 95 serum samples from patients suspected TBE was 98.8% (82/83) and the specificity was 100% (12/12) as compared with the neutralization results (Table 1). All of 21 samples which were negative by neutralization were negative on the SP-IgG ELISA. Only 57 of the 83 NT-positive samples were positive on the commercial IgG ELISA (Table 2), whereas five samples were negative and 21 samples were inconclusive because the results were close to the boundary values. The 12 samples that had neutralizing titers <1:20 were also negative on both the SP-IgG ELISA and the commercial IgG ELISA. IgG antibody was detected with SP-IgG ELISA in the samples from 1 to 72 days after onset of illness. On the other hand, the samples were positive with commercial IgG ELISA from 2 to 72 days.

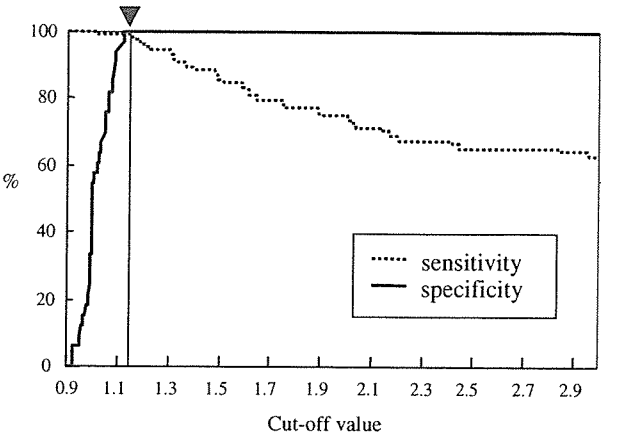

Fig. 1. Relationship between cut-off value, sensitivity, and specificity for the SP-IgG ELISA: 95 serum samples collected from 43 patients who were suspected TBE and 21 negative control serum samples were tested. The cut-off value was set as the point at which the difference in the sensitivity and specificity was minimal.

Table 1
Comparison of the results obtained by neutralization and SP-IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	SP-IgG ELISA		Total
	Positive	Negative	
Positive	82	1	83
Negative	0	12	12
Total	82	13	95

3.2. SP-IgM ELISA

The distribution of the P/N ratios resulting from the analysis of the serum samples using the SP-IgM ELISA is shown in Fig. 2. The samples tested were clearly separated into two groups: one group of sera had P/N values between <1.0 and <1.25 and were presumed to be negative for TBE-specific IgM antibodies, and the other group had P/N values between <1.9 and <50 and were presumed to be positive for TBE-specific IgM antibodies. The mean of the maximum P/N ratio of the negative group (1.224)

Table 2
Comparison of the results obtained by the neutralization test and commercial IgG ELISA (95 serum samples collected from 43 patients who were suspected TBE)

Neutralization test	Commercial IgG ELISA			Total
	Positive	Negative	Inconclusive[a]	
Positive	57	5	21	83
Negative	0	12	0	12
Total	57	17	21	95

[a] Inconclusive results due to boundary values.

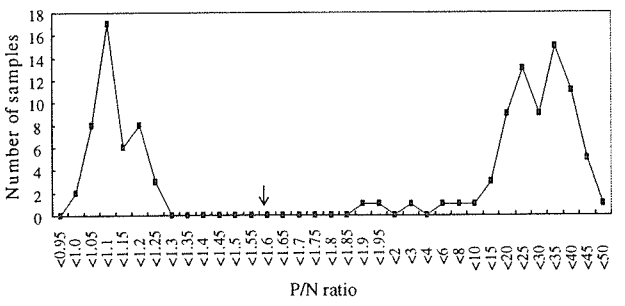

Fig. 2. The distribution of the P/N ratios for 95 serum samples tested with the SP-IgM ELISA: selected cut-off value.

and the minimum P/N ratio of the positive group (1.891) were selected as the cut-off value (i.e., P/N = 1.557).

Paired serum samples were collected from 34 of the patients suspected of having TBE; 17 of the 34 patients were diagnosed with TBE infection based on a greater than four-fold increase in the NT titer in the convalescent phase compared with the acute phase of infection. Two of the 34 patients had convalescent antibody titers that remained <1:20 and were thus found not to have been infected with TBE virus. Fifteen of the 34 patients did not have a conclusive diagnosis because the neutralizing titers of the paired sera did not increase significantly.

Table 3 shows the comparison of the testing results with the SP-IgM ELISA and the neutralization test for these paired serum samples from 34 patients. Of the 17 patients that tested positive for TBE infection by neutralization, 16 patients were positive for TBE-specific IgM antibodies on both the SP-IgM ELISA and the commercial IgM ELISA, and one patient were negative by both the SP-IgM ELISA and the commercial IgM ELISA. These serum samples of this one patient were collected on 2, 13 and 33 days after the onset of illness and neutralizing titers were 1:80, 80 and 320. These serum samples were all positive by both the SP-IgG ELISA and the commercial IgG ELISA. The two patients that were negative for TBE infection by neutralization also tested negative on both the SP-IgM ELISA and commercial IgM ELISA. Of the 15 patients that gave equivocal results by the neutralization test, 11 were positive for TBE-specific IgM antibodies by the SP-IgM ELISA.

Paired serum samples were not available for nine patients, of which seven were not diagnosed with TBE infection despite a high neutralizing titers (from 1:160 to 1:20,480) because the results for acute and convalescent phase sera could not be compared. Six of these seven patients were found to have TBE-specific IgM antibodies according to the SP-IgM ELISA. One patient whose neutralizing antibody titer was 1:160 was negative by the SP-IgM ELISA. The remaining two patient sera had neutralizing antibody titers <1:20 and were also negative by the SP-IgM ELISA.

Table 3
Comparison of the results obtained in by the neutralization test and SP-IgM ELISA (86 paired serum samples from 34 patients)

Neutralization test	SP-IgM ELISA		Total
	Positive	Negative	
Positive[a]	16	1	17
Negative[b]	0	2	2
Undetermined[c]	11	4	15
Total	27	7	34

[a] Neutralizing titers in the convalescent phase increased greater than four-fold compared with the acute phase.
[b] Neutralizing titers in both acute and convalescent samples were 1:<20.
[c] Undetermined due to no significant increase of neutralizing titers.

Table 4
Comparison of results obtained by commercial IgM ELISA and SP-IgM ELISA

Commercial IgM ELISA	SP-IgM ELISA		Total
	Positive	Negative	
Positive	52	0	52
Negative	13	23	36
Inconclusive[a]	7	0	7
Total	72	23	95

[a] Inconclusive results due to boundary values.

The results from the SP-IgM ELISA and the commercial IgM ELISA were compared (Table 4). Of the 95 serum samples tested, 52 were considered positive for TBE-specific IgM antibodies by both tests. Of the 36 samples that were negative with the commercial IgM ELISA, 13 were positive and 23 were negative by the SP-IgM ELISA; the 13 positive samples also had positive neutralizing antibody titers. Seven samples that gave inconclusive results by the commercial IgM ELISA were positive on the SP-IgM ELISA. IgM antibody was detected with SP-IgM ELISA in the samples from 1 to 72 days after onset of illness. The samples from 2 to 64 days were positive with commercial IgM ELISA. These results indicated that the sensitivity of the SP-IgM ELISA was better than that of the commercial IgM ELISA.

3.3. *Cross-reactivity with Japanese encephalitis virus*

Ten serum samples from patients with Japanese encephalitis virus infections, which were known to contain JE-specific IgM antibodies (Akiba et al., 2001), were tested to examine the

Table 5
Cross-reactivity of JE patient sera to TBE virus by commercial ELISAs and SP-ELISAs

Patient no.	Commercial ELISAs		SP-ELISAs	
	IgG	IgM	IgG	IgM
1	±[a]	±	–	–
2	–[b]	–	–	–
3	–	–	–	–
4	–	–	–	–
5	–	–	–	–
6	–	–	–	–
7	–	±	–	–
8	–	–	–	–
9	±	–	–	–
10	±	±	–	–

[a] ±: Boundary.
[b] –: Negative.

cross-reactivities of the SP-based and commercial ELISAs. Whereas 3 of 10 samples gave inconclusive results by the commercial IgG and IgM ELISAs, respectively (Table 5), all 10 samples were negative by both the SP-IgG ELISA and the SP-IgM ELISA. These results indicated that the SP-IgG and SP-IgM ELISAs were specific to antibodies against TBE virus.

4. Discussion

The virus neutralization test is often used as a specific serological diagnostic test for TBE infection. However, the test is time-consuming and must be carried out in a high-level biosafety facility. ELISA tests using inactivated whole virus as antigen have also been widely used for the serological diagnosis of TBE virus infection, but the production and inactivation of live TBE virus for this application is also restricted by safety considerations. Thus, the generation of recombinant viral proteins is an important approach for the development of alternative antigens that are both less expensive and less hazardous to prepare and use.

During in vitro infection of cells with TBE virus, subviral particles with no nucleocapsids are released from cells at the same time as mature virions (Allison et al., 1995; Russell et al., 1980). Likewise, when recombinant flaviviral prM and E proteins are co-expressed in mammalian cells, SPs are secreted into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). Similarly, it has been confirmed that transfection of the pCAGprME plasmid, which encodes the prM and E proteins of TBE virus, into 293T cells results in the release of TBE SPs into the culture medium (Yoshii et al., 2003). The E proteins in SPs are considered to have almost the same structure and function as those in complete virions (Allison et al., 1995). As SPs do not have genomic RNA, they cannot replicate even if they enter cells and can thus be handled in laboratories with no biosafety facilities. For these reasons, SPs are gradually replacing viruses for a variety of applications. ELISAs using SPs from Japanese encephalitis virus, West Nile virus, and European subtype TBE virus have been presented as useful serological diagnostic methods (sequentially, Davis et al., 2001; Hunt et al., 2001; Konishi et al., 1996; Jaaskelainen et al., 2003).

As presented in a previous report, ELISAs have been developed for the serological diagnosis of TBE infection based on recombinant viral proteins (Yoshii et al., 2003). In that study, lysates of cells transfected with pCAGprME were used as the ELISA antigen. In the present study, new ELISA tests were developed using antigens prepared from TBE virus SPs harvested from the supernatant of transfected cells and compared these ELISAs with other testing methods.

The SP-IgG ELISA was found to have a high sensitivity (82/83, 98.8%) and specificity (12/12, 100%) as compared with the neutralization test when the cut-off value for the ELISA was set at a P/N ratio of 1.155 (Table 1). In contrast, the commercial IgG ELISA had a relatively low sensitivity and generated many inconclusive test results (Table 2). These findings suggest that the SP-IgG ELISA can substitute for the neutralization test and a commercial IgG ELISA for the detection of anti-TBE virus IgG antibodies. The SPs used as antigen in the SP-IgG ELISA were concentrated from culture supernatants without further purification or fixation, and an E-protein-specific mAb was used as the capture antibody. This ELISA may have led to the high degree of sensitivity and specificity of the SP-IgG ELISA.

The distribution of the P/N ratios from the SP-IgM ELISA indicated that the serum samples were clearly separated into a low P/N group and a high P/N group, which were presumably IgM-negative and IgM-positive, respectively (Fig. 2). This distribution curve permitted a cut-off value at the mean of the maximum P/N ratio of the negative group and the minimum P/N ratio of the positive group.

The diagnosis of TBE infection using the neutralization test requires paired serum samples and the measurement of a significant (greater than four-fold) increase in the neutralizing antibody titer. We found that the SP-IgM ELISA was superior to the neutralization test for diagnostic testing using paired and single serum samples. Only one of 17 neutralization-positive patients was negative according to the SP-IgM ELISA. Of the 15 paired sera that yielded equivocal results by the neutralization test owing to the lack of significant increases in antibody titers, 11 were positive and 4 were negative using the SP-IgM ELISA. The SP-IgM ELISA could also be applied to single serum samples for the diagnosis of TBE infection. Of seven single serum samples with NT titers $\geq$1:160, six (NT titers were from 1:1280 to 1:20,480) were judged to be positive by the SP-IgM ELISA.

The SP-IgM ELISA was both more sensitive and more specific than the commercial IgM ELISA when both tests were compared (Table 4). Of 95 samples tested, 52 were positive by both tests. Eleven (21.2%) of these 52 samples were negative by the IgM ELISA using recombinant antigens (Yoshii et al., 2003). However, of 36 samples that were negative by both the commercial IgM ELISA and the IgM ELISA using recombinant antigens, 23 were negative and 13 were positive by the SP-IgM ELISA. The 13 samples that were positive by the SP-IgM ELISA also were positive by the neutralization. Seven serum samples that yielded inconclusive results with the commercial IgM ELISA were positive with the SP-IgM ELISA. The discrepancy between the two ELISA tests may be attributed to differences in the antigens used. The SP-IgM ELISA uses unfixed SPs, whereas the commercial ELISA uses formalin-fixed virions. Formalin fixation may cause a loss of antigenicity of the virion proteins (Heinz et al., 1995). In addition, the two tests use different strains of TBE virus as the antigen source; the SP-IgM ELISA uses the prM and E proteins of a Far Eastern subtype strain, whereas the commercial ELISA uses a European subtype strain.

It has been reported that infection and/or vaccination with other flaviviruses, including yellow fever virus, dengue virus, West Nile virus, and JE virus, can induce cross-reactive antibodies (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Both the IgG and IgM TBE-specific SP ELISAs had little cross-reaction with antibodies against the JE virus. Whereas 3 of 10 serum samples from JE patients had marginal cross-reactivity on the commercial IgG and IgM ELISAs, respectively, all 10 samples were negative on both the SP-IgG and SP-IgM ELISAs (Table 5). Again, although the reason for this difference is not

known, it may be attributed to differences in the antigens, as discussed above.

These newly developed ELISA systems based on safe and inexpensive SPs are potential alternatives to the conventional diagnostic ELISA methods based on inactivated whole virions. These new methods had high sensitivity and specificity and no cross-reactivity with anti-JE virus antibodies. Therefore, these SP-IgG and SP-IgM ELISAs can be applied to epidemiological research and the diagnosis of TBE in Japan, where TBE virus and JE virus are both endemic.

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Dengue tetravalent DNA vaccine increases its immunogenicity in mice when mixed with a dengue type 2 subunit vaccine or an inactivated Japanese encephalitis vaccine

Jun-ichi Imoto, Eiji Konishi *

Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan

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Abstract

We previously developed a dengue tetravalent DNA vaccine that can induce neutralizing antibodies against four dengue viruses in mice. Here, we demonstrated that immunogenicity of our tetravalent vaccine is synergistically increased in mice by co-immunization with dengue type 2 virus (DENV2) subviral extracellular particles (D2EPs) or inactivated Japanese encephalitis vaccine (JEVAX). A single immunization with a mixture of 100 μg of the tetravalent vaccine and 150 ng of D2EPs or a 1/10 dose of JEVAX induced moderate levels of neutralizing antibodies in a 90% plaque reduction assay. Immunized mice were protected from "artificial" viremia created by intravenous injection with DENV2.

Keywords: Dengue tetravalent DNA vaccine; Combined immunization; Needle-free injection

1. Introduction

Dengue type 1–4 viruses (DENV1–DENV4) cause dengue fever (DF) and dengue hemorrhagic fever (DHF) [1–3]. DF and DHF are endemic in at least 100 countries in tropical and subtropical regions. An estimated 50–100 million DF sufferers and hundreds of thousands of DHF cases occur annually with >2.5 billion people at risk of infection. One of the most promising strategies for preventing DF and DHF is vaccination [4–6].

Live-attenuated tetravalent dengue vaccines developed in Thailand [7,8] or the U.S.A. [9,10] are at advanced stages of evaluation, and several new strategies have so far been reported [11–16]. Attenuated vaccines are inexpensive and suitable for distribution into endemic areas. However, a combination of infectious vaccines may cause "interference" among the four dengue viruses in terms of virus propagation in vaccinated hosts. Such interference is a particularly important concern for dengue vaccines, since imbalanced immune responses may cause increased disease severity when the vaccinated host acquires an infection with one of the four dengue viruses to which induction of immunity is insufficient.

DNA vaccines [17–20] are an appropriate strategy to overcome the concerns of "interference" with combined use, since these vaccines are not infectious. DNA vaccines also have features suitable for use in endemic areas: low cost of production and long-term duration of immune responses. We have demonstrated that a dengue tetravalent DNA vaccine can induce neutralizing antibodies against each of the four dengue viruses in mice without interference [21].

The four dengue viruses are members of the genus *flavivirus* and the family *Flaviviridae* [22]. The flavivirus genome contains genes coding for three structural and seven non-structural proteins. The dengue tetravalent DNA vaccine we have constructed [21] consists of four DNA vaccines expressing premembrane (prM) and envelope (E) proteins under the control of the human cytomegalovirus promoter. prM is the precursor of membrane (M), a small-sized

* Corresponding author. Tel.: +81 78 796 4594; fax: +81 78 796 4594.
E-mail address: ekon@kobe-u.ac.jp (E. Konishi).

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protein existing on the virion surface, and E is the major surface protein containing important neutralizing epitopes. Co-expression of flavivirus *prM* and *E* genes in mammalian cells can induce production of non-infectious subviral extracellular particles (EPs) containing the same compositions (prM/M and E) on the surface as possessed in the infectious virion particles [22]. We have demonstrated that EPs obtained from cells expressing the *prM* and *E* genes of DENV2 (designated D2EPs) are immunogenic in mice [23].

Increase in efficiency of DNA delivery [24,25] and administration with different types of vaccine [26,27] are effective approaches for enhancing immunogenicity of DNA vaccines. In our laboratory, the ability of DNA vaccines to induce neutralizing antibodies in mice has been successfully increased by simultaneous immunization with a protein vaccine [28] and by injection of DNA vaccines using a needle-free system [29]. In the former study [28], we used EPs of Japanese encephalitis virus (JEV) or D2EPs as protein vaccines in homologous combinations with DNA vaccines expressing JEV or DENV2 antigens. Further, we have demonstrated that a needle-free inoculation with a mixture of DNA and protein vaccines induced significantly effective immune responses in a JEV model using an inactivated JE vaccine (JEVAX) as a protein vaccine in addition to JEV EPs [30].

In the present report, we assessed the effect of co-immunization with protein vaccines on the immunogenicity of our dengue tetravalent DNA vaccine using the needle-free injection system. Protein vaccines used were either with D2EPs as an antigen of the same flavivirus serocomplex or with JEVAX as an antigen of a different flavivirus serocomplex. The results demonstrated that both protein vaccines increased immunogenicity of the dengue tetravalent DNA vaccine. In addition, mice immunized with a mixture of the tetravalent DNA vaccine and a protein vaccine were protected from artificial viremia created by intravenous (i.v.) injection with DENV2.

2. Materials and methods

2.1. Viruses

The Mochizuki strain of DENV1 [29], the New Guinea C (NGC) strain of DENV2 [31], the H87 strain of DENV3 [21], the H241 strain of DENV4 [21] and the Nakayama strain of JEV [32], were used. Culture fluids harvested from Vero cells infected with each of these viruses were used for neutralization tests and protection experiments.

2.2. Antibodies

Hyperimmune mouse ascitic fluids have been described previously [21]. Briefly, these were collected from mice repeatedly immunized with DENV1 (Mochizuki), DENV2 (NGC), DENV3 (H87), DENV4 (H241) or JEV (Nakayama) in the form of 10% homogenate of suckling mouse brain. Hyperimmune rabbit sera have been described previously [21]. These were collected from rabbits repeatedly immunized with the purified virion fraction of each dengue virus.

2.3. DNA vaccines

The pcDNA3-based vaccine plasmids encoding the *prM* and *E* genes of the DENV1 Mochizuki strain (pcD1ME [29]), the DENV2 NGC strain (pcD2ME [31]), the DENV3 H87 strain (pcD3ME [21]), the DENV4 H241 strain (pcD4ME [21]) and the JEV Nakayama strain (pcJEME [32]) have been described. A mixture of 25 μg of each of pcD1ME, pcD2ME, pcD3ME and pcD4ME (totaling 100 μg) was used as the dengue tetravalent DNA vaccine. All DNAs were purified using a plasmid DNA Purification Kit (Qiagen, Hilden, Germany).

2.4. Protein vaccines

D2EPs were produced from a cell line stably transfected with pcD2ME containing the *prM* and *E* genes of DENV2, and purified from culture fluids by polyethylene glycol precipitation and sucrose density gradient centrifugation as described previously [23]. JEVAX, a mouse brain-derived inactivated JE vaccine, was purchased from Takeda Chemical Industries (Osaka, Japan).

2.5. Mouse experiments

Groups of five 4-week-old male ddY mice (Japan SLC, Shizuoka, Japan) or BALB/c mice (CLEA Japan, Tokyo, Japan) were immunized once or twice with DNA and/or protein vaccines by inoculation using a spring-powered needle-free jet injector (ShimaJET; Shimadzu, Kyoto, Japan [29]). The volume of the vaccine solution injected into each thigh was adjusted to 50 μl (100 μl per mouse) with phosphate-buffered saline. Because of this size limitation and since the commercial JEVAX used in the present study is available in a dose of 0.5 ml, we used a 1/10 human dose of JEVAX to prepare a mixture with the tetravalent DNA vaccine. Mice were retroorbitally bled at 3-week intervals during periods of 3–9 or 3–18 weeks after the first immunization. Pooled sera were examined for neutralizing antibody titers and individual sera were examined for ELISA antibody levels. In some experiments, individual neutralizing antibody titers were determined to evaluate statistical differences between titers obtained with pooled sera.

Protection experiments were performed by monitoring levels of viremia in mice that received an i.v. injection of DENV2. Following blood collection to test serum neutralizing antibody titers, mice were injected with 0.5 ml of culture fluid harvested from C6/36 cells infected with the NGC strain. The injection was given into the tail vein within approximately 10 s under anesthesia. The dose of virus injected per mouse was 6.6 $\log_{10}$ PFU/ml. One, 2, 3 and 5 min after virus injection, 100 μl of heparinized blood were collected retroor-

bitally. Blood samples were immediately cooled on ice and the plasmas were titrated for infectivity on Vero cells.

All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine.

2.6. Neutralization test

Neutralizing antibodies elicited in immunized mice were titrated using plaque reduction assays performed with DENV1 (Mochizuki), DENV2 (NGC), DENV3 (H87), DENV4 (H241) and JEV (Nakayama) in the presence of complement essentially as previously described [21]. Plaques were visualized by the immunostaining method using polyclonal antibodies specific for each dengue virus or JEV. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 90% reduction in plaque number, unless otherwise specified. Since neutralization tests for titrating dengue antibodies occasionally showed inter-day variations, a positive control serum was examined in each test for standardization.

2.7. ELISA

A conventional ELISA was performed as described previously [21]. Briefly, microplates were sensitized with hyperimmune rabbit sera against each dengue virus and incubated with infected culture fluids containing antigens of the corresponding virus. Sensitized plates were incubated serially with test sera, alkaline phosphatase-conjugated anti-mouse IgG and *p*-nitrophenyl phosphate. A constant positive control was included in every plate to standardize absorbances obtained with test samples, which were expressed as ELISA values.

2.8. Statistical analysis

Significance of differences in geometric mean antibody levels/titers or infective titers was evaluated by the Student's *t*-test. The probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. Immunization with a mixture of pcD2ME and JEVAX

We previously studied the effect of co-immunization with JEVAX on the immunogenicity of pcJEME [30]. Then, though not described in that report [30], we included a group of mice immunized with pcD2ME and JEVAX in preliminary tests to examine if a synergistic increase in immunogenicity shown by pcJEME and JEVAX would be shown by pcD2ME and JEVAX, a combination of different flavivirus serocomplexes. Groups of five ddY mice were immunized once with

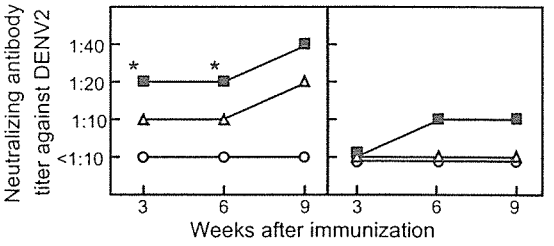

Fig. 1. Effect of co-immunization with JEVAX on the immunogenicity of pcD2ME. Mice were immunized with a mixture of 10 μg of pcD2ME and a 1/10 dose of JEVAX (left panel) or 1 μg of pcD2ME and a 1/100 dose of JEVAX (right panel). The pcD2ME–JEVAX mixture (closed squares) was compared with pcD2ME (open triangles) or JEVAX (open circles) alone, in ability to induce neutralizing antibodies against DENV2, 3–9 weeks after immunization. The ordinate indicates neutralizing antibody titers in sera pooled from five mice. Asterisks indicate significant differences between neutralizing antibody titers induced by the pcD2ME–JEVAX mixture and those induced by pcD2ME or JEVAX alone ($P < 0.05$), when mean antibody titers calculated from individual titers in each group of mice were compared.

a mixture of pcD2ME and JEVAX and examined for neutralizing antibody against DENV2 (Fig. 1, closed squares). As controls, mice were immunized with either of pcD2ME (Fig. 1, open triangles) or JEVAX (Fig. 1, open circles) alone. The doses of pcD2ME and JEVAX were 10 μg and a 1/10 human dose (Fig. 1, left panel) or 1 μg and a 1/100 dose (Fig. 1, right panel), respectively; these doses followed our previous study [30].

Neutralizing antibody levels induced by 10 μg of pcD2ME were increased 2-fold by co-immunization with a 1/10 dose of JEVAX during the experimental period (3–9 weeks after immunization), during which immunization with JEVAX alone did not induce detectable levels of neutralizing antibodies (Fig. 1, left panel). Statistical evaluation using individual titers detected significance between these 2-fold differences at weeks 3 and 6 (1:20 versus 1:10 in pooled sera at both time points). Specifically, the geometric means [range of 1 standard deviation] of individual antibody titers obtained by the pcD2ME–JEVAX mixture (1:15 [1:10–1:22] at week 3 and 1:19 [1:10–1:28] at week 6) were significantly higher than those obtained by pcD2ME alone (1:8 [1:5–1:11] at week 3 and 1:8 [1:5–1:10] at week 6; $P < 0.05$), when titers below the detection limit (<1:10) were assigned 1:5. Furthermore, co-immunization with 1 μg of pcD2ME and a 1/100 dose of JEVAX induced detectable levels of neutralizing antibody at weeks 6 and 9, which were not induced by pcD2ME or JEVAX alone (Fig. 1, right panel). Although the level of increase was small, these results encouraged us to examine the effects of co-immunization with JEVAX on the immunogenicity of the dengue tetravalent DNA vaccine. In this experiment, we also examined these mice for induction of neutralizing antibodies against JEV. The titers induced by 10 μg of pcD2ME and a 1/10 dose of JEVAX were 1:40–1:80, which were higher than those induced by the same dose of JEVAX alone (<1:10 or 1:10; data not shown). The increase suggests the effect of CpG adjuvant contained in the vaccine plasmid as was shown in our previous study [30].

In the present study, we used at maximum a 1/10 human dose of JEVAX, due to the limitation of the inoculum size (100 μl) adopted per mouse. Although data were not shown, pilot experiments indicated that an increasing effect of JEVAX on the immunogenicity of pcD2ME depended on the dose of the protein vaccine. Specifically, ddY mice immunized simultaneously with a 1/5 dose of JEVAX by a subcutaneous route and 10 μg of pcD2ME using a needle-free injector developed a neutralizing antibody titer of 1:80 against DENV2 in pooled serum at 6 weeks after immunization, whereas those immunized with 10 μg of pcD2ME alone or a 1/5 dose of JEVAX alone did not develop detectable levels of neutralizing antibody. The level of neutralizing antibody shown by co-immunization with a 1/5 dose of JEVAX (1:80) was much higher than that shown by co-immunization with a 1/10 dose of JEVAX (1:20) under the same inoculation conditions (subcutaneous route).

3.2. Cross-neutralization among JEV and four dengue viruses

Since the protein vaccines examined for their ability to increase the immunogenicity of the dengue tetravalent DNA vaccine in the present study were derived from a different serocomplex (JEVAX) or a virus species of the same serocomplex (D2EPs), we evaluated cross-neutralization among JEV and the four dengue viruses. Hyperimmune mouse ascetic fluids against each of the five viruses were examined for neutralizing antibody titers against each virus (Fig. 2). Overall, neutralizing antibody titers of 1:5120 or 1:10240 were shown against the homologous virus, whereas antibody titers against heterologous viruses were ≤1:40; thus the cross-neutralization titers were ≤1/128 of the specific titer. These results indicated that the neutralization test was specific enough to distinguish the antibody raised by the homologous virus from those by heterologous viruses within the five viruses used in the present study. In addition, hyperimmune ascitic fluids against a dengue virus showed higher cross-neutralization titers against other viruses of the same serocomplex than the virus of a different serocomplex (JEV) in most combinations.

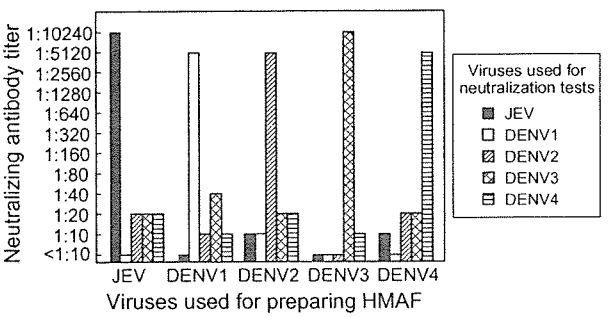

Fig. 2. Cross-neutralization among JEV and four dengue viruses. Neutralizing antibody titers against JEV (Nakayama), DENV1 (Mochizuki), DENV2 (NGC), DENV3 (H87) and DENV4 (H241) are shown for each of the hyperimmune mouse ascitic fluids against JEV and four dengue viruses. The abscissa indicates virus species used for preparing hyperimmune mouse ascitic fluids and the box indicates types of the bar corresponding to virus species used for neutralization tests.

3.3. Immunization with a mixture of the dengue tetravalent DNA vaccine and JEVAX

To evaluate the effect of co-immunization with JEVAX on the immunogenicity of the dengue tetravalent DNA vaccine, we mixed 100 μg of the tetravalent vaccine with a 1/10 dose of JEVAX. This dose of the DNA vaccine (100 μg) was used in our first evaluation of the tetravalent vaccine [21]. Groups of five ddY mice were immunized with the DNA–JEVAX mixture and neutralizing antibody titers were compared with those induced by DNA or JEVAX alone. Further, to examine if the effect of co-immunization with JEVAX would be shown after booster immunization, mice were immunized with the same immunogens at 13 weeks after the first immunization. For a group of mice immunized with JEVAX alone, 73 μg of pcDNA3 was mixed with JEVAX to adjust the molarity of the vector plasmid DNA containing CpG motifs to that contained in 100 μg of the tetravalent vaccine. Time courses of the neutralizing antibody titers obtained with pooled sera and the mean ELISA antibody levels obtained with individual sera against each of the four dengue viruses are shown in Fig. 3. A control group of mice immunized with 73 μg of pcDNA3 showed neutralizing antibody titers of <1:10 and low ELISA antibody levels at all time points until 18 weeks after the first immunization; not shown in Fig. 3.

Neutralizing antibody titers induced by immunization with the DNA–JEVAX mixture were ≥2-fold higher than those induced by DNA alone at almost all time points against the four dengue viruses (Fig. 3, upper panels). Although the titers increased only 2-fold against DENV3 at weeks 9, 12 and 15, the increasing levels were ≥4-fold against DENV3 at weeks 6 and 18 and against other dengue viruses at most time points. After booster immunization, titers induced by the DNA–JEVAX mixture increased 2–4 times against all the dengue viruses, whereas immunization with DNA alone showed booster effects on neutralizing antibodies against only two of the dengue viruses. Since antibody titers obtained with JEVAX were all <1:10 as expected from the specific feature of the neutralization tests shown in Fig. 2, higher titers induced by the DNA–JEVAX mixture than DNA alone suggest an increasing effect from JEVAX. All titers showing ≥4-fold differences in the 90% plaque reduction neutralization test showed ≥4-fold differences in the 70% plaque reduction neutralization test, supporting the results obtained with the 90% plaque reduction assay.

The effect of co-immunization with JEVAX was further evaluated by comparing mean ELISA antibody levels obtained in each group (Fig. 3, lower panels). For this evaluation, we obtained the sum of the mean ELISA antibody levels in two groups of mice immunized with either DNA or JEVAX alone (Fig. 3, closed circles). Mean ELISA antibody levels induced by the DNA–JEVAX mixture were significantly

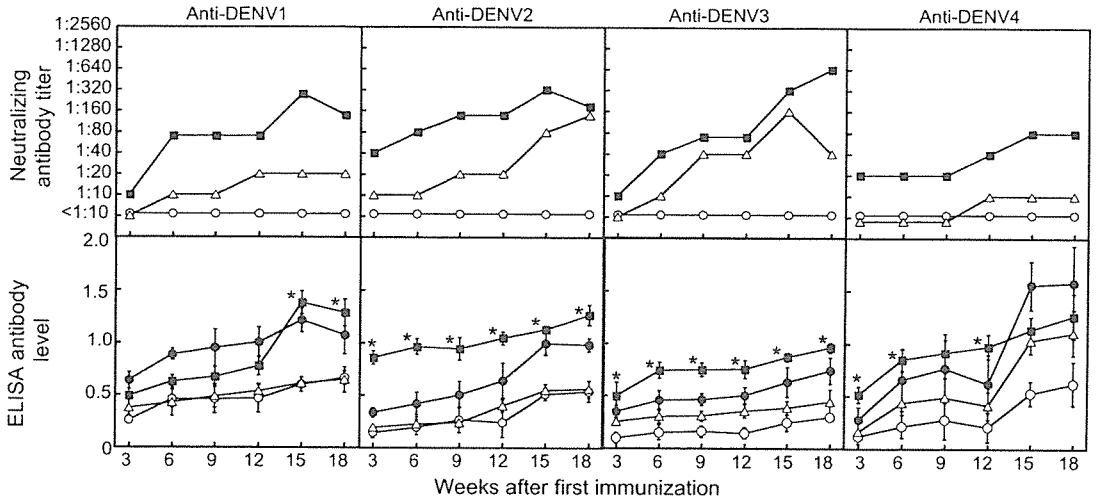

Fig. 3. Effect of co-immunization with JEVAX on the immunogenicity of the dengue tetravalent DNA vaccine in ddY mice. Neutralizing antibody titers (upper panels) and ELISA antibody levels (lower panels) were obtained from mice immunized with a mixture of 100 μg of the tetravalent DNA vaccine and a 1/10 dose of JEVAX (closed squares), 100 μg of the tetravalent DNA vaccine (open triangles) or a 1/10 dose of JEVAX (open circles). To adjust the molarity of CpG immuno-stimulatory motifs on antibody responses to JEVAX, 73 μg of pcDNA3 was mixed with JEVAX. Mice were boosted with the same immunogen as used for the first immunization 13 weeks after the first immunization. Neutralizing antibody titers 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, DENV2, DENV3 and DENV4. Closed circles in lower panels indicate the sum of ELISA antibody levels induced by DNA and JEVAX alone. Asterisks indicate significant differences between ELISA antibody levels induced by the DNA–JEVAX mixture and the sum of those induced by DNA and JEVAX alone ($P < 0.05$).

higher than the sums of those induced by the corresponding single components at all time points against DENV2 and DENV3 and some time points against DENV1 and DENV4 (asterisks in lower panels in Fig. 3). Although significant differences were not obtained with ELISA antibody levels against DENV1 and DENV4 at other time points probably due to cross-reactivity between JEV and the dengue viruses, neutralizing antibody levels induced by the DNA–JEVAX mixture were ≥4-fold higher than those induced by DNA alone at most of these other time points. These results indicate that co-immunization with JEVAX provided a synergistic increase in the immunogenicity of the dengue tetravalent DNA vaccine.

3.4. Immunization with a mixture of the dengue tetravalent DNA vaccine and D2EPs

In addition to JEVAX, an antigen that is different from those of the dengue serocomplex, we used D2EPs, an antigen of the same serocomplex to evaluate its effects on the immunogenicity of the dengue tetravalent DNA vaccine. Immunization of groups of five ddY mice with a mixture of 100 μg of the tetravalent DNA vaccine and 150 ng of D2EPs or with D2EPs alone was performed in parallel to the experiment to evaluate effects of co-immunization with JEVAX shown in Fig. 3.

Neutralizing antibody titers induced by the DNA–D2EPs mixture were ≥4-fold higher than those induced by DNA alone throughout the experimental period against any of the four dengue viruses, except DENV3 and DENV4 at week 12, a week before booster immunization (Fig. 4, upper panels). The titers against DENV2 induced by the DNA–D2EPs mixture showed ≥8-fold increase from those induced by DNA alone at all time points. After booster immunization, titers induced by the DNA–D2EPs mixture were increased 4–32 times against all dengue viruses. Mean ELISA antibody levels induced by the DNA–D2EPs mixture were significantly higher than the sums of those induced by the corresponding DNA or D2EPs alone at all time points against DENV2, DENV3 and DENV4 and some time points against DENV1 (Fig. 4, lower panels).

Although the levels of increase in antibody responses by co-immunization with D2EPs varied according to the assay method, either a ≥4-fold increase in neutralizing antibody titers or a significant increase in mean ELISA antibody levels were shown in all time points against the four dengue viruses. These results indicate that the immunogenicity of the dengue tetravalent DNA vaccine was synergistically increased by co-immunization with D2EPs and that the increase in the effect was larger than the effect of JEVAX.

To examine if the increasing effect of D2EPs would be shown in another strain of mice, we immunized BALB/c mice with the same immunogens used for the ddY mice. Since BALB/c mice showed lower antibody responses against dengue viral antigens than ddY mice, neutralizing antibody titers after booster immunization were used for comparison. Mice immunized with the DNA–D2EPs mixture developed ≥4-fold higher neutralizing antibody titers against all four dengue viruses than those immunized with DNA or D2EPs alone 2 weeks after the booster immunization (data not shown). This result shown in BALB/c mice supported the increasing effect of co-immunization with D2EPs on

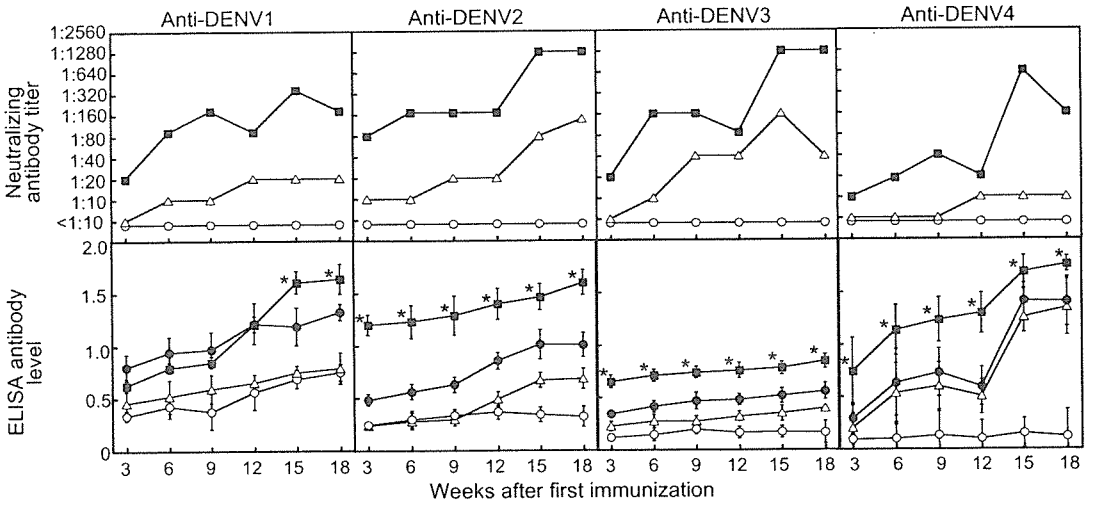

Fig. 4. Effect of co-immunization with D2EPs on the immunogenicity of the dengue tetravalent DNA vaccine in ddY mice. Neutralizing antibody titers (upper panels) and ELISA antibody levels (lower panels) were obtained from mice immunized with a mixture of 100 μg of the tetravalent DNA vaccine and 150 ng of D2EPs (closed squares), 100 μg of the tetravalent DNA vaccine (open triangles) or 150 ng of D2EPs (open circles). To adjust the molarity of CpG immuno-stimulatory motifs on antibody responses to D2EPs, 73 μg of pcDNA3 was mixed with D2EPs. Mice were boosted with the same immunogen as used for the first immunization 13 weeks after the first immunization. Neutralizing antibody titers 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, DENV2, DENV3 and DENV4. Closed circles in lower panels indicate the sum of ELISA antibody levels induced by DNA and D2EPs alone. Asterisks indicate significant differences between ELISA antibody levels induced by the DNA–D2EPs mixture and the sum of those induced by DNA and D2EPs alone ($P < 0.05$).

immunogenicity of the tetravalent DNA vaccine shown in ddY mice.

3.5. Protection based on reduction of viremia levels

To evaluate the protective capacities of the tetravalent DNA vaccine mixed with the protein vaccine in mice, we compared viremia levels in immunized and unimmunized mice following injection of live DENV2 by the i.v. route. Since pilot experiments had indicated that no detectable or very low infectivities were shown at 30 or 10 min, respectively, after virus injection in naïve ddY mice, we decided to monitor viremia levels within 5 min after the injection. Groups of four ddY mice inoculated only with pcDNA3 or immunized with the tetravalent vaccine mixed with JEVAX or D2EPs, which were kept for 8 weeks after the experiment shown in Figs. 3 and 4, were injected i.v. with 6.6 $\log_{10}$ PFU/ml of the DENV2 NGC strain. Retroorbital blood was collected successively at 1, 2, 3 and 5 min after injection, and the plasmas were titrated for infectivity. Individual neutralizing antibody titers prior to virus injection were <1:10 in pcDNA3-inoculated mice, 1:40–1:80 in mice immunized with the tetravalent vaccine and JEVAX, and 1:640–1:1280 in mice immunized with the tetravalent vaccine and D2EPs (Fig. 5).

Infective titers in unimmunized mice were 4.6–4.8 $\log_{10}$ PFU/ml at 1 min after injection without considerable individual variations. Relatively high titers around 4 $\log_{10}$ PFU/ml were maintained in all unimmunized mice 2–3 min after injection. Five min after injection, three of four mice showed 3.5–4.0 $\log_{10}$ PFU/ml and one mouse

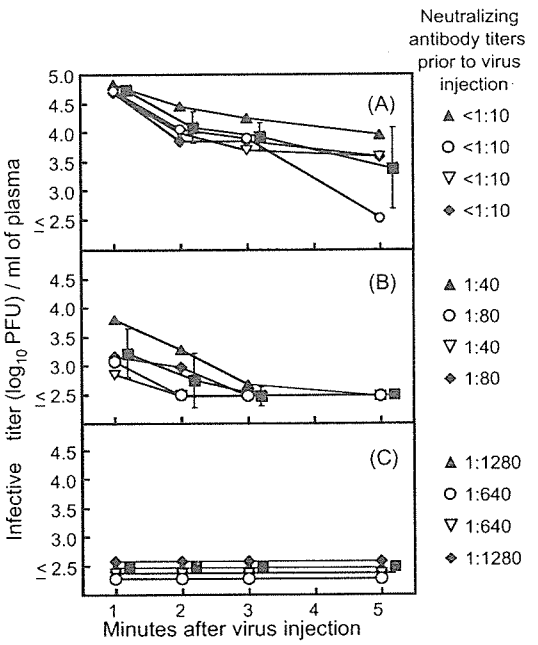

Fig. 5. Levels of viremia following i.v. injection of DENV2 in vaccinated and unvaccinated mice. Groups of four mice inoculated with 73 μg of pcDNA3 (A), immunized with a mixture of 100 μg of the tetravalent vaccine and a 1/10 dose of JEVAX (B) and immunized with a mixture of 100 μg of the tetravalent DNA vaccine and 150 ng of D2EPs (C), were injected with 6.6 $\log_{10}$ PFU/ml of DENV2 and monitored for infective titers contained in plasma samples. Individual neutralizing antibody titers before virus injection are indicated at the right side of each panel. The average infective titers are indicated by closed squares with the standard deviations by bars.

showed an undetectable titer. On the other hand, none of the mice immunized with the tetravalent vaccine and D2EPs showed any detectable titers during the experimental period. Although detectable titers were shown at least in one mouse immunized with the tetravalent vaccine and JEVAX until 3 min after injection, their mean infective titers were significantly lower than those obtained with unimmunized mice at each time point ($P < 0.05$). These results indicated that levels of neutralizing antibodies are strongly associated with a reduction in viremia levels in mice and that the neutralizing antibody levels induced by immunization with the tetravalent vaccine and a protein vaccine can protect mice from artificial viremia created by i.v. injection of DENV2.

4. Discussion

The use of combinations of different types of vaccines has been demonstrated to increase their respective immunogenicities, irrespective of their use at different times as in a prime-boost strategy [26,27] or at the same time as in simultaneous immunization [33,34]. We have previously reported that immunogenicity of DNA vaccines was synergistically increased by co-immunization with protein vaccines in systems of DENV2 and JEV [28]. The synergistic increase is probably based on strong priming with a relatively large amount of the protein vaccine followed by long-term boosting with a small amount of the immunogen continuously produced by cells expressing vaccine plasmid-encoded genes. As demonstrated in a JEV system [30], needle-free injection can enhance the efficiency of vaccine plasmid delivery into muscle cells, providing higher levels of antigen production than those achieved by the normal needle/syringe injection.

Most members of the genus *Flavivirus* are grouped into eight antigenic complexes. JEV belongs to the JE virus serocomplex, while the four dengue viruses belong to the dengue virus serocomplex [1]. In the present study, co-immunization with D2EPs provided a synergistic increase in the ability of the dengue tetravalent DNA vaccine to induce neutralizing antibodies against all four dengue viruses. In addition to a DNA vaccine component homologous to the protein vaccine (pcD2ME), heterologous DNA vaccine components (pcD1ME, pcD3ME, pcD4ME) increased in their immunogenicity by co-immunization with D2EPs, probably based on the relatively high serological cross-reactivity within the dengue virus serocomplex. However, co-immunization with JEVAX, an antigen of a different serocomplex, also synergistically increased the immunogenicity of the dengue tetravalent DNA vaccine, although the increasing levels were generally lower than co-immunization with D2EPs.

The increase in the immunogenicity of the dengue tetravalent DNA vaccine by co-immunization with JEVAX is considered an advantage of the present immunization strategy using a DNA–protein mixture. An attempt to increase neutralizing antibody responses by co-immunization with the same type of vaccine (DNA vaccine) against JEV and DENV2 failed (data not shown). Specifically, neutralizing antibody titers against DENV2 in mice immunized with a mixture of 10 μg of pcD2ME and 10 μg of pcJEME (1:10 at both 6 and 9 weeks after immunization) were comparable to those immunized with 10 μg of pcD2ME plus 7.3 μg of pcDNA3 (1:10 and 1:20 at 6 and 9 weeks after immunization, respectively). Thus, co-immunization with different types of vaccine (DNA and protein) may increase immune responses to cross-reactive antigens, the levels of which are primarily low as determined by the neutralization test. It seems likely that cross-reactive antigens contained in the protein vaccine co-immunized with the DNA vaccine could effectively enhance specific immune responses to the antigen expressed by the plasmid DNA. In this sense, booster immunization only with the protein vaccine also may increase specific immune responses to each of four dengue viruses, although we only used the DNA–protein mixture for booster immunization in the present study.

The neutralizing antibody titer against DENV2 obtained with serum pooled from mice immunized with the tetravalent vaccine and D2EPs at the end of the experimental period in Fig. 4 (1:1280) were comparable to the individual titers shown prior to virus injection in Fig. 5 (1:640–1:1280). This indicates that neutralizing antibody responses induced by the tetravalent vaccine and D2EPs were maintained for 8 weeks, the interval between the end of the experiment shown in Fig. 4 and the start of the experiment shown in Fig. 5. On the other hand, neutralizing antibody titers induced by the tetravalent vaccine and JEVAX were decreased from 1:160 (Fig. 3) to 1:40–1:80 (Fig. 5) during the 8 weeks. The neutralizing antibodies induced by the DNA–protein combination of the same virus species may be maintained longer than those induced by the combination of different serocomplexes.

The only animal that has reliably been used for evaluating the protective efficacy of candidates as dengue vaccines is the monkey, which resembles humans in both viremia and antibody induction in response to dengue virus infections. The protection has usually been evaluated by monitoring viremia shown 2–5 days after subcutaneous inoculation with the challenge virus. As a simple alternative, mice have been used in most cases, in which lethal encephalitis following intracranial challenge has been a marker of protection. However, the murine model does not seem to reflect dengue virus infections in humans, since humans are infected peripherally via infective mosquito bites and encephalitis cases are very rare [35]. In the present study, we monitored reduction in levels of "artificial" viremia created by i.v. injection of live DENV2. Since mice have a heart rate of 300–800 beats/min in general [36], it is likely that inoculated viruses are evenly distributed in the host body within 1 min. Although the virus titers in the circulation became undetectable within 10–30 min, we could use the titers shown at 1–3 min for evaluating the protective capacity against viremia. The rapid decrease in viremia level