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Ⅳ. 研究成果の刊行物・別刷

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Co-immunization with West Nile DNA and inactivated vaccines provides synergistic increases in their immunogenicities in mice

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Abstract

West Nile virus is now distributed throughout many temperate, subtropical and tropical areas: vaccines need to be developed that are affordable for developed and developing countries. Here, we constructed and evaluated a DNA vaccine expressing the premembrane and envelope proteins of West Nile virus (pcWNME). Mice immunized twice with 100 or 10 μg of pcWNME developed high or moderate levels of neutralizing antibodies, respectively. These mice were protected from viremia and death after lethal challenge. Mice immunized with a mixture of 1 μg of pcWNME and a small amount (1/10 dose) of a commercial inactivated vaccine developed moderate levels of neutralizing antibodies, whereas immunization with pcWNME or the inactivated vaccine alone induced only low or undetectable levels: co-immunization with the DNA and protein vaccines synergistically increased their own immunogenicities. The synergism reduced the amount of DNA sufficient to induce neutralizing antibodies: a single immunization with doses as low as 0.1 μg induced a titer of 1:40 at a 90% plaque reduction 6 or 9 weeks after immunization. Both IgG1 and IgG2a antibodies were induced in mice by co-immunization with the DNA and protein vaccines.

Keywords: DNA vaccine; Co-immunization; West Nile virus

1. Introduction

West Nile virus (WNV) causes a self-limited febrile illness in mild cases, but meningitis or encephalitis in severe cases [1]. The geographical distribution of WNV was limited to Africa, Middle East, Europe, and central Asia before 1999. In the summer of 1999, WNV was introduced into the North American continent [2]. The virus then progressively spread across the United States; nearly 10,000 human cases and 264 deaths were reported in 2003 [1]. In addition, WNV also reached Canada [3], Central America [4] and most recently Argentina [5]. Further, Kunjin virus, which is widespread in Australia, was reclassified as a subtype of WNV. Thus, WNV is currently distributed in most areas of the temperate, subtropical and tropical zones throughout the world. Therefore, development of preventive vaccines that are affordable for both developed and developing countries is urgently needed.

Although several strategies for developing WNV vaccines are ongoing [1], there is no vaccine currently approved for human use. For veterinary use, formalin-inactivated and recombinant canarypox vaccines were approved in the United

Abbreviations: WNV, West Nile virus; E, envelope; prM, precursor membrane; EPs, extracellular particles; SHA, slowly-sedimenting hemagglutinin; JEV, Japanese encephalitis virus; HMAF, hyperimmune mouse ascitic fluids; HA, hemagglutination; HAI, hemagglutination-inhibiting; Th1, T-helper 1; Th2, T-helper 2.

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States and/or Europe. Recently, a DNA vaccine for veterinary use was also approved in the United States. Of the several vaccine strategies available, DNA vaccines are the most promising candidates as they are affordable for developed and developing countries (since development and production is simple and relatively inexpensive) and the DNA vaccine can induce long-lasting immune responses.

WNV, belonging to the genus *Flavivirus*, is a positive-strand enveloped RNA virus [1]. The envelope of virion particles contains the envelope (E) and membrane proteins: the latter is synthesized as a precursor membrane (prM) protein in infected cells. The E protein plays important roles in receptor binding and fusion to the cell membrane and, more importantly for vaccine strategy, possesses most of the neutralizing epitopes [6]. Cells expressing prM and E are known to secrete the subviral extracellular particles (EPs), which are similar to slowly sedimenting hemagglutinin (SHA) particles secreted from flavivirus-infected cells [7]. Since EPs are an excellent immunogen, most of the gene-engineering flavivirus vaccines, including DNA vaccines, have been developed using the *prM* and *E* genes [8].

Neutralizing antibodies are the major factor involved in protection from diseases caused by flaviviruses [9]. We have demonstrated in models of Japanese encephalitis virus (JEV) and dengue viruses that co-administration with a protein-based vaccine [10], administration using a needle-free jet injector [11] and needle-free administration of mixture with a protein vaccine [12,13] can enhance the immunogenicity of DNA vaccines in mice. The increase in immunogenicity makes a decrease in vaccine dose possible, which is sufficient to induce protective levels of antibodies. Our recent study using a JEV model indicates that a single DNA vaccination with a dose of 1 μg could induce neutralizing antibodies [12]. Reduction of the vaccine dose is critical for vaccine introduction for both developed and developing countries for safety and cost effectiveness.

In the present study, we constructed a DNA vaccine against WNV (designated pcWNME) and evaluated the immunogenicity in a murine model. The results indicated that pcWNME at as low as 0.1 μg could induce neutralizing antibodies when its mixture with a 1/10 dose of the formalin-inactivated WNV vaccine was administered using a needle-free jet injector.

2. Materials and methods

2.1. Cells and viruses

BHK-21 [14] and C6/36 [15] cells were grown at 37 °C and 28 °C, respectively, in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 10 mM non-essential amino acids (Invitrogen, Carlsbad, CA). Vero cells [15] were grown in the same medium without non-essential amino acids at 37 °C. The NY99-6922 strain of WNV [16] was used for construction of pcWNME and challenge experiments. The Eg101 strain of WNV, which had been passaged through suckling mouse brains 34 times [16], was further passaged twice through Vero cells before use in all other experiments.

2.2. Construction of pcWNME

RNA of the NY99-6922 strain was extracted from culture fluids of infected Vero cells with a High Pure RNA Extraction Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. To obtain the cDNA encoding the signal sequence of prM, prM and E from the RNA, reverse transcriptase PCR was performed using Thermoscript RT-PCR (Invitrogen) with the anti-sense primer 5′-GCT CTA GAT TAA GCG TGC ACG TTC ACG GAG AG-3′ containing the 3′ end of the *E* gene corresponding to the C-terminal seven amino acids. The genes coding for the signal-prM-E were amplified from the cDNA using the anti-sense primer described above and the sense primer 5′-CCG GAA TTC ACC ATG AAA AGA GGA GGA AAG ACC GGA-3′ containing a sequence corresponding to the 3′-terminal 20 amino acid sequence of the *C* gene. The amplified signal-*prM-E* gene cassette was inserted into a vector plasmid, pcDNA3 (Invitrogen) at the EcoRI/XbaI site, to construct pcWNME. The nucleotide sequence of the cassette was proven to be identical to the same region of the NY99-6922 strain. Plasmid DNAs (pcWNME, pcDNA3) were purified using a plasmid DNA purification kit (Qiagen, Hilden, Germany).

2.3. Antibodies

Hyperimmune mouse ascitic fluids (HMAF) against WNV were obtained from adult ICR mice by repeated immunization with the virion and SHA fractions purified from culture fluids of WNV-infected Vero cells, followed by inoculation with sarcoma 180 cells.

2.4. Sucrose density gradient centrifugation

Sedimentation analysis was performed as described previously [15]. Culture fluids from pcWNME-transfected BHK-21 cells or Eg101-infected C6/36 cells were precipitated with polyethylene glycol and applied to a 10–40% continuous sucrose gradient. Following ultracentrifugation, fractions were collected from the bottom. Each fraction was examined for E antigen levels, infective titers and hemagglutination (HA) titers.

2.5. ELISA for measuring E antigen levels

The antigen level of each fraction was measured by a direct ELISA as described previously [15]. Briefly, microplates were sensitized with each fraction sample at a 1:10 dilution, and then incubated with HMAF, alkaline phosphatase-conjugated anti-mouse IgG and *p*-nitrophenyl phosphate.

2.6. Focus assay

Infective titers were determined on a monolayer of Vero cells as described previously [11]. For counting foci, cells were immunostained with HMAF.

2.7. Mouse experiments

Groups of six 4 or 5-week-old female ddY or ICR mice were inoculated with pcWNME and/or the formalin-inactivated WNV vaccine (Fort Dodge Animal Health, Fort Dodge, IA; hereinafter WNVAX) by a spring-powered needle-free jet injector, ShimaJET (Shimazu, Kyoto, Japan). The dose of pcWNME ranged from 0.01 to 100 μg, whereas the dose of WNVAX was fixed at a 1/10 dose. The vaccine was inoculated into the thigh and the volume of the inoculum was 100 μl (50 μl for each thigh). As a control, pcDNA3 was used instead of pcWNME. When the effect of co-immunization with WNVAX on immunogenicity of pcWNME was assessed, the amount of pcDNA3 mixed with WNVAX was adjusted to contain the same molarity of CpG motifs as those contained in the corresponding amount of pcWNME. These mice were bled three times at intervals of 2–3 weeks after immunization. The pooled sera were examined for neutralizing and HA-inhibiting (HAI) antibodies.

For protection studies, mice were challenged intraperitoneally with 500 LD_{50} of the NY99-6922 strain. The mice were bled at 3 and 6 days after challenge, and the pooled blood was examined by TaqMan RT-PCR to detect viral RNA. The mice were observed for survival for 14 days.

All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University and National Institute of Infectious Diseases.

2.8. Neutralization test

Neutralizing antibody titers were determined against the WNV Eg101 strain, using plaque reduction assays on Vero cells, essentially as described previously [10]. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 90% reduction in plaque number.

2.9. HA and HAI tests

HA and HAI titers were determined by a micro-modification of the method described by Clarke and Casals [17].

2.10. TaqMan RT-PCR

RNA was isolated from the pooled blood using the High Pure RNA Extraction Kit. Five microliters of the RNA solution was subjected to the TaqMan RT-PCR assay in an ABI Prism 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA). The forward primer, reverse primer and probe were: TCA GCG ATC TCT CCA CCA AAG, GGG TCA GCA CGT TTG TCA TTG and TGC CCG ACC ATG GGA GAA GCTC, respectively [18].

2.11. ELISA for IgG isotyping

The IgG isotype was determined by ELISA essentially as previously described [10]. Microplates sensitized with culture fluids from Eg101-infected Vero cells were incubated with two-fold serial dilutions (from 1:10 to 1:1280) of sera, alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a (Southern Biotechnology Associations, Birmingham, AL), and *p*-nitrophenyl phosphate. The antibody titer was expressed as the maximum serum dilution showing an absorbance greater than the average plus two times the standard deviation of absorbance obtained with normal mouse serum.

3. Results

3.1. In vitro expression of pcWNME

To evaluate pcWNME for antigen expression, BHK cells were transfected with pcWNME, incubated for 48 h and examined by immunostaining with monoclonal antibodies against WNV E or prM (data not shown). The cells were stained with both antibodies, indicating that WNV prM and E were expressed in pcWNME-transfected cells.

Next, the extracellular antigens produced by pcWNME-transfected BHK cells were analyzed by sucrose density gradient centrifugation, in comparison with those produced by WNV-infected C6/36 cells (Fig. 1). Culture fluids from WNV-infected C6/36 cells showed that the virion fraction coincided with the peak of infectivity and the first peak of HA

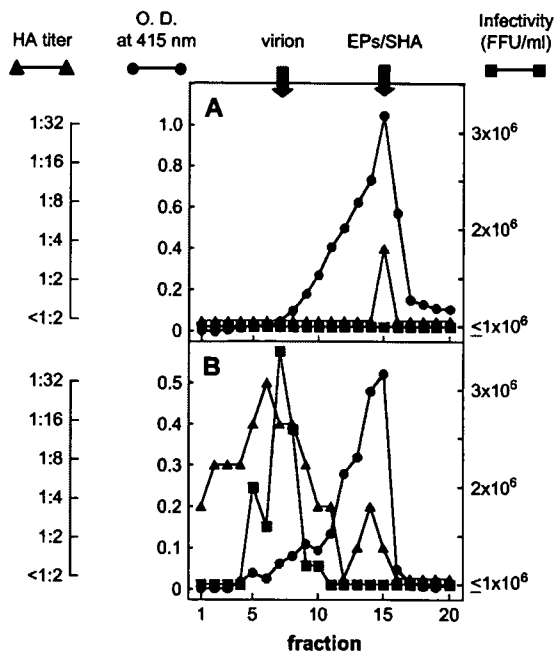

Fig. 1. Sedimentation profiles of culture fluids from pcWNME-transfected BHK cells and WNV-infected C6/36 cells. Culture fluids harvested at 48 h after transfection or infection were fractionated on sucrose density gradients. Each fraction was examined for E antigen level (circle), infectivity (square) and HA activity (triangle). (A) pcWNME-transfected BHK cells, (B) WNV-infected C6/36 cells.

activity, which was clearly separated from the SHA fraction coinciding with the second peak of HA activity; the peak of E antigen corresponded to the SHA fraction but the virion fraction was present in the broad shoulder. On the other hand, culture fluids from pcWNME-transfected BHK cells showed a single peak of HA activity and E antigen, which was co-sedimented with the SHA particles shown by the sample from WNV-infected C6/36 cells. These results indicate that pcWNME can produce prM and E antigens in transfected cells and that the antigens are released from the cells in a SHA-like particulate form.

3.2. Induction of protective immunity by pcWNME

To assess the immunogenicity of pcWNME in a murine model, groups of six 5-week-old female ddY mice were immunized twice at a 3-week intervals with 100 μg or 10 μg of pcWNME or with 100 μg of pcDNA3 (Table 1). Neutralizing antibodies were detected in both groups of pcWNME-immunized mice at week 4 (1 week after the second immunization) and 100 μg of pcWNME induced a higher neutralizing antibody titer than 10 μg did. Titers were increased at week 6 and then leveled off in both pcWNME-immunized groups. In comparison, mice inoculated with pcDNA3 did not develop detectable levels of neutralizing antibodies until week 8 (5 weeks after the second immunization).

Mice were challenged intraperitoneally with 500 LD_{50} of the WNV NY99-6922 strain at week 8 and then monitored for viremia and survival. The viremia was assessed by detecting viral RNA in the blood, as determined by TaqMan RT-PCR analysis (the detection limit: 0.1 PFU [18]). In this challenge experiment, a group of six naïve mice of the corresponding age was used as a "real" non-immune control in addition to the pcDNA3-inoculated control, since mice inoculated with pcDNA3 occasionally showed CpG-induced non-specific protection in other flavivirus systems [19].

The viremia was detected in both pcDNA3-inoculated and non-immune controls, whereas mice immunized with 100 or 10 μg of pcWNME did not show detectable levels of viremia at 3 and 6 days post challenge (Table 2). As expected, a small number of mice in the pcDNA3-inoculated group survived the challenge, although neutralizing antibodies were undetectable (Table 1) and the viremia was detected in this group (Table 2).

Table 1
Neutralizing antibody titers induced in ddY mice immunized with pcWNME

Group	Immunogen (dose)[a]	Neutralizing antibody titer[b]		
		Week 4[c]	Week 6	Week 8
1	pcWNME (100 μg)	1:160	1:2560	1:2560
2	pcWNME (10 μg)	1:20	1:80	1:80
3	pcDNA3 (100 μg)	<1:10	NT[d]	<1:10

[a] Groups of six 5-week-old female ddY mice were immunized twice at a 3-week interval with indicated doses of pcWNME or the pcDNA3 vector.
[b] Represented as the maximum serum dilution yielding a 90% reduction in plaque number.
[c] Week after the 1st immunization.
[d] Not tested.

Table 2
Protection of pcWNME-immunized mice from lethal WNV challenge

Group[a]	Immunogen	TaqMan PCR threshold cycle[b]		Number of mice[e]	
		3 dpc[c]	6 dpc	Dead	Alive
1	pcWNME	–[d]	–	0	6
2	pcWNME	–	–	0	6
3	pcDNA3	30.0	38.5	4	2
4	None	29.0	36.8	6	0

[a] For groups 1–3, mice used in the experiment described in Table 1 were challenged 8 weeks after the first immunization. For group 4, six naïve mice of the corresponding age were used as a control of the challenge experiment.
[b] Represented as the cycle number, the signal intensity at which was greater than the detection threshold of 0.5.
[c] Days postchallenge. The challenge was performed by intraperitoneal inoculation with 500 LD_{50} of the WNV NY99-6922 strain.
[d] Negative for the presence of RNA in pooled blood as determined by the TaqMan PCR assay. When the signal intensity was less than the detection threshold of 0.5 at 40 cycles, the result was regarded as negative.
[e] Observation at 14 days after challenge.

However, all non-immune mice died from challenge, showing the ability of the challenge virus to kill all mice at this dose (500 LD_{50}). In contrast, all mice immunized with 100 or 10 μg of pcWNME were protected (Table 2). The full protection was consistent with no detectable viremia in these groups, in which virus dissemination to the brain following the peripheral virus challenge was likely prevented. These results indicate that pcWNME was able to induce neutralizing antibodies in mice and protect them from lethal WNV challenge.

3.3. Co-immunization of ICR mice with pcWNME and WNVAX

To examine if immunogenicity of pcWNME would be increased by co-immunization with a protein vaccine, ICR mice were immunized with a mixture of pcWNME and WNVAX (Table 3). Since our previous study showed that 1 μg of a JE DNA vaccine induced neutralizing antibodies in mice when co-immunized with protein vaccines [12], in the present study, 1 μg and 0.1 μg of pcWNME were used for the first evaluation of the co-immunization strategy, with a constant dose (1/10 dose) of WNVAX. As a control, mice were immunized with either pcWNME or WNVAX. For the WNVAX control, the 1/10 dose of WNVAX was mixed with pcDNA3, since our previous study using a JEV system [12] indicated that CpG molecules contained in the vaccine vector enhanced immunogenicity of protein vaccines in the co-immunization system. The amounts of pcDNA3, 0.73 and 0.073 μg, corresponded to 1 and 0.1 μg of pcWNME, respectively, in terms of molarity of CpG molecules.

Neutralizing antibodies were detected at 6 and 9 weeks after immunization. Mice co-immunized with 1 μg of pcWNME and WNVAX developed a much higher neutralizing antibody titer (1:160) than those immunized with 1 μg of pcWNME alone (1:10 or <1:10) or WNVAX alone (1:20 or 1:10). In addition, mice co-immunized with 0.1 μg of pcWNME and WNVAX developed neutralizing antibody titers (1:40 or

Table 3
Effect of co-immunization with WNVAX on immunogenicity of pcWNME in ICR mice

Dose of immunogen[a]			Neutralizing antibody titer[c]			HAI titer		
pcWNME (μg)	WNVAX (dose)	pcDNA3[b] (μg)	Week 3[d]	Week 6	Week 9	Week 3	Week 6	Week 9
1	1/10	–	<1:10	1:160	1:160	1:80	1:320	1:320
1	–	–	<1:10	1:10	<1:10	1:20	1:40	1:40
–	1/10	0.73	<1:10	1:20	1:10	1:20	1:80	1:40
0.1	1/10	–	<1:10	1:40	1:10	1:20	1:40	1:20
0.1	–	–	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
–	1/10	0.073	<1:10	1:10	<1:10	1:10	1:40	1:40

[a] Groups of six 4-week-old female ICR mice received a single immunization with a mixture of pcWNME and WNVAX at indicated doses.
[b] The amount of pcDNA3 mixed with WNVAX was adjusted to contain the same molarity of CpG motifs as those contained in the corresponding amount of pcWNME.
[c] Represented as the maximum serum dilution yielding a 90% reduction in plaque number.
[d] Weeks after immunization.

1:10) higher than those immunized with 0.1 μg of pcWNME alone (<1:10) or WNVAX alone (1:10 or <1:10). On the other hand, HAI antibodies were detected at 3 weeks after immunization, probably due to higher sensitivity of the HAI than neutralization tests in our assay system. Similar to the results obtained with neutralization tests, mice co-immunized with 1 μg of pcWNME and WNVAX developed much higher HAI antibody titers (1:80 at week 3 and 1:320 at weeks 6 and 9) than those immunized with 1 μg of pcWNME alone (1:20 at week 3 and 1:40 at weeks 6 and 9) or WNVAX alone (1:20, 1:80 and 1:40 at weeks 3, 6 and 9, respectively). These results indicate that co-immunization with WNVAX exerts a synergistic (more than additive) effect on the immunogenicity of 1 μg of pcWNME. Although the synergism was not clearly shown with 0.1 μg of pcWNME, even this low dose of pcWNME could induce neutralizing antibodies in mice.

3.4. Co-immunization of ddY mice with pcWNME and WNVAX

The co-immunization experiment using ICR mice showed that 0.1 μg of pcWNME could induce neutralizing antibodies, when mixed with a 1/10 dose of pcWNME. To examine the immunogenicity at lower vaccine dose and to confirm the co-immunization effect in another mouse strain, we immunized ddY mice with 0.1 or 0.01 μg of pcWNME (Table 4). The WNVAX was used at the same dose as in the above experiment (1/10 dose).

Consistent with the results obtained with ICR mice immunized with 0.1 μg of pcWNME and WNVAX, ddY mice immunized with the same dose of the DNA–protein mixture developed detectable levels of neutralizing antibodies. However ddY mice immunized with 0.01 μg of pcWNME and WNVAX did not show detectable neutralizing antibody titers. HAI titers, which were higher overall than neutralizing antibody titers, were detected in mice 3 weeks after immunization with 0.01 μg of pcWNME and WNVAX. These results obtained with ddY mice supported the results obtained with ICR mice, and indicate that 0.1 μg of pcWNME mixed with WNVAX was able to induce neutralizing antibodies in mice. The results suggest that 0.1 μg was the minimum dose that could induce neutralizing antibodies under the present immunization conditions. Again, consistent with the results obtained with ICR mice immunized with 0.1 μg of pcWNME and WNVAX, the synergistic effect was not clearly shown at these doses (0.1 and 0.01 μg) of pcWNME in mice.

3.5. IgG profiles induced by co-immunization with pcWNME and WNVAX in mice

To assess the type of immune responses (T-helper-1 (Th1) or T-helper-2 (Th2)) induced by co-immunization with

Table 4
Effect of co-immunization with WNVAX on immunogenicity of pcWNME in ddY mice

Dose of immunogen[a]			Neutralizing antibody titer[c]			HAI titer		
pcWNME (μg)	WNVAX (dose)	pcDNA3[b] (μg)	Week 3[d]	Week 6	Week 9	Week 3	Week 6	Week 9
0.1	1/10	–	<1:10	1:10	1:40	1:20	1:80	1:80
0.1	–	–	<1:10	<1:10	<1:10	1:10	1:20	1:10
–	1/10	0.073	<1:10	1:10	<1:10	1:40	1:40	1:20
0.01	1/10	–	<1:10	<1:10	<1:10	1:20	1:40	1:20
0.01	–	–	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
–	1/10	0.0073	<1:10	<1:10	<1:10	1:10	1:10	1:20

[a] Groups of six 4-week-old female ddy mice received a single immunization with a mixture of pcWNME and WNVAX at indicated doses.
[b] The amount of pcDNA3 mixed with WNVAX was adjusted to contain the same molarity of CpG motifs as those contained in the corresponding amount of pcWNME.
[c] Represented as the maximum serum dilution yielding a 90% reduction in plaque number.
[d] Weeks after immunization.

Table 5
IgG1/IgG2a profiles induced by co-immunization with pcWNME and WNVAX

Dose of immunogen[a]			Antibody titer[b]	
pcWNME (μg)	WNVAX (dose)	pcDNA3 (μg)	IgG1	IgG2a
0.1	1/10	–	1:640	1:160
0.1	–	–	1:10	1:20
–	1/10	0.073	1:160	1:320

[a] Sera pooled from ddY mice 9 weeks after immunization with indicated doses of immunogens, which were obtained in experiments shown in Table 4, were used for this experiment.
[b] Antibody titer was expressed as the maximum serum dilution showing the absorbance greater than absorbance obtained with normal mouse serum (average +2 × standard deviation).

pcWNME and WNVAX, relative concentrations of antibodies belonging to IgG1 and IgG2a isotypes were determined, using sera obtained from ddY mice 9 weeks after immunization with 0.1 μg of pcWNME and/or WNVAX, which were obtained in experiments shown in Table 4. As shown in Table 5, mice co-immunized with pcWNME and WNVAX showed relatively high levels of both IgG1 and IgG2a antibodies. In addition, IgG1 antibody levels were similar to IgG2a antibody levels in mice immunized with pcWNME alone or WNVAX alone (but with pcDNA3). These results suggest that co-immunization with pcWNME and WNVAX induce both Th1 and Th2 immune responses in mice.

4. Discussion

Development of flavivirus DNA vaccines has been mainly based on expression of the signal-*prM-E* gene cassette [8]. Although DNA vaccines are generally less effective at inducing humoral immunity, EPs produced by cells expressing the signal-*prM-E* gene cassette are sufficiently immunogenic to induce neutralizing antibodies, which are important for protection against flavivirus diseases. We have demonstrated the ability of DNA vaccines to induce neutralizing antibodies in mice, pigs and/or monkeys in systems of JEV and dengue viruses [20–23]. A DNA vaccine using the signal-*prM-E* gene cassette against WNV has been reported [24], but it was different at the signal sequence upstream the *prM* gene: we used the sequence derived from the WNV gene, whereas the other study [24] used a sequence derived from the JEV gene.

The susceptibility of mice to viral infections including those with flaviviruses is associated with their genetic background [25]. In addition, immune responses to DNA immunization sometimes vary according to the strains of mice [26,27]. Thus, the use of more than one mouse strain provides more reliable evaluation of vaccine candidates, particularly for determining the minimum dose required to induce immune responses. In the present study, our immunization strategy was evaluated using ddY and ICR strains, in both of which detectable levels of neutralizing antibodies were induced by 0.1 μg of pcWNME upon co-immunization with a small dose of WNVAX.

Immunization with the DNA–protein mixture has been demonstrated to synergistically increase their immunogenicities in our JEV [12] and dengue virus [13] systems. In the present study, synergism was also shown by co-immunization with pcWNME (1 μg) and WNVAX, when antibody levels induced by co-immunization were compared with those induced by pcWNME alone or WNVAX mixed with pcDNA3. A possible explanation for this synergistic effect is considered to be that the protein vaccine works as a priming immunogen and the DNA vaccine as a boosting immunogen. Specifically, the host immune responses can be quickly induced against the protein vaccine and then later against the antigen expressed by the DNA vaccine. The synergistic effect was not clearly shown with 0.1 μg of pcWNME, probably due to smaller amounts of expressed antigens than those expressed by 1 μg of pcWNME.

An adjuvant effect of CpG motifs present in the DNA vaccine plasmid on immunogenicity of the protein vaccine is considered to be another explanation. Although the dose-dependent effect of CpG adjuvant was not precisely investigated in the present study, neutralizing antibodies were not detected in ddY mice immunized with the mixture of WNVAX with 0.0073 μg of pcDNA3 (Table 4), suggesting that a 1/10 dose of WNVAX without CpG adjuvant is not able to induce detectable levels of neutralizing antibodies. On the other hand, neutralizing antibodies were detected in ddY or ICR mice immunized with WNVAX mixed with 0.073 and 0.73 μg of pcDNA3 (Tables 4 and 5). The immunogenicity of WNVAX is considered to be effectively increased by CpG adjuvant, contributing to a further increase in immune responses to booster antigens expressed by pcWNME, as described above.

In a murine model, it has been well established that Th1 and Th2 immune responses relate to induction of IgG2a and IgG1 antibodies, respectively [28]. In general, the types of immune responses induced by DNA vaccines depend on the administration route. Studies using DNA vaccines expressing flavivirus EPs have indicated that intramuscular inoculations by a normal needle/syringe injection induce Th1-dominant responses [10,29], whereas needle-free jet injections induce mixed Th1/Th2 responses [12]. In comparison, inactivated JE vaccines induced mixed Th1/Th2 responses, irrespective of the inoculation route [12,19,29]. Further, co-immunization of a DNA–protein mixture by needle-free jet injection induced mixed Th1/Th2 responses in a JEV system [12]. Consistently, the present study suggested that co-immunization of pcWNME and WNVAX induced both Th1/Th2 responses.

In conclusion, our WN DNA vaccine induced neutralizing antibodies in mice at a dose as low as 0.1 μg, when the DNA vaccine is co-administrated with a protein vaccine by the needle-free jet injector. This low DNA dose can be achieved by administration using a gene gun [30] or by in vivo electroporation [25]. However, the spring-powered needle-free jet injector is simpler, quicker and more convenient than other devices, and has a background of having been developed for drug delivery as a safe alternative to the needle and syringe. Reduction of the DNA vaccine dose and the time required for immunization may contribute to vaccination against a large number of people not in only developed, but also in developing countries.

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Epitope-Blocking Enzyme-Linked Immunosorbent Assay To Differentiate West Nile Virus from Japanese Encephalitis Virus Infections in Equine Sera▽

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West Nile virus (WNV) is now widely distributed worldwide, except in most areas of Asia where Japanese encephalitis virus (JEV) is distributed. Considering the movement and migration of reservoir birds, there is concern that WNV may be introduced in Asian countries. Although manuals and guidelines for serological tests have been created in Japan in preparedness for the introduction of WNV, differential diagnosis between WNV and JEV may be complicated by antigenic cross-reactivities between these flaviviruses. Here, we generated a monoclonal antibody specific for the nonstructural protein 1 (NS1) of WNV and established an epitope-blocking enzyme-linked immunosorbent assay that can differentiate WNV from JEV infections in horse sera. Under conditions well suited for our assay system, samples collected from 95 horses in Japan (regarded as negative for WNV antibodies), including those collected from horses naturally infected with JEV, showed a mean inhibition value of 8.2% and a standard deviation (SD) of 6.5%. However, inhibition values obtained with serum used as a positive control (obtained after 28 days from a horse experimentally infected with WNV) in nine separate experiments showed a mean of 54.4% and an SD of 7.1%. We tentatively determined 27.6% (mean + 3 × SD obtained with 95 negative samples) as the cutoff value to differentiate positive from negative samples. Under this criterion, two horses experimentally infected with WNV were diagnosed as positive at 12 and 14 days, respectively, after infection.

Before 1999, the geographic distribution of West Nile virus (WNV) was limited to Africa, the Middle East, and parts of Europe and Asia (4, 35). That year, WNV was detected in New York City. This was the first recognition of WNV in the Western Hemisphere. WNV has since spread rapidly across the United States and has extended its range to Canada, the Central American countries (12), and most recently to Argentina (39). In Eastern Europe, an outbreak of WNV infection with 40 deaths occurred in the Volgograd region of Russia in 1999 (34, 41). Reports have described WNV detection in birds in Vladivostok in 2003 (43) and in the Far Eastern region of Russia in 2004 (42). Kunjin virus that is distributed in Australia has now been reclassified as a subtype of WNV (5, 10). Thus, WNV is now more widely distributed worldwide than are the other flaviviruses.

WNV belongs to the genus *Flavivirus* of the family *Flaviviridae* and is a member of the Japanese encephalitis serocomplex (4). The Japanese encephalitis serocomplex includes four antigenically related human pathogens, which include members Murray Valley encephalitis virus (MVEV), Saint Louis encephalitis virus (SLEV), and Japanese encephalitis virus (JEV). MVEV and SLEV are distributed in the Australian and American continents, respectively. JEV has been described as distributed in the Far East, East, Southeast, and South Asia, and recently in Australia (11). Thus, WNV is itself distributed or with either MVEV or SLEV in many regions of the world. However, for areas in Asia, JEV is the sole flavivirus distributed there.

WNV is maintained in nature through a transmission cycle between vector mosquitoes and reservoir birds (16, 35). Many different wild bird species act as reservoir hosts for WNV. The movement and migration of birds are considered to be major causes of the dramatic spread in America (45). Since migratory birds move both north and south (38), WNV distributed in either the Russian Far East or Australia might be transported by migratory birds and introduced into WNV-free areas in Asia that include Japan. Once introduced, WNV is considered endemic/epizootic, since several species of vector mosquitoes (49) and reservoir birds (45) are commonly found in WNV-endemic/epizootic areas.

Infection with WNV results in a spectrum of clinical features in humans and horses (12, 16, 40). Until the mid-1970s, human outbreaks had been associated mainly with mild febrile illness, but outbreaks over the last decade have involved severe neurologic diseases such as meningitis and encephalitis. Since the clinical features caused by WNV are similar to those of JEV in humans (48) and horses (6, 31), laboratory tests are essential for the differential diagnosis of WNV from JEV disease. In general, laboratory diagnosis of WNV disease can be achieved by virus isolation/viral RNA detection and serological tests (4, 6, 36). Although the former method provides a firm diagnosis, the use of this method is limited to the period of viremia/RNAemia. Importantly, the virus is not often detectable in the blood at the time of illness onset (46). Therefore, serological tests are important since they can cover this limitation for the

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diagnosis of WNV disease and are also applicable to epidemiological surveys of WNV infections.

Serological tests for WNV infections (47) include the neutralization test, the hemagglutination-inhibiting test, the enzyme-linked immunosorbent assay (ELISA), and the immunofluorescence assay (IFA). A critical issue in serological tests in areas where several flaviviruses coexist is serological cross-reactivity between flaviviruses (28). Among these tests, neutralization tests are recognized as the "gold standard," providing the highest specificity. ELISA and IFA are also known to detect specific immunoglobulin M (IgM) antibodies. However, even with these tests, cross-reaction between members of the JE serocomplex may affect the diagnostic result (26, 36, 51). In Australia and the United States, where WNV coexists with MVEV and SLEV, respectively, epitope-blocking ELISAs have been established and are successfully used for differentiation between these flavivirus infections (1, 2, 8, 17).

The present study sought to establish an epitope-blocking ELISA that could differentiate WNV from JEV infections in horses. A monoclonal antibody specific for WNV was generated from mice immunized with the nonstructural protein 1 (NS1). We selected a nonstructural protein, since an assay system based on antibodies to the nonstructural protein would be useful even in the future when WNV vaccines are introduced. Indeed, we previously demonstrated in a JEV system (22–25) that antibodies to NS1 can be used for detecting natural infections among vaccinated populations. The epitope-blocking ELISA established in the present study is able to differentiate horses experimentally infected with WNV from those naturally infected with JEV.

MATERIALS AND METHODS

Viruses. The NY99 strain of WNV isolated from an infected horse was obtained from the National Veterinary Services Laboratories, United States Department of Agriculture, Ames, IA. This strain was passaged two times through Vero cells in our laboratory. The culture fluids harvested from infected Vero cells were used for experimental infection of horses and neutralization tests. The Eg101 strain of WNV (50) was provided by Tomohiko Takasaki of the National Institute of Infectious Diseases (NIID), Japan. This strain, which had been passaged 34 times through suckling mouse brains, was passaged two times through Vero cells in our laboratory. The culture fluids harvested from Vero cells infected with WNV 72 h earlier, containing approximately 2×10^8 PFU/ml, were used for construction of the plasmid and for purification of NS1 to produce polyclonal and monoclonal antibodies to the NS1 protein of WNV (hereinafter WNV-NS1). WNV strains FCG and G2266, also provided by Takasaki, were used for antigens for immunostaining. JEV strains Nakayama, Beijing 1, Beijing 3, JaTH-160, KE-093, and JaGAr-01 (14), the Mochizuki strain of dengue type 1 virus, the New Guinea C strain of dengue type 2 virus, the H87 strain of dengue type 3 virus, and the H241 strain of dengue type 4 virus (21) were also used for antigens for immunostaining.

Antibodies. Production of JE-2D5, a monoclonal to the NS1 protein of JEV (hereinafter JEV-NS1), was described previously (22). Another monoclonal to JEV-NS1, JE-6H4, was produced by a method similar to that used for production of JE-2D5 (unpublished data). A monoclonal to the envelope (E) protein, D1-4G2 (flavivirus group cross-reactive [13]), was provided by Takasaki of NIID. A monoclonal to NS1, D2-7E11 (dengue serocomplex cross-reactive [53]), was provided by Mary K. Gentry of the Walter Reed Army Institute of Research, Washington, DC. Monoclonal antibodies to WNV-NS1 were generated based on a method previously described (18). Briefly, BALB/c mice were immunized repeatedly with WNV-NS1 that was affinity purified from culture fluids of WNV-infected Vero cells by using JE-6H4 (cross-reactive with WNV-NS1). Spleen cells were collected from mice showing high antibody levels and fused with mouse myeloma P3U1 cells. Hybridoma cells were screened by ELISA for the production of antibodies to WNV-NS1 and cloned by limiting dilution. Hybridoma clones were grown as ascites tumors by intraperitoneal injection of pristan-primed BALB/c mice with 10^7 cells from culture. A monoclonal WN-2H4 specific for WNV-NS1 as determined by immunostaining (see Table 1) was used for the blocking ELISA established in the present study. Rabbit anti-WNV-NS1 hyperimmune serum was obtained by repeated immunization of a Japanese white rabbit with the WNV-NS1 antigen that was affinity purified from culture fluids of WNV-infected Vero cells.

TABLE 1. Reactivity of monoclonal antibodies to viral antigens by immunostaining[a]

Virus	Strain	Reactivity of:					
		WN-2H4	JE-6H4	JE-2D5	D1-4G2	D2-7E11	NMS
WNV	Eg101	+	+	−	+	NT	−
	NY99	+	+	−	+	NT	−
	FCG	+	+	−	+	NT	−
	G2266	+	+	−	+	NT	−
JEV	Nakayama	−	+	+	+	NT	−
	Beijing 1	−	+	+	+	NT	−
	Beijing 3	−	+	+	+	NT	−
	JaTH-160	−	+	+	+	NT	−
	KE-093	−	+	+	+	NT	−
	JaGAr-01	−	+	+	+	NT	−
DENV1	Mochizuki	−	−	−	+	+	−
DENV2	New Guinea C	−	+	−	+	+	−
DENV3	H87	−	−	−	+	+	−
DENV4	H241	−	+	−	+	+	−

[a] Vero cells were infected with indicated virus strains and used as antigens for immunostaining. DENV, dengue virus types 1 to 4. Monoclonal antibodies and normal mouse serum (NMS) were used at a dilution of 1:400. NT, not tested.

Serum samples. Sera were obtained from horses experimentally infected with WNV. Two thoroughbred horses (horse 1 and horse 2) were infected subcutaneously with 1×10^7 PFU of the NY99 strain of WNV and bled periodically until 35 days after infection. This experimental infection resulted in subclinical infection in both horses (unpublished data). Serum collected from horse 2 at 28 days after experimental infection with WNV was used as the positive control in experiments to determine basic conditions best suited for the blocking ELISA. As controls negative for antibodies to WNV, a total of 95 sera collected from individual thoroughbred racehorses stabled in Japan were used. Since WNV is not distributed in Japan, these horses were regarded as negative for antibodies to WNV. Of 95 sera, 60 were negative for JEV-NS1 antibodies and 35 positive as determined by ELISA for detecting JEV-NS1 antibodies in horse sera (22). The sera negative for JEV-NS1 antibodies included 40 sera from yearlings born and kept in an area of northern Japan where JEV is not endemic and 20 sera from horses aged 3 to 12 years that were selected from those previously used for a survey of natural JEV infections (23, 24). The 40 yearling sera were collected from 20 yearlings with and 20 without a vaccination history; it was confirmed by ELISA that all vaccinated yearlings were negative for JEV-NS1 antibodies and all unvaccinated yearlings were negative for JEV-E antibodies. Thirty-five sera positive for JEV-NS1 antibodies were selected from the horses aged 3 to 12 years used in our earlier survey (23, 24).

All animal experiments were conducted according to the Guidelines for Animal Experimentation at the Equine Research Institute, Tochigi Prefecture, Japan.

Plasmids. The cDNA encoding the signal sequence of NS1 and NS1 with or without NS2A of the Eg101 strain was produced from purified viral RNA by reverse transcriptase-PCR (RT-PCR) using a Thermo Script RT-PCR system (Invitrogen, San Diego, CA). The primers used in this RT-PCR were designed based on the nucleotide sequence of the WNV (Eg101 strain) genome registered in GenBank (accession number AF260968). The antisense primers used for production of the cDNAs with or without the NS2A gene were 5′-GCTCTAG ATTATCGTTTACGGTTGGGATCACATGC-3′, including the C-terminal eight codons of NS2A, or 5′-GCTCTAGATTAAGCATTCACTTGTGACTGC ACAAG-3′, including the C-terminal eight codons of NS1: the C-terminal codon of each of NS2A and NS1 was adjacent to a termination codon and an XbaI site. The sense primer 5′-GATATCACCATGGCTCTCACGTTTCTCGCAGTTGG A-3′ included an EcoRV site, an efficient eukaryotic initiation site (27), and a start codon, followed by the codons encoding Ala-Leu-Thr-Phe-Leu-Ala-Val-Gly

of the NS1 signal sequence. The amplified cDNA was inserted into the pcDNA3 vector (Invitrogen) at the EcoRV/XbaI site between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from bovine growth hormone. The constructs were designated pcWNNS1NS2A (with the *NS2A* gene) and pcWNNS1 (without the *NS2A* gene). Proper insertion of the *NS1* gene in both constructs was confirmed by sequencing. Although there was one nucleotide difference accompanied by the amino acid substitution from the reported sequence of the Eg101 strain within the N-terminal 70 amino acids of the *NS2A* gene, we used pcWNNS1NS2A for production of NS1 and NS1', since the level of NS1' production was significantly lower than that of NS1 in WNV (Fig. 2 and Fig. 3). The N-terminal 70 amino acids of the NS2A protein are thought to be utilized for the biosynthesis of flavivirus NS1' in infected mammalian cells, based on the description for MVEV (3) and JEV (32). Both of the plasmid DNA were purified using a Quantum Prep plasmid miniprep kit (Bio-Rad Laboratories, Hercules, CA) and used for the transfection of cells.

Generation of cells stably expressing the WNV-NS1 antigen. Cell clones stably transfected with pcWNNS1 or pcWNNS1NS2A were generated essentially as previously described (20). Briefly, CHO cells were transfected with 1 μg of the plasmid DNA and then selected with medium containing G418, followed by limiting dilution cloning to obtain transfected cells displaying high-level NS1 protein expression.

Immunoprecipitation. Viral antigens contained in culture fluids of JEV- or WNV-infected Vero cells or cells stably expressing WNV-NS1 antigens were immunoprecipitated with monoclonal antibodies coupled to protein A agarose (Invitrogen), essentially as previously described (19). Following immunoprecipitation, viral antigens were heated at 100°C for 2 min under nonreducing conditions and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by detection by silver staining (silver staining kit; GE Healthcare Bio-Science, Piscataway, NJ).

Affinity purification. The WNV-NS1 protein was affinity purified from culture fluids of cells infected with WNV by a monoclonal JE-6H4 coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare Bio-Science), followed by elution with 0.2 M glycine (pH 3.0).

Sandwich ELISA for quantification of WNV-NS1 antigens. WNV-NS1 antigens in culture fluids of cells stably transfected with pcWNNS1NS2A or pcWNNS1 were quantified using a sandwich ELISA basically as previously described (20). Briefly, microplates sensitized with rabbit anti-WNV-NS1 hyperimmune serum were serially incubated with test samples, a monoclonal WN-2H4, alkaline phosphatase-conjugated goat anti-mouse IgG, and *p*-nitrophenyl phosphate. Antigen levels were calculated from absorbancies obtained with the sample and a reference standard and expressed as the amount of NS1 protein in ng per ml. The reference standard was prepared with the WNV-NS1 protein affinity purified from WNV-infected Vero cells. The amount of purified WNV-NS1 was estimated by comparison with that of bovine serum albumin (BSA) samples in silver-stained gels.

Conventional ELISA for quantification of WNV-NS1 antibodies in horse sera. WNV-NS1 antibody levels were measured by a conventional ELISA essentially as previously described (20). Briefly, microplates were sensitized at 4°C overnight with the affinity-purified WNV-NS1 antigen at 50 ng/ml. The sensitized plates were serially incubated with test sera at a 1:100 dilution at 37°C for 1 h, with alkaline phosphatase-conjugated affinity-purified rabbit anti-horse IgG (gamma chain-specific; Rockland, Gilbertsville, PA) at a 1:1,000 dilution at 37°C for 1 h, and with *p*-nitrophenyl phosphate at 1 mg/ml. To minimize interplate variations, a constant positive control serum (collected from horse 2 at 28 days after experimental infection with WNV) was included in every plate, and absorbancies obtained with test samples were adjusted with the value for the positive control as 1.0. Specifically, absorbancies of the test samples were divided by the value of the positive control included in the same plate.

Blocking ELISA for differentiating WNV-NS1 from JEV-NS1 antibodies in horse sera. The principle of the blocking ELISA is shown in Fig. 1. Microplates (Maxisorp; Nunc A/S, Roskilde, Denmark) were incubated serially in the following steps with (i) rabbit anti-WNV-NS1 hyperimmune serum at a 1:10,000 dilution in 0.1 M sodium carbonate buffer (pH 9.6) at 4°C overnight; (ii) culture fluids of pcWNNS1NS2A-transfected cells, adjusted to 100 ng/ml of NS1 with ELISA diluent (phosphate-buffered saline containing 0.05% Tween 20 and 1% BSA) at 37°C for 1 h; (iii) test sera at a 1:5 dilution or ELISA diluent at 37°C for 1 h; (iv) WN-2H4 at a 1:1,000 dilution or affinity-purified mouse IgG1 (1 mg/ml; Bethyl, Montgomery, TX) at an appropriate dilution at 37°C for 1 h; (v) alkaline phosphatase-conjugated goat anti-mouse IgG at a 1:1,000 dilution at 37°C for 1 h; and (vi) *p*-nitrophenyl phosphate at 1 mg/ml. In this system, test sera were incubated in parallel with the ELISA diluent in step iii and WN-2H4 (subclass, IgG1) with mouse IgG1 (without any anti-WNV activity) in step iv, to minimize nonspecific reactions. The concentration of mouse IgG1 was adjusted to the IgG1 concentration contained in the 1:1,000 dilution of WN-2H4 in an ascites form. The percentage of inhibition of monoclonal antibody binding was calculated from absorbancies at 415 nm by the formula $100 - 100 \times (A - B)/(C - D)$, where A is an absorbance obtained with a combination of steps iii and iv with test sera and WN-2H4, B is obtained with test sera and purified IgG1, C is obtained with ELISA diluent and WN-2H4, and D is obtained with ELISA diluent and purified IgG1, respectively.

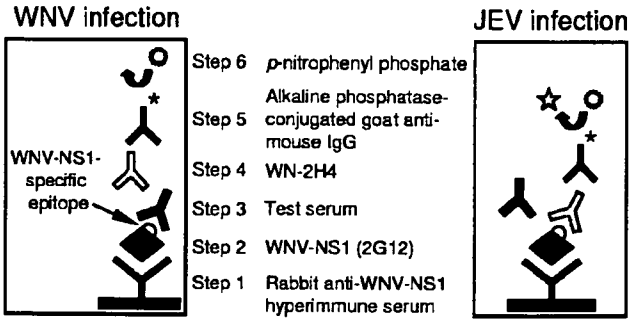

FIG. 1. Principle of the epitope-blocking ELISA to differentiate WNV from JEV infections. The procedure consists of six incubation steps from 1 to 6 (see Materials and Methods for details). (Left panel) When the test serum contains antibodies specific for WNV-NS1, these antibodies cover the specific epitope on WNV-NS1 antigens in step iii (3), thus blocking the binding of WN-2H4 antibody in step iv (4) and subsequent reactions in steps 5 and 6. (Right panel) When the test serum does not contain antibodies specific for WNV-NS1, the WN-2H4 antibody can bind to the specific epitope in step iv (4), thus allowing the subsequent reactions in steps 5 and 6.

Statistical analysis. Significance of differences of mean inhibition values was evaluated by Student's *t* test. Probability levels (P) of less than 0.05 were considered significant.

RESULTS

Characterization of monoclonal antibodies. Fourteen hybridoma clones secreting antibodies to WNV-NS1 were collected from two mice immunized with affinity-purified WNV-NS1 antigen of the Eg101 strain. From the 14 clones, WN-2H4 was selected for use in the blocking ELISA, based on its specificity, antibody productivity, and cell growth rate. Table 1 shows the reactivities of monoclonal WN-2H4 against WNV, JEV, and dengue virus antigens as determined by immunostaining; four strains including Eg101 were used for WNV, and six strains were used for JEV. For comparison, reactivities of monoclonal antibodies JE-6H4 and JE-2D5, both of which were obtained from mice immunized with JEV-NS1 (Nakayama strain), are also shown in Table 1. For immunostaining controls, E-specific flavivirus group-cross-reactive monoclonal D1-4G2 and NS1-specific dengue serocomplex-cross-reactive monoclonal D2-7E11, as well as normal mouse serum, were used. The results indicate that WN-2H4 was reactive with all strains of WNV but with none of the JEV and dengue virus strains. In contrast, JE-2D5 was reactive only with JEV, and JE-6H4 was cross-reactive with WNV, JEV, and dengue type 2 and 4 viruses.

The viral protein(s) recognized by monoclonal WN-2H4 was characterized by immunoprecipitation using culture fluids of Vero cells infected with the Eg101 strain of WNV. For reference, culture fluids of Vero cells infected with the Nakayama strain of JEV were subjected to immunoprecipitation with the

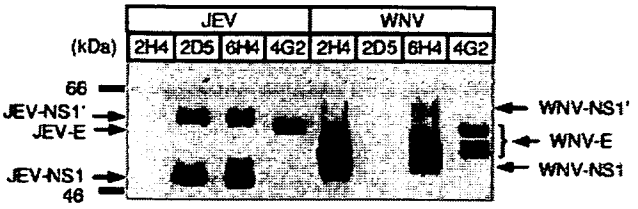

FIG. 2. Immunoprecipitation of culture fluids from JEV- or WNV-infected Vero cells with monoclonal antibodies (JE-2D5, JE-6H4, D1-4G2, and WN-2H4). Samples heated under nonreducing conditions were run on an 8% polyacrylamide gel and detected by silver staining.

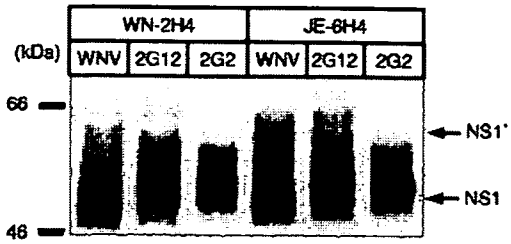

FIG. 3. Immunoprecipitation of culture fluids from Vero cells infected with WNV (WNV) and CHO cells transfected with pcWNNS1NS2A (2G12) or pcWNNS1 (2G2) with monoclonal antibodies (WN-2H4 and JE-6H4). Samples heated under nonreducing conditions were run on an 8% polyacrylamide gel and detected by silver staining.

monoclonal antibody JE-2D5, JE-6H4, or D1-4G2. As shown in Fig. 2, JEV-NS1 and JEV-NS1′ were precipitated with JE-2D5 and JE-6H4 in a pattern similar to that which we previously obtained with the same JEV antigen and JE-2D5 (22). Consistent with the JEV antigens, WNV antigens precipitated with WN-2H4 and JE-6H4 showed two bands corresponding to the WNV-NS1 and WNV-NS1′ proteins: these bands of approximately 50 and 60 kDa corresponded to the predicted molecular mass of 46 and 55 kDa calculated from the amino acid composition of the WNV-NS1 and WNV-NS1′ proteins, respectively. Different from JEV antigens, the band corresponding to WNV-NS1 was broader than the JEV-NS1 band, while the band corresponding to WNV-NS1′ was fainter than the JEV-NS1′ band. These differences might be attributable to the differences in heterogenicity of glycosylation and productivity of NS1′ between WNV and JEV. Since JE-6H4 is cross-reactive to the JEV and WNV antigens, these results indicate that WN-2H4 was directed to WNV-NS1 and WNV-NS1′. Figure 2 also indicated that WN-2H4 did not react with JEV-NS1, consistent with the result shown in Table 1.

Generation of a cell line stably producing extracellular WNV-NS1 antigen. To produce WNV-NS1 antigens used for the blocking ELISA, CHO cells were transfected with pcWNNS1 or pcWNNS1NS2A to generate cell lines stably expressing WNV-NS1. Although only 10 to 20% of the cells expressed NS1 antigen following five passages in G418-containing medium, one cloning step of these cells increased the percentage of NS1-expressing cells to nearly 100% as determined by immunostaining using monoclonal WN-2H4. Among those transfected with pcWNNS1, the highest yield of extracellular NS1 antigen was shown with the clone 2G2 (designated 2G2 cells), while among those transfected with pcWNNS1NS2A, it was the clone 2G12 (designated 2G12 cells), as determined by the sandwich ELISA to measure NS1 antigen in the culture fluid.

Next, NS1 antigens released from 2G2 or 2G12 cells were analyzed by immunoprecipitation with monoclonal antibodies WN-2H4 and JE-6H4. Silver staining of a polyacrylamide gel (Fig. 3) revealed a broad band corresponding to WNV-NS1 and a faint band corresponding to WNV-NS1′ in samples from WNV-infected Vero cells, consistent with the pattern shown in Fig. 2. Both WNV-NS1 and WNV-NS1′ were produced from 2G12 cells, whereas 2G2 cells produced only WNV-NS1. Although these two NS1 protein species produced by WNV-infected Vero cells comigrated with those produced by CHO-derived 2G12 or 2G2 cells, the former migrated slightly faster than the latter, similar to the differences previously shown between the JEV-NS1 proteins released from Vero cells infected with JEV and CHO cells stably expressing JEV-NS1 (22). The difference is probably due to the difference in cell type. Since 2G12 cells could produce WNV-NS1 and WNV-NS1′ in patterns similar to those shown by WNV-infected Vero cells, we decided to use 2G12 cells for the production of the NS1 antigen used for our blocking ELISA to differentiate WNV-NS1 from JEV-NS1 antibodies.

Determination of assay conditions best suited for blocking ELISA. The blocking ELISA consisted of six incubation steps with (i) anti-WNV-NS1 hyperimmune serum, (ii) WNV-NS1 antigen, (iii) test sera, (iv) WN-2H4, (v) enzyme-conjugated goat anti-mouse IgG, and (vi) substrate (see Materials and Methods for details). Assay conditions for each step were investigated using various conditions in a single step with conditions in the other steps constant, except for steps v and vi, which were fixed to a dilution of 1:1,000 and a concentration of 1 mg/ml, respectively.

The dilution factor of anti-WNV-NS1 hyperimmune serum in step i was investigated by comparing absorbancies obtained with $1:10^3$ to $1:10^6$ dilutions of the hyperimmune serum, in the above ELISA protocol with step iii skipped. Since absorbancies obtained at 10^3 and 10^4 dilutions were considerably higher than those obtained at 10^5 and 10^6 dilutions (data not shown), we decided to use the 10^4 dilution of the hyperimmune serum.

The concentration of WNV-NS1 antigens in step ii was investigated by comparing absorbancies obtained with several dilutions of the culture fluid of 2G12 cells containing WNV-NS1 antigens at 0 to 200 ng/ml. When ELISA diluent was used in step iii (Fig. 4, closed circles), absorbancies increased with an increase of antigen concentration and leveled off at 100 ng/ml, indicating the saturation of antigens bound to the capture antibody at this dilution. As well, as a preliminary examination of the inhibition of WN-2H4 binding by serum from a WNV-infected horse, a 1:10 dilution of the positive control serum (collected from horse 2 at 28 days after experimental infection with WNV) was used in step iii (Fig. 4, open circles). With the use of the positive control, absorbancies were lower than those obtained without its use, but roughly at constant inhibition values within the antigen concentrations of 10 to 200 ng/ml. Based on these results we decided to use 100 ng/ml of NS1 antigens.

The dilution factor of the test sera in step iii was investigated by comparing inhibition values obtained with sera from WNV-

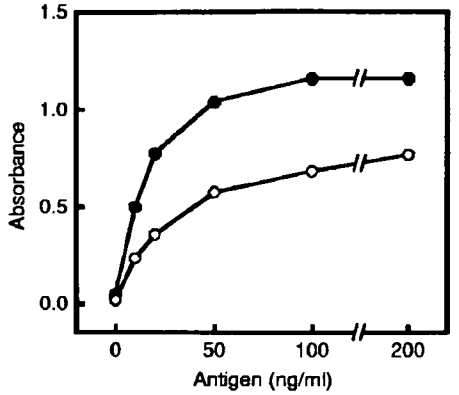

FIG. 4. Dose-response absorbance curves of NS1 antigens contained in culture fluids of 2G12 cells (see text for details). Absorbancies were obtained with (open circles) or without (closed circles) a 1:10 dilution of the positive control serum in step iii.

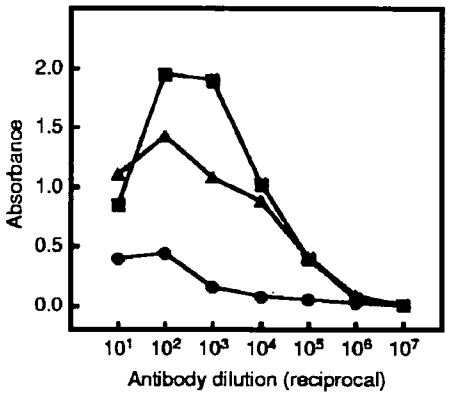

FIG. 6. Comparison of dose-response absorbance curves obtained with monoclonal antibodies WN-2H4 (squares), JE-2D5 (circles), and JE-6H4 (triangles; see text for details).

or JEV-infected horses at 1:2 to 1:1,000 dilutions (Fig. 5). The serum from a WNV-infected horse (the positive control) was repeatedly examined six or three times for each of 1:2 to 1:10 or 1:20 to 1:1,000 dilutions, respectively, whereas sera from 35 or 3 JEV-infected horses were examined in the range of 1:2 to 1:10 or 1:20 to 1:1,000 dilutions, respectively. The serum from the WNV-infected horse showed dose-dependent inhibition values with higher mean values at lower serum dilutions (Fig. 5, closed circles). On the other hand, sera from JEV-infected horses showed constantly low mean inhibition values of less than 5% at dilutions of 1:5 or more with a relatively high mean inhibition value of 15% at a dilution of 1:2 (Fig. 5, open circles). We therefore decided to use a 1:5 dilution of the test sera.

The dilution factor of WN-2H4 in step iv was investigated within the range of $1:10^1$ to $1:10^7$ dilutions, in the protocol with step iii skipped (Fig. 6). Absorbancies were increased with an increase of antibody concentration at dilutions over $1:10^3$ with a "prozone" effect at dilutions below $1:10^3$ (Fig. 6, squares). We therefore decided to use a 1:1,000 dilution of WN-2H4, as it was considered that the amount of WN-2H4 antibody at this dilution is enough to bind to the WNV-NS1 antigen at the solid phase. Also, the binding would be effectively inhibited by the presence of WNV-NS1-specific antibodies contained in test sera. Figure 6 also shows dose-response curves obtained with JE-2D5 (Fig. 6, circles) and JE-6H4 (Fig. 6, triangles) antibodies, supporting the specificity of these monoclonal antibodies shown in Table 1.

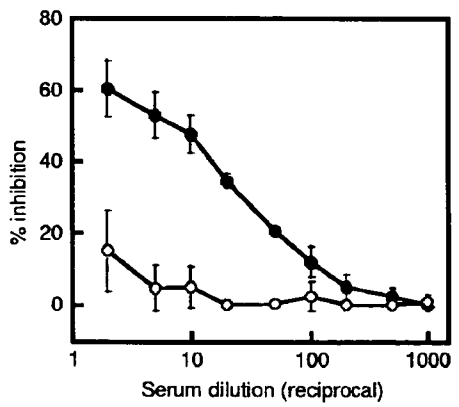

FIG. 5. Effect of serum dilutions on the percentage of inhibition of WN-2H4 binding, using sera from horses experimentally infected with WNV (closed circles; used as the positive control serum; see Materials and Methods for details) or naturally infected with JEV (open circles) in blocking ELISA. Each plot shows a mean inhibition value with an SD (indicated by bars) obtained with the WNV-infected horse serum by six (for each of 1:2 to 1:10 dilutions) or three (for each of 1:20 to 1:1,000 dilutions) repeated experiments and with 35 (for 1:2 to 1:10 dilutions) or 3 (for 1:20 to 1:1,000 dilutions) JEV-infected horse samples.

Determination of the cutoff value. The cutoff value used to differentiate positive from negative samples was investigated using four groups of horse sera collected in Japan. The sera included samples from 3- to 12-year-old horses positive or negative for antibodies to JEV-NS1 and yearlings with or without JE vaccination histories (Fig. 7, groups A to D). The mean inhibition values in these groups were 8.0 to 8.5%, without significant differences between groups ($P > 0.05$). We therefore used all groups of sera for determining the cutoff value in our blocking ELISA. These sera (95 samples) showed inhibition of WN-2H4 binding ranging from 0.0 to 27.6%, with a mean of 8.2% and a standard deviation (SD) of 6.5%. We tentatively determined 27.6% (mean + 3 × SD) as the cutoff value to differentiate positive from negative samples for antibodies to WNV-NS1. Theoretically, the probability of negative samples to show greater than this cutoff value is calculated to be 0.14%.

Figure 7 also shows inhibition values obtained with the positive control serum in nine separate experiments. The inhibition values varied from 43.7% to 67.5%, with a mean of 54.4% and an SD of 7.1% with a coefficient of variation of 13.1%, indicating reproducibility of this blocking ELISA.

Time course of inhibition values in horses after experimental infection. Sera collected from two horses periodically until 35 days after experimental infection with WNV were tested with the blocking ELISA to determine when antibodies specific for WNV-NS1 could be detected during the course of infection. Sera were also tested with the neutralization test that measures antibodies to viral surface proteins but not NS1, as

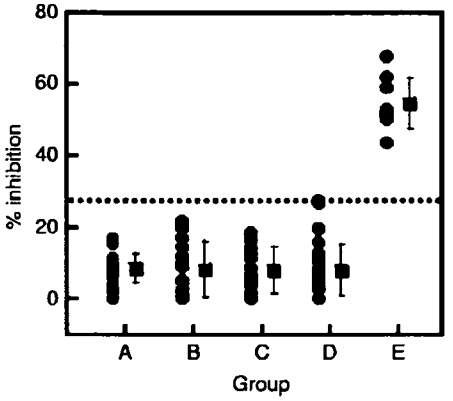

FIG. 7. Comparison of the percentage of inhibition of WN-2H4 binding among groups of sera in blocking ELISA. These groups included sera from yearlings born and kept in Hokkaido without (A, 20 samples) or with (B, 20 samples) JE vaccination, from 3- to 12-year-old horses negative (C, 20 samples) or positive (D, 35 samples) for JEV-NS1 antibodies, and from horse 2 at 28 days after experimental infection with WNV (E, data obtained from nine repeated experiments). Circles indicate individual inhibition values, and squares with bars indicate means and standard deviations of the corresponding groups. A dotted line indicates the cutoff value calculated for the blocking ELISA (27.6%).

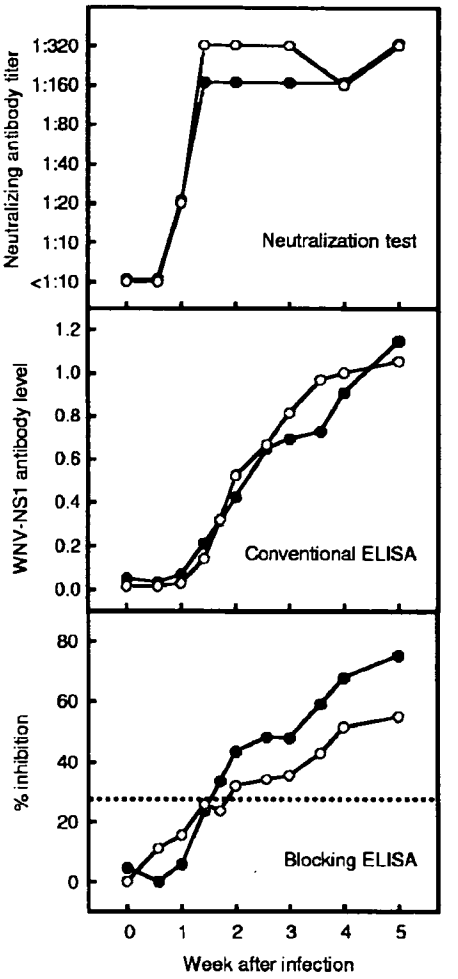

FIG. 8. Time courses of the percentage of inhibition of WN-2H4 binding in two horses experimentally infected with WNV: horse 1 (closed circles) and horse 2 (open circles). For references, neutralizing antibody titers and WNV-NS1 antibody levels are shown. A dotted line indicates the cutoff value used to differentiate positive from negative samples in the blocking ELISA (27.6%).

well as the conventional ELISA that measures both specific and cross-reactive WNV-NS1 antibodies (Fig. 8). Time courses of neutralizing antibody titers in two horses were similar: titers were detectable on day 7, increased until day 10, and then leveled off. In the conventional ELISA, levels of antibodies to WNV-NS1 began to increase on day 10 and continued to increase until the end of the experimental period in both horses. In the blocking ELISA, infected horses became positive for the presence of specific antibodies on days 12 (horse 1) and 14 (horse 2). The inhibition value continued to increase until day 35, with horse 1 showing higher inhibition values than horse 2. These results indicate that the blocking ELISA could be used for differentiating WNV from JEV infections approximately 2 weeks after horses were infected with WNV.

DISCUSSION

Serological cross-reactivities between flaviviruses often complicate differential diagnoses (28). This is especially the case for members of the JE serocomplex in which differentiation is often difficult even using the neutralization test, recognized to have the highest specificity among the currently available serological tests (36, 51). In areas where more than a single flavivirus coexists, sequential infection with the second virus induces anamnestic responses against cross-reactive antigens produced by infection with the first virus. In Japan, almost all humans and horses have immunity against JEV through vaccination and/or natural infections. Upon infection with WNV, it is highly probable that anamnestic antibody responses to common epitopes can produce JEV antibody levels equivalent to or even higher than WNV antibody levels produced by the primary antibody responses to WNV antigens.

Establishment of a blocking ELISA depends primarily on the availability of specific antibodies. To the best of our knowledge, there have been no reports of monoclonal antibodies that are reactive with WNV but not JEV antigens. Although the present study used horse sera infected with the NY99 strain, this blocking ELISA is considered to correctly detect horses infected with other wild strains, since the monoclonal WN-2H4 produced from the prototype Eg101 antigen reacted with antigens of two other WNV strains but not with those of six JEV strains. Based on the principle of this assay system, the blocking ELISA established in the present study for use in equine sera is probably applicable to use with sera from humans, birds, and other animals. Furthermore, since the monoclonal WN-2H4 did not react with four dengue viruses, this blocking ELISA may be applied in Asian countries where dengue viruses are endemic. Identification of the amino acid sequence of the WN-2H4 epitope will contribute to the future development of virus type-specific diagnosis of flavivirus infections.

The Japanese government has created manuals and guide-

lines as part of its preparedness for the arrival of WNV in Japan (15, 29, 37). Serological diagnosis of human and equine WNV disease described in these documents includes the detection of WNV-specific IgM antibodies by ELISA and the detection of neutralizing antibodies. A fourfold or higher rise in neutralizing antibody titer in paired sera collected from acute and convalescent phases, as well as higher antibody titers against WNV than JEV, is a critical factor for diagnosis. However, only a comparison between levels of JEV and WNV antibodies is considered insufficient, as described above. The blocking ELISA established in the present study constitutes another powerful tool for differential diagnosis.

A monoclonal antibody to a nonstructural protein (WNV-NS1) was used in our blocking ELISA to differentiate WNV from JEV infections. At present, there is no licensed vaccine against WNV for human use, whereas three WNV vaccines have been licensed for equine use in the United States and/or Europe, consisting of inactivated, canarypox virus-based recombinant, and DNA vaccines (7). Since most of the recent recombinant flavivirus vaccines have been developed using the *prM* and *E* genes, these vaccines, besides inactivated ones, can induce antibodies to structural but not nonstructural proteins (30, 44). On the other hand, WNV infection can induce antibodies to both structural and nonstructural proteins. Therefore, even in the future, when vaccines may be introduced for humans and horses in Asian countries, by demonstrating antibodies to nonstructural proteins, vaccinated individuals who acquire infections are considered to be distinguishable from those uninfected. Since NS1 is the only nonstructural protein secreted from flavivirus-infected mammalian cells (33), NS1 is considered to induce the highest antibody responses in infected humans or animals among seven nonstructural proteins. Furthermore, the monoclonal antibody used in blocking ELISAs established for differentiating WNV from MVEV (in Australia) or from SLEV (in America) infections is also directed to NS1 (2, 8). It has been demonstrated that NS1 has more virus-specific epitopes than cross-reactive ones in contrast to E, which has more cross-reactive than specific epitopes (9). Thus, NS1 is considered an appropriate target for blocking ELISAs for differentiation between flavivirus infections. In relation, an immunoassay targeting nonstructural protein 5 can differentiate WNV from SLEV and dengue virus infections and also from prior vaccination against flavivirus diseases (52).

Our blocking ELISA detected WNV-specific antibodies in sera from two horses at the latest 14 days after their experimental infection with WNV. Since these horses did not show any symptoms after virus inoculation, it is likely that the blocking ELISA can detect horses exposed to natural infections, as well as horses with clinical infections, demonstrating its applicability to epidemiological surveys in addition to serodiagnosis. In conclusion, we developed an easy, sensitive, and specific NS1-based epitope-blocking ELISA for differentiating WNV from JEV infections in horses. Our ELISA can be used in JEV-endemic areas after the introduction of WNV, as well as for diagnosis of travelers returning from areas where WNV and JEV coexist.

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Infection-Enhancing and -Neutralizing Activities of Mouse Monoclonal Antibodies against Dengue Type 2 and 4 Viruses Are Controlled by Complement Levels[▽]

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Dengue viruses are distributed widely in the tropical and subtropical areas of the world and cause dengue fever and its severer form, dengue hemorrhagic fever. While neutralizing antibodies are considered to play a major role in protection from these diseases, antibody-dependent enhancement (ADE) of infection is an important mechanism involved in disease severity, in addition to the involvement of T lymphocytes. Here, we analyzed relationships between neutralizing and enhancing activities at a clonal level using models of dengue type 2 virus (DENV2) and dengue type 4 virus (DENV4). Totals of 33 monoclonal antibodies (MAbs) against DENV2 and 43 against DENV4 were generated, all directed to the envelope protein. In these MAbs, enhancing activities were shown at subneutralizing doses under normal ADE assay conditions where test samples were heat inactivated. However, the inclusion of commercial rabbit complement or fresh sera from healthy humans in the ADE assay system abolished the enhancing activities of all these MAbs. The reductive effect of fresh sera on enhancing activities was significantly reduced by their heat inactivation or the use of C1q- or C3-depleted sera. In some fresh sera, enhancing activities were shown within a range of 20 to 80% of normal complement levels in a dose-dependent fashion. These results indicate that a single antibody species possesses two distinct activities (neutralizing/enhancing), which are controlled by the level of complement, suggesting the involvement of complement in dengue disease severity. Fresh human sera also tended to reduce enhancing activities more effectively in homologous than heterologous combinations of viruses (DENV2/DENV4) and MAbs (against DENV2/DENV4).

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are mosquito-borne diseases caused by infection with any one of the four dengue viruses, dengue virus types 1 to 4 (DENV1 to DENV4) (2, 12, 15). These viruses are distributed throughout the tropical and subtropical areas of the world, with an estimated 50 to 100 million DF cases and 250 to 500 thousand DHF cases reported every year (31, 47). Most infections with dengue viruses result in asymptomatic infections. Clinical cases usually take a benign form (DF) and occasionally a severe form (DHF). DF patients develop high fever, headache, and muscle and joint pain from which almost all recover, whereas DHF patients, though the occurrence and progress of disease are similar to those of DF patients, develop mainly plasma leakage and hemorrhagic manifestations, which may lead to shock (11). The case-fatality rate of DHF is roughly 5% (31), and a large proportion (90%) of DHF patients are children under 15 years of age (53).

Four dengue viruses belong to the genus *Flavivirus* of the family *Flaviviridae*. The flavivirus virion consists of a nucleocapsid structure surrounded by a lipid bilayer containing an envelope (E) and a membrane (M) protein (34). The E protein is the major surface protein, containing many neutralizing epitopes. Most members of the genus *Flavivirus* are grouped into eight antigenic complexes, and four dengue viruses belong to the dengue virus serocomplex (2). Four dengue viruses are antigenically cross-reactive with each other, while they also possess type-specific epitopes as recognized by neutralization tests that provide the highest specificity among existing serological tests (29). Thus, antibodies induced by dengue virus infections can be roughly divided into type-specific neutralizing antibodies, cross-reactive nonneutralizing antibodies, and cross-neutralizing antibodies among the four dengue viruses (31, 47).

Epidemiological evidence indicates that people once infected with one type of dengue virus are usually protected from subsequent infection with the same type of dengue virus (hereafter called homotypic infection) (13, 15, 44). Cross-protection against infections with different types (hereafter called heterotypic infection) is only shown in the short term following infection. It has been considered that neutralizing antibody is important for protection against dengue virus infection, since passive transfer of neutralizing monoclonal antibodies (MAbs) can confer protection from lethal challenge in a murine model (22, 23). On the other hand, secondary infection with a different type of dengue virus may cause DHF, as demonstrated by several epidemiological studies (reviewed in reference 15): secondary infections cause 40 times more DHF cases than primary infections. Therefore, cross-reactive nonneutralizing antibodies induced by the primary infection have been considered to serve as "enhancing" antibodies causing increased disease severity upon secondary infection with a different type of dengue virus.

The initial targets of dengue virus infections following infective mosquito bites have been reported to be immature monocyte-derived dendritic cells (39, 54). A virus replicated and

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released from these cells may enter the bloodstream and circulate throughout the host body, where monocytes/macrophages (4, 14) and the liver (9) are considered to be principal targets. Several different hypotheses have been proposed for the mechanisms of DHF development (11, 31), including virus virulence (40, 42), cross-reactive T lymphocytes (8, 36), etc. However, most agree that the level of viremia correlates to disease severity (33, 47, 52). One of the host factors relating to increased viremia levels is antibody-dependent enhancement (ADE) of infection (16, 24), which is mediated by Fc gamma receptors on the monocytes/macrophages in the presence of "enhancing" antibodies (30).

It is widely believed that neutralizing antibodies can reduce viremia levels, whereas cross-reactive nonneutralizing (enhancing) antibodies may increase them. Therefore, the balance of these antibody species has been considered important for determining the outcome of the disease; that is, protection or pathogenesis. However, a recent paper reported that some individuals who had neutralizing antibodies against DENV2 develop symptoms upon infection with DENV2, suggesting that neutralizing antibodies do not always work for protection from the subsequent homologous infection (7). Another study reported that enhancing antibodies do not correlate to the viremia level (32). Thus, detailed analyses of the relationships between neutralizing and enhancing activities are needed to elucidate the mechanisms increasing disease severity.

Most previous studies dealing with neutralizing and/or enhancing activities have used serum specimens. However, approaches at a polyclonal level seem to limit detailed analyses. In the present study, MAbs against DENV2 (D2MAbs) or DENV4 (D4MAbs) were generated from immune mice and characterized for enhancing and neutralizing activities against homologous and heterologous types. The results indicated that all MAbs showing enhancing activities showed neutralizing activities. Furthermore, the addition of commercial rabbit complement or fresh human sera into the ADE assay systems abolished the enhancing activities of all MAbs.

MATERIALS AND METHODS

Cells. Vero cells (26) were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 60 μg/ml kanamycin. C6/36 cells (26) were cultivated in MEM supplemented with 10% FBS, nonessential amino acids, and 60 μg/ml kanamycin. The U937 human monocytic cell line (48) and the K562 erythroleukemia cell line (35), both provided by Ichiro Kurane of the National Institute of Infectious Diseases (NIID), Japan, were cultivated in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI-10% FBS). The P3-X63-Ag8-U1 (P3U1) mouse myeloma cell line and hybridoma cells were cultivated in RPMI 1640 medium supplemented with 10 to 20% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. All cells were cultivated in a humidified atmosphere of 5% CO_2 95% air at 37°C, except for the C6/36 cells, which were cultivated at 28°C.

Viruses. The New Guinea C strain of DENV2 and the H241 strain of DENV4 (27) were used. Viruses harvested from culture fluids of infected Vero cells were used as antigens for the competition assay, hemagglutination inhibition (HAI) test, and enzyme-linked immunosorbent assay (ELISA) to quantify antibody levels. Viruses harvested from culture fluids of infected C6/36 cells were used for immunoprecipitation, the neutralization test, and the ADE assay. Viruses in the form of an infected mouse brain homogenate at a 10% emulsion in 7.5% bovine serum albumin in phosphate-buffered saline were used for booster immunization of mice to generate hybridomas.

Rabbit hyperimmune sera. Sera from rabbits hyperimmune to the New Guinea C strain of DENV2 (26) or the H241 strain of DENV4 (27) have been described previously.

Human sera. Serum samples were collected from 14 healthy humans aged 21 to 52 years, average 26.1 years, with no history of travel in countries where dengue virus is endemic. Heat inactivation of sera was performed at 56°C for 30 min. Complement C1q-depleted human serum and C3-depleted human serum were purchased from Merck, Darmstadt, Germany. All human sera used in the present study were negative for neutralizing antibodies against DENV2 and DENV4 as determined by 50% focus reduction assay (data not shown). The use of human sera was approved by the ethical committee of Kobe University School of Medicine.

Generation of mouse MAbs. Four-week-old female BALB/c mice were immunized twice at intervals of 3 weeks by inoculation with 100 μg of a DNA vaccine using a needle-free jet injector (ShimaJET; Shimadzu, Kyoto, Japan). The DNA vaccines were pcDNA3-based plasmids expressing the premembrane and E proteins of DENV2 (26) or DENV4 (27). One to 2 months after the second immunization, the mice were boosted with dengue virus antigens of the corresponding type (infected mouse brain homogenate; 1×10^7 PFU/mouse), and spleen cells were collected 3 to 4 days after the booster immunization. Hybridoma cells were generated essentially as described by Kohler and Milstein (25) with some modifications (28). Briefly, spleen cells were fused to P3U1 cells using polyethylene glycol-4000 (Merck, Darmstadt, Germany). Hybridoma cells were screened by ELISA for the production of specific antibodies (see below) and cloned by limiting dilution. For MAb production, hybridoma clones were grown as ascites tumors by intraperitoneal inoculation of 6- to 8-week-old pristane-primed male BALB/c mice with 10^7 cells from culture. One to three weeks later, ascitic fluids were collected, clarified, and stored at −30°C until use as D2MAbs or D4MAbs.

Affinity purification of IgG from ascitic fluids and biotinylation. Immunoglobulin G (IgG) was purified from ascitic fluids by using protein G Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences, Piscataway, NJ) and dialyzed against phosphate-buffered saline. Purified IgG was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) following the instructions provided by the manufacturer.

Competition assay. Microplates were sensitized by incubation at 4°C overnight with a 1:1,000 dilution of rabbit hyperimmune serum against DENV2 or DENV4. The sensitized plates were then incubated with culture fluids of Vero cells infected with the corresponding virus (DENV2 or DENV4), a 1:1,000 dilution of the first antibody (ascitic fluid), a 1:100 dilution of the biotinylated second antibody (IgG fraction), a 1:1,000 dilution of avidin-alkaline phosphatase conjugate, and then *p*-nitrophenyl phosphate. When the absorbance was less than 70% of the control absorbance obtained without incubation with the first antibody, the reaction was determined to be competitive.

ELISA for measuring antibodies to DENV2 or DENV4. Antibody levels in hybridoma culture fluids or mouse ascitic fluids were measured by a conventional ELISA as described previously (27). Briefly, microplates sensitized with rabbit hyperimmune sera against DENV2 or DENV4 were incubated serially with the corresponding (DENV2 or DENV4) antigen, ascitic fluid samples at a 1:1,000 dilution or culture fluid samples at the original dilution, alkaline phosphatase-conjugated antimouse IgG, and then *p*-nitrophenyl phosphate. Samples were determined to be positive when the absorbance was higher than the average plus two times the standard deviation (SD) of absorbances obtained with six negative control samples.

ELISA for measuring IgG concentrations. A sandwich ELISA was performed as described previously (28). Briefly, microplates sensitized with goat anti-mouse IgG were incubated with serial 10-fold dilutions of test samples, alkaline phosphatase-conjugated goat anti-mouse IgG, and then *p*-nitrophenyl phosphate. The IgG concentrations were calculated by comparing the absorbances with those obtained for the standard mouse sera with known IgG concentrations and expressed as ng/ml.

Determination of isotypes. The isotype of each MAb was determined by using an ImmunoPure monoclonal antibody isotyping kit II (Pierce, Rockford, IL) according to the manufacturer's instructions.

Immunoprecipitation. The immunoprecipitation of viral proteins with MAbs was performed by using protein A agarose (Invitrogen, Carlsbad, CA) as described previously (26). Briefly, protein A-coated agarose beads were incubated with each MAb sample, rinsed, and then incubated with culture fluids of C6/36 cells infected with DENV2 or DENV4. Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions and detected by silver staining. E and M proteins were confirmed as bands with predicted molecular masses of approximately 63 and 10 kDa, respectively, which were calculated from the amino acid compositions of the E and M proteins based on the nucleotide sequences of the dengue virus genomes registered in GenBank: accession number M19197 for DENV2 NGC and AF326825 for DENV4 H241.