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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.

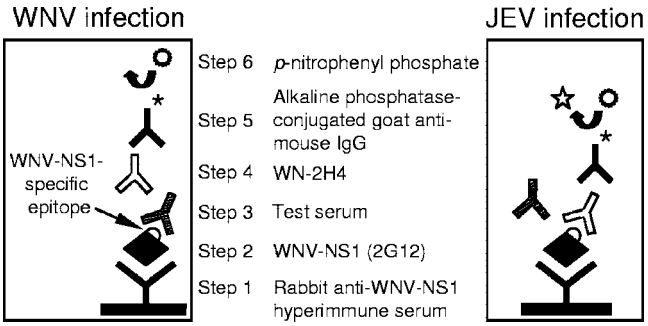

FIG. 1. Principle of the epitope-blocking ELISA to differentiate WNV from JEV infections. The procedure consists of six incubation steps from 1 to 6 (see Materials and Methods for details). (Left panel) When the test serum contains antibodies specific for WNV-NS1, these antibodies cover the specific 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

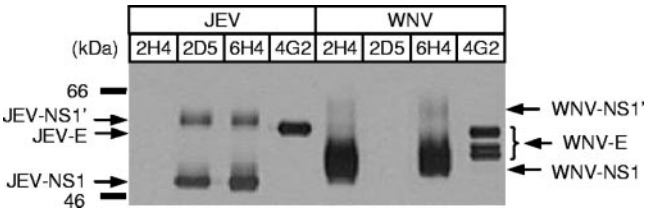

FIG. 2. Immunoprecipitation of culture fluids from JEV- or WNV-infected Vero cells with monoclonal antibodies (JE-2D5, JE-6H4, D1-4G2, and WN-2H4). Samples heated under nonreducing conditions were run on an 8% polyacrylamide gel and detected by silver staining.

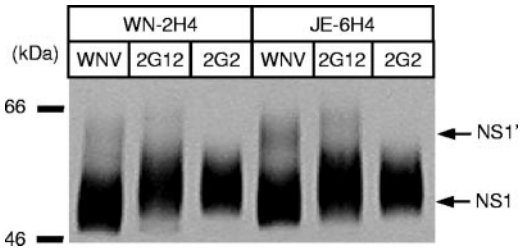

FIG. 3. Immunoprecipitation of culture fluids from Vero cells infected with WNV (WNV) and CHO cells transfected with pcWNNS1NS2A (2G12) or pcWNNS1 (2G2) with monoclonal antibodies (WN-2H4 and JE-6H4). Samples heated under nonreducing conditions were run on an 8% polyacrylamide gel and detected by silver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

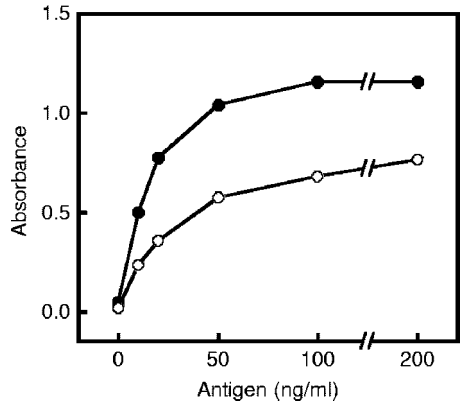

FIG. 4. Dose-response absorbance curves of NS1 antigens contained in culture fluids of 2G12 cells (see text for details). Absorbancies were obtained with (open circles) or without (closed circles) a 1:10 dilution of the positive control serum in step iii.

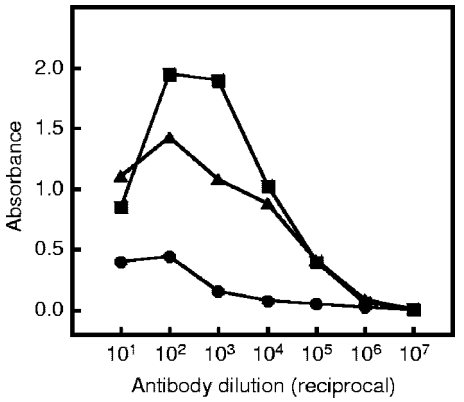

FIG. 6. Comparison of dose-response absorbance curves obtained with monoclonal antibodies WN-2H4 (squares), JE-2D5 (circles), and JE-6H4 (triangles; see text for details).

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.

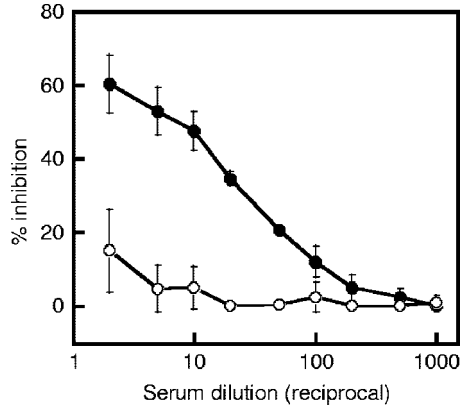

FIG. 5. Effect of serum dilutions on the percentage of inhibition of WN-2H4 binding, using sera from horses experimentally infected with WNV (closed circles; used as the positive control serum; see Materials and Methods for details) or naturally infected with JEV (open circles) in blocking ELISA. Each 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

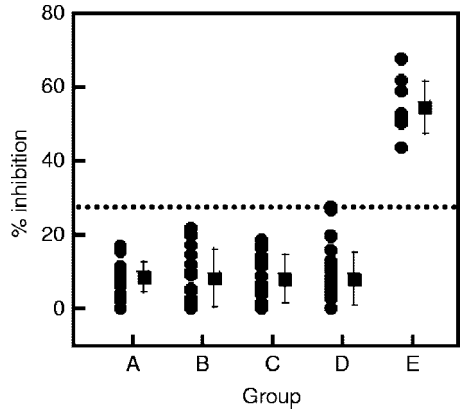

FIG. 7. Comparison of the percentage of inhibition of WN-2H4 binding among groups of sera in blocking ELISA. These groups included sera from yearlings born and kept in Hokkaido without (A, 20 samples) or with (B, 20 samples) JE vaccination, from 3- to 12-year-old horses negative (C, 20 samples) or positive (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%).

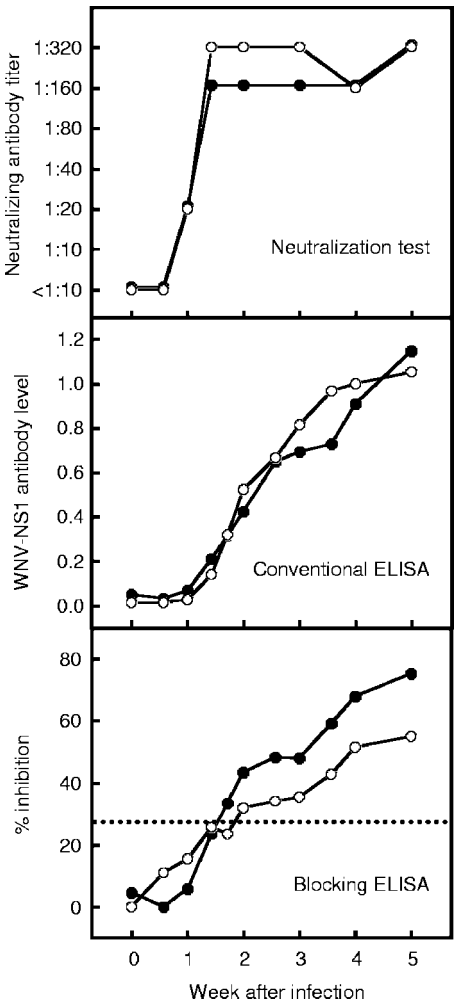

FIG. 8. Time courses of the percentage of inhibition of WN-2H4 binding in two horses experimentally infected with WNV: horse 1 (closed circles) and horse 2 (open circles). For references, neutralizing antibody titers and WNV-NS1 antibody levels are shown. A dotted line indicates the cutoff value used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