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Topographical Analysis of Antigenic Determinants on Envelope Glycoprotein V3 (E) of Japanese Encephalitis Virus, Using Monoclonal Antibodies

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Ten monoclonal antibodies directed against envelope glycoprotein V3 (E) of Japanese encephalitis virus were obtained. They were characterized by hemagglutination inhibition (HI), neutralization, and enzyme-linked immunosorbent assay and divided into four types: flavivirus-cross-reactive HI and non-neutralizing antibody (group 1), subgroup-specific HI and non-neutralizing antibody (group 2), low HI and neutralizing antibody (group 3), and non-HI and neutralizing antibody (groups 4 and 5, respectively). Competitive binding assays were performed to analyze the topography of antigenic determinants by enzyme-linked immunosorbent assay. The results of the competitive binding assay separated non-HI and neutralizing antibody into groups 4 and 5, respectively, and demonstrated the existence of at least five distinct antigenic determinants on V3. The site of group 1 was distinct from any other site. The sites of groups 2 and 3 seemed to be located close together. Our results suggest the following relationship between HI and neutralization: (i) The HI sites are separated from the neutralization sites, and (ii) there are two distinct HI sites, one of which is flavivirus cross-reactive, the other subgroup specific.

Japanese encephalitis virus (JEV) is a flavivirus which belongs to the togaviruses and contains three structural proteins, V1 (M), V2 (C), and V3 (E) (8; T. Takegami, H. Miyamoto, H. Nakamura, and K. Yasui, Acta Virol., in press). It has been suggested that the major surface glycoprotein of flaviviruses, V3, contains at least three antigenic determinants, one of which is flavivirus cross-reactive, one which is complex (subgroup) specific, and one which is serotype specific (22, 23). These determinants seem to be correlated with the important biological properties hemagglutination (HA) and neutralization. In the preceding investigations, the biological functions of the JEV structural proteins were examined, and it was suggested that the envelope protein V3 is related to HA and neutralizing activity (8, 20; Takegami et al., in press). The relationships between antigenic determinants and biological activities have not been studied in detail with monospecific antiserum.

Monoclonal antibodies would be useful for studying the biological functions of the antigenic determinants of the structural proteins of viruses. In the case of flaviviruses, monoclonal antibodies against dengue virus (Den) and West Nile virus (WNV) have been reported recently (6, 16). However, details of the relationship between the antigenic determinant sites and the portions or domains on the structural protein V3 concerning biological activity remain to be elucidated with monoclonal antibody techniques.

In the present study, we isolated 10 monoclonal antibodies directed against V3 glycoprotein. Monoclonal antibodies were characterized by hemagglutination inhibition (HI), neutralization, and enzyme-linked immunosorbent assay (ELISA) against the homologous virus and other flaviviruses, West Nile virus (WNV) and Den type 2 (Den-2). The topography of the antigenic determinants reacting with monoclonal antibodies was also determined by using competitive binding assays. Based on these results, we were able to demonstrate the existence of at least five topographically distinct antigenic determinant regions on V3. HI and neutralizing activities of these antibodies were not parallel, in contrast with the monoclonal antibodies against Sindbis virus or Venezuelan equine encephalomyelitis virus, which are alphaviruses belonging to the togaviruses (3, 19). In this paper, we discuss the relationship between the five antigenic determinants and the biological functions HA and neutralization.

MATERIALS AND METHODS

Viruses. JEV strains JaGAr-01 and Nakayama-NIH were grown in suckling mouse brains or in C6/36

(*Aedes albopictus*) cells. C6/36 cells were obtained through the kindness of A. Igarashi, Nagasaki University, Nagasaki, Japan, and S. Inoue, National Institutes of Health of Japan, Tokyo. They were cultivated in Eagle minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and nonessential amino acids. Other flaviviruses, WNV strain Egypt 101 and Den-2 strain Tr. 1751, were kindly provided by A. Oya and H. Kobayashi, National Institutes of Health of Japan. The viruses were propagated in suckling mouse brains or in C6/36 cells. The viruses in culture fluids were purified by 60% Angio-conray (Daiichi Seiyaku Co., Ltd.)–10% sucrose discontinuous density gradient centrifugation. In the case of virus grown in suckling mouse brains, the virus band on 60% Angio-conray was further purified after discontinuous density gradient centrifugation by banding in isopycnic gradients of 60% Angio-conray–20% sucrose.

Preparation of spleen and myeloma cells for hybrids. BALB/c mice were primed by an intravenous injection of 400 PFU of JEV strain JaGAr-01. At 9 and 14 weeks after priming, the mice received an intraperitoneal inoculation of 8×10^3 HA units of purified JEV derived from suckling mouse brains. The mice were sacrificed 3 days after the last inoculation, and a suspension of immune spleen cells was prepared with RPMI 1640 medium supplemented with 3% heat-inactivated fetal bovine serum. Myeloma (NS-1) cells were obtained through the kindness of K. Yokomuro, Nippon Medical School, Tokyo, Japan, and cultivated in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum–1 mM pyruvic acid (growth medium).

Production of hybrid cell lines. Hybrid cell lines producing antibodies against JEV were prepared by the fusion of 10^8 NS-1 cells with 3×10^8 immune spleen cells in RPMI 1640 medium without serum by established fusion procedures (9, 24). Polyethylene glycol 2000 was used as the fusing agent. The hybrid cells were seeded in 96-well microplates (Falcon Plastics, Oxnard, Calif.) with HAT medium (complete growth medium supplemented with 1.0×10^{-4} M hypoxanthine–4.0×10^{-7} M aminopterin–1.6×10^{-5} M thymidine). After 10 to 14 days, the hybrid cells were tested for anti-JEV antibodies by ELISA and HI tests as described below. Hybrids that produced anti-JEV antibodies were cloned at least twice more by the limiting dilution method. The resulting monoclonal hybrid cells were maintained in growth medium. Monoclonal antibodies were concentrated 20 times from the culture fluids by precipitation with $(NH_4)_2SO_4$ at 50% saturation. A high concentration of monoclonal antibodies was obtained from the ascitic fluid of BALB/c nu/nu mice inoculated intraperitoneally with approximately 10^7 hybrid cells.

ELISA. Screening and quantitation of anti-JEV antibodies were performed by ELISA. Purified virus from the culture fluids of C6/36 cells was used as antigen. The protein concentration of the virus solution was determined by the method of Lowry et al. (11). After dilution in 0.1 M carbonate buffer at pH 9.6 to a concentration of 1 μg of protein per ml, the antigen was adsorbed to the individual wells of a plastic Immulon U-microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). Fifty microliters of the antigen solution was dispensed into each well, and the plate was incubated overnight at 4°C. The plate was washed four times with 0.1% Tween 40 in phosphate-buffered saline (PBS), pH 7.2. The following ELISA was performed in three steps. (i) Fifty microliters of culture fluid or ascitic fluid, diluted in PBS–Tween 40 containing 10% heat-inactivated newborn calf serum, was incubated in each of the virus-adsorbed wells for 2 h at room temperature. Nonbound immunoglobulins were then removed from the wells by washing four times with PBS–Tween 40. (ii) Goat peroxidase-conjugated anti-mouse gamma globulin fraction (E. Y. Laboratories, San Mateo, Calif.) in 50 μl of PBS–Tween 40 containing 10% heat-inactivated newborn calf serum was added to each well, and the plate was held at room temperature for 2 h. The wells were then washed as described above. (iii) A substrate solution containing 0.006% H_2O_2 and 0.4 mg of *o*-phenylenediamine per ml in 0.1 M citrate buffer (pH 5.0) was added at 100 μl per well, and the plate was incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 50 μl of 2 N H_2SO_4 to each well, and the optical density at 490 nm (OD_{490}) was observed with a spectrophotometer. The ELISA endpoint titer was estimated from the dose-response curve by serial dilutions of monoclonal antibodies (Fig. 1). Hybridoma growth medium concentrated by $(NH_4)_2SO_4$ precipitation was used as a control, and its OD_{490} was always less than 0.05. The ELISA endpoint titer was expressed as the maximum dilution of monoclonal antibody that showed an OD_{490} of at least 0.1. The titer of normal mouse serum was 10 to 20 U because of its nonspecific binding.

Serological test. Antibodies were treated with cold acetone to remove nonspecific inhibition before HI assay. HI was assayed by the method of Clark and

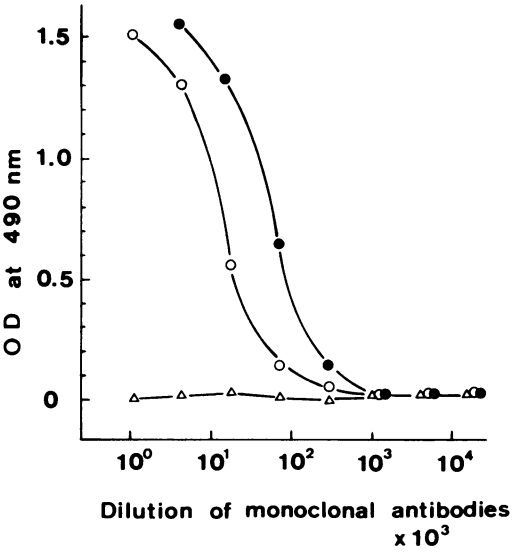

FIG. 1. ELISA titration curves of monoclonal antibodies. See text for detailed description. ELISA endpoint titers are expressed as the maximum dilution of antibody showing an OD_{490} of at least 0.1; the titers of clones 110 and 112 from culture fluids are about 1×10^5 and 5×10^5 units, respectively. Symbols: ○, clone 110; ●, clone 112; △, control (growth medium).

Cassals (4), using 8 HA units of antigen. HA antigens of other togaviruses were obtained from A. Oya, H. Kobayashi, and S. Inoue, National Institutes of Health of Japan.

Ascitic fluid was heat-inactivated at 56°C before neutralization assays. A plaque reduction neutralization test (PRNT) of the antiviral monoclonal antibodies was performed by the Vero cell plaque assay. Vero cells were cultured in Eagle MEM containing 5% fetal bovine serum. Monoclonal antibodies were serially diluted in Eagle MEM containing 20% Haemaccell (Hoechst-Roussell Pharmaceuticals Inc., Somerville, N.J.) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid [pH 7.2]), and each dilution was incubated with an equal volume of virus solution containing about 50 to 70 PFU at 37°C for 1 h. Infectivity remaining in the samples was then assayed on a confluent Vero cell monolayer overlaid with Eagle MEM containing 5% gamma globulin-free fetal bovine serum and 0.6% methylcellulose on 24-well plastic trays (NUNC Laboratories, Roskilde, Denmark). After 2 days of incubation at 37°C, monolayers were treated with rabbit anti-JEV hyperimmune antibody (500 HI U) and guinea pig complement solution in Eagle MEM buffered with 25 mM HEPES (pH 7.2). Rabbit anti-JEV hyperimmune serum was obtained by immunization with the purified JEV JaGAr-01 strain, followed by one or two booster injections. Guinea pig serum at a dilution of 1/10 served as the complement source. After 2 h, the cells were stained with 0.3% trypan blue in PBS. The blue-colored plaques of virus-infected cells were counted. The antibody titer was expressed as the maximum dilution of ascitic or culture fluid that yielded a 50% reduction in the infectivity of the virus inoculum.

Anti-mouse IgG serum. Anti-mouse immunoglobulin G (IgG) serum was obtained from rabbits injected with purified mouse IgG fraction. Its activity was 1:16 by the Ouchterlony gel diffusion test against mouse IgG.

Isotype characterization of monoclonal antibodies. The immunoglobulin class and subclass were determined by the Ouchterlony gel diffusion test against antisera specific for each immunoglobulin isotype obtained from Meloy Laboratories Inc., Springfield, Va.; Serotec Japan Co., Sapporo, Japan; and Cappel Laboratories, Downingtown, Pa. These results were confirmed by a competitive binding assay, a modification of the ELISA described above. Monoclonal antibodies at a constant concentration (approximately 10^3 ELISA units) were adsorbed to the JEV-coated wells of microtiter plates. Each class-specific antiserum was used to compete with the binding of peroxidase-conjugated anti-mouse gamma globulin. Isotypes of the monoclonal antibodies were determined by the reduction in OD_{490} compared with the value for an unblocked control.

Radioimmunoprecipitation of the JEV structural protein by monoclonal antibodies. JEV strain JaGAr-01 labeled with [^{3}H]leucine was prepared from infected Vero cells and purified by density gradient centrifugation (Takegami et al., in press.). The purified virus was incubated in 1% Nonidet P-40–0.5% sodium deoxycholate–0.01% sodium dodecyl sulfate (SDS) for 30 min at room temperature. After centrifugation for 30 min at 15,000 rpm, the supernatants containing [^{3}H]leucine-labeled JEV structural proteins were mixed with each sample of monoclonal antibodies in ascitic fluid and allowed to stand overnight at 4°C. The resultant antibody-antigen complexes were precipitated by adding Formalin-killed cells of *Staphylococcus aureus* Cowan 1. After incubation overnight at 4°C, the washed bacterium-antibody-antigen complexes were resuspended in 0.5% SDS to elute immunoprecipitated antigens from the bacteria. After centrifugation, the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Peroxidase-labeled monoclonal antibody. Peroxidase-labeled monoclonal antibodies were prepared by the method of Nakane and Kawaoi (15). Briefly, monoclonal antibodies from ascitic fluid were purified by precipitation three times with $(NH_4)_2SO_4$ at 35% saturation. Then, 10 mg of monoclonal antibody was mixed with 4 mg of horseradish peroxidase (HRPO), oxidized with 0.1 M sodium *m*-periodate ($NaIO_4$) in 0.01 M carbonate buffer (pH 9.6), and incubated for 2 h at room temperature. After addition of sodium borohydride solution ($NaBH_4$, 4 mg) and standing at 4°C for 2 h, the mixture was dialyzed against 0.02 M PBS (pH 7.2). HRPO-conjugated antibody was separated from HRPO and from antibody that had not formed antibody-enzyme complexes by passing the mixture through a Sephadex G-200 column (1.5 by 38 cm).

Competitive binding assay. The competitive binding assay was performed by a modified ELISA. The purified virus at the limiting concentration was used as antigen. Serial dilutions of each competing antibody from culture fluid were added to the JEV-adsorbed wells in microplates. Growth medium concentrated by $(NH_4)_2SO_4$ precipitation was used as control. After standing for 2 h at room temperature, the wells were washed as previously described, and HRPO-labeled monoclonal antibody, which typically showed an OD_{490} of 1.0, was added. The amount of competitive binding was estimated from the absorbance at 490 nm in the presence or absence of unlabeled competing antibodies. The percentage of competition was determined by the formula $[100(A - n)]/(A - B)$, where A is OD in the absence of competing antibody, B is OD in the presence of homologous antibody (10^4 ELISA units), and n is OD in the presence of competitor.

RESULTS

Formation of antibody-producing hybridomas. At 10 to 14 days after fusion, culture fluids from 106 of 229 wells of microplates showed positive activity by ELISA (46%), and those from 34 of the 229 wells gave positive HI with a titer of >1:24 (15%). Hybrid cells in the ELISA-positive wells were cloned two to five times in 96-well microtiter plates at the limiting dilution. All antibodies used in this study were derived from the same fusion with BALB/c mouse spleen cells, except for clone 23, which was derived from the fusion of NS-1 cells with immunized C57BL/6 mouse spleen cells.

JEV polypeptide specificity of monoclonal antibodies. The specificity of the monoclonal antibodies was determined by immune precipitation of [^{3}H]leucine-labeled purified virus preparations. To precipitate the immune complexes, *S. aureus* Cowan 1 was used. Purified virions con-

tained V1, V2, NV2, and V3 proteins as determined by SDS-PAGE (Fig. 2A). Only V3 was precipitated by the monoclonal antibodies of clones 23, 112, and 303 (Fig. 2B). The specificity of the other monoclonal antibodies was determined in this way, and of more than 20 clones tested, only 10 stable clones directed against the V3 virion surface glycoprotein were used.

Characterization of monoclonal antibodies. The immunoglobulin class of the monoclonal antibodies from culture fluids was determined with anti-mouse immunoglobulin class-specific serum. All of the monoclonal antibodies were IgG (IgG2a, IgG2b, or IgG3) (Table 1).

Table 1 shows the characterization of 10 monoclonal antibodies directed against V3. Each monoclonal antibody was assayed by ELISA, HI, and PRNT. All of the clones had high ELISA titers against JEV.

The antibodies of clones 110, 111, 301, and 23 (group 1) had high HI titers against JEV but did not show any neutralizing activity. They also showed no neutralizing activity in the chicken embryo cell plaque assay (data not shown). When we used highly concentrated antibodies from ascitic fluid, they sometimes showed a trace of neutralizing activity; for example, clone 301 showed a PRNT titer of 50 per 2.7×10^7 ELISA units. These HI antibodies showed high cross-reactivity with WNV and Den-2 by ELISA and HI. ELISA and HI titers of these cross-reactive monoclonal antibodies against WNV were similar to those of antibodies against JEV, whereas slightly lower titers were exhibited against Den-2. HI cross-reactivity of the clone 23 antibody against flaviviruses and alphaviruses was examined in detail. The clone 23 antibody cross-reacted with all flaviviruses examined but did not react with the two kinds of alphaviruses, Sindbis virus and Semliki Forest virus (Table 2). It is clear from the above results that these antibodies are flavivirus cross-reactive HI antibodies.

Clone 109 antibody (group 2) also had high HI titers and no neutralizing activity against JEV. This antibody reacted significantly with WNV, which is of the same subgroup of flaviviruses as JEV, but their titers were low compared with those against JEV in ELISA and HI tests. The differences in the titers between JEV and WNV were about 4-fold by ELISA and 10^2-fold by HI. Antibody 109 was therefore assigned to group 2 as a subgroup-specific HI antibody.

The antibodies of clones 112, 203, 302, 303, and 204 (groups 3, 4, and 5) neutralized the infectivity of JEV and showed high neutralization titers. All of these clones showed reaction in various degrees with WNV but not with Den-2 by ELISA. These neutralizing antibodies were subdivided into three groups as follows.

Only group 3 antibody (clone 112) had low HI titers against JEV and showed very low affinity for WNV. The clone 112 antibody showed 4×10^3-fold higher affinity for JEV than for WNV by ELISA and did not show any HI activity against WNV.

Both group 4 (clones 203, 302, and 303) and group 5 (clone 204) antibodies neutralized JEV but gave no HI reaction. ELISA titers of group 4 antibodies against WNV were also lower than

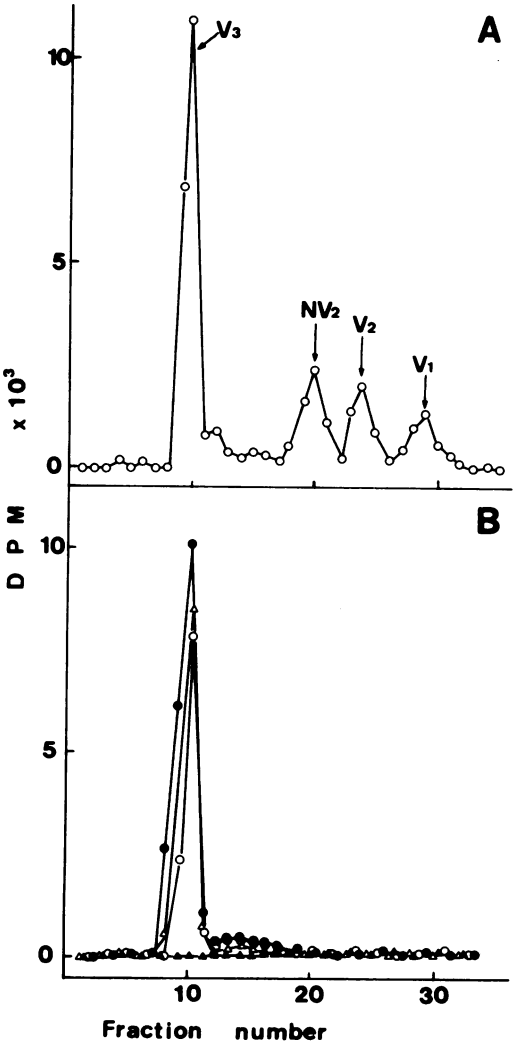

FIG. 2. JEV polypeptide specificity of monoclonal antibodies. Immunoprecipitation was performed with [^{3}H]leucine-labeled virions. (A) [^{3}H]leucine-labeled virions were dissociated and analyzed by SDS-PAGE as described in the text. The arrows show V3, V2, NV2, and V1 proteins of JEV. (B) [^{3}H]leucine-labeled virions were dissociated, immunoprecipitated with each monoclonal antibody from ascitic fluid and *S. aureus*, and analyzed by SDS-PAGE. Symbols: ○, clone 23; ●, clone 112; △, clone 303; ▲, normal mouse serum.

TABLE 1. Characterization of JEV monoclonal antibodies[a]

Group	Clone no.	IgG class	Titer as determined by: ELISA JEV	ELISA WNV	ELISA Den-2	HI JEV	HI WNV	HI Den-2	JEV PRNT titer per 10^6 ELISA units
1	110	3	1.3×10^6	2.6×10^6	4.0×10^4	2.0×10^4	1.0×10^4	2.5×10^3	ND[b]
	111	2a	6.8×10^6	3.5×10^6	6.4×10^5	5.7×10^4	5.7×10^4	1.0×10^4	<50
	301	2a	2.7×10^7	1.4×10^7	2.6×10^6	1.6×10^5	1.1×10^5	7.1×10^3	<50
	23	2	2.4×10^6	2.4×10^6	2.6×10^5	8.0×10^4	4.0×10^4	2.8×10^4	<50
2	109	2a	1.7×10^6	4.3×10^5	<20	2.0×10^4	2.3×10^2	<10	<50
3	112	2a	2.0×10^7	5.0×10^3	<20	9.0×10^2	<10	<10	4.3×10^2
4	203	2a	1.2×10^5	1.9×10^3	<20	<10	<10	<10	1.0×10^3
	302	2b	2.0×10^7	9.8×10^6	<20	<10	<10	<10	3.1×10^2
	303	2a	2.0×10^7	5.0×10^6	<20	<10	<10	<10	3.5×10^2
5	204	2a	4.0×10^5	4.0×10^5	<20	<10	ND	ND	1.5×10^2

[a] Monoclonal antibodies were obtained from culture fluids by precipitation with $(NH_4)_2SO_4$ at 50% saturation or from ascites fluid of a BALB/c nu/nu mouse injected intraperitonealy with hybrid cells. The strain of JEV used was JaGAr-01.

[b] ND, Not done.

those against JEV, especially in the case of clone 203. The clone 204 antibody (group 5) showed similar ELISA titers against both JEV and WNV. Group 4 and 5 antibodies did not react with Den-2. There were no significant differences in antibody characteristics between groups 4 and 5, although distinct topographical differences in antigenic sites were observed by the competitive binding assay, as described below.

The antibodies of groups 3, 4, and 5, which could neutralize JEV, showed significant ELISA titers against WNV, so WNV was also assayed by ELISA. When we used highly concentrated monoclonal antibodies from ascitic fluid, clones 112, 302, and 303 showed low neutralization titers against WNV. The neutralization titers (PRNT titers) per ELISA endpoint titers were as follows: clone 112, 80 per 2.7×10^7 ELISA units; clone 302, 40 per 2.0×10^7 ELISA units; clone 303, 40 per 2.0×10^7 ELISA units.

Effects of anti-mouse IgG on neutralizing activity of monoclonal antibody. We examined the effects of adding anti-mouse IgG on the neutralizing activity of each monoclonal antibody by a modification of PRNT. After 1 h of incubation of the antibody-virus mixtures at 37°C, rabbit anti-mouse IgG serum at a dilution of 1/10 was added to them. The resultant mixtures were incubated for 1 h at 37°C and inoculated to Vero cells. Then, all of the monoclonal antibodies from culture fluids neutralized JEV completely. Group 1 and group 2 antibodies, which had no neutralizing activity themselves, showed high neutralization titers of 5×10^3 or more per 10^5 ELISA endpoint titers when the antibody-virus mixtures were treated with anti-mouse IgG serum. The neutralizing activity of groups 3, 4, and 5 antibodies were enhanced extremely by the addition of anti-mouse IgG serum. The neutralization titers of these antibodies were 2×10^4 or more per 10^5 ELISA endpoint titers. The results indicate that all of the monoclonal antibodies can bind an infectious virion surface.

Strain differences of monoclonal antibodies. Many strains of JEV have been isolated and divided into several antigenic types. We compared the characteristics of all monoclonal antibodies against two typical strains, JaGAr-01 and Nakayama-NIH, by ELISA, HI, and PRNT.

TABLE 2. HI cross-reactivity of clone 23 against other togaviruses

Virus antigens	Antigen treatment[a]	HI titer
JEV JaGAr-01	AEFD	1.0×10^4
JEV Nakayama-NIH	AEFD	5.1×10^3
WNV Eg 101	AEFD	1.0×10^4
Den-1	SAFD	3.6×10^3
Den-2 Tr 1751	NT	2.6×10^3
Den-3	SAFD	3.6×10^3
Den-4	SAFD	5.1×10^3
Yellow fever 17D	AEFD	2.6×10^3
Sindbis	NT	<20
Semliki Forest	SAPFD	≤20

[a] Antigens were treated as follows: freeze-dried after treatment with acetone and ether (AEFD); freeze-dried after treatment with sucrose and acetone (SAFD); freeze-dried after treatment with sucrose, acetone and protamine (SAPFD); or not treated (NT) (whole virion).

There were no significant differences between JaGAr-01 and Nakayama-NIH, except in the case of the clone 112 antibody. This antibody had similar ELISA and PRNT titers against both strains, but the HI titer against strain Nakayama-NIH (1:14) was significantly lower than that against strain JaGAr-01 (1:900). The results show that neutralizing activity of the antibody does not parallel the HI activity of strains JaGAr-01 and Nakayama-NIH.

Neutralizing activities of monoclonal antibody mixtures. Neutralizing activities of mixtures of the different groups of monoclonal antibodies are shown in Table 3. Mixtures of antibodies within group 1 (clones 301, 110, 111, and 23), none of the clones of which neutralized JEV but all of which had similar HI activities, showed no neutralizing activity. However, when we mixed clones 301 (group 1) and 109 (group 2), which had different types of HI cross-reactivity but no neutralizing activity themselves, the mixture did show a considerable neutralizing activity (Table 3). The neutralizing activities of the mixtures of neutralizing antibodies (clone 112 or 303) and non-neutralizing antibodies (group 1 or 2 or both) were increased approximately 3- to 10-fold higher than that expected by simple dilution of the activity of the neutralizing antibody (shown in parentheses in Table 3).

Topographical analysis of antigenic determinants of V3 protein. Competitive binding assays were used to analyze the topography of the epitopes to which the monoclonal antibodies reacted. The competitive binding assay was developed on the premise that if two epitopes are close to each other, the binding of antibody to one of the epitopes will sterically hinder the binding of antibody to the other epitope. Complete inhibition of antibody binding would be caused by another antibody, which defined the same antigenic determinant. Competition of antibody binding can also be caused by another mechanism. Binding of an antibody may allosterically alter a second antigenic site, which causes inhibition or enhancement of the binding of the antibody to the second antigenic site. Because of this, a topographical map based on competitive binding analyses would be expected to overestimate the physical overlap between individual antigenic sites. It was further assumed that, if the viral proteins are univalent for each of the antigen epitopes, a single antibody molecule would bind and project from the surface of each of the viral proteins. Variation in antibody avidity would also affect competitive binding. Therefore, competing monoclonal antibodies were used at a limiting concentration (10 to 10^4 ELISA units per well). With careful consideration of these factors, it is possible to estimate the topographical maps of antigenic determinants (1, 12, 14, 17, 19, 21).

TABLE 3. Mixture of monoclonal antibodies and their neutralizing activities[a]

Group of mixed monoclonal antibodies				PRNT titer[b]
1	2	3	4	
301, 110, 111, 23				<20
301	109			1.2×10^2 (<20)
301		112		>4.8×10^2 (1.5×10^2)
301	109,	112		4.8×10^2 (1.0×10^2)
301	109,	112,	303	1.3×10^3 (1.2×10^2)

[a] JEV JaGAr-01 strain was used as target.

[b] Neutralization titers of each clone were as follows: clones 301, 110, 111, 23, and 109, <20; clone 112, 3.0×10^2; clone 303, 1.8×10^2. Titers expected from simple dilutions of neutralizing antibodies are shown in parentheses.

To analyze epitopes of V3 protein, a competitive binding assay was performed with five types of HRPO-labeled monoclonal antibodies: clone 23 (group 1), clone 109 (group 2), clone 112 (group 3), clone 303 (group 4), and clone 204 (group 5) (Fig. 3). Ten monoclonal antibodies were tested for competition with the binding of each HRPO-labeled antibody (Table 4).

All group 1, i.e., flavivirus-cross-reactive HI but non-neutralizing, monoclonal antibodies completely inhibited the binding of HRPO-23. However, none of the antibodies from the other groups did so. Even clone 109 antibody, which had high HI activity but a different cross-reactive HI pattern, did not inhibit the binding of HRPO-23.

HRPO-109 was blocked completely by homologous and group 3 antibodies (clone 112) and partially by group 4 antibodies (27 to 58%). In contrast, group 1 and group 5 antibodies did not block HRPO-109.

HRPO-112 was completely blocked only by homologous antibody. Partial competition by group 2 antibodies (57%) was higher than that by group 4 antibodies (30 to 38%).

HRPO-303 was blocked completely by clones 203, 302, and 303. Group 4 antibodies showed reciprocal partial competition with groups 1, 2, and 3 antibodies. When the ELISA plates were incubated for 5 to 6 h at room temperature after the addition of HRPO-labeled monoclonal antibody, partial competition between antibodies from group 4 and those from the other goups decreased until, finally, no competition was observed.

Clone 204, but none of the other antibodies, completely blocked the binding of HRPO-204. Further, clone 204 did not block any type of HRPO-labeled antibodies in these experiments. Based on these results, we divided non-HI,

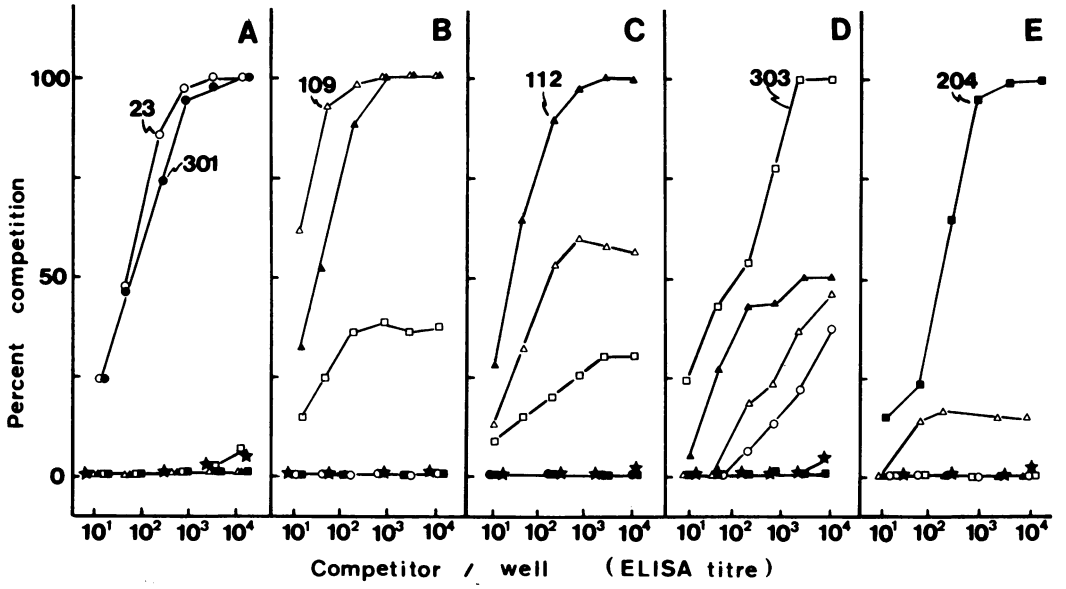

FIG. 3. Competitive binding assay with HRPO-labeled monoclonal antibodies. JEV-adsorbed wells of microplates were treated with unlabeled monoclonal antibodies as competitor, and then HRPO-labeled monoclonal antibodies were added. (A) HRPO-23; (B) HRPO-109; (C) HRPO-112; (D) HRPO-303; (E) HRPO-204. The percentage of competition was estimated from OD_{490} in the presence of competitor compared with that in the absence of competitor as described in the text. Symbols: ○, clone 23; ●, clone 301; △, clone 109; ▲, clone 112; □, clone 303; ■, clone 204; ★, control (growth medium).

neutralizing antibodies (clone 203, 302, 303, and 204) into groups 4 and 5.

These results were confirmed by tests in which a constant amount of competing monoclonal antibodies and various concentrations of HRPO-labeled monoclonal antibodies were used, and the results were not affected by the use of another virus strain, Nakayama-NIH, as antigen (data not shown).

DISCUSSION

Ten monoclonal antibodies directed against the V3 protein of JEV strain JaGAr-01 were isolated. The monoclonal antibodies were characterized by HI, PRNT, and ELISA (Table 1) and divided into four types: group 1 (clones 110, 111, 301, 23), flavivirus-cross-reactive HI and non-neutralizing antibodies; group 2 (clone 109), subgroup-specific HI and non-neutralizing antibody; group 3 (clone 112), low HI and high neutralizing antibodies; group 4 (clones 203, 302, 303) and group 5 (clone 204), non-HI and neutralizing antibodies, respectively.

Competitive binding assays suggest the presence of at least five antigenic determinants on the V3 protein of JEV. Non-HI, neutralizing antibodies were separated into two groups (groups 4 and 5) on the basis of the results shown in Table 4. In some cases, partial competition was observed. Partial competition may be due to a low degree of structural overlap of determinants or a conformational change by the binding of competing antibody and is difficult to explain by suggesting heterogeneity of antigen preparation, because all monoclonal antibodies of any

TABLE 4. Results of competitive binding assay

Competitor[a]	% Inhibition of binding with peroxidase-conjugated clone[b]				
	23	109	112	303	204
Group 1					
301	**100**	9	0	37	14
110	**100**	11	0	45	0
111	**100**	2	0	27	3
23	**100**	0	0	38	0
Group 2, 109	0	**100**	57	46	15
Group 3, 112	0	100	**100**	49	0
Group 4					
203	2	27	30	**89**	0
302	12	58	38	**97**	5
303	5	45	30	**100**	0
Group 5, 204	0	0	0	0	**100**

[a] Competitors are used at the limit concentration of 10^4 ELISA units.

[b] Numbers in boldface indicate percent inhibition between homogeneous antibodies or antibodies from the same group.

group neutralized JEV completely by the addition of anti-mouse IgG serum. This observation indicates that all monoclonal antibodies react with a single type of infectious particle and is confirmed by the fact that enhancement of neutralization occurred by mixing antibodies from different groups. Furthermore, these results suggest that monoclonal antibodies from each group can react with different antigenic sites on the same V3 protein. Partial competition between group 4 antibodies and group 1, 2, or 3 antibodies was decreased by longer incubation with HRPO-labeled monoclonal antibodies, suggesting that the site of group 4 may be slightly related to the sites of groups 1, 2, and 3. Considerable reciprocal competition was observed between groups 2 and 3 antibodies. This partial competition might be caused by differences in affinity of each monoclonal antibody. In this case, complete or partial competition was not affected by longer incubation with HRPO-labled clone 109 or 112. Another possible explanation for this result is that HA spikes of JEV may be a polymeric form of V3 protein. In any case, this result suggests that the binding sites of group 2 or 3 antibodies might be overlapped or adjacent to each other on the V3 protein, even though their serological characteristics are quite different from each other.

These topographical analyses provide two new indications about the relationship between antigenic determinants and the biological functions HA and neutralization. First, the sites of HI and neutralization on the V3 protein of JEV seem to be separated from each other. Group 1 cross-reactive HI antibodies did not neutralize JEV by themselves, and their binding site is expected to be located at a position distant from those of any other neutralizing antibodies. Group 2 subgroup-specific HI antibody also had no neutralizing activity by itself, although its site seems to be adjacent to that of group 3 neutralizing antibody, which have a low HI titer, because the antibodies of groups 2 and 3 showed considerable reciprocal competition. Both cases suggest separation of the HI site from the neutralizing site. It has been reported that the neutralization test for flaviviruses separates one flavivirus from another, but the HI test shows high cross-reactivity (2, 18). These reports agree with our observations. On the other hand, it has been reported that some monoclonal antibodies against Sindbis virus or Venezuelan equine encephalomyelitis virus possess both HI and neutralizing activities (3, 19).

Second, the flavivirus-cross-reactive HI site (group 1) seems to be distinct from the subgroup-specific HI site (group 2). Both group 1 and group 2 antibodies had high HI titers against JEV (Table 1), but their cross-reactivity with other flaviviruses showed distinct differences, and they did not compete at all with each other in the competitive binding assay. Thus, we can demonstrate the presence of at least two sites that inhibit HA. It is interesting to note that only the subgroup-specific HI site (group 2) seems to be adjacent to the binding site of neutralizing antibodies (group 3). It is not likely that there are two receptors for HA, because either group 1 or group 2 antibodies completely inhibit HA. It has been reported that the binding site of erythrocytes is flavivirus cross-reactive (25), and this suggests that it seems to be the binding site of group 1. Therefore, group 2 antibody might induce an allosteric conformational change and result in HI. A more precise analysis of topographical mapping of antigenic determinants on V3 protein will require the interpretation of data obtained by a variety of experimental approaches, for example, those using single-point mutants of JEV. This approach is in progress now in our laboratory.

To understand the mechanisms of neutralization, further experiments were performed with respect to inhibitory activities of monoclonal antibodies on virus-mediated hemolysis and cell fusion (data not shown). Our preliminary experiments showed that group 3 antibodies had high inhibitory activity against hemolysis and cell fusion caused by JEV, and group 1 had no such activity. The domain or region of V3 where the group 3 antibodies bind may be important for infection of JEV. We recently obtained a monoclonal antibody against JEV Nakayama-NIH which bound only to JEV and had high neutralizing activity. The competitive binding assay showed that this antibody might bind to the same domain of V3 to which clone 112 binds.

Groups 2, 3, 4, and 5 antibodies showed significant affinity for WNV, but this affinity did not always parallel their biological functions, HI and neutralization, in regard to WNV, suggesting that similar antigenic determinants may play different roles in WNV or even that the antibodies that bind to WNV may not effectively inhibit the biological activities of WNV. Similar evidence has been reported in the case of rabies virus (10) and influenza virus (7).

Mixtures of monoclonal antibodies potentiated the neutralizing activity (Table 3). Similar evidence has been obtained by using monoclonal antibodies against WNV (16) and Sindbis virus (3). Lubeck and Gerhard reported that the binding of an antibody induces conformational changes in the HA molecule of influenza virus which result in the increased avidity of other antibody-HA interactions (13). Similar effects might cause these effects of mixed antibodies. In fact, enhancement of binding of group 2 or 3 antibodies caused by the binding of group 1

antibodies, and vice versa, was observed when HRPO-labeled monoclonal antibodies were used at a low concentration. These effects of the mixtures may be reflected in cross-neutralization. Mixtures of several types of anti-JEV monoclonal antibodies neutralized WNV, and their titers were higher than the expected titers (data not shown). It has been reported that significant cross-neutralization occurs among the same subgroup of flaviviruses (5). The in vivo role of these mixing effects is interesting and requires further investigation.

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