TABLE 1. Characterization of D2MAbs and D4MAbs[a]

Hybridoma clone group	Clone code	Isotype	Neutralizing Ab titer[b]	HAI titer[c]	ELISA reactivity[d]				No. of clones[e]
					1	2	3	4	
D2-1	D2-II-1B3	IgG2a	1:11,449	1:2,560	+	+	−	+	2
D2-2	D2-II-6D6	IgG2a	1:1,810	<1:10	+	+	−	+	1
D2-3	D2-II-15A4	IgG2a	1:1,280	1:320	−	+	−	+	1
D2-4	D2-III-1B6	IgG2a	1:90,510	1:10	−	+	−	+	1
D2-5	D2-III-13F2	IgG2a	1:10,119	1:1,280	+	+	−	+	2
D2-6	D2-II-13A3	IgG2b	1:160	1:20	−	+	+	+	1
D2-7	D2-V-10F1	IgG2a	1:3,620	1:640	+	+	−	+	1
D2-8	D2-IX-5B10	IgG2a	1:7,241	1:10	−	+	−	−	1
D2-9	D2-II-9A7	IgG2b	1:28	<1:10	−	+	−	−	1
D2-10	D2-II-11H4	IgG2a	1:40	1:20	−	+	+	+	4
D2-11	D2-II-11D1	IgG1	1:40	<1:10	−	+	+	+	3
D2-12	D2-II-11E1	IgG1	<1:10	<1:10	−	+	+	+	6
D2-13	D2-VI-4A1	IgG1	<1:10	<1:10	−	+	−	−	9
D4-1	D4-I-1D6	IgG2a	1:40	1:160	−	+	−	+	1
D4-2	D4-II-12B2	IgG2a	1:2,024	1:10	−	−	−	+	3
D4-3	D4-IV-10E10	IgG2a	1:160	1:80	−	−	−	+	3
D4-4	D4-IV-10E5	IgG2a	1:28	1:20	−	+	+	+	3
D4-5	D4-III-10E10	IgG2b	1:113	1:10	−	−	−	+	1
D4-6	D4-I-11D11	IgG2a	1:5,634	<1:10	−	−	−	+	3
D4-7	D4-III-1A12	IgG1	1:113	<1:10	+	+	+	+	2
D4-8	D4-IV-3G12	IgG2a	1:505	<1:10	+	+	−	+	1
D4-9	D4-I-14F1	IgG2a	<1:10	1:160	−	−	−	+	3
D4-10	D4-III-2F1	IgG2a	<1:10	1:10	+	+	+	+	4
D4-11	D4-IV-9D4	IgG1	<1:10	<1:10	−	−	−	+	7
D4-12	D4-I-9B4	IgG2a	<1:10	<1:10	−	−	−	+	5
D4-13	D4-III-5C8	IgG1	<1:10	<1:10	−	+	−	+	7

[a] All MAbs were used in ascitic form.
[b] Neutralizing Ab titer obtained by a 50% focus reduction assay against the homologous virus type in the absence of complement. Geometric means of the results obtained in two separate experiments are shown.
[c] HAI antibody titer against the homologous virus type.
[d] ELISA reactivities to dengue virus antigens. Numbers 1 to 4 correspond to DENV1 to DENV4.
[e] The number of clones generated in each group.

HAI test. HAI tests were performed by using a microplate modification of the method of Clarke and Casals (5).

Titration of viral infectivity and neutralization test. Infective titers were determined on Vero cells by counting infectious foci after immunostaining (see below) and expressed as focus-forming units (FFU). Neutralizing antibody titers were determined by using focus reduction assays performed with DENV2 or DENV4 essentially as described previously (27). Briefly, the virus-antibody mixture was incubated with rabbit complement at a final concentration of 5%. In some experiments, the neutralization test was performed without complement. The neutralizing activities were expressed as percentages of focus reduction or neutralizing antibody titers. The percentage of focus reduction was calculated relative to the results for virus controls without test samples. The neutralizing antibody titer was expressed as the maximum sample dilution yielding a 50% reduction in focus number, unless otherwise specified.

Immunostaining. Immunochemical staining was performed essentially as described previously (26). Briefly, cells infected with DENV2 or DENV4 were fixed with acetone-methanol (1:1) for use as antigens in the immunostaining. These cells were incubated serially with MAbs to DENV2 or DENV4, biotinylated anti-mouse IgG, ABC (avidin-biotinylated peroxidase complex) reagents, and VIP substrate (Vector Laboratories, Burlingame, CA). This method was used for the screening of MAbs, titration of viral infectivity, and ADE assays. The MAbs used for the virus titration and ADE assays were D2-4G2 (E specific, flavivirus group cross-reactive [19]; provided by Tomohiko Takasaki of NIID, Japan) and D2-II-11H4 (E specific, reactive with both DENV2 and DENV4), which was generated from a DENV2-immune mouse in the present study (refer to Table 1).

ADE assay. Three assay methods, based on the infection rate, yield, and number of infectious centers, were used to evaluate the enhancing activities of MAbs.

(i) Infection rate assay. Based on the method described by Wu et al. (54), enhancing activity was assessed by the percentage of infected cells 4 to 6 days after cells were infected with virus in the presence of antibody. U937 or K562 cells, 1×10^5 in number, were suspended in 50 μl of MAb diluted in RPMI–10% FBS and immediately mixed with 150 μl of DENV2 or DENV4 containing 1×10^5 FFU. The IgG concentrations of MAbs included in the virus-antibody-cell mixture ranged from 10^0 to 10^6 ng/ml, and concentrations that showed the highest enhancing activities were mainly used. Following incubation at 37°C for 2 h, cells were washed three times and cultivated at 37°C for 4 to 6 days in RPMI–10% FBS including the MAb identical to that used in the virus-antibody-cell mixture at the same concentration. Then, cells were washed, fixed on a slide glass, and immunostained. Stained and unstained cells contained in three random microscopic fields were counted to calculate percentages of infected cells: the mean number of the total cell counts per field was approximately 600. As a negative control, ascitic fluids from mice inoculated with P3U1 cells were used. The borderline differentiating enhancing from nonenhancing activities was the average plus two times the SD of the percentages of infected cells obtained with six negative controls. In experiments where rabbit complement, fresh human serum, or C1q/C3-depleted human serum was added to the virus-antibody-cell mixture, complement or serum was added to the cell-antibody mixture before being mixed with the virus. To make up the final volume of the virus-antibody-cell mixture to 200 μl, the volume of the virus was adjusted. In experiments to prepare the cell-antibody mixture under dense serum conditions, the volumes of both the MAb and virus were adjusted to achieve the final concentration of the serum at a maximum of 80%. Rabbit complement or fresh human serum was not included in RPMI–10% FBS during cultivation for 4 to 6 days because of the rapid inactivation of the complement at 37°C and the limited amount of human sera available for this experiment.

(ii) Yield assay. U937 cells mixed with MAb and virus were incubated, washed, and cultivated as described above. Following the method described by Halstead and O'Rourke (17), the enhancing activity was evaluated by the infective titer contained in the culture fluid.

(iii) Infectious center assay. U937 cells were used for this assay. Based on the method described by Halstead and O'Rourke (18), the virus-antibody-cell mixture prepared as described above for the infection rate assay was serially diluted twofold and incubated at 37°C for 2 h. The mixture was then mixed with 2.5×10^5 Vero cells in wells of a 24-well microplate and further incubated at 37°C for 3 h, allowing cells to attach onto the bottoms of wells. An overlay medium (1%

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methyl cellulose in MEM) replaced the culture fluid, and the cells were cultivated at 37°C for 3 to 4 days. The cells were then fixed and immunostained to count the foci. The number of infectious centers was expressed as the number of foci included in 1×10^5 cells used for the virus-antibody-cell mixture.

Complement, complement component, and measurement of complement levels. Low-Tox-M rabbit complement (designated "rabbit complement") (Cedarlane, Hornby, Canada) was used as a source of complement. Complement C1q and C3 components were purchased from Merck, Darmstadt, Germany. Complement hemolytic activities were measured using sheep red blood cells treated with antibodies to sheep red blood cells in a CH50 "SEIKEN" kit (Denka Seiken, Niigata, Japan) according to the manufacturer's instructions. Absorbances measured following hemolysis were expressed as a 50% hemolytic unit of complement (CH50). Levels of C1q or C3 in C1q- or C3-depleted sera were measured by a sandwich ELISA essentially as described for measuring IgG concentrations (see above). Microplates sensitized with goat anticomplement C1q or C3 were incubated with serial 10-fold dilutions of test samples, peroxidase-conjugated anticomplement C1q or C3 (sheep anti-human C1q:horseradish peroxidase [AbD Serotec, Oxford, United Kingdom] or peroxidase-conjugated goat IgG fraction to human complement C3 [Cappel, West Chester, PA]), and then *o*-phenylenediamine dihydrochloride. The C1q and C3 concentrations were calculated by comparing absorbances with those obtained from the standard human complement component: C1q and C3 (Merck, Darmstadt, Germany) with known concentrations.

Statistical analysis. The significances of differences in percentages of infected cells were evaluated by the Student's *t* test. Probability values (*P*) of less than 0.05 were considered significant.

RESULTS

Generation and basic characterization of D2MAbs and D4MAbs. BALB/c mice immunized twice with 100 μg of DENV2 or DENV4 DNA vaccine developed low neutralizing antibody titers of 1:40 to 1:80 in a 70% focus reduction assay (<1:10 to 1:10 in a 90% focus reduction assay). Booster immunization with homologous types of dengue viruses at a dose of 1×10^7 PFU elicited significantly higher neutralizing antibody titers (1:320 or more in a 90% focus reduction assay), which were considered enough for generating a relatively large number of hybridoma clones.

Following screening by ELISA using homologous antigens, we obtained 33 hybridoma clones from nine DENV2-immune mice and 43 clones from four DENV4-immune mice. These clones were grouped into 13 distinct groups each for D2MAbs and D4MAbs, based on neutralizing and HAI activities against DENV2 or DENV4 antigens and ELISA reactivities to four dengue virus antigens, as well as competition assays. Table 1 lists the results of these basic characterizations of representative MAbs in each group. Neutralizing activities were shown in MAbs from 11 (D2MAbs) or 8 (D4MAbs) of the 13 groups, and the MAbs showing neutralizing activities were mostly of the IgG2a subclass in both D2MAbs and D4MAbs. The total numbers of MAbs showing neutralizing activities were 18 of the 33 (55%) for D2MAbs and 17 of the 43 (40%) for D4MAbs. Although the data are not described here, all MAbs recognized the E protein as determined by immunoprecipitation.

Comparison of three ADE assay methods. Three representative methods based on the infection rate (54), yield (17), and number of infectious centers (18) have been reported for measuring enhancing activities. In comparison to the infection rate assay, the yield assay represents the actual production of progeny viruses. The infectious center assay represents the number of cells with primary infection releasing progeny viruses, different from the infection rate or yield assay that represents the outcome of secondary infections or later ones. To select indicator cells (K562 or U937) and one ADE assay method to be used for the subsequent characterization of our MAbs, we compared the usefulness of these two indicator cells and three ADE assays. The MAbs used for this comparison were D2-II-1B3, D4-I-1D6, and D4-IV-10E5, all of which showed relatively high enhancing activities in a pilot experiment.

The comparison of two indicator cells for an ADE assay using DENV2 indicated a higher sensitivity of U937 than of K562 cells in the infection rate assay. Specifically, the maximum percentages of infected cells obtained with D4-I-1D6 at optimal concentrations (1:10^3 to 1:10^4 dilutions of ascitic fluids and IgG concentrations of 2,800 to 280 ng/ml) were 30 to 40% in K562 cells but approximately 90% in U937 cells, whereas the average percentages of infected cells obtained without enhancing antibodies were 1 to 5% in both cells (data not shown). The comparison of U937 with K562 cells using DENV4 provided results consistent with those obtained with DENV2 (data not shown). We therefore used U937 cells for most of the subsequent experiments.

Three ADE assay methods were compared at various dilutions of MAbs (IgG concentrations ranging from 10^0 to 10^6 ng/ml). After the virus-antibody-cell mixture was incubated at 37°C for 2 h, half of the cells were used for infection rate and yield assays and the other half for the infectious center assay. For infection rate and yield assays, cells were cultivated at 37°C for 4 days for DENV2 and 6 days for DENV4. Half of the cells were used for the infection rate assay, while the other half were kept in cultivation for one more day and the culture fluids used for the yield assay. For the infectious center assay, cells were cultivated for 3 days for DENV2 and 4 days for DENV4. A pilot experiment using DENV2 and two MAbs in homologous (D2-II-1B3) and heterologous (D4-I-1D6) combinations indicated that the highest enhancing activities were shown at 32 or 2,800 ng/ml in homologous and heterologous combinations, respectively, in all assay methods (data not shown). Then, we examined each of the MAbs against DENV2 (D2-II-1B3) and DENV4 (D4-I-1D6 and D4-IV-10E5) for enhancing activities against DENV2 or DENV4; thus, ADE assays were performed in two homologous and two heterologous combinations.

As shown in Fig. 1, three dose-dependent curves were roughly in parallel in any combination. Particularly, the highest enhancing activities were shown at the same IgG concentration in all three ADE assays in both homologous and heterologous combinations. The consistent results for the three methods indicate the reliability of each assay system. Although the data are not shown, examinations using affinity-purified IgG fractions of these MAbs showed dose-response curves similar to those obtained with the ascitic fluids that are shown in Fig. 1.

Since the infection rate assay took less time than the yield assay and allowed more samples to be tested at one time than the infectious center assay, we selected the infection rate assay to test enhancing activities. In this assay, we included MAbs in the medium during cultivation for 4 to 6 days at the same concentration as used in the virus-antibody-cell mixture, since the addition of MAbs in the culture provided clearer differentiation between enhancing and nonenhancing activities.

Relationship between enhancing and neutralizing activities. All representative D2MAbs and D4MAbs were tested for enhancing activities with the infection rate assay using DENV2

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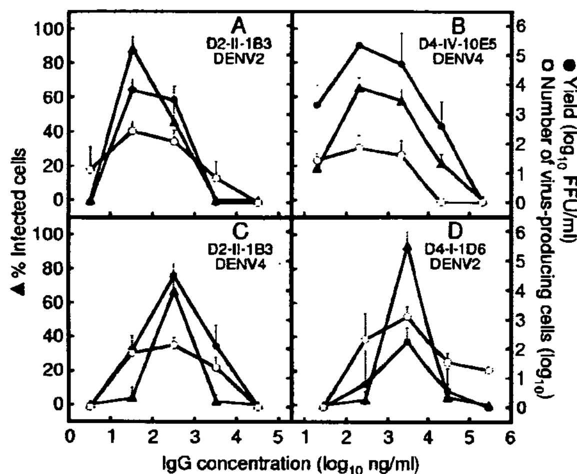

FIG. 1. Comparison of three assay methods for evaluating enhancing activities of MAbs against DENV2 or DENV4 using U937 cells. One D2MAb (D2-II-1B3) and two D4MAbs (D4-I-1D6 and D4-IV-10E5) were used in homologous (panels A and B) and heterologous (panels C and D) combinations with DENV2 and DENV4. The left ordinate indicates percentages of infected cells obtained by the infection rate assay (closed triangles). The right ordinate indicates infective titers (FFU/ml) contained in the culture fluid obtained by the yield assay (closed circles) and the number of virus-producing cells included in 10^5 cells obtained by the infectious center assay (open circles). The abscissa indicates the final concentration of IgG included in the virus-antibody-cell mixture. The assays were done in triplicate (infection rate assay) or duplicate (other assays). Each datum represents an average obtained in two separate assays, with SDs indicated by error bars.

and DENV4 in homologous and heterologous combinations (see closed triangles in Fig. 2). Of 13 D2MAbs, 10 showed enhancing activities against DENV2, including 4 also showing activities against DENV4. On the other hand, only 4 of the 13 D4MAbs showed enhancing activities against the homologous type, none of which showed activities against the heterologous type. One MAb (D4-I-1D6) showed enhancing activities only against the heterologous, but not the homologous, type. In the subsequent ADE assays, IgG concentrations showing the highest ADE activities, as shown in Fig. 2, were used for each MAb.

Comparison with neutralizing activities (see open circles in Fig. 2) showed that all MAbs showing enhancing activities against homologous types showed neutralizing activities against homologous types. As well, all MAbs showing enhancing activities against heterologous types showed neutralizing activities against heterologous types. Most MAbs showed enhancing activities at subneutralizing doses; however, some exceptions (e.g., D2-II-9A7 and D4-IV-10E5) showed the highest enhancing activities at IgG concentrations approximately 100-fold different from those showing neutralizing activities at 50% focus reduction. In addition, some MAbs (e.g., D2-II-11D1 and D4-IV-3G12) showed neutralizing activities in a certain range of IgG concentrations but did not show enhancing activities at any IgG concentrations examined in the present study.

Effect of rabbit complement on enhancing and neutralizing activities. Since rabbit complement was included in the virus-antibody mixture to increase sensitivity in our neutralization test, we attempted to include rabbit complement in the virus-antibody-cell mixture in the ADE assay at a final concentration of 5%. As shown in Fig. 2, enhancing activities shown in the absence of complement were abolished when complement was added to the assay system in all MAbs that showed enhancing activities. This abolishment was shown in both homologous and heterologous combinations.

We also attempted not including the rabbit complement in the virus-antibody mixture in neutralization tests. In the absence of the complement, neutralizing activities were decreased approximately 2- to 50-fold in all MAbs (Fig. 2).

Effect of fresh human serum on enhancing activities. Based on the results obtained by the inclusion of rabbit complement, we next included fresh human serum in the ADE assay system. Heterologous combinations of MAbs and dengue virus types were used: D2-II-1B3 with DENV4 and D4-I-1D6 with DENV2 (Fig. 3). The enhancing activity was reduced depending on the final concentration of fresh serum in the virus-antibody-cell mixture, within a range of 0.1 to 10%. As a reference, rabbit complement was included in the virus-antibody-cell mixture at various concentrations and showed a pattern of dose-dependent effects similar to those obtained by the inclusion of fresh human serum (Fig. 3).

Heat-inactivated or complement component-depleted sera reduced the effect on enhancing activities. To confirm that the complement contained in fresh human serum could be a factor involved in the effect on enhancing activities, heat-inactivated human serum was included in the ADE assay system using D4-I-1D6 and DENV2. As a reference, heat-inactivated rabbit complement was tested in parallel. As shown in Fig. 4A, there was no significant reduction of enhancing activities by the addition of rabbit complement when the complement was heat inactivated ($P > 0.05$). Although heat-inactivated fresh human serum showed reduction of enhancing activities with low statistical significance ($P < 0.05$), this reduction was considerably smaller than the reduction shown with noninactivated fresh human serum.

To confirm that the heat-labile factor associated with reduction in enhancing activity is the complement, commercial C1q- or C3-depleted human sera were used for an infection rate assay comparing them with nondepleted human sera (fresh human sera used in the experiments whose results are shown in Fig. 3 and 4A). These commercial sera did not contain detectable antibodies to DENV2 or DENV4 as determined by a 50% focus reduction neutralization test (data not shown). As shown in Fig. 4B, the significant reduction in enhancing activities shown with the use of nondepleted serum was not shown with the use of C1q- or C3-depleted serum.

Furthermore, the addition of purified C1q or C3 to the C1q- or C3-depleted serum, respectively, reduced enhancing activities in a dose-dependent manner (Fig. 4C). In this experiment, C1q levels of 7.5 to 240 μg/ml and C3 levels of 75 to 2,400 μg/ml were used, based on the standard range for normal individuals (mostly 70 to 150 μg/ml for C1q and 850 to 1,500 μg/ml for C3) since the original C1q or C3 levels in the commercial C1q- or C3-depleted sera were unknown. Although complementation by C1q was more effective than that by C3, the decrease in enhancing activities was closely related to the increase in CH50 values (Fig. 4C). Specifically, enhancing activities were significantly reduced when CH50 values were within the normal range of 25 to 45. These results indicated

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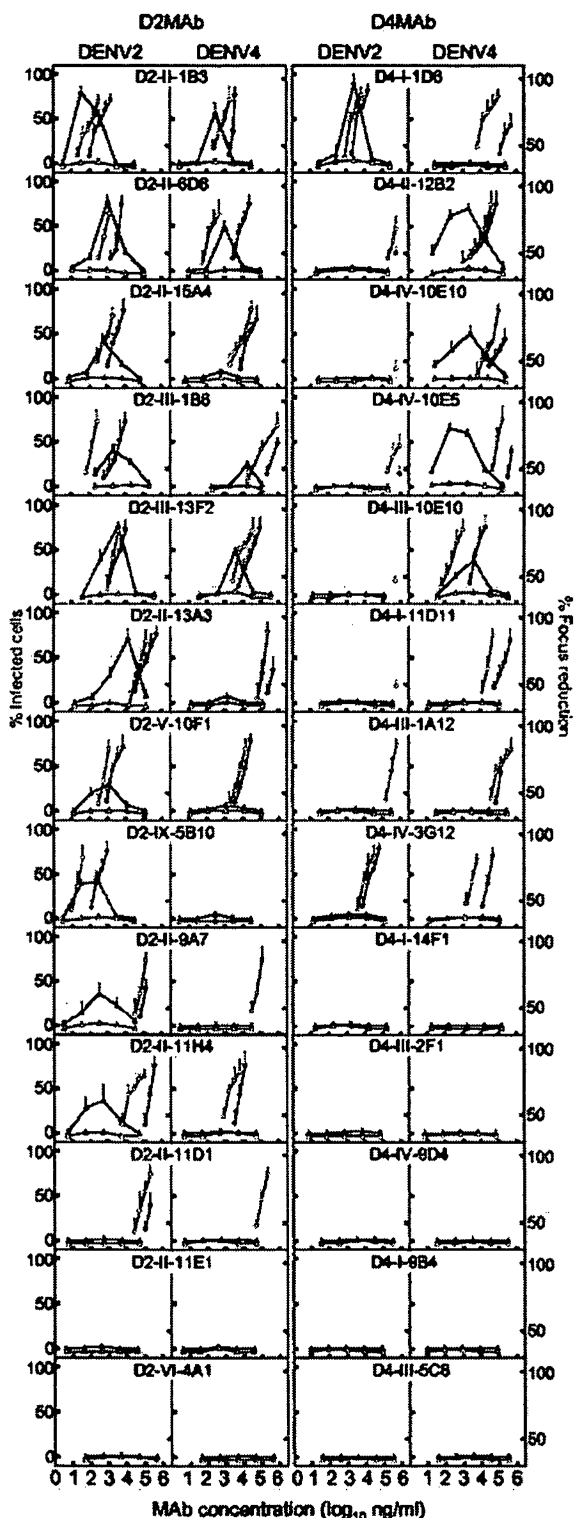

FIG. 2. Relationship between enhancing and neutralizing activities in 13 representative D2MAbs and 13 representative D4MAbs. Enhancing activities were determined by an infection rate assay using U937 cells mixed with virus and serial 10-fold dilutions of each MAb and expressed as percentages of infected cells (open and closed triangles; left ordinate). Neutralizing activities were determined by a focus reduction assay using Vero cells infected with mixtures of virus and serial twofold dilutions of each MAb and expressed as percentages of focus reduction (open and closed circles; right ordinate). Only percentages of focus reduction between 40 and 100% were plotted. IgG fractions purified from ascitic fluids were used in neutralization tests when neutralizing antibody titers in ascitic fluids were 1:40 or lower as determined by a 90% focus reduction assay, since control ascitic fluids obtained from P3U1-inoculated mice frequently showed positive results in a 50% focus reduction assay. Closed triangles and circles indicate results obtained without inclusion of rabbit complement in the assay system, whereas open triangles and circles indicate results obtained in the presence of rabbit complement at a final concentration of 5%. Both assays were done in duplicate. Each datum represents an average obtained in three (for the infection rate assay) or two (for the focus reduction assay) separate experiments, with SDs indicated by error bars. The mean borderlines for enhancing activities obtained from three experiments using negative controls without MAbs were 18.0% for DENV2 and 14.3% for DENV4 in three separate assays.

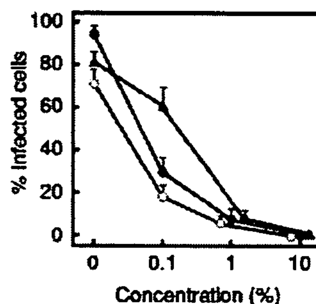

FIG. 3. Effect of fresh human serum on enhancing activities of MAbs. Enhancing activities were evaluated in the infection rate assay using U937 cells and expressed as percentages of infected cells. Heterologous combinations of MAbs and the viruses were used: D2-II-1B3 and DENV4 (open circles) and D4-I-1D6 and DENV2 (closed circles). The final concentrations of IgG included in the virus-antibody-cell mixture for D2-II-1B3 and D4-I-1D6 were 32 and 2,800 ng/ml, respectively, which showed the highest enhancing activities in the experiments whose results are shown in Fig. 2. Rabbit complement was also examined for its effect on enhancing activities in a heterologous combination of D4-I-1D6 and DENV2 (closed triangles). The abscissa indicates the final concentration of human serum or rabbit complement in the virus-antibody-cell mixture. The assays were done in duplicate, and each datum represents an average obtained in three separate assays, with SDs indicated by error bars.

that the complement was responsible for the effect of fresh human serum on enhancing activities.

Effect of fresh human serum on enhancing activities under dense serum conditions. To create an assay condition closer to the in vivo environment, the serum concentration in the virus-antibody-cell mixture was increased to 80%. To achieve different complement levels at a constant serum concentration, heat-inactivated serum was mixed with fresh serum at various ratios. The complement level in each inactivated/fresh serum mixture was confirmed by measuring CH50 values. Four fresh human sera were examined under a combination of D4-I-1D6 and DENV2 (Fig. 5A). Under the dense serum condition, the percentages of infected cells shown in the absence of complement activity (40 to 70%) were lower than those shown under the normal assay condition without the inclusion of serum in the virus-antibody-cell mixture (over 90%) (Fig. 3). Accordingly, the borderline provided under the dense serum condition (8.0%) (Fig. 5) was lower than that obtained under the normal condition (18.0%) (Fig. 2).

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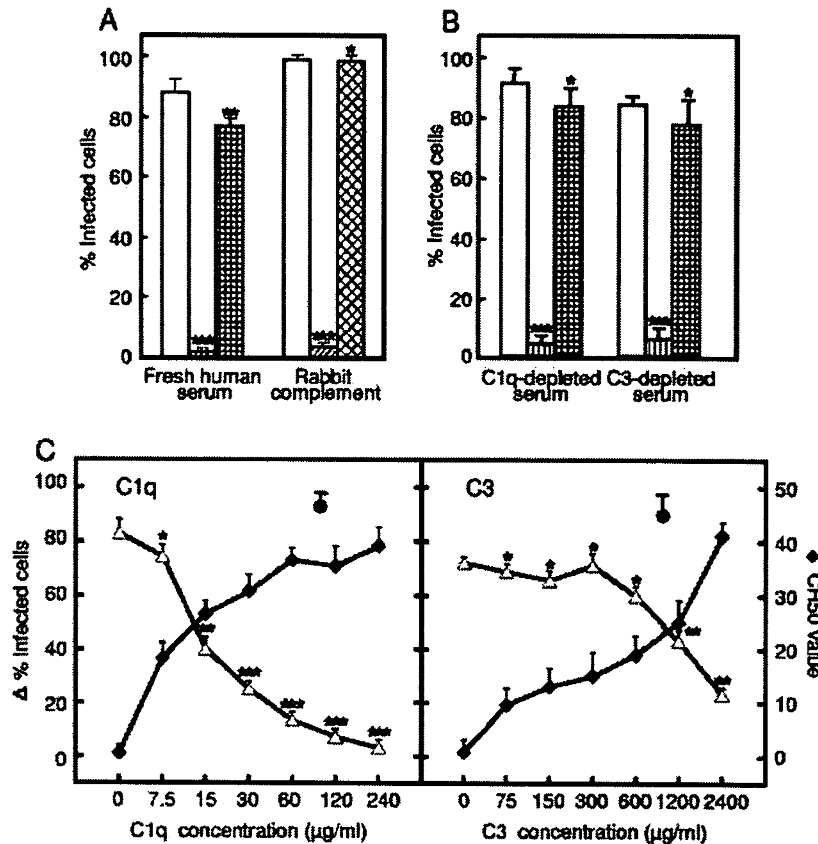

FIG. 4. Effect of heat inactivation or depletion of Clq or C3 on fresh human serum's capacity to reduce enhancing activities. Infection rate assay was performed using U937 cells with a combination of D4-I-1D6 and DENV2. The D4-I-1D6 was included in the virus-antibody-cell mixture at a final IgG concentration of 2,800 ng/ml. (A) Effect of heat inactivation. Rabbit complement was used as a reference. Fresh human serum and rabbit complement were inactivated at 56°C for 30 min. The assay was performed in the absence (open bars) or presence of fresh (hatched bars) or inactivated (cross-hatched bars) human serum or rabbit complement at a final concentration of 5%. (B) Effect of depletion of Clq or C3. The assay was performed in the absence (open bars) or presence of fresh (hatched bars) or Clq- or C3-depleted (cross-hatched bars) human serum at a final concentration of 1%. (C) Effect of complementation by addition of purified Clq or C3 to Clq- or C3-depleted human serum. Enhancing activities were obtained with the virus-antibody-cell mixture including Clq- or C3-depleted serum at a final concentration of 1% and various concentrations of Clq or C3 (open triangles). The abscissa indicates Clq or C3 concentrations adjusted to those contained in depleted and complemented human sera. The depleted and complemented human sera were also examined for CH50 values (closed diamonds). As controls, enhancing activities obtained with the virus-antibody-cell mixture including only Clq or C3 at a final concentration of 100 or 1,000 μg/ml, respectively, are shown (closed circles). The Clq and C3 in Clq- and C3-depleted sera, respectively, were not detectable in a sandwich ELISA (<0.02 μg/ml for both). All assays were done in duplicate. Each datum in panel A represents an average obtained in three separate assays, and each datum shown in panels B and C represents an average obtained in two separate assays, with SDs indicated by error bars. For panels A and B, asterisks indicate significant differences from percentages of infected cells obtained in the absence of serum in each experimental group: *, $P > 0.05$; **, $P < 0.05$; ***, $P < 0.001$. For panel C, asterisks indicate significant differences from percentages of infected cells obtained without addition of Clq or C3 in each experimental group: *, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Two of four fresh human sera showed enhancing activities within complement levels of 20 to 80%, whereas two other sera did not show enhancing activities in any complement levels, except at 0% (Fig. 5A). One of the two sera that showed enhancing activities showed higher activities at 50 and 75% than at 10 and 30% for the fresh serum concentration, whereas another serum showed higher activities at 20 and 40% than at 60 and 80%. These results indicated that the dose-dependent curves under dense serum conditions differed according to the individual sera and that a decrease in the complement level to 50 to 75% can induce enhancing activities in some sera.

To seek the reason for enhancing activities with relatively high complement levels under dense serum conditions, one of the sera that showed enhancing activities when using U937 cells (closed triangles, Fig. 5A) was examined for enhancing activities when using K562 cells. As shown in Fig. 5B, enhancing activities were not detectable within complement levels of 30 to 100% when K562 cells were used. Since K562 cells do not have complement receptor 3 (CR3), which is possessed by U937 cells, this result suggested that enhancing activities shown with relatively high complement levels under dense serum conditions were associated with a mechanism of CR3-mediated enhancement (3).

Comparison between effects of fresh human sera on enhancing activities of homologous and heterologous MAb-virus combinations. The experiment detailed above was performed in a heterologous MAb-virus combination. To compare the effect of fresh sera on enhancing activities between homologous and

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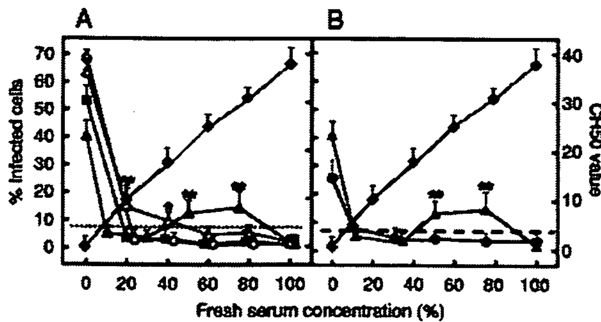

FIG. 5. Dose-dependent reduction of enhancing activities by inclusion of fresh human serum in the virus-antibody-cell mixture under dense serum conditions. Enhancing activities were evaluated in an infection rate assay using U937 or K562 cells mixed with D4-I-1D6 and DENV2 and expressed as percentages of infected cells (left ordinate). The D4-I-1D6 was included in the virus-antibody-cell mixture at a final IgG concentration of 2,800 ng/ml. The dense serum condition was achieved by including the fresh/inactivated serum mixture in the virus-antibody-cell mixture at a final concentration of 80%. Fresh human serum was included in the fresh/inactivated serum mixture at various concentrations as indicated by the abscissa. The complement activity in the fresh/inactivated serum mixture was measured by a standard CH50 hemolytic assay and expressed as CH50 values (right ordinate). (A) Evaluation with sera from four healthy humans using U937 cells. Enhancing activities obtained with these sera are indicated by open circles and triangles and closed triangles and squares. The dotted line indicates the mean borderline (8.0%) for enhancing activities, obtained from three separate assays using negative controls without MAbs. The CH50 values were similar in four serum samples; one result is shown (closed diamonds). (B) Evaluation using K562 cells. One serum used in the above experiment (indicated by closed triangles in panel A) was used for this evaluation. Enhancing activities obtained using K562 cells are indicated by closed circles, while those obtained using U937 cells (the same data as shown in panel A) are indicated by closed triangles for reference. The assays were done in duplicate, and each datum represents an average obtained in three (for data shown in closed triangles) or two (for other data) separate assays, with SDs indicated by error bars. The broken line indicates the mean borderline (7.4%) for enhancing activities obtained from two separate assays using K562 cells. Asterisks indicate significant differences from percentages of infected cells obtained at a fresh serum concentration of 100% in each serum sample, except for data obtained at 0%: *, $P < 0.05$; **, $P < 0.01$.

heterologous combinations, MAbs D2-II-1B3, D4-I-1D6, and D4-IV-10E5 were used in various combinations with DENV2 and DENV4, using 14 fresh human sera. In this experiment, each fresh serum was included in the virus-antibody-cell mixture at a final concentration of 50%. Although there were individual variations, the heterologous combinations (Fig. 6B) showed significantly higher enhancing activities than the homologous combinations (Fig. 6A) in 8 of 28 (29%) MAb-virus combinations ($P < 0.05$ or $P < 0.01$). These 14 sera had similar complement levels, as determined by the CH50 assay (data not shown).

Among the 14 sera, serum number 4 was selected for further investigation of the effect of fresh sera on enhancing activities under dense serum conditions in homologous and heterologous combinations with DENV2. The fresh serum was mixed with heat-inactivated serum at various ratios as in the experiment whose results are shown in Fig. 5. D2-II-1B3 and D2-IX-5B10 were used for homologous combinations and D4-I-1D6 for a heterologous combination. As shown in Fig. 6C, the

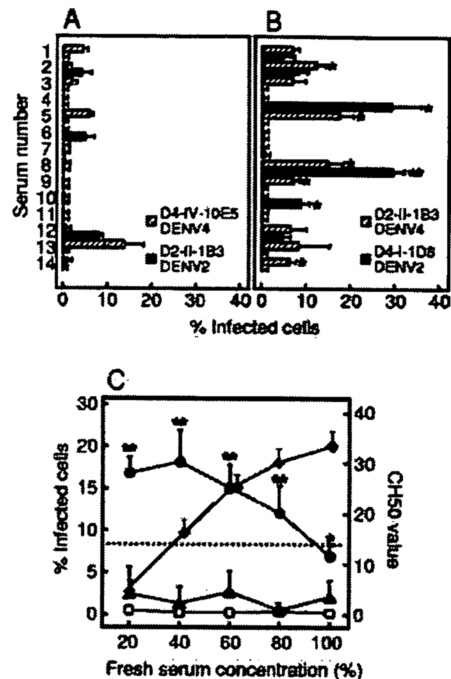

FIG. 6. Effect of fresh human sera on enhancing activities under dense serum conditions in homologous and heterologous MAb-virus combinations. Infection rate assay was performed in duplicate using U937 cells. (A and B) Effect of sera from 14 healthy humans. One D2MAb (D2-II-1B3) and two D4MAbs (D4-I-1D6 and D4-IV-10E5) were used in homologous (A) and heterologous (B) combinations with DENV2 and DENV4. The final concentrations of IgG in the virus-antibody-cell mixture were 32 (for D2-II-1B3), 2,800 (for D4-I-1D6), and 200 (for D4-IV-10E5) ng/ml. Fresh human sera were included in the virus-antibody-cell mixture at a final concentration of 50%. Closed and hatched bars indicate percentages of infected cells obtained with DENV2 and DENV4, respectively. Each datum represents an average obtained in two separate assays, with SDs indicated by error bars. Asterisks indicate significant differences between percentages of infected cells shown in homologous (A) and heterologous (B) combinations: *, $P < 0.05$; **, $P < 0.01$. For controls, mean percentages of infected cells obtained from two separate experiments without inclusion of human sera were 98.6% for DENV2 and 72.2% for DENV4 in homologous combinations and 97.3% for DENV2 and 71.6% for DENV4 in heterologous combinations, while those obtained with negative controls without MAbs were 4.6% for DENV2 and 1.4% for DENV4. (C) Comparison of dose-dependent enhancing activities under various fresh serum concentrations. Serum number 4 used in the experiment shown in panels A and B was used in this experiment. The abscissa indicates concentrations of fresh serum in the fresh/inactivated serum mixture. The left ordinate indicates percentages of infected cells obtained with D2-II-1B3 (closed triangles), D2-IX-5B10 (open squares), and D4-I-1D6 (closed circles). The final concentrations of IgG in the virus-antibody-cell mixture for D2-II-1B3 and D4-I-1D6 were the same as described above, and that for D2-IX-5B10 was 300 ng/ml. The right ordinate indicates complement activities in the fresh/inactivated serum mixture as determined by the CH50 assay (closed diamonds). Each datum represents an average obtained in four separate experiments, with SDs indicated by error bars. Asterisks indicate significant differences between percentages of infected cells shown in homologous (closed triangles) and heterologous (closed circles) combinations: *, $P < 0.05$; **, $P < 0.001$. The mean baseline enhancement in the presence of MAb and the absence of fresh serum was 60.7% for D2-II-1B3, 50.5% for D2-IX-5B10, and 71.0% for D4-I-1D6. The dotted line indicates the mean borderline (7.9%) for enhancing activities obtained from four separate assays using negative controls without MAbs.

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heterologous combination showed higher percentages of infected cells than did the homologous combinations under all experimental conditions for this serum sample (within the fresh serum concentrations of 20 to 100%). The percentages of infected cells were higher than the borderline within a range of 20 to 80% in the heterologous combination: even at 100%, the percentage of infected cells was close to the borderline. These results indicate that the heterologous combination provided higher enhancing activities than the homologous combination in some serum samples under dense serum conditions, so long as DENV2 was used for the assay.

DISCUSSION

Infection enhancement is a critical factor involved in the pathogenesis of many viral infections (49). An enhancing phenomenon was first reported with flaviviruses and, thereafter, with human immunodeficiency virus (HIV), Ebola virus, etc. Flaviviruses usually have a mechanism of Fc receptor-dependent enhancement (30, 41, 45), whereas HIV has a mechanism of Fc receptor-independent, complement receptor-mediated enhancement, in addition to an Fc receptor-dependent one (10). In Ebola virus, antibody-dependent, C1q receptor-mediated mechanisms are involved in the enhancement (50). In some HIV and most Ebola virus studies, enhancing assays have been performed using fresh sera. In the present study, we used fresh sera in ADE assays, although, to date, heat-inactivated sera have been almost exclusively used for dengue virus ADE studies. Use of fresh sera in an in vitro assay is considered to provide an assay condition closer to the in vivo environment.

Cross-reactive nonneutralizing antibodies have been generally considered a major factor involved in enhancing activities in dengue virus infections (15, 31, 47). In the present study, however, all MAbs showing enhancing activities had neutralizing activities irrespective of the homologous or heterologous combination of the MAb and virus used in the ADE assay. That is, nonneutralizing antibody species showing enhancing activities could not be found for the MAbs generated against DENV2 or DENV4. A similar result has been reported in an influenza A virus system in which all MAbs showing enhancing activities showed high or low levels of neutralizing activities (51). As well, the enhancing activities at subneutralizing doses were abolished or dramatically decreased when the assay was performed in the presence of fresh sera, although enhancing activities exhibited at subneutralizing doses were consistent with the results of previous reports where test samples were heat inactivated before the ADE assay (15). A similar result has been reported in a measles virus system in which ADE was almost completely blocked by the addition of guinea pig or rabbit complement in the virus-antibody mixture (21). Further, MAbs that showed neutralizing, but not enhancing, activities irrespective of the presence or absence of complement were generated in the present study. Although most of these MAbs had relatively low neutralizing activities, these might be new antibody species that have a protective role, since MAbs showing neutralizing activities have been generally considered to show enhancing activities (37).

Several experimental results in the present study demonstrated that the reductive effect of fresh sera on enhancing activities is attributed to the complement. Specifically, the reductive effects of fresh sera were reduced by their heat inactivation or by depletion of the complement component, C1q or C3. The most probable mechanism underlying this effect is virolysis, since MAbs showing enhancing activity had neutralizing activity that was increased in the presence of complement in all cases. Immune complexes consisting of MAb and the E protein on the surface of virus particles may activate complement pathways, followed by formation of the C5b-C9 membrane attack complex that may have lysed the viral envelope (20). On the other hand, the possibility that cell lysis may occur through complement activation on the cell surface to which the virus is attached along with the immune complex can be considered negligible, since over 99% of cells survived after incubation with the virus, antibody, and rabbit complement as determined by trypan blue inclusion (data not shown). Under both the normal and dense serum conditions, fresh serum reduced enhancing activities in a dose-dependent manner (Fig. 3, 5), indicating that the function of antibodies (neutralizing or enhancing activities) is regulated by the level of complement in the assay system. Specifically, neutralizing/enhancing antibody shows neutralizing activities under normal complement levels, in contrast to the enhancing activities under reduced complement levels. Since some sera showed enhancing activities with relatively high complement levels under dense serum conditions (Fig. 5), a decrease in complement levels to a certain extent within the physiological range may enhance infection by a CR3-mediated mechanism in some individuals, through the binding of iC3b, a major cleavage product of activated C3 (3).

Although there have been only a few reports describing complement levels in dengue virus-infected patients, marked reductions in levels of the complement component C3 have been shown in DHF patients (1, 38). Additionally, increasing levels of immune complexes have been reported in dengue virus-infected patients (43): antibodies induced by infection may form immune complexes with viral antigens that may consume the complement (20). Finally, complement hemolytic activities (CH50 values) have been shown to be low in the acute phase of DHF (38), in contrast to most DF patients, in whom the complement activities were not altered (46). As well, mass complement components are produced mainly in the liver and monocytes/macrophages (6), both known as principal targets of dengue virus infection (4, 9, 14). The infection may decrease the level of complement production, possibly facilitating further decreases in the complement levels in circulation.

Most of the MAbs generated in the present study had higher neutralizing antibody titers in homologous than heterologous combinations with the viruses (Fig. 2). As well, using 14 healthy human sera, cross-reactive antibodies provided higher enhancing activities than did specific antibodies under dense serum conditions in 29% of MAb-virus combinations (Fig. 6A and B). Further, in one particular serum, cross-reactive antibodies provided significantly higher enhancing activities than did specific antibodies within the complement levels of 20 to 80% of the original fresh serum in the assay system using DENV2 (Fig. 6C). These results suggested that, although there are wide individual variations, some sera give an environment that provides higher enhancing activities against heterologous than homologous antibodies, consistent with epidemiological evi-

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dence showing that disease severity is usually greater upon heterologous secondary infection (15).

In conclusion, the present study demonstrated in DENV2 and DENV4 models that antibody species that have enhancing activities also have neutralizing activities and that these two distinct activities are controlled in vitro by levels of complement within the physiological range. Therefore, complement levels are considered an additional factor involved in the in vivo ADE phenomenon, suggesting that the disease outcome may be determined by the complement level in the early stage of the disease: protection at a normal level, but increased severity at a reduced level. However, the existence of other mechanisms or factors, in particular, nonneutralizing cross-reactive antibody species against DENV1 and DENV3, is not ruled out. Reports concerning the inclusion of complement in an ADE assay system, though limited in early studies, have described its boosting (14) or not detectable (17) effects on enhancing activities in a polyclonal system. We are currently investigating this complement-dependent ADE phenomenon using a mouse model and also planning to monitor the serum complement level in DF and DHF patients to confirm the relationship of the complement level to the level of viremia.

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Utilization of Complement-Dependent Cytotoxicity To Measure Low Levels of Antibodies: Application to Nonstructural Protein 1 in a Model of Japanese Encephalitis Virus[∇]

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Enzyme-linked immunosorbent assay (ELISA) and related assays are representative of methods currently used for antibody tests. However, they occasionally produce nonspecific reactions, thus making it difficult to reliably measure low levels of specific antibodies. To find a test method that minimizes nonspecific reactions, we introduced the principle of antibody-mediated complement-dependent cytotoxicity (CDC) into an antibody assay. The procedure has three steps: (i) the mixing of test samples with a suspension of cells expressing the antigen of interest on their surfaces, (ii) the addition of rabbit complement, and (iii) the measurement of lactose dehydrogenase (LDH) activities by adding a chromogenic substrate to the reaction mixture. When the specific antibodies exist in the sample, complement activation triggered by antibody binding on the surface of the antigen-expressing cells may lyse the cells, releasing LDH into the medium. Mouse and rabbit sera hyperimmune to nonstructural protein 1 (NS1) of Japanese encephalitis virus (JEV) lysed NS1-expressing cells in a dose-dependent manner. Evaluations using sera from horses naturally infected with JEV showed that the CDC assay had quantitative correlation and qualitative agreement with previously established NS1 antibody-detecting immunostaining and ELISA methods. The assay method also detected NS1 antibodies in sera of mice 2 days after experimental infection with JEV; specific, but not natural, immunoglobulin M antibodies were detected. Since almost all sera examined in this study showed no nonspecific reactions, the CDC assay was shown to be a reliable method for measuring low levels of specific antibodies.

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Enzyme-linked immunosorbent assay (ELISA) and related assays are representative of methods currently used for testing antibodies induced by viral infections (22). These assays are based on measurements of antibody molecules of a certain immunoglobulin class(es) bound to antigen molecules, irrespective of the biological functions of the antibody. Although these methods are simple, easy, and rapid, they also detect antibodies that are not specifically bound to the antigen, resulting in nonspecific reactions. These include naturally occurring low-affinity polyreactive antibodies (natural antibodies) that are secreted by a subset of long-lived B cells termed B-1 cells, many of which are CD5 positive (6, 9). This nonspecific reaction is thought to make it difficult for these methods to reliably detect low levels of specific antibodies.

Our laboratory has developed methods to measure relatively low levels of antibodies to the nonstructural protein 1 (NS1) of Japanese encephalitis virus (JEV) elicited by natural infections with JEV (12–14). The test methods we have developed to measure NS1 antibodies are useful for surveying natural JEV infections in populations vaccinated with inactivated JE vaccine. Since levels of NS1 antibodies induced by asymptomatic infections are considerably lower than those induced in JE patients, an ELISA established for measuring NS1 antibodies induced in JE patients (21) cannot detect those induced by natural infections. We therefore established a method based on immunostaining that was sufficiently sensitive to measure NS1 antibodies induced in naturally infected humans (14) and horses (13). We have established an ELISA method for horses (12); however, because of the relatively high levels of nonspecific reactions, even this ELISA was unable to detect NS1 antibodies induced in naturally infected humans. The success in establishing an ELISA for horse sera seems to be attributed to the relatively high levels of NS1 antibodies in this animal species, which is more frequently exposed to infective mosquito bites in nature than are humans, though the levels of exposure are not so high as to cause disease.

Antibody-mediated complement-dependent cytotoxicity (CDC) frequently has been used for specific cell depletion (3). The mechanism is based on complement activation triggered by a specific antibody binding to the antigen appearing on the cell surface and the subsequent formation of the C5b-9 membrane attack complex that may lyse the cells. CDC also is likely to be a mechanism of host defense against viral infections (24). For JEV infection, protection from a lethal challenge in mice that have JEV NS1 antibodies but not neutralizing ones is considered to be related in part to this mechanism (16). This also has been assumed for NS1 antibody-induced protection of mice from infection with other flaviviruses, such as yellow fever (19, 20), dengue (4), and tick-borne encephalitis (8) viruses; however, a complement-independent mechanism in protection by NS1 antibodies with a West Nile virus system recently has been reported (2). Considering the specificity of the CDC phenomenon, its principle is applicable to antibody testing.

This study aimed to utilize the principle of CDC to establish a

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novel method for testing JEV NS1 antibodies. Although CDC assays originally were performed for functional evaluations of antibodies to estimate an in vivo role of the CDC mechanism in flaviviruses (4, 16, 20) and other systems (1, 5, 7, 17, 18, 23), the present study sought to use CDC to measure low levels of specific antibodies with high reliability in general antibody assays. For our initial evaluation of this method, we used sera from mice and rabbits hyperimmune to NS1, along with sera from horses naturally infected with JEV that we had used in our earlier study to establish immunostaining and ELISA methods. We also used sera of mice experimentally infected with JEV. The results we obtained indicated that the principle of CDC could be applied to testing for NS1 antibodies.

MATERIALS AND METHODS

Serum samples. With the exception of naïve mouse sera, all sera used in the present study also were used in or prepared for our earlier studies (10, 12, 13, 15) and were stored at −30°C. Naïve mouse sera were prepared for this study and used as normal mouse sera (NMS) or for obtaining cutoff values to differentiate positive from negative samples for the CDC assay and ELISA. Hyperimmune mouse serum (HMS) (10) was obtained by repeated immunization of adult BALB/c mice with an affinity-purified NS1 antigen obtained from culture fluids of Vero cells infected with the Nakayama strain of JEV. A monoclonal antibody to JEV NS1, JE-2D5 (12), was used for the affinity purification. Hyperimmune rabbit serum (HRS) (12) was obtained by repeated immunization of a Japan White rabbit with an NS1 antigen affinity purified from culture fluids of Nakayama-infected Vero cells. Normal rabbit serum (NRS) was obtained from the same rabbit prior to immunization. Horse sera (13) were collected from 1996 through 2000 from thoroughbred yearlings that had been born and kept on Hokkaido Island (a northern nonenzootic area). These horses were without JEV vaccination and were negative for hemagglutination-inhibiting (HAI) antibody. Sera also were collected at this time from clinically healthy thoroughbred racehorses aged 2 to 4 years and kept in the prefectures Hokkaido, Gumma, Saitama, Chiba, Aichi, Oita, and Saga. Experimentally infected mouse sera (15) were obtained from ICR mice immunized with 10 or 100 μg of a pcDNA3-based plasmid expressing premembrane and envelope genes of the JEV Nakayama strain (pcJEME) (15) or inoculated with phosphate-buffered saline (PBS); all mice then were infected with 10,000 50% lethal doses of the Beijing 3 strain. All sera were heat inactivated prior to use for antibody testing.

Monoclonal antibodies. Hybridoma clones 1D12, 5A5, and 6A11, which secrete natural immunoglobulin M (IgM) antibodies, have been described previously (11). Briefly, spleen cells were collected from naïve mice, fused with mouse myeloma P3U1 cells, screened for the production of antibodies to the protozoan parasite *Toxoplasma gondii*, and then cloned. Antibodies were obtained in an ascites form from pristane-primed BALB/c mice. Control ascitic fluids were obtained from pristane-primed mice inoculated with P3U1 cells.

NS1-expressing cells. The generation of cells stably transfected with the *NS1* gene (MF6 cells [14]) or *NS1* and *NS2A* genes (3G8 cells [12]) of JEV has been described already. Briefly, CHO-K1 cells were transfected with a pcDNA3-based plasmid expressing NS1 or NS1/NS2A genes of the JEV Nakayama strain, selected in G418-containing medium, and then cloned by limiting dilution. Approximately 80 to 100% of cells expressed NS1 antigen in both MF6 and 3G8 cells, as determined by immunostaining. Live MF6 cells were used as the antigen for the CDC assay, while the fixed MF6 cells for the immunostaining assay (13) and culture fluids of 3G8 cells for ELISA (12) had been used in our earlier studies to measure NS1 antibodies in horse sera.

Fluorescent antibody staining. Cells were fixed with 2% paraformaldehyde in PBS. Next, they were incubated with a monoclonal against NS1 (JE-2D5) and then with fluorescein isothiocyanate-labeled anti-mouse IgG. Cells then were observed under a fluorescent microscope.

ELISA to measure NS1 antibody levels. All ELISA data from horse sera were obtained as previously described (12). ELISAs for detecting NS1 antibodies in mouse and rabbit sera were performed essentially by following the method previously described for measuring NS1 antibody levels in horse sera (12). Briefly, microplates sensitized with NS1 antigen affinity purified from culture fluids of MF6 cells were incubated serially with test sera, conjugates, and *p*-nitrophenyl phosphate. The amount of NS1 antigen used for sensitization was 3 ng/well, unless otherwise specified. Conjugates were alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Zymed, San Francisco, CA) or goat anti-rabbit IgG (Sigma Chemical, St. Louis, MO). Tests were done in duplicate, and absorbances obtained from the two wells were averaged. To minimize nonspecific reactions, a nonsensitized control plate was run in parallel and the difference in absorbances from antigen-sensitized wells was obtained, unless otherwise specified. When the subtraction provided a negative value, 0.000 was assigned to the result.

CDC assay. For the CDC assay, an MF6 cell suspension containing 5×10^4 cells in 50 μl of serum-free minimal essential medium (SF-MEM) was mixed with an equal volume of test serum diluted in SF-MEM and incubated on ice for 30 min. Eleven microliters of rabbit complement (Low-Tox-M rabbit complement; Cedarlane, Hornby, Canada) was added to make a final concentration of 10% and was incubated at 37°C for 2 h. Following centrifugation at 500 rpm for 5 min, 50 μl of the supernatant was mixed with 50 μl of a lactose dehydrogenase (LDH) substrate (cytotoxicity detection kit plus [LDH]; Roche, Mannheim, Germany) and incubated at room temperature for 15 min, followed by spectrophotometry at 490 nm. All procedures were done in duplicate in 96-well microplates, and absorbances obtained from the two wells were averaged. The percentage of specific cell lysis was calculated according to the manufacturer's instructions by using the following formula: $100 \times [(A - C)/(B - C)]$, where A represents an absorbance obtained with test serum (experimental release), B represents an absorbance obtained by lysing all of the target cells with 1% Triton X-100 (maximum release), and C represents an absorbance obtained with target cells incubated in SF-MEM containing rabbit complement at 10% (minimum release). When this calculation provided a negative value, 0.00% was assigned to the result. Reactions showing greater than 4.32 or 5% specific lysis were determined to be positive for NS1 antibodies in horse and mouse sera, respectively. In the one-dilution method, the percentage of specific cell lysis obtained at a 1:10 dilution of sera was used as the NS1 antibody level. In the endpoint method, the NS1 antibody titer was expressed as the highest serum dilution giving greater than 4.32 or 5% specific lysis for horse and mouse sera, respectively.

Depletion of IgG or IgM from serum. A 1:250 dilution of HMS or a 1:40 dilution of serum pooled from mice 4 days after infection with JEV was used for the depletion of IgG or IgM. Fifty microliters of each serum sample was mixed with an equal volume of serial 10-fold dilutions (from $1{:}10^1$ to $1{:}10^4$) of goat anti-mouse IgG (Chemicon, Temecula, CA) or sheep anti-mouse IgM (Binding Site, Birmingham, England). Following incubation at 37°C for 1 h and then at 4°C overnight, the mixture was centrifuged at $16{,}000 \times g$ for 1 h, and the supernatant was examined for IgG or IgM levels by ELISA and for NS1 antibody levels by the CDC assay.

ELISA to measure IgG or IgM levels. A sandwich ELISA was performed essentially as previously described (10). Briefly, microplates sensitized with a goat anti-mouse IgG or sheep anti-mouse IgM were incubated with a 1:2 dilution of depleted sera (described above) and then alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Zymed, San Francisco, CA), respectively. The enzyme activity bound on the plate was measured with *p*-nitrophenyl phosphate. The absorbances obtained with depleted sera were divided by the control absorbance obtained with nondepleted sera and were expressed as relative IgG or IgM levels.

Statistical analysis. Correlation coefficients were calculated by means of the Microsoft Excel statistical package. Probability levels (P) of less than 0.05 were considered significant.

RESULTS

Selection of antigen-expressing cells used for the CDC assay. Since the CDC assay uses a mechanism involving complement-dependent cytotoxicity, cells expressing the antigen of interest on their surfaces are a prerequisite. We therefore looked for the presence of the NS1 antigen on the surfaces of cells stably expressing the *NS1* gene (MF6 cells) and the *NS1* and *NS2A* genes (3G8 cells). Cells were fixed without permeabilization and incubated with JE-2D5 and fluorescein isothiocyanate conjugate to stain their surfaces. Since MF6 cells stained more strongly than 3G8 cells (data not shown), we selected MF6 cells for the antigens in the CDC assay. The stronger surface staining of cells expressing the *NS1* gene alone than of those expressing the *NS1* and *NS2A* genes was consistent with a report that examined the expression of JEV NS1 on

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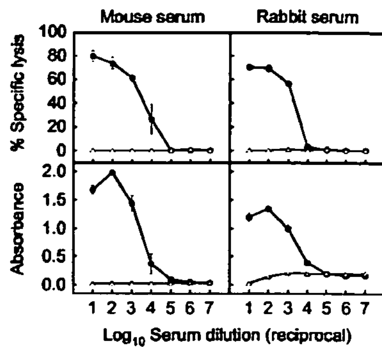

FIG. 1. Comparison between the CDC assay and ELISA for measuring antibodies to JEV NS1 in mouse and rabbit sera. Percentages of specific cell lysis in the CDC assay (upper graphs) and ELISA absorbances (lower graphs) were obtained with serial 10-fold dilutions of hyperimmune (closed circles) or nonimmune (open triangles) serum from a mouse (left graphs) or rabbit (right graphs). Each datum represents an average obtained in two separate experiments (standard deviations are indicated by bars).

the surface of cells expressing the *NS1* gene with or without a part of the *NS2A* gene (16).

Preliminary evaluation of the CDC assay using HMS and HRS. The preliminary evaluation of the applicability of CDC for the antibody assay was done using hyperimmune animal sera. MF6 cells were incubated with serial 10-fold dilutions of HMS or HRS and then with complement. Following brief centrifugation, the supernatant was incubated with the LDH substrate. Nonimmune animal sera (NMS or NRS) were used as a control. For comparison, the same dilutions of these specimens were examined by an ELISA for measuring NS1 antibodies.

As shown in Fig. 1, high percentages of specific cell lysis (approximately 60 to 80%) were found in highly concentrated sera (dilutions of $1:10^1$ to $1:10^3$), which steeply declined in the dilution range between $1:10^3$ and $1:10^5$ in both HMS and HRS. In contrast, NMS and NRS showed 0.00% specific lysis even at high concentrations. The dose-response curves obtained from the CDC assay were essentially similar to those obtained by ELISA. The results indicated that the CDC mechanism can be used for antibody testing.

Evaluation of the CDC assay using sera from naturally infected horses. Since we had previously developed two methods, based on immunostaining (13) and ELISA (12), for measuring NS1 antibodies in horse sera, the CDC assay could be evaluated using horse sera with known NS1 antibody levels/titers.

First, we obtained the percentages of specific cell lysis using serial twofold dilutions of horse sera with different NS1 antibody levels as determined by ELISA (Fig. 2). The ELISA values for these sera were 1.362 (highly positive), 0.883 (moderately positive), 0.258 (weakly positive), and 0.058 (negative). The dose-response curves were consistent with those obtained with mouse and rabbit sera, as shown in Fig. 1. Specifically, highly and moderately positive sera showed high percentages of specific lysis at high concentrations followed by steep declines, whereas the negative serum did not show detectable levels of specific lysis even at the highest concentration. The steep declines in the dose-response curves were nearly parallel

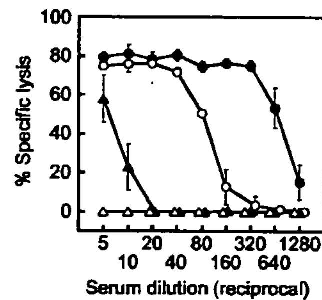

FIG. 2. Percentages of specific cell lysis obtained with serial twofold dilutions of highly positive (closed circles), moderately positive (open circles), weakly positive (closed triangles), and negative (open triangles) horse sera as determined by the CDC assay for measuring antibodies to JEV NS1. Each datum represents an average obtained in two separate experiments (standard deviations are indicated by bars).

in three positive samples and shifted to the right with increasing ELISA antibody levels. For sera showing percentages of specific lysis between the minimum (0%) and maximum (75 to 81%) values, higher percentages of specific lysis were shown for sera with higher ELISA antibody levels at any dilution point. These results indicated that the CDC assay also can measure NS1 antibody levels in equine sera and that the results can be expressed by both the one-dilution and the endpoint methods.

Second, we used 40 horse sera for comparisons of results obtained by the one-dilution and endpoint methods (Fig. 3). Since the weakly positive serum used in the above-described experiment did not show detectable specific lysis at a dilution of 1:20 (Fig. 2), we tentatively used a dilution of 1:10 in the one-dilution method. The cutoff value differentiating positive from negative samples was obtained with negative control sera from 42 horses that were negative for NS1 antibodies in ELISA, aged 1 to 4 years, and born and kept in a nonepizootic area of northern Japan (Hokkaido Island). Most samples (40 of 42 samples; 95.2%) showed 0.00% specific lysis, while the other two showed 1.57 and 6.40%, respectively, at a dilution of 1:10 (data not shown); the mean percentage of specific lysis

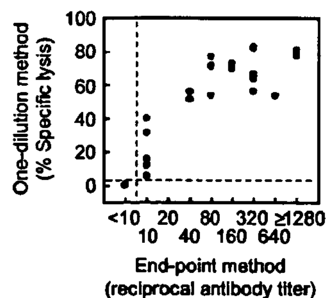

FIG. 3. Comparison between the one-dilution and endpoint methods of the CDC assay for measuring antibodies to JEV NS1 in horse sera. Percentages of specific cell lysis obtained by the one-dilution method were compared to NS1 antibody titers obtained by the endpoint method. Dotted lines indicate cutoff values for differentiating positive from negative samples. In the one-dilution method, 4.32% specific cell lysis was calculated from the averages and standard deviations obtained with negative samples; in the endpoint method, titers of 1:10 or higher were determined to be positive.

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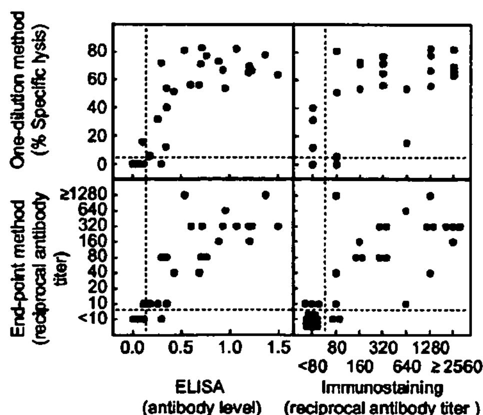

FIG. 4. Comparison of the CDC assay to two previously developed methods for measuring JEV NS1 antibodies in horse sera. Percentages of specific cell lysis shown by the one-dilution method (upper graphs) and NS1 antibody titers shown by the endpoint method (lower graphs) obtained in the present study using the CDC assay were compared to NS1 antibody levels shown by ELISA (left graphs) or NS1 antibody titers shown by the immunostaining method (right graphs), which were obtained in our earlier studies (12, 13). Dotted lines indicate cutoff values for differentiating positive from negative samples. NS1 antibody levels/titers of 4.32% (the one-dilution method), 1:10 (the endpoint method), 0.122 (ELISA), and 1:80 (the immunostaining method) or higher were determined to be positive.

was 0.19%, and the standard deviation was 1.01%. In line with a method used to obtain the cutoff value in ELISA (12), the confidence limit at a probability level of 0.01% was used for the cutoff value in the one-dilution method and was calculated to be 4.32%. We also used this value for determining the NS1 antibody titer for the endpoint method: the NS1 antibody titer was expressed as the highest serum dilution providing greater than 4.32% specific lysis. Since a 1:10 dilution was used in the one-dilution method, serum samples showing NS1 antibody titers of 1:10 or higher were determined to be positive for NS1 antibodies by the endpoint method. As shown in Fig. 3, the percentages of specific lysis obtained by the one-dilution method significantly correlated with antibody titers obtained by the endpoint method, with a correlation coefficient of 0.933 ($P < 0.001$).

Third, we compared the CDC assay (both one-dilution and endpoint methods) to each of the two previously established methods of ELISA (12) and immunostaining (13). Quantitative comparisons (Fig. 4) provided significant correlation coefficients in all combinations between the one-dilution method and ELISA (0.848; $P < 0.001$) or immunostaining (0.784; $P <$ 0.001) and between the endpoint method and ELISA (0.884; $P < 0.001$) or immunostaining (0.799; $P < 0.001$). Higher correlation coefficients were shown with ELISA than with the immunostaining method, and the correlation coefficients of the endpoint method were higher than those of the one-dilution method. Additionally, qualitative comparisons (Table 1) showed high levels of agreement between the CDC assay and ELISA (38 of 40; 95.0%) or the CDC assay and the immunostaining method (35 of 40; 87.5%). Consistent with quantitative comparisons (Fig. 4), the CDC assay results showed higher agreement with those of the ELISA than did the immunostaining method (Table 1). We did not separate the results of the

TABLE 1. Qualitative comparison between the CDC assay and the ELISA or immunostaining method for measuring JEV NS1 antibodies in horse sera

CDC assay result	No. of sera with indicated result by:					
	ELISA			Immunostaining		
	Positive	Negative	Total	Positive	Negative	Total
Positive	22	1	23	20	3	23
Negative	1	16	17	2	15	17
Total	23	17	40	22	18	40

qualitative comparison by one-dilution and endpoint methods, since the qualitative agreement between these methods was 100% (Fig. 3). The sensitivity and specificity of the CDC assay were, respectively, 95.7% (22 of 23) and 94.1% (16 of 17) against ELISA and 90.9% (20 of 22) and 83.3% (15 of 18) against the immunostaining method (Table 1). These results indicated that the CDC assay correctly measured NS1 antibodies in horse sera.

Finally, NS1 antibody titers obtained by the endpoint method of the CDC assay were compared to HAI antibody titers, using 40 sera used in the experiments shown in Fig. 3 and 4 and Table 1; HAI antibody titers of these sera were previously determined (13). Although racehorses in Japan are vaccinated with inactivated JE vaccine every year, it is considered that natural infections inducing NS1 antibodies increase HAI antibody titers. As shown in Fig. 5, a significant correlation was obtained between NS1 and HAI antibodies, with a correlation coefficient of 0.711 ($P < 0.001$). All sera positive for NS1 antibodies were positive for HAI antibodies, while all of the sera negative for HAI antibodies were negative for NS1 antibodies. These results suggested that the increase in NS1 antibody titers as determined by the CDC assay was accompanied by an increase in HAI antibody titers.

In addition, five repeated antibody titrations by the endpoint method using highly and moderately positive sera provided assay variations within just a twofold range (data not shown), indicating the reproducibility of the CDC assay.

Evaluation of the CDC assay using sera from experimentally infected mice. To evaluate the CDC assay for its ability to detect antibodies in an early phase of infection, we measured NS1 antibody levels/titers in sera successively collected from

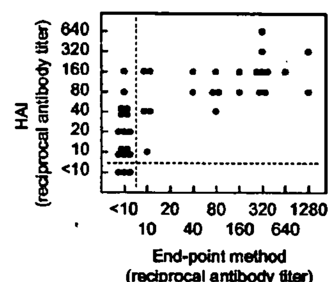

FIG. 5. Comparison between the CDC assay (endpoint method) and HAI test. NS1 antibody titers shown by the endpoint method obtained in the present study were compared to HAI antibody titers obtained in our earlier studies (13). Dotted lines indicate cutoff values for differentiating positive from negative samples for both tests.

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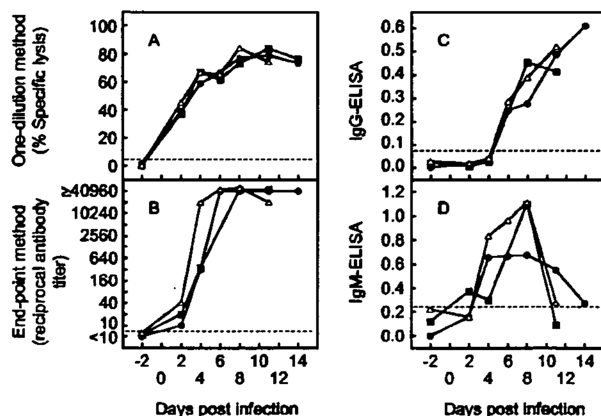

FIG. 6. Time course of NS1 antibody levels/titers in mice infected with JEV. Sera were examined by the one-dilution (A) or endpoint (B) method of the CDC assay and ELISA for measuring IgG (C) or IgM (D) antibodies. The sera used in this experiment were collected in a previous study (15), in which five mice in one group were immunized with 100 μg (closed circles) or 10 μg (closed squares) of pcJEME or were inoculated with PBS (open triangles), followed by infection with JEV. Because of the small volumes of stored sera, sera pooled from the five mice were used for this experiment. Sera were not available from PBS-inoculated mice on day 14, since the mice died of JEV infection. The volume of stored sera from mice immunized with 10 μg of pcJEME on days 6 and 14 was not enough for examination by all assay methods. For ELISA, the antigen amount used for sensitization was 3 or 20 ng/well to measure IgG or IgM antibodies, respectively, and test sera were used at a 1:200 dilution. Dotted lines indicate cutoff values to differentiate positive from negative samples. Antibody levels/titers of 5% (the one-dilution method), 1:10 (the endpoint method), 0.076 (IgG ELISA), and 0.217 (IgM ELISA) or higher were determined to be positive (see the text for details).

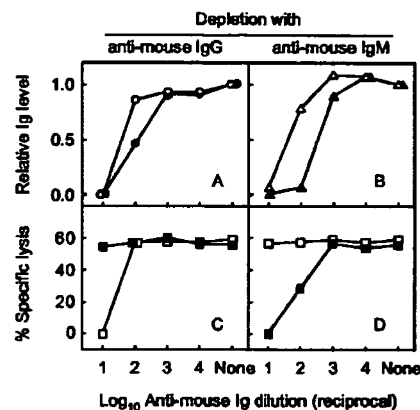

FIG. 7. Percentages of specific cell lysis obtained with the day-4 serum or HMS depleted of IgG or IgM as determined by the one-dilution method of the CDC assay. Serum was incubated with anti-mouse IgG or IgM and examined for depletion by a sandwich ELISA for measuring IgG (A) or IgM (B) levels and for percentages of specific lysis in sera depleted of IgG (C) or IgM (D). IgG levels obtained from the day-4 serum (closed circles) or HMS (open circles), IgM levels obtained from the day-4 serum (closed triangles) or HMS (open triangles), and percentages of specific lysis obtained from the IgG/IgM-depleted day-4 sera (closed squares) or HMS (open squares) are shown. The abscissa indicates dilutions of anti-mouse IgG or IgM used for depletion. None indicates a control in which sera were incubated with PBS in place of diluted anti-mouse IgG or IgM. The ordinate indicates relative IgG or IgM levels contained in depleted serum as determined by a sandwich ELISA (upper graphs) or percentages of specific lysis obtained with depleted serum by the CDC assay (lower graphs).

mice after experimental infection with JEV (Fig. 6). These sera were the same as those obtained in our earlier study to evaluate a candidate vaccine that did not contain or express NS1 antigens (15); thus, sera collected following viral infection could be used to show increases in NS1 antibody levels/titers. There were three groups of mice, one immunized with 100 μg of pcJEME, one immunized with 10 μg of pcJEME, and one inoculated with PBS, after which all three groups were infected with JEV. Sera pooled from five mice in each group were evaluated. As a reference, these pooled sera were tested by ELISA to measure IgG and IgM class antibodies to NS1. Cutoff values differentiating positive from negative samples were obtained using sera from 10 naïve mice. Since all of the sera showed 0.00% specific lysis, we set the arbitrary cutoff value as 5% for the CDC assay. For ELISA, 0.076 and 0.217 cutoff values were calculated for IgG and IgM antibodies, respectively, from the averages (plus two times the standard deviation) of ELISA antibody levels obtained with the naive mouse sera.

NS1 antibodies were detectable by the CDC assay in sera 2 days after infection in all groups of mice (Fig. 6). Percentages of specific lysis obtained by the one-dilution method increased sharply until day 4 and then increased gradually until day 8 in all groups. However, NS1 antibody titers obtained by the endpoint method increased to high levels by day 4 in the PBS-inoculated group and by day 6 or 8 in the two pcJEME-immunized groups. By ELISA, IgG antibodies were first detected on day 6 in all groups, whereas IgM antibodies were first detected on day 2 in one of the three groups and on day 4 in all three groups. These results suggested that the CDC assay can detect IgM antibodies.

Depletion experiments were performed to determine if the antibodies detected by the CDC assay in the early phase of infection were of the IgM class. Sera pooled from pcJEME (100 μg)-immunized mice 4 days after infection (hereafter termed day-4 serum) were incubated with serial 10-fold dilutions of anti-mouse IgG or IgM, centrifuged, and examined for Ig levels by a sandwich ELISA and for NS1 antibodies by the CDC assay (Fig. 7). As a reference, HMS was examined in parallel. The dose-dependent curve obtained from the sandwich ELISA indicated that IgG or IgM fractions were almost completely depleted from the day-4 serum and HMS by incubation with anti-mouse IgG or IgM at a 1:10 dilution (Fig. 7A, B). Under this condition, the day-4 serum depleted of IgM did not show any specific cell lysis (Fig. 7D), whereas the day-4 serum depleted of IgG showed a percentage of specific cell lysis similar to that shown by the nondepleted control (Fig. 7C). In contrast, depleted HMS showed the opposite pattern: no detectable specific lysis was shown by depletion of IgG (Fig. 7C), but depletion of IgM did not change the value (Fig. 7D). As controls, anti-mouse IgG or IgM used for depletion showed 0.00% specific lysis at a 1:10 dilution. These results indicated that the CDC assay can detect IgM class NS1 antibodies.

No detectable nonspecific reactions against natural IgM antibodies. The results depicted in Fig. 6 show that ELISA was able to detect IgM antibodies in only one of the three groups

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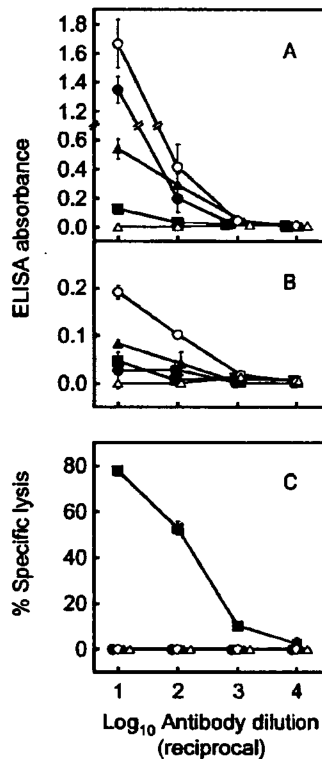

FIG. 8. Comparison of ELISA (A and B) to the CDC assay (C) using natural IgM antibodies. The ELISA results show absorbances obtained with antigen-sensitized wells (A) and differences from those obtained with nonsensitized wells (B). Natural antibodies were obtained in an ascites form from mice inoculated with hybridoma clones 1D12 (closed circles), 5A5 (open circles), and 6A11 (closed triangles), as well as with control P3U1 cells (open triangles). For comparison, the day-4 serum (closed squares) was used. Each datum represents an average obtained in two separate experiments (standard deviations are indicated by bars).

on day 2, whereas the CDC assay determined that all three groups were positive. A probable factor affecting the ELISA result is the natural IgM antibodies that are contained in almost all sera (6). To investigate the effects of the natural IgM antibodies on the assay results, three monoclonal antibodies generated from naïve mice, as well as the day-4 serum, were examined for NS1 antibodies by the CDC assay and ELISA (Fig. 8). In Fig. 8, ELISA absorbances obtained only with antigen-sensitized wells, which more effectively exhibit net reactivities caused by natural antibodies (Fig. 8A), are shown, as well as the absorbance differences from nonsensitized wells (Fig. 8B).

All three monoclonal antibodies showed ELISA reactions at higher levels than did the day-4 serum at dilutions of $1:10^1$ and $1:10^2$ in antigen-sensitized wells (Fig. 8A), whereas these monoclonal antibodies did not show any specific lysis. This is in contrast to the day-4 serum, which showed high percentages of specific lysis (Fig. 8C). Subtraction of absorbances obtained with nonsensitized wells from those obtained with antigen-sensitized wells still showed positive values (Fig. 8B). Ascites from P3U1-inoculated mice used as controls did not show any reactions on either ELISA or the CDC assay. These results clearly indicated that the CDC assay detects specific, but not natural, IgM antibodies.

DISCUSSION

The present study demonstrated that the antibody-mediated complement-dependent cytotoxicity mechanism can be used for measuring low levels of antibodies to JEV NS1 induced in naturally infected horses or during the early phase of experimental infection in mice. Specific NS1 antibodies, when present in a test sample, may bind to the NS1 antigen expressed on the surface of MF6 cells. The antigen-antibody complex may induce complement activation, and the final product (the C5b-9 membrane attack complex) may lyse the cells. Thus, the LDH activity released into the medium represents the level of NS1 antibodies in the test sample. In the present experiments, the percentages of specific cell lysis depended on the level of dilution of the serum samples. Quantitative and qualitative comparisons using horse sera indicated that the CDC assay correlated and agreed with two different previously established methods for measuring NS1 antibodies.

Almost all sera from nonimmune animals (10 of 10 mice and 40 of 42 horses) showed no nonspecific cell lysis in the CDC assay, at least at the dilution used for the one-dilution method (1:10). In addition, monoclonal antibodies exhibiting a low-affinity and multireactive nature (natural antibodies) did not show any detectable specific lysis. CDC is considered a specific host immune mechanism against viral infections (24), and here it seems to have brought that high specificity to the antibody assays. That is, the CDC assay established in the present study for measuring NS1 antibodies is based on the ability of the complement to differentiate specific from nonspecific antigen-antibody reactions. The specificity of the assay system increases the reliability for measurements of low levels of antibodies.

It has been reported that CDC is induced by antibodies of the IgM class as well as of the IgG class (1, 18). The assay method developed here, which utilized the principle of CDC, measured specific IgM antibodies with consistency, suggesting that this CDC assay can be applied to early serodiagnoses. Experiments using sera successively collected from mice after infection indicated that, on day 2, all three of the pooled sera showed detectable specific lysis, but only one showed a positive result in the ELISA that measured IgM antibodies. The basic reason for this difference is the level of the cutoff value. More precisely, nonspecific reactions were completely blocked in all nonimmune mouse sera in the CDC assay, whereas some levels of nonspecific reactions provided by nonimmune mouse sera in ELISA increased the cutoff value, which was calculated from their average values and the standard deviations.

Subtraction of the absorbances obtained with nonsensitized wells from those obtained with antigen-sensitized wells generally has been the method used to minimize nonspecific reactions occurring in ELISA. The ELISA results shown in Fig. 8A are absorbances obtained only with antigen-sensitized wells, which decreased when those obtained with nonsensitized wells were subtracted (Fig. 8B). However, even when such a subtraction method is used, the experimental variations shown in both antigen-sensitized and nonsensitized wells lead to positive values, resulting in relatively high cutoff values for ELISA, as shown in Fig. 6. However, the CDC assay detects specific but

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not natural IgM antibodies, making it a more reliable testing method for detecting low levels of IgM antibodies in an early phase of infection. An advantage of the CDC assay is that it can measure both IgG and IgM antibodies at the same time; a disadvantage is that IgM antibodies are not differentiated from IgG ones. However, a simple depletion of IgG or IgM from the serum, for example, by incubation with anti-IgM or anti-IgG at a 1:10 dilution, as performed in the present study (Fig. 7), can overcome this disadvantage.

The CDC assay presented here has some other advantages over the two previously established methods for measuring NS1 antibodies, immunostaining (13, 14) and ELISA (12). Since the results are obtained as numerals, the CDC assay is more objective than the immunostaining method; this is probably the reason for the finding that the CDC assay was more significantly correlated and consistent with ELISA than with the immunostaining method (Fig. 4, Table 1). Unlike the procedure for ELISA, the CDC assay does have a cumbersome element in its procedure, since the method requires live cells. However, the number of incubation steps is smaller for the CDC assay (three steps) than the ELISA (five steps), and the time period required for the CDC assay (3 to 4 h) is shorter than that for the ELISA (approximately 5 h). Thus, this procedure is considered suitable for testing large numbers of samples in a limited time period. We are currently undertaking a survey of JEV NS1 antibodies from recently collected equine sera and also are investigating the establishment of a CDC assay to detect NS1 antibodies in human sera.

Reliable measurements of low levels of specific antibodies greatly contribute to diagnoses of infectious diseases, such as those for identifying asymptomatic infections for safety tests of donated blood samples and for the early screening of infections with teratogenic agents in pregnant women. Theoretically, the newly developed method utilizing complement-dependent cytotoxicity can measure antibodies in any animal species by using the same test reagents, thus being useful for applications in both medical and veterinary fields.

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REVIEW ARTICLE

A comparative epidemiological study of hantavirus infection in Japan and Far East Russia

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Abstract

Hantaviruses are causative agents of some severe human illnesses, including hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The viruses are maintained by rodent hosts, and humans acquire infection by inhaling virus-contaminated excreta from infected animals. To examine the epidemiology of hantavirus infections in Japan and Far East Russia, we conducted epidemiological surveys in these regions. In Japan, anti-hantavirus antibodies were found in four rodent species, *Clethrionomys rufocanus, Rattus norvegicus, R. rattus,* and *Apodemus speciosus.* Although no new HFRS cases have been officially reported over the past 20 years in Japan, one member of the Japan Ground Self-Defense Force did test positive for hantavirus antibody. Repeated surveys in Far East Russia have revealed that two distinct hantavirus types cause severe HFRS in this region. Hantavirus sequences identified from *A. peninsulae,* fetal HFRS cases in Vladivostok, and Amur virus are highly similar to each other (>92% identity), but they are less similar (~84% identity) to the prototypical Hantaan virus, which is carried by *A. agrarius.* Phylogenetic analysis also indicates that Amur and *A. peninsulae*-associated viruses are distinct from Hantaan virus, suggesting that *A. peninsulae* is the reservoir animal for Amur virus,

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which causes severe HFRS. From HFRS patients in the Khabarovsk region, we identified viruses with nucleotide sequences that are more similar to Far East virus (>96%identity) than to the Hantaan (88-89%identity) or Amur (81-83%identity) viruses. Phylogenetic analysis also indicates that the viruses from Khabarovsk HFRS patients are closely related to the Far East virus, and distinct from Amur virus.

Key Words : Hantavirus, rodent, epidemiology, epizootiology, zoonosis

Introduction

Hantaviruses are known causative agents of two human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The animal reservoirs of hantaviruses are various rodent species, which, when infected, are asymptomatic and carry the virus for long periods. Humans acquire hantavirus infection by inhaling virus-containing excreta from infected animals.

Hantaviruses belong to the genus *Hantavirus* of the family *Bunyaviridae*, are enveloped, and contain three single-stranded antisense RNA segments. The large (L), medium (M), and small (S) RNA segments encode a viral polymerase, surface glycoproteins G1 and G2, and a nucleocapsid protein (NP), respectively[6,34,35,38]. More than 20 serotypes or genotypes of hantaviruses have been reported, and each of these viruses has a specific rodent reservoir (although one hantavirus strain has been isolated from the insectivore *Suncus murinus*)[36]. Because the phylogenies of the viruses and their reservoir rodents are topologically identical, hantaviruses and rodents are generally believed to have coevolved[39]. The emergence of human hantavirus infections may be a result of changes in both ecological factors and human activities. The most important risk factor is close contact with rodents as a result of agricultural, forestry, or military activities[16].

HFRS is caused by Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV) in Eurasia ; these viruses are carried by *Apodemus agrarius, Rattus norvegicus and R. rattus, Clethrionomys glareolus,* and *A. flavicollis,* respectively[5]. Recently, Saaremaa virus (SAAV) was identified as the causative agent of a mild form of HFRS in Europe, and *A. agrarius* was found to be the carrier[15,20]. Serological evidence indicates that human SAAV infections occur in Estonia[7], and SAAV may have been the cause of an HFRS outbreak in Russia in the 1990s[26]. On the American continent, HPS is caused by Sin Nombre virus (SNV), New York virus (NYV), Black Creek Canal virus (BCCV), Bayou virus (BAYV), and Andes virus (ANDV), which are carried by *Peromyscus maniculatus, P. leucopus, Sigmodon hispidus, Oryzomys palustris,* and *Oligoryzomys longicaudatus,* respectively[23,24,30].

About 200,000 HFRS cases are reported annually throughout the world[15]. A wide variety of hantaviruses responsible for HRFS have been found in East Asia[33]. About 50,000 to 100,000 HFRS cases are reported annually in China, where HTNV and SEOV are responsible for most of the cases[32]. In addition, about 100 to 200 HFRS patients are reported annually in Far East Russia, a well-known endemic area for HFRS. Recently, a distinct type of hantavirus, Amur virus (AMRV), was identified in HFRS patients in Far East Russia[40]. Our previous studies revealed that *A.*

peninsulae is the reservoir animal for AMRV, and revealed antigenic and genetic evidence of a distinct hantavirus serotype[17,18]. Furthermore, Khabarovsk and Vladivostok viruses have also been identified in the same region[9,11].

Two outbreaks of HFRS have occurred in Japan since the 1960s. One outbreak was reported in the Umeda district of Osaka in the 1960s[4,6]. The source of the infection is believed to have been urban rats (*R. norvegicus*). The other outbreak was reported in various Japanese animal facilities between 1970 and 1984, and the human infections were related to contact with laboratory rats (*R. norvegicus*)[17,19]. No new HFRS cases have been reported since 1985, although seropositive *R. norvegicus* specimens have been identified in ports and reclaimed areas in various locations throughout the country[3]. In addition, Puumala-related viruses are widely distributed in *C. rufocanus* on Hokkaido, the northern-most major island of Japan[10,11].

Although we recently identified anti-hantavirus antibodies among patients with hepatitis of unknown etiology in Japan, the prevalence of the antibody is very low in the general population[20]. Why Japan has relatively few HFRS patients, in spite of the highly endemic nature of the disease in the surrounding countries, is unknown. To investigate this issue, a large-scale epizootiological study targeting indigenous rodents was essential. Therefore, we carried out epizootiological surveys in rodents from various areas of Japan, including the four major islands (Hokkaido, Honshu, Shikoku, and Kyushu), to determine the endemic areas and the reservoir animals. In addition, we carried out epidemiological surveys in Vladivostok and Khabarovsk to examine the ecology of hantaviruses in Far East Russia, a highly endemic area of HFRS.

Epidemiology of hantavirus infection in Japan

Rodent epidemiology

Despite its location amidst countries endemic for HFRS, Japan has not had a reported case of HFRS for about twenty years. During this period, however, anti-hantavirus antibodies have been detected in *R. norvegicus* specimens captured in various Japanese ports. Our previous epizootiological surveys revealed that 10% of *C. rufocanus* specimens on Hokkaido have anti-hantavirus antibodies, and this species carried PUUV-related viruses[10,11].

Epizootiological studies of hantavirus infection among wild rodents were conducted in various locations in Japan, including the four major islands (Honshu, Kyushu, Shikoku, and Hokkaido), from 2000 to 2003. A total of 806 rodents and insectivores were captured from 11 wild settings on Honshu, Shikoku, Kyushu, and Tsushima Islands, and from six sites on Hokkaido. The geographical locations of the survey sites are shown in Figure 1. The sera were screened for anti-hantavirus antibodies using immunofluorescent antibody assay (IFA).

Serum samples from a total of 592 rodents collected from wild settings in the southern regions of Japan, including Honshu, Shikoku, Kyushu, and Tsushima Islands, were screened for antibodies to HTNV, SEOV, and PUUV. Seropositive animals were detected in Toyama and Shimane (Table 1). Of 471 *A. speciosus* individuals, five (1.1%) were seropositive by IFA. Two *R. norvegicus* individuals (5.1%) from Toyama were positive for SEOV. No antibodies to PUUV were detected in any rodent species from the southern region of Japan. Some of the IFA-positive sera from *A. speciosus* neutralized HTNV (1 : 20), but not SEOV. Using RT-PCR, we attempted

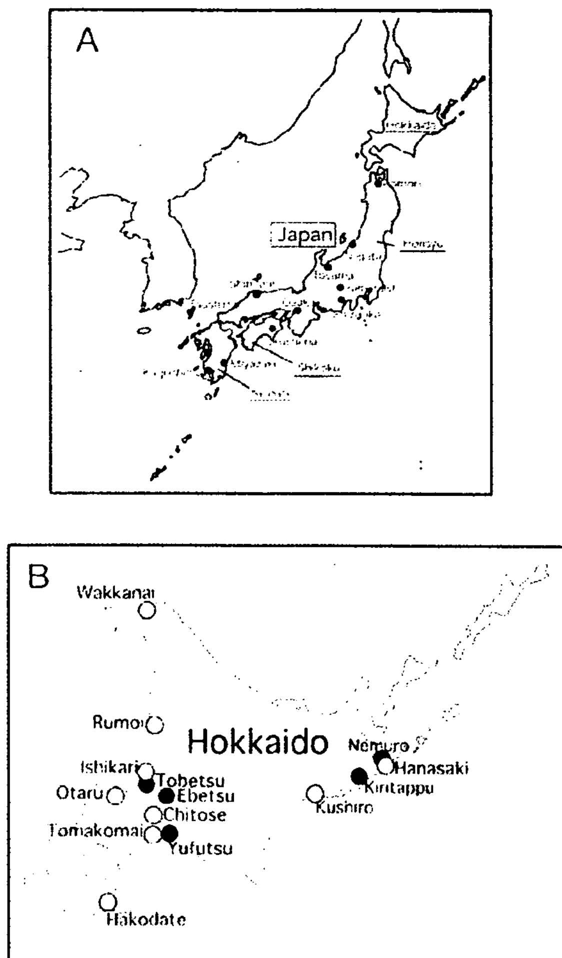

Fig. 1. Geographical location of epizootiological survey sites in Japan. A: Surveys were carried out in Aomori, Niigata, Toyama, Yamanashi, Shizuoka, Osaka, Tokushima, Shimane, Tsushima, Miyazaki, and Kagoshima prefectures. B: Surveys were carried out in Wakkani, Rumoi, Ishikari, Otaru, Tomakomai, Hakodate, Tobetsu, Ebetsu, Chitose, Yufutsu, Kushiro, Kiritappu, Hanasaki, and Nemuro on Hokkaido, the northernmost main island of Japan. Survey sites were (○) seaports and airports or (●) wild settings such as forests.

to detect viral genes in seropositive *A. speciosus,* but none of the samples produced virus-specific PCR product bands (data not shown).

In contrast, of the 214 animals captured in wild settings in Hokkaido, five out of the 153 (3.26%) *C. rufocanus* individuals from Nemuro, Ebetsu, and Tobetsu were found to be seropositive (Table 2). No other rodent species captured in wild settings had detectable anti-hantavirus antibodies. In rodents captured in urban or semi-urban settings (ports and a Hokkaido airport), 4.5% (2/44) of *C. rufocanus,* 0.62% (2/321) of *R. norvegicus,* and 6.7% (3/45) of *R. rattus individuals* were seropositive (Table 3). *C. rufocanus* from both wild settings and the Chitose Airport had anti-PUUV IFA titers ranging from 1:32 to 1:128 and lower or undetectable anti-HTNV titers. Seropositive *R. norvegicus* and *R. rattus* were found in the port areas of Rumoi, Otaru, and Hakodate. The anti-SEOV and-HTNV IFA titers in these samples were almost equivalent, but antibodies to PUUV were undetectable.

We detected seropositive specimens of *A. speciosus* (5/482, 1.0%), *R. norvegicus* (4/364, 1.1%), *R. rattus* (3/45, 6.7%), and *C. rufocanus* (7/197, 3.6%) among 1,221 animals captured in various areas and settings of Japan. These four rodent species may play important roles as reservoir animals of hantavirus in Japan.

Further epizootiological surveys should be conducted to determine the type of hantavirus carried by *A. speciosus*. Furthermore, since seropositive *R. rattus* and *R. norvegicus* found in seaports and at the Chitose airport could be sources of human SEOV infection, their presence could pose a threat to people working in these facilities, to travelers, and to quarantine office employees. A higher seroprevalence was reported in workers employed in a reclaimed area where seropositive urban rats were detected [34]. Therefore, a larger-scale epidemiological study of hantavirus infection among people associated with the seaports and airports in Japan is warranted.

Hantavirus infection in humans

To examine the epidemiology of human