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Antibody-Mediated Enhancement of Dengue Virus Infection in Mouse Macrophage Cell Lines, Mk1 and Mm1 (41802)

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Abstract. Antibody-mediated enhancement of dengue type 2 virus (D2V) replication in murine macrophage cell lines (Mk1 and Mm1) was studied. While both Mk1 and Mm1 supported D2V replication in the absence of enhancing antibodies, virus production was enhanced when both cell lines were inoculated with D2V in the presence of dengue type 1 virus (D1V)-hyperimmune rabbit IgG, D1V-hyperimmune mouse ascitic fluids, or D2V-hyperimmune mouse ascitic fluids at subneutralizing concentrations. The enhancement ratios were greater in Mk1 than in Mm1.

Type-specific neutralizing monoclonal anti-D2V antibody also mediated D2V replication enhancement in Mk1 to the same extent as mediated by three other enhancing antibodies described above. In contrast, however, the same monoclonal antibody mediated only a slight and smaller magnitude of D2V replication enhancement in Mm1 than did the other enhancing antibodies.

Fluorescent antibody observations revealed that virus replication enhancement in both Mk1 and Mm1 was due primarily to an increase in the numbers of virus-infected cells. D2V infection enhancement in Mk1 by the anti-D2V mouse ascitic fluids at a dilution showing nearly 50% plaque-reduction activity was markedly suppressed by addition of complement to the inocula, whereas that by the monoclonal antibody, which has been identified as mouse IgG1, was not.

Phagocytoses of tritiated thymidine-labeled bacteria by Mk1 and Mm1 were also enhanced when the bacteria had been opsonized with antibody. The phagocytosis enhancement ratios were again greater in Mk1 than in Mm1.

It has been known that dengue virus multiplies in peripheral blood monocytes, a member of the mononuclear phagocyte system (1), of humans and monkeys, and that the virus permissiveness of the cells as well as that of human monocyte cell line cells are enhanced when those cells are inoculated with dengue virus in the presence of non-neutralizing, cross-reactive antibodies (2–8). The virus replication enhancement is undoubtedly interesting from biological and virological viewpoints, and may play an important role in the pathogenesis of severe dengue infection such as dengue hemorrhagic fever and shock syndrome.

In our previous studies (9, 10), a possibility was pointed out that mononuclear phagocytes in athymic nude mice supported dengue virus replication *in vivo*. We have further shown that peritoneal macrophage cultures obtained from dengue type 1 virus (D1V)-immune mice showed increased permissiveness to dengue type 2 virus (D2V), producing larger amounts of virus than did those from nonimmune control mice (11, 12).

More recently, we found that D2V multiplied in two murine macrophage cell lines, namely Mk1 and Mm1 (13, 14), both of which were derived from the same parent leukemic cell line. In the present study, we investigated D2V infection enhancement in Mk1 and Mm1 by enhancing antibodies including type-specific neutralizing monoclonal anti-D2V antibody.

Materials and Methods. *Virus.* Dengue type 2 virus (D2V; Trinidad 1751 strain), in the form of supernatant from a 10% infected mouse brain emulsion in Eagle's minimum essential medium supplemented with 4% heat-inactivated calf serum unless otherwise stated, was used in virus replication experiments as well as for immunizing mice to obtain virus replication-enhancing antibody as described below. Dengue type 1 virus (D1V; Mochizuki strain), either prepared in the same manner as D2V or purified by sucrose density gradient centrifugation (15), was used for obtaining enhancing antibodies.

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Virus replication-enhancing antibodies. (i) D1V-hyperimmune rabbit IgG. Serum obtained from a rabbit hyperimmunized with purified D1V was treated with 33% saturated ammonium sulfate to precipitate crude IgG fraction. The precipitate was dissolved in phosphate-buffered saline (PBS; free of Ca^{2+} and Mg^{2+}), and was dialyzed with large amounts of PBS (pH 7.2). (ii) D1V-hyperimmune mouse ascitic fluids (D1V-HMAF) were prepared by the method of Cardiff *et al.* (16). Briefly, ICR mice were injected with D1V on Days 1, 3, 25, and 28. On the same day of the last immunization, Ehrlich ascitic carcinoma cells were inoculated into the peritoneal cavity of the mice to induce ascites. After about 10 days, the ascites obtained were centrifuged and the supernatants were heated at 56°C for 30 min. (iii) D2V-HMAF were prepared in the same manner as in preparing D1V-HMAF except for using D2V. (iv) Monoclonal anti-D2V antibody. A hybridoma cell line (3H5) (17, 18) producing type-specific neutralizing monoclonal anti-D2V antibody was kindly supplied by Dr. W. E. Brandt of the Walter Reed Army Institute of Research, Washington, D.C. The hybridoma cells were inoculated intraperitoneally into BALB/c mice which had received 0.5 ml of Pristane (2,6,10,14-tetramethyl-pentadecane; Wako Pure Chemical Industries, Ltd., Osaka, Japan) 5 days before the cell inoculation. After 12 days, ascites fluids were centrifuged and the supernatants were heated at 56°C for 30 min. The monoclonal antibody has been identified as murine IgG1 (17).

The 50% plaque-reduction-neutralizing titers ($PRNT_{50}$) of the antibody preparations against D2V and D1V, and dilutions optimum for D2V infection enhancement determined by a representative experiment are summarized in Table I.

Cell cultures. Mk1 and Mm1 cells, kindly supplied by Dr. T. Masuda of the Institute for Immunology, Faculty of Medicine, Kyoto University, Kyoto, Japan (13, 14), were maintained in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal calf serum. Both Mk1 and Mm1 were seeded at concentrations of $0.5–1.0 \times 10^5$ cells per well of a plastic tissue-culture plate (Falcon 3047, 24 flat-bottom wells, 16 mm in diameter, Becton Dickinson & Co., Oxnard, Calif.). When the seeded cells formed subconfluent monolayers on the next day, D2V was inoculated into the cultures. According to our experience, cultures of these cells too densely populated were apparently unsuitable, yielding smaller amounts of virus in culture fluids than those of the cultures as indicated above.

Inoculation of D2V to Mk1 and Mm1. One day after the cell seeding, Mk1 and Mm1 were inoculated with D2V at a multiplicity of infection of 5 plaque-forming units (PFU) per cell, either in the presence or absence of the enhancing antibodies described above. After cell–virus adsorption at 35°C for 90 min, the cells were washed with Hanks' balanced salt solution (HBSS) to remove unadsorbed virus, and the complete medium was added to the cultures. Sampling of culture fluids was periodically done, and the samples were titrated for virus infectivity by a plaque method using BHK-21 monolayer cultures (11, 12, 19).

Treatment of Mk1 and Mm1 with trypsin. Mk1 and Mm1 were treated with 0.1% trypsin in HBSS at 36°C for 30 min before D2V inoculation, in order to examine effects of trypsin on virus receptors of those cells (6).

Fluorescent antibody (FA) observations. Mk1 and Mm1 were inoculated with D2V as described above. Three days after infection in experiments with Mk1, and 2 days after infection with Mm1, the cultured cells were scraped off by a rubber policeman, suspended in small amounts of PBS, and smeared on a slide glass. FA observations were done using the monoclonal anti-D2V antibody (3H5) and fluorescent isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Medical and Biological Lab. Ltd., Nagoya, Japan). It was confirmed that uninfected control cells were negative for the reaction.

Effects of complement on antibody-mediated D2V infection enhancement in Mk1. In this series of experiments, D2V had been passed once in BHK-21 cell cultures and supernatants of the infected culture media were used, because the supernatants of infected suckling mouse brain emulsion as a D2V preparation exerted complement-inhibitory effect. It was confirmed that the tissue culture-derived D2V preparation did not inhibit complement activity.

D2V, diluted antibodies and fresh guinea pig serum as a complement source (at a final

TABLE I. CHARACTERIZATION OF D2V REPLICATION-ENHANCING ANTIBODIES

Enhancing antibodies	$PRNT_{50}$ against: D2V	D1V	Dilution optimum for D2V replication enhancement
Anti-D1V rabbit IgG	15	400	80
Anti-D1V HMAF	<10	80	20
Anti-D2V HMAF	160	<10	2,000
Monoclonal anti-D2V (3H5)	1600	<10	20,000

dilution of 1:10; Kyokuto Seiyaku Industries, Co. Ltd., Tokyo, Japan) were mixed and incubated at 35°C for 60 min in order that complement-dependent neutralization of virus infectivity proceeded well. The mixtures were then inoculated to Mk1 and incubated again at 35°C for 90 min. After the cell–virus adsorption, the Mk1 cultures were washed three times with HBSS to remove unadsorbed virus and the complete medium was added to the cultures.

Phagocytoses of opsonized or unopsonized bacteria by Mk1 and Mm1. (i) Bacteria. *Staphylococcus epidermidis* was cultivated in brain–heart-infusion broth at 36°C for 18 hr, and heat-killed at 60°C for 60 min. (ii) Opsonizing antibody. ICR mice were injected intraperitoneally with heat-killed *S. epidermidis* (ca. 5×10^8 organisms per mouse) four times at weekly intervals. Seven days after the last immunization, sera were obtained and heated at 56°C for 30 min. Agglutinating titer of the serum samples used for test was 1:200. (iii) Labeling of *S. epidermidis* with [^{3}H]thymidine. The organisms were cultivated in the broth containing 1.0 or 10 μCi of [*methyl*-^{3}H]-thymidine (Amersham International Ltd., Buckinghamshire, England) per ml. After an overnight incubation at 36°C, the labeled bacteria were heat-killed at 60°C for 60 min and washed three times with PBS to remove unincorporated radioisotope. (iv) Phagocytoses by Mk1 and Mm1. The ^{3}H-labeled bacteria, either alone or mixed with the opsonizing antibody (at a final dilution of 1:20), were added to Mk1 and Mm1 which were cultured in a glass tube (13 mm × 100 mm) with a rubber stopper. After 60 min of incubation at 36°C, nonphagocytized bacteria were washed off with PBS, and 0.5 ml of 1 *N* KOH was added to dissolve the cultured cells. The dissolutions were mixed with Bray's solution (New England Nuclear, Boston, Mass.) and the radioactivities were measured by a liquid scintillation spectrophotometer (Beckman LS 9000).

Results. *Infection enhancement by anti-D1V rabbit IgG in cultures of Mk1 and Mm1.* Both Mk1 and Mm1, when inoculated with D2V even in the absence of enhancing antibodies, supported D2V replication, producing ca. 10^4 PFU/ml or more in culture fluids (Fig. 1). The D2V production was enhanced in both cell lines, when the cell cultures were inoculated with D2V in the presence of the anti-D1V rabbit IgG (Fig. 1). The enhancement ratios were greater in Mk1 (ca. 100-fold) than in Mm1 (ca. 10-fold).

Unlike human peripheral blood monocytes or another mouse macrophage-like cell line,

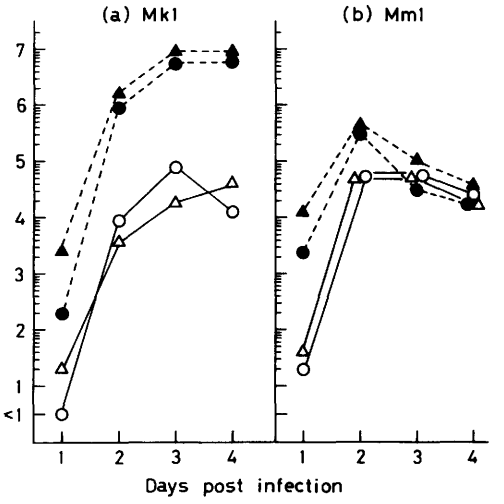

FIG. 1. Growth curves of D2V in Mk1 and Mm1. (a) Mk1 and (b) Mm1, either treated with 0.1% trypsin (▲, △) or untreated (●, ○), were inoculated with D2V in the presence (closed symbols) or absence (open symbols) of rabbit anti-D1V IgG.

$P388D_1$ ((6, 8) and Hotta *et al.*, manuscript submitted for publication), treatment of Mm1 with 0.1% trypsin did not reduce the D2V permissiveness in the absence of the enhancing antibodies.

Infection enhancement by type-specific neutralizing monoclonal anti-D2V antibody. Type-specific monoclonal antibody was compared with conventional polyclonal antibodies in the ability to enhance D2V replication in Mk1 and Mm1. The monoclonal antibody mediated more than 100-fold enhancement of D2V replication in Mk1, and the enhancement ratio was practically the same as those mediated by three other polyclonal conventional antibodies (Table II). In Mm1, however, the enhancement ratio mediated by the same monoclonal antibody was only 2-fold and smaller than those mediated by the other antibodies (Table II).

Infection efficiencies determined by the FA observations. Three or four days after infection, many atrophied cells were observed and numbers of viable cells apparently decreased in both Mk1 and Mm1 cultures inoculated with D2V in the presence of the enhancing antibodies. In cultures inoculated with D2V in the absence of the antibodies, however, the cell numbers constantly increased. These observations indicated that cytopathic effect (CPE) was brought about by D2V infection in both cell lines, and that infection efficiencies were comparatively low in the absence of the enhancing antibodies. In connection with the latter, it was shown in FA observations that only a few percent of the cultured cells were infected with D2V to produce viral antigens, and that the infection efficiencies of both Mk1 and Mm1 were enhanced when virus inocula contained the enhancing antibody (Table III).

In Mm1, increases in percentages of virus-infected cells by addition of the enhancing antibody were parallel with increases in virus yields in culture fluids (Table III). In Mk1, however, enhancement ratios determined by the FA tests were smaller than those in virus yields (Table III).

Effects of complement on antibody-mediated D2V infection enhancement in Mk1. The infection enhancement by the anti-D2V HMAF at a dilution showing nearly 50% plaque-reduction activity (1:200) was markedly suppressed when the inocula had been incubated with complement, although some enhancement was still observed at higher dilutions of the antibody (Table IV). In contrast, as had been expected from a fact that mouse IgG1 does not activate complement system via clas-

TABLE II. COMPARISON AMONG TYPE-SPECIFIC NEUTRALIZING MONOCLONAL ANTI-D2V ANTIBODY AND OTHER CONVENTIONAL ANTI-DENGUE ANTIBODIES IN THE ABILITY TO ENHANCE D2V REPLICATION IN Mk1 AND Mm1

Cells	Enhancing antibodies[a]	Virus yield[b]	
		Expt 1	Expt 2
Mk1	None	4.09 (1)[c]	3.84 ± 0.09[d] (1)
	Monoclonal anti-D2V	6.25 (144)	5.96 ± 0.07 (133)
	Anti-D2V HMAF	6.26 (148)	NT[e]
	Anti-D1V HMAF	6.08 (98)	NT
	Anti-D1V rabbit IgG	6.22 (136)	NT
Mm1	None	4.64 (1)	4.92 ± 0.03 (1)
	Monoclonal anti-D2V	4.99 (2.2)	5.20 ± 0.07 (1.9)
	Anti-D2V HMAF	5.45 (6.4)	NT
	Anti-D1V HMAF	5.42 (5.9)	NT
	Anti-D1V rabbit IgG	5.51 (7.3)	NT

[a] The cultured cells were inoculated with D2V in the presence or absence of the enhancing antibodies.
[b] Virus yield as expressed by Log_{10}(PFU/ml), on the third day of infection in Mk1 cultures, and the second day of infection in Mm1 cultures.
[c] Parenthesis indicates enhancement ratio.
[d] Mean value ± standard deviation of triplicate cultures.
[e] Not tested.

TABLE III. D2V INFECTION EFFICIENCIES IN CULTURES OF Mk1 AND Mm1 DETERMINED BY IMMUNOFLUORESCENCE

Cells	Trypsin treatment[a]	Enhancing antibody[b]	Percentage of FA-positive cells[c]	Log_{10}(PFU/ml)[d]
Mk1	−	−	1.6 ± 0.0[e] (1)[f]	2.63 (1)
	−	+	14.7 ± 1.5 (9.2)	4.73 (135)
	+	−	1.0 ± 0.9 (0.6)	2.10 (0.3)
	+	+	31.9 ± 2.0 (20.0)	5.34 (535)
Mm1	−	−	3.1 ± 0.4 (1)	3.83 (1)
	−	+	30.8 ± 3.5 (9.9)	4.95 (13.2)
	+	−	1.2 ± 0.3 (0.4)	3.87 (1.1)
	+	+	32.5 ± 3.9 (10.0)	4.83 (10.0)

[a] The cultured cells were treated with 0.1% trypsin just before D2V inoculation.
[b] The cultured cells were inoculated with D2V in the presence or absence of the anti-D1V rabbit IgG.
[c] D2V-infected cultures were submitted to the FA observations 3 days after infection in Mk1 experiments, and 2 days after infection in Mm1 experiments.
[d] Virus titers in culture fluids.
[e] Mean value ± standard deviation of duplicate cultures.
[f] Parenthesis indicates enhancement ratio.

sical pathway (20), addition of complement to inocula consisting of D2V and the monoclonal antibody (IgG1) even at a dilution showing nearly 50% plaque-reduction activity (1:2000) did not exert significant effect on the antibody-mediated D2V infection in Mk1 (Table IV).

Phagocytoses of opsonized or unopsonized S. epidermidis by Mk1 and Mm1. Phagocytic ability of Mk1 for unopsonized bacteria was much weaker than that of Mm1. When the bacteria had been opsonized with antibody, phagocytic activities were enhanced in both Mk1 and Mm1, and the phagocytosis enhancement ratios were again greater in Mk1 than in Mm1 (Table V). Trypsin-treated cells of both cell lines also showed antibody-mediated phagocytosis enhancement, although a tendency was noted that the enhancement ratio was a little smaller in the trypsin-treated Mk1 than in the untreated controls (Table V).

Discussion. In the present study, we demonstrated antibody-mediated enhancement of D2V replication in two murine macrophage cell lines, Mk1 and Mm1, both of which were derived from the same parent leukemic cell line. The enhancement ratios were greater in Mk1 than in Mm1. The D2V replication enhancement was mediated also by type-specific neutralizing monoclonal antibody in Mk1

TABLE IV. EFFECTS OF COMPLEMENT ON ANTIBODY-MEDIATED D2V INFECTION ENHANCEMENT IN Mk1

Enhancing antibodies[a]	Dilution of antibody	Virus yield[b]	
		−Complement	+Complement[c]
None	—	2.48 (1)[d]	2.00 (1)
Anti-D2V HMAF	1:20,000	5.99 (3267)	5.26 (1840)
	1:2,000	5.93 (2867)	4.88 (760)
	1:200	5.73 (1800)	2.97 (9)
Monoclonal anti-D2V	1:20,000	6.06 (3867)	5.68 (4800)
	1:2,000	5.95 (3000)	5.20 (1600)

[a] Mk1 cultures were inoculated with D2V in the presence or absence of the enhancing antibodies.
[b] Virus yield as expressed by Log_{10}(PFU/ml) on the third day of infection.
[c] Inocula consisting of D2V and the enhancing antibodies were incubated with complement at 35°C for 60 min before inoculated to Mk1.
[d] Parenthesis indicates enhancement ratio.

TABLE V. PHAGOCYTOSIS OF ANTIBODY-OPSONIZED OR UNOPSONIZED *S. epidermidis* BY Mk1 AND Mm1

Cells	Trypsin treatment[a]	Opsonization of bacteria[b]	cpm per culture	
			Expt 1[c]	Expt 2[d]
Mk1	−	−	Negligible	32.3 ± 9.5 (1)
	−	+	24.5 ± 1.4	527.0 ± 18.4 (16.3)
	+	−	Negligible	44.5 ± 17.7 (1.4)
	+	+	15.5 ± 3.5	265.5 ± 6.4 (8.2)
Mm1	−	−	29.0 ± 4.0[e] (1)[f]	369.5 ± 130.1
	−	+	101.0 ± 7.1 (3.5)	NT[g]
	+	−	20.5 ± 3.5 (0.7)	NT
	+	+	93.5 ± 17.7 (3.2)	NT

[a] The cultured cells were treated with 0.1% trypsin just before being incubated with [^{3}H]thymidine-labeled *S. epidermidis*.
[b] [^{3}H]Thymidine-labeled and heat-killed *S. epidermidis* were mixed with mouse anti-*S. epidermidis* antiserum.
[c] *Staphylococcus epidermidis* were labeled by incubation with 1 μCi of [^{3}H]thymidine/ml for 18 hr.
[d] *Staphylococcus epidermidis* were labeled by incubation with 10 μCi of [^{3}H]thymidine/ml for 18 hr.
[e] Mean value ± standard deviation of duplicate cultures.
[f] Parenthesis indicates enhancement ratio.
[g] Not tested.

cultures to the same extent as by conventional cross-reactive antibodies, whereas, in Mm1 cultures, the monoclonal antibody enhanced D2V replication only slightly and to a lesser extent than did the conventional polyclonal antibodies.

A conventional antibody preparation is heterogeneous in IgG subclasses as well as in Fab portions, but a monoclonal antibody preparation, by nature, consists of only one IgG subclass whose Fab portions react with a single antigenic determinant. On the other hand, it has been reported that mouse macrophages possess two kinds of Fc receptors for IgG, Fc receptor I for mouse IgG2a and Fc receptor II for IgG1 and IgG2b (21, 22). Therefore, an assumption may be made that the different responses of Mk1 and Mm1 to D2V mixed with the enhancing antibodies are due to different distribution patterns, e.g., density and localization on the cell membrane, of Fc receptors for IgG subclasses.

In connection with the above, Brandt *et al.* (7) reported that type-specific monoclonal antibodies, either IgG1 or IgG2a, did not enhance replication of New Guinea C strain of dengue type 2 virus in human monocyte cell line, U-937, while flavivirus cross-reactive monoclonal antibodies, irrespective of IgG subclass, caused infection enhancement. However, we observed that D2V replication enhancement was mediated by the type-specific monoclonal antibody (3H5) in human peripheral blood monocyte cultures (Hotta *et al.*, manuscript submitted for publication). One of the explanations for the discrepancy may be that we inoculated D2V to the cells at an input multiplicity of 5 PFU per cell, whereas they inoculated at an input multiplicity of 0.01. Further studies are needed to elucidate the infection enhancement in mononuclear phagocytes.

The infection enhancement by the neutralizing monoclonal antibody also indicated that D2V particles are still infective even if an antigenic determinant closely associated with viral infectivity is covered with the neutralizing antibody.

In Mk1, D2V infection enhancement ratios determined by the FA tests were smaller than those determined by virus infectivity assay. While it was certain that the infection enhancement in Mk1 as well as in Mm1 was due primarily, if not, at least partly, to an increase in the numbers of virus-infected cells, possibilities could not be excluded that Mk1 cells were still heterogeneous in the ability to support D2V replication, and that some of Mk1 cells, when infected with D2V in the presence of the enhancing antibodies, produced infective progeny virus more efficiently than did cells infected with D2V in the absence of the enhancing antibodies. This point is now under investigation.

D2V infection enhancement in Mk1 by the anti-D2V HMAF at a dilution showing nearly 50% plaque-reduction activity was markedly suppressed by addition of complement to the inocula, although some enhancement was still observed at higher dilutions of the antibody. It is likely that the suppression was brought about by complement-dependent neutralization of virus infectivity. On the contrary, the infection enhancement by the monoclonal antibody, which has been identified as murine IgG1 (17), was not affected by the addition of complement. This is also compatible with a fact that murine IgG1 does not activate complement system via classical pathway (20). While it is unequivocal that complement-dependent neutralization of virus infectivity plays a role in protection against infections, the above observations imply a possibility that antibody-mediated D2V infection enhancement occurs even *in vivo* where complement system is available, giving a supporting evidence for *in vivo* infection enhancement by passively transferred antiserum (23).

Phagocytoses of bacteria by Mk1 and Mm1 were also enhanced when the bacteria had been opsonized with antibody, and the phagocytosis-enhancement ratios were greater in Mk1 than in Mm1. These observations did not conflict with the results that ratios of D2V replication enhancement by the enhancing antibodies were greater in Mk1 than in Mm1. However, it is not yet certain how the antibody-opsonized virus particles behave after they attach Fc receptors of the cells.

The experimental system using Mk1 and Mm1 is a useful and convenient tool for studying the mechanism(s) of virus infection enhancement by enhancing antibodies.

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