PROTECTIVE EFFECT OF THE $F(ab')_2$ FRAGMENTS OF MONOCLONAL ANTIBODIES TO MOUSE HEPATITIS VIRUS

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ABSTRACT

The $F(ab')_2$ fragments were prepared from three monoclonal antibodies (MAbs) reactive with either peplomer glycoprotein (E2) or nucleocapsid protein (NP) of a low-virulence mouse hepatitis virus (MHV), MHV-NuU. All the three MAbs could protect mice from challenge infection with virulent MHV-2, whereas only one of the anti-E2 MAbs was capable of neutralizing the virus in vitro. The $F(ab')_2$ fragment of neutralizing anti-E2 MAb was shown to protect mice from challenge infection, but those of non-neutralizing anti-E2 and anti-NP MAbs were not protective.

INTRODUCTION

Mouse hepatitis virus (MHV) is a member of the coronavirus group, producing both acute and chronic diseases in various species of animals (Siddell et al., 1983; Wege et al., 1982; Robb and Bond, 1979). Most MHV strains were shown to have the nucleocapsid protein (NP) and the minor (E1) and major (E2) envelope glycoproteins (Siddell et al., 1982; Sturman and Holmes, 1983). The E1 may be of the viral matrix controlling maturation and stability of the virion, while the E2 forms the projecting spikes or peplomers having important roles for the virus to attach the host cell (Siddell et al., 1982; Sturman and Holmes, 1983).

In our previous paper (Nakanaga et al., 1985), were described the production of monoclonal antibodies (MAbs) to a low-virulence isolate MHV-NuU from a wasted nude mouse (Hirano et al., 1975) and their specific binding with viral polypeptides. These MAbs were shown to be cross-reactive in vitro with other MHV strains than MHV-NuU, including highly virulent

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and hepatitogenic MHV-2 (Nelson, 1952; Piazza, 1969; Hirano et al., 1981). Both neutralizing and non-neutralizing anti-E2 or anti-NP MAbs protected mice from lethal MHV-2 infection. Histpathologically, more remarkable inflammatory responses were seen in the livers of protective MAb-treated mice (Nakanaga et al., 1986).

To see the mechanisms by which the MAbs protect mice against viral infection, especially the role of the Fc portion, the $F(ab')_2$ fragments of MAbs were prepared and examined for protective ability in mice.

MATERIALS AND METHODS

Mice. Five- to 6-week-old male and female BALB/c mice from a commercial breeder (Charles River Japan, Atsugi) were used. The breeder colony was proven to be free from MHV infection by seromonitoring (Fujiwara et al., 1976).

Virus and inoculation. MHV-2 and MHV-NuU passaged through DBT cells (Taguchi et al., 1982) were used. Culture supernatant of DBT cells was harvested at 12 hr postinoculation, centrifuged at 10,000 x g for 20 min at 4°C, and stored at -70°C as a virus stock. Mouse inoculation was made intravenously (i.v.) with 0.2 ml dilutions of the virus stock in chilled minimum essential medium (MEM)(Nissui, Tokyo).

MAbs to MHV-NuU. Male BALB/c mice received intraperitoneal (i.p.) injections with 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane) (Wako, Tokyo) and then with 5×10^6 to 2×10^7 cells of 23-2:C2, 7-2:A2 or 10-1:D2 hybridoma producing anti-MHV MAbs (Nakanaga et al., 1985). Six to 15 days later, ascitic fluid was collected and centrifuged at 10,000 x g for 20 min, and the supernatant was heated at 56°C for 30 min, filter-sterilized and then stored at -20°C before using as MAb samples. The biological properties of the MAbs are shown in Table 1 (Nakanaga et al., 1986). As controls, ascitic fluid was collected from mice injected with the parental myeloma and processed in the same manner.

$F(ab')_2$ fragments of MAbs. MAbs were purified by column chromatography using protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Sweden) (Ey et al., 1978), and the purified samples were digested with pepsin (Sigma, U.S.A.) in 0.1 M sodium acetate buffer (pH 4.5) for 18 hr at 37°C (Garvey et al., 1977). The sample protein:enzyme ratio (total protein weight) was about 10:1 . The digest was then adjusted to pH 7.2 and applied to a Sephadex G-150 (Pharmacia Fine Chemicals, Sweden) column equilibrated with 0.02 M phosphate buffered saline, pH 7.2 (PBS). $F(ab')_2$-rich fractions were separated from intact IgG or Fc by repeated passage

Table 1. Biological characteristics of MAbs

MAb	Isotype	Viral Polypeptide[a]	VN titer[b] to MHV-2	Mortality after challenge[c]
23-2:C2	IgG1	E2	1:20,000	0/17[d]
7-2:A2	IgG2a	E2	1:<5	0/10
10-1:D2	IgG2a	NP	1:<5	0/19
Control[e]	-	-	1:<5	59/59

[a]Determined by immunoprecipitation and SDS-PAGE with infected DBT cell extract.
[b]Using DBT cells.
[c]MAb sample (1:1) was given i.p. and 30 PFU of MHV-2 were inoculated i.v. 24 hr later.
[d]No. dead/no. tested; on day 10 postchallenge.
[e]Ascitic fluid from BALB/c mice infected with parental myeloma cells.

through the protein A-Sepharose CL-4B column and brought into a visking tube (Sanko pure chem., Tokyo). It was concentrated by keeping the tube in a bottle which was vaccumized by pumping. The final preparation was checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)(Laemmli, 1970), filter-sterilized and stored at -20°C.

Mouse protection test. Five-week-old female BALB/c mice received i.p. injection with 0.1 ml of a dilution of each MAb or $F(ab')_2$ fragment samples and 24 hr later, they were challenged i.v. with 30 PFU of virulent MHV-2, a dose killing mice within 6 days.

Virus neutralizing (VN) test. MAbs or their $F(ab')_2$ fragments were examined for VN activity by 50% plaque reduction assay using DBT cells as described previously (Taguchi et al., 1979; Nakanaga et al., 1983).

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed as described before (Nakanaga et al., 1985). In brief, DBT cells infected with MHV-NuU were brought into PBS containing 0.5% Nonidet P40 (BDH chemicals, U.K.), and the supernatant of the resulting cell lysate was used as ELISA antigen. Test materials were injected into microtiter wells coated with 5 unit-antigen and the plates were incubated at 37°C for 45 min. The plates were then examined for specific binding of antibodies using peroxidase-conjugated anti-mouse rabbit IgG (Cappel, U.S.A.) and 5 aminosalicylic acid (Tokyokasei, Tokyo) at 450 nm.

RESULTS

As shown in Table 2, the three $F(ab')_2$ preparations had binding activity to MHV antigen in ELISA. The $F(ab')_2$ of anti-E2 MAb 23-2:C2 had neu-

Table 2. Virus binding activity in vitro and passive protection in mice by $F(ab')_2$ of MAbs

Antibody	Dilution	O.D. in ELISA[a]	VN titer to MHV-2	Mortality after challenge[b]	Mean time-to-death (days)
23-2:C2	1:1	0.130	1:20,000	0/10[c]	-
	1:10	0.092	1:2,000	0/10	-
	1:100	0.071	1:200	4/10	6.5
23-2:C2[$F(ab')_2$]	1:1	0.130	1:100,000	0/10	-
	1:5	0.107	1:20,000	0/10	-
	1:50	0.075	1:2,000	0/10	-
7-2:A2	1:1	0.090	1:$<$5	0/10	-
	1:10	0.074	NT[e]	3/10	6.3
	1:100	0.062	NT	5/10	6.4
7-2:A2[$F(ab')_2$]	1:1	0.092	1:$<$5	10/10	5.0
	1:10	0.078	NT	10/10	4.7
10-1:D2	1:1	0.070	1:$<$5	0/10	-
	1:10	0.053	NT	8/10	6.0
10-1:D2[$F(ab')_2$]	1:1	0.090	1:$<$5	10/10	4.8
	1:10	0.064	NT	10/10	4.2
Control[d]	1:1	NT	1:$<$5	10/10	5.2

[a]Assayed using an immunosorbent of MHV-NuU infected DBT cell extract at 450 nm.

[b]MAb preparations or $F(ab')_2$ fragments were given i.p. 24 hr before i.v. challenge with 30 PFU of MHV-2.

[c]No. of dead/no. of tested; on day 10 postchallenge.

[d]Ascitic fluid from BALB/c mice infected with parental myeloma cells.

[e]Not tested.

tralizing activity five times as high as the intact MAb when compared at the same O.D. level (0.130). The O.D. value of $F(ab')_2$ seemed to be decreased by removal of antigenic determinants on the Fc portion recognized by the peroxidase-conjugated anti-mouse rabbit IgG.

The $F(ab')_2$ preparations as well as intact MAbs were tested for passive protection in mice against challenge infection with virulent MHV-2. They were compared on the bases of equivalent binding activity measured by VN test or ELISA, because they possibly contained other murine $F(ab')_2$ fragments not specific for MHV. Though challenged control animals died within 6 days postchallenge, all of those having received a 1:10 dilution of MAb 23-2:C2

which was equivalent to a VN titer of 1:2,000 survived the challenge. The MAb was effective at a dilution of 1:100. The neutralizing $F(ab')_2$ preparation of MAb 23-2:C2 was also shown to be protective at 1:1 to 1:50 dilutions, being equivalent to VN titers of 1:100,000 to 1:2,000.

However, the $F(ab')_2$ of 7-2:A2, non-neutralizing anti-E2 MAb, was not protective at a 1:1 dilution showing O.D. 0.092, although the intact MAb was able to protect some animals at 1:100 showing O.D. 0.062. Another $F(ab')_2$ of non-neutralizing anti-NP MAb 10-1:D2 had no protective effect. These findings suggested that the Fc portion of antibody molecules might be required for non-neutralizing MAb but not for neutralizing MAb to protect mice.

DISCUSSION

The present results indicated that the $F(ab')_2$ fragments of non-neutralizing MAbs 10-1:D2 and 7-2:A2 were shown to have no protective effect in mice, suggesting that the intact Fc portion of the MAbs was required for protecting mice from challenge infection. However, neutralizing anti-E2 MAb 23-2:C2 was protective in mice without Fc. With other virus infections non-neutralizing MAbs protecting in vivo were shown to be non-protective in mice after pepsin digestion, which did not affect their binding activity to viral antigen in vitro (Lefrancois, 1984; Boere et al., 1985; Mathews et al., 1985). Thus the Fc portion of antibodies might be of importance for protecting mice by non-neutralizing protective MAbs.

The Fc-mediated protection of non-neutralizing antibodies might result from antibody-dependent cytolysis of infected host cells due to either cell-mediated or complement-mediated cytotoxicity, and possibly inducing local accumulation of inflammatory cells (Rawls and Tompkins, 1975). This might be supported by our previous observations that cellular infiltration was more remarkable in liver necrotic foci in mice treated with protective MAbs (Nakanaga et al., 1986). In addition, antibody-coated virions were found to be phagocytized within macrophages or polymorphonuclear leukocytes, probably being mediated by Fc receptor (Halstead and O'Rourke, 1977; Peiris et al., 1982). Such mechanisms may also operate, though not indispensably, in case of MAb 23-2:C2 which was neutralizing in vitro and protecting in vivo. The ability of passive protection in mice by the neutralizing MAb as well as its $F(ab')_2$ fragment was apparently related to its VN titers (Table 2).

There has been no reports that anti-NP MAb is effective in protecting mice from infection with enveloped viruses. Our previous as well as present studies revealed that an IgG2a MAb recognizing the viral NP (10-1:D2) ren-

dered mice resistant to lethal MHV-2 challenge (Nakanaga et al., 1986) and that it was not effective without the Fc portion. By immunofluorescence MAb 10-1:D2 was found to combine with the surface of DBT cells infected with MHV-2, while anti-NP MAb 23-1:G8 directing to the cytoplasm of infected cells was unable to protect mice (unpublished observation). These findings suggested that certain conservative NP epitope(s) common to most MHV strains (Nakanaga et al., 1985; Fleming et al., 1983) might be expressed on the surface of MHV-infected cells, triggering the host immune system as in case of NP-specific cytotoxic T cells in influenza A virus infection (Townsend and Skehel, 1985; Yewdell et al., 1985). Further studies should focus on the significance of the viral NP expression on the infected cell surface.

REFERENCES

Boere, W.A.M., Benaissa-Trouw, B.J., Harmsen, T., Erich, T., Kraaijeveld, C.A., and Snippe, H., 1985, Mechanisms of monoclonal antibody-mediated protection against virulent Semiliki forest virus, J. Virol., 54:546.

Ey, P.L., Prowse, S.J., and Jenkin, C.R., 1978, Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-sepharose, Immunochemistry, 15:429.

Fujiwara, K., Takenaka, S., and Shumiya, S., 1976, Carrier state of antibody and viruses in a mouse breeding colony persistently infected with Sendai and mouse hepatitis viruses, Lab. Anim. Sci., 26:153.

Garvey, J.S., Cremer, N.E., and Sussdorf, D.H., 1977, "Methods in immunology, Third Edition," W.A. Benjamin, Inc., Advanced Book Program Reading, Massachusetts.

Halstead, S.B., and O'Rourke, E.J., 1977, Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody, J. Exp. Med., 146:201.

Hirano, N., Murakami, T., Taguchi, F., Fujiwara, K., and Matsumoto, M., 1981, Comparison of mouse hepatitis virus strains for pathogenicity in weanling mice infected by various routes, Arch. Virol., 70:69.

Hirano, N., Tamura, T., Taguchi, F., Ueda, K., and Fujiwara, K., 1975, Isolation of low-virulent mouse hepatitis virus from nude mice with wasting syndrome and hepatitis, Jpn. J. Exp. Med., 45:429.

Laemmli, U.K., 1970, Cleavage of the structural proteins during the assembly of the head of bacteriophage T4, Nature (London), 227:680.

Lefrancois, L., 1984, Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: Distinct mechanisms of action in vivo, J. Virol., 51:208.

Mathews, J.H., Roehrig, J.T., and Trent, D.W., 1985, Role of complement and the Fc portion of immunoglobulin G in immunity to Venezuelan equine encephalomyelitis virus infection with glycoprotein-specific monoclonal antibodies, J. Virol., 55:594.

Nakanaga, K., Ishida, T., and Fujiwara, K., 1983, Difference in antibody production against mouse hepatitis virus (MHV) among mouse strains, Lab. Anim., 17:90.

Nakanaga, K., Kitagawa, T., Kiuchi, Y., Makino, S., Hayami, M., and Fujiwara, K., 1985, Production and characterization of monoclonal antibodies to mouse hepatitis virus, MHV-NuU, Jpn. J. Vet. Sci., 47:423.

Nakanaga, K., Yamanouchi, K., and Fujiwara, K., 1986, Protective effect of

monoclonal antibodies on lethal mouse hepatitis virus infection in mice, J. Virol., 59:168.
Nelson, J.B., 1952, Acute hepatitis associated with mouse leukemia. I. Pathological features and transmission of the disease, J. Exp. Med., 96:293.
Peiris, J.S.M., Porterfield, J.S., and Roehrig, J.T., 1982, Monoclonal antibodies against the flavivirus West Nile, J. Gen. Virol., 58:283.
Piazza, M., 1969, "Experimental viral hepatitis," Charles, C. Thomas, Publisher, Springfield, Ill.
Robb, J.A., and Bond, C.W., 1979, Coronaviridae, Compr. Virol., 14:193.
Rawls, W.E., and Tompkins, W.A.F., 1975, Destruction of virus-infected cells by antibody and complement, in:"Viral immunology and immunopathology," A.L. Notkins, ed., Academic Press, Inc., New York.
Siddell, S., Anderson, R., Cavanagh, D., Fujiwara, K., Klenk, H.D., Macnaughton, M.R., Pensaert, M., Stohlman, S.A., Sturman, L.S., and van der Zeijst, B.A.M., 1983, Coronaviridae, Intervirology, 20:181.
Siddell, S., Wege, H., and ter Meulen, V., 1982, The structure and replication of coronaviruses, Curr. Top. Microbiol. Immunol., 99:131.
Sturman, L.S., and Holmes, K.V., 1983, The molecular biology of coronaviruses, Adv. Virus Res., 28:35.
Taguchi, F., Goto, Y., Aiuchi, M., Hayashi, T., and Fujiwara, K., 1979, Pathogenesis of mouse hepatitis virus infection. The role of nasal epithelial cells as a primary target of low-virulence virus, MHV-S, Microbiol. Immunol., 23:246.
Taguchi, F., Makino, S., and Fujiwara, K., 1982, Antigenic differentiation of mouse hepatitis viruses by neutralization test, Microbiol. Immunol., 26:741.
Townsend, A.R.M., and Skehel, J.J., 1984, The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells, J. Exp. Med., 160:552.
Wege, H., Siddell, S., and ter Meulen, V., 1982, The biology and pathogenesis of coronaviruses, Curr. Top. Microbiol. Immunol., 99:165.
Yewdell, J.W., Bennink, J.R., Smith, G.L., and Moss, B., 1985, Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes, Proc. Natl. Acad. Sci. USA, 82:1785.