Protection from mouse hepatitis virus type 3-induced acute disease by an anti-nucleoprotein monoclonal antibody

Brief Report

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Summary

Fusion of MHV-3-immune splenocytes from MHV-3-resistant A/J murine strain, with NS myeloma cells produced several hybridomas. Among eight hybridoma clones, the 1E7A4H1 clone secreted kappa IgG2a apparently directed against the nucleoprotein of the MHV-3 virion. The monoclonal antibody was able to neutralize the in vitro cytopathic effect of MHV-3 on cultured L2 cells, and was detected by indirect immunofluorescence on MHV-3-infected cultured YAC cells. In addition, it conferred a significant protection against MHV-3-induced acute disease, if injected intraperitoneally to C57BL/6 mice before inoculation with MHV-3.

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Mouse hepatitis viruses (MHVs), members of the coronavirus family, produce various diseases in the mouse and other animal species [16]. They exhibit different tissue tropisms and patterns of infection [1]. The susceptibility of the animals to the virus-induced diseases appears to be genetically controlled at different levels [16]. Serological neutralization patterns of several of the MHVs are very complex [11, 15] and the three structural proteins demonstrated variable degrees of conservation [4, 13]. Coronaviruses are frequently endemic in laboratory mouse colonies [8]. Neutralizing monoclonal antibodies have been recently prepared against the JHM (MHV-4) [2, 4, 17] and MHV-2 [9] strains. In this brief note, we report on a monoclonal antibody, apparently specific for the nucleo-

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protein (N), that can neutralize the cytopathic activity of the virus on cultured cells and protect susceptible mice from the acute disease resulting from an infection with type 3 murine hepatitis virus (MHV-3).

For hybridoma production, MHV-3-resistant A/J mice [7], 10-12 week old, were infected intraperitoneally with in vivo propagated MHV-3 virus ($10^3 LD_{50}$). Two weeks later, the mice received a second virus challenge, ($10^5 LD_{50}$, intraperitoneally). Animals were killed 3 days later and the spleens removed. Fusions with the splenocytes and P3/NSI/1-Ag4-1 ("NS-1") myeloma cells, of BALB/c origin, were performed. Screening of monoclonal antibodies specific for MHV-3 antigens was done, by both enzyme-linked immunosorbent (ELISA) and dot-immunoblotting assays [3]. After two successive preliminary screenings with ELISA, 8 hybridomas positive in both ELISA and dot-immunoblotting assays, were selected.

Eight cloned hybridoma lines were established after two clonings at limiting dilutions from two hybridomas: 1E7 (one clone) and 2F4 (seven clones). The clones were characterized and tested for reactivity with MVH-3 virus proteins. All were positive by ELISA, whereas only 1E7A4H1 was positive by ELISA and a dot immunoblotting test. All were of the IgG2a kappa subtype. All but one gave workable amounts of ascitic fluid, after transplantation of the hybridomas in (A/J X BALB/ c) F_1 hybrid mice. Since all the seven subclones of the 2F4 series gave similar data by neutralization we report here results with the monoclonal antibodies 1E7A4H1 and 2F4H9F4.

The polypeptide specificities of the monoclonal antibodies were determined by Western immunoblotting [14] using concentrated supernatants of MHV-3-infected L2 cells. Figure 1 shows that both the 1E7A4H1 and 2F4H9F4 reacted with a protein of 51kDa with minor bands showing at 62kDa and 47kDa and that the 1E7A4H1 monoclonal antibody gave the most intense reaction (lane 3). The latter antibody gave a similar imunoblotting profile with the A59 strain of MHV and showed strong cross-reactivity by ELISA with MHV-A59 and MHV-4, whereas the epitope recognized by monoclonal antibody 2F4H9F4 appeared to be conserved on MHV-4 but lost on MHV-A59 (data not shown). It was therefore established that both monoclonal antibodies were most likely specific for two different epitopes on the structural N protein.

The viral specificity of the monoclonal antibodies was also established by indirect immunofluorescence. Chromatographically purified immunoglobulins from ascitic fluid were used for reaction with MHV-3-infected live YAC cells [5]. The expression of MHV-3 antigens on live infected cells was detected with a FITC-conjugated rabbit anti-mouse immunoglobulin serum (Miles Laboratories Inc., Elkhart, Ind.). Figure 2A shows that the 1E7A4H1 ascitic fluid reacted with the surface of MHV-3-infected YAC cells, but not with the membrane of non-infected cells
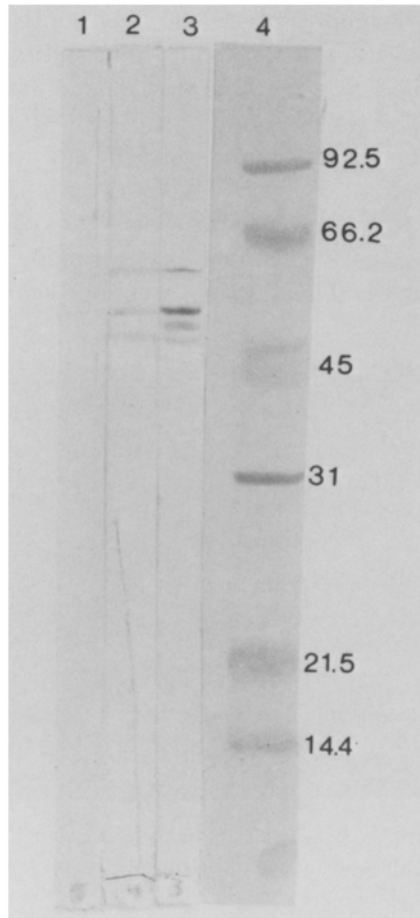
Fig. 1. Western immunoblots of MHV-3 with monoclonal antibodies. L2 cells were infected at a MOI of 0.1 until cytopathic effect occurred at 72 h post-infection. Supernatants of infected cells were clarified at $1000 \times g$ and concentrated by ultracentrifugation at $90,000 \times g$ for 90 min. Pelleted virus from 25 m supernatant was resuspended in 1 m of 0.625M Tris-HCl pH 6.8, 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol and heated at 37 °C for 15 min. Ten μL were applied on 10% resolving SDS-Page. Transfer onto nitrocellulose membrane was carried out according to Towbin et al. [14]. Strips of nitrocellulose (5 mm wide) were reacted with a 1 : 10 dilution of ascitic fluid of: *1* irrelevant monoclonal antibody to human herpes virus 1; *2* 2F4H9F4: *3* 1E7A4H; *4* Bio-Rad Laboratories (Richmond, CA) molecular weight standards Phosphorylase-B (92500), Bovine Serum Albumin (66 200), Ovalbumin (45000), Carbonic anhydrase (31000) Soybean trypsin inhibitor (21 500) and lysozyme (14400 Da) stained with 0.1% Amido Black in sodium acetate 0.6%, acetic acid 0.25% (v/v) and glycerol 5% (v/v). The antigen-antibody reaction on nitrocellulose strips was revealed with a rabbit anti-mouse IgG conjugated to horseradish peroxidase diluted 1/400 (Miles Laboratories)

(Fig. 2 B). The intensity of fluorescence was comparable to that of a polyclonal mouse anti-MHV-3 serum and no reaction was observed with the monoclonal antibodies of the 2F4 series or an irrelevant anti-herpes monoclonal antibody (data not shown).

Both cultures supernatants and ascitic fluids were tested for virus neutralization in vitro as well as in vivo. For in vitro neutralization, hybridoma supernatants were mixed with virus dilutions. To the antibody-virus mixture was added guinea-pig complement diluted 1:10 and incubated at 37 °C for 1 h. This mixture was then plated in quadruplicate
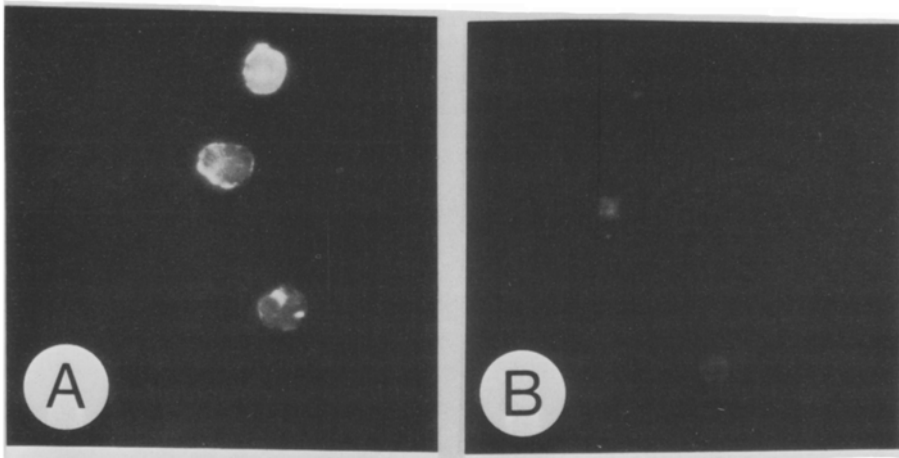
Fig. 2. Indirect immunofluorescence staining of the surface of YAC cells, with 1E7A4H1 anti-MHV-3 monoclonal antibody (chromatographically purified ascitic fluid). A MHV-3-infected cells. B Non-infected cells

on monolayers of L2 cells in 96 well Linbro culture plates. Infections with several dilutions of MHV-3 could be suppressed with 1E7A4H1 monoclonal antibody in the presence of guinea-pig complement and not with the 2F4 derived clones (data not shown).

In vivo neutralizations were performed by intraperitoneal injections of C57BL/6 mice with 0.5 mL of ascitic fluid, 4h before challenge with 1000 LD$_{50}$ of MHV-3 as described [7]. Mice surviving the acute, normally lethal disease, recovered healthy appearance within 2 weeks, and the ratios surviving mice to infected mice were determined at 15 days after infection. Only 1E7A4H1 provided significant protection when injected 4h before infection (Table 1). No protection was obtained with the non-neutralizing ascitic fluid from the 2F4H9F4 hybridoma clone or with an irrelevant monoclonal antibody.

It was of some interest to look for histological evidence of the protection conferred by the 1E7A4H1 monoclonal antibody in susceptible

**Table 1.** Occurence of MHV-3-induced acute disease in C57BL/6 mice, as a function of preinjection treatment

Expt. No	Preinjection treatment	Number f survivors: numbers of injected (% survivors)	Difference with untreated controls
1	None (control)	4/22 (18%)	—
	1E7A4H1 ascitic fluid (undiluted)	17/23 (73%)	p<0.01
	Other ascitic fluids* (undiluted)	4/18 (22%)	not significant
2	None (control)	3/29 (10%)	—
	1E7A4H1 ascitic fluid (diluted 1:5)	10/26 (38%)	p<0.05

* Non-neutralizing 2F4H9F4 anti-MHV-3 monoclonal antibody (5 mice) or unrelated (6D4-II) anti-herpes-1 virus monoclonal antibody (13 mice)

C57BL/6 mice. For this, another group of 5 mice was injected as above with 0.5 mL of ascitic fluid, 4 h before challenge with 1000 LD_{50} of MHV-3. Five other C57BL/6 mice were injected with a similar amount of an irrelevant monoclonal antibody against the human herpes type-1 virus. Intraperitoneally inoculated C57BL/6 mice are very sensitive to MHV-3. Infection of the liver results in rapidly lethal hepatitis within 6-7 days [16].

For histologic examination, brains and livers were removed by day 6, fixed in histologic fixative (Perfix, Fisher Scientific Co., Fair Lawn, NJ), sectioned (6 µm) and stained with hematoxilin and eosin. At this time two out of 5 mice, having received an irrelevant monoclonal antibody, had died from the MHV-3 challenge. For the three other surviving mice, multifocal necrotizing hepatitis was evident in the liver of surviving mice sacrificed six days post-inoculation. Numerous randomly distributed foci were present in which hepatocytes had undergone coagulative necrosis with a marked infiltration of mixed leukocytes (Fig. 3A). In contrast, at 6 days after challenge, in mice having received the 1E7A4H1 monoclonal antibody, small scattered lesions were observed consisting mainly of a mixed reaction of nuclear cells. No lesions were observed in the brain of either passively protected or infected mice (Fig. 3B).

To our knowledge, 1E7A4H1 is the first anti-MHV-3 monoclonal antibody reported to be specific for the N protein, and to confer in vivo protection. Furthermore, it was shown that it reacts with an epitope which is expressed on the surface of infected YAC cells. In contrast, the 2F4 monoclonal antibodies, although specific for the N protein, did not
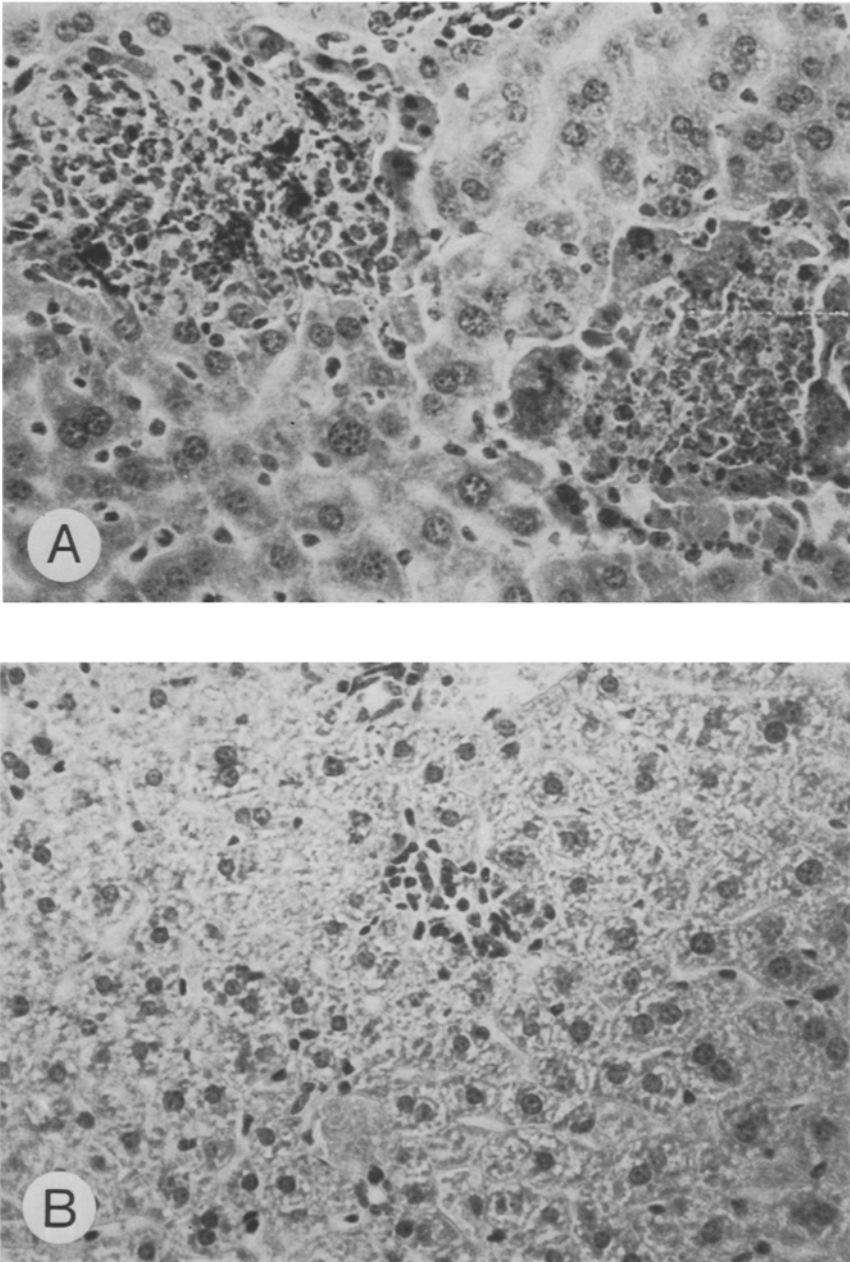
Fig. 3. Multifocal necrotizing hepatitis in the liver at 6 days after challenge in mice having received an irrelevant monoclonal antibody (**A**). Mild inflammatory reaction in a protected mouse without evidence of necrosis (**B**). Hematoxylin and eosin stain 250 ×

show any complement mediated neutralization in vitro or protective activity in vivo and did not react with infected cell plasma membranes. An anti-N protein monoclonal antibody without in vitro neutralizing activity against MHV-2, that could confer protection in MHV-2-infected mice, has been reported previously [9]. On the other hand, monoclonal antibody against the N protein of MHV-JHM (MHV-4), like our 2F4 monoclonal antibodies, did not have any protective activity against a lethal dose of virus [2]. It can be assumed that the 1E7A4H1 and 2F4 monoclonal antibodies are reacting against different epitopes, which would also explain the different patterns of cross-reactivities shown by ELISA with MHV-A59 and MHV-4. Obviously because of the small number of monoclonal antibodies in this series exhibiting different patterns of reaction it is not possible to analyse virus structures in relation to protection mechanisms, as has been done for other MHVs [2, 11, 12, 17].

However, the present study demonstrates that at least one epitope on the N protein is accessible to antibodies on the surface of infected cells. This has not yet been shown for MHVs but it has been shown for the N protein of influenza virus [18]. It can be envisaged that although an anti-N protein monoclonal antibody cannot prevent adsorption of the virus to initiate a first cycle of replication, it could confer protection by reacting with the surface of infected cells causing antibody-dependent complement or cell-mediated cytolysis, as has been shown for various virus infections [6, 10].

Acknowledgements

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