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Protection from Lethal Coronavirus Infection by Affinity-Purified Spike Glycoprotein of Murine Hepatitis Virus, Strain A59

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Murine hepatitis viruses provide excellent animal models for the study of virus-induced diseases of the central nervous system and gastrointestinal tract. Several studies have indirectly provided evidence that the spike glycoprotein (S) of these coronaviruses bears determinants for pathogenesis and the induction of protective immunity. In order to directly evaluate the immunogenicity of this protein, it was purified by affinity chromatography with an in vitro neutralizing and in vivo protective monoclonal antibody which immunoprecipitated the 180-kDa spike glycoprotein of the neurotropic A59 strain of murine hepatitis virus (MHV-A59). Mice immunized twice with approximately 1 μg of purified S in Freund's adjuvant developed high titers of neutralizing and fusion inhibiting antibodies, even though the protein was at least partially denatured after elution from the affinity column. Moreover, these mice were protected from lethal encephalitis when challenged intracerebrally with 10 LD₅₀ of MHV-A59. This study provides a direct demonstration of the importance of the coronavirus spike glycoprotein in the induction of a protective immune response.

INTRODUCTION

Murine hepatitis viruses (MHV) are enveloped positive-stranded RNA viruses which belong to the Coronaviridae (Siddell et al., 1983). These viruses are known for their broad tissue tropism, which makes them responsible for a variety of acute and chronic diseases in humans and animals, such as respiratory, gastrointestinal, and neurological disorders (Wege et al., 1982). Moreover, the neurotropic A59 and JHM strains of MHV provide an animal model of virus-induced diseases of the central nervous system (CNS), such as multiple sclerosis (Erlich and Fleming, 1985; Johnson, 1985).

Three major proteins were identified on MHV virions (Sturman and Holmes, 1983). They are the nucleocapsid protein (N) and the two envelope glycoproteins, M (previously designated E1) and S (previously designated E2). The N protein (50 kDa) is phosphorylated and associated with the genomic RNA. The M glycoprotein (23 kDa), which contains O-linked carbohydrates, is a membrane protein whereas the S glycoprotein (180 kDa), which contains N-linked carbohydrates, constitutes the characteristic surface projections of the virions. The S molecule mediates many of the biological properties of the virus, such as attachment to cell receptors, penetration, and spread by virus-induced cell to cell fusion, which is activated by cleavage of the 180-kDa glycoprotein into two subunits of 90 kDa (Sturman et al., 1985). The presence of a third glycoprotein of 65 kDa, designated HE (hemagglutination and/or esterase activity), and its biological functions remain to be clarified for MHV (Luytjes, 1989).

S also plays an important role in the immune response against the virus since neutralizing complement-independent antibodies (Collins et al., 1982; Wege et al., 1984; Talbot et al., 1984b; Gilmore et al., 1987), passive antibody protection (Buchmeier et al., 1984; Wege et al., 1984), and cell-mediated immunity (Holmes et al., 1986; Stohlman et al., 1986) have been related to S. Recently, protective neutralizing antibodies were induced by immunization with a synthetic decapetide of S (Talbot et al., 1988). In order to directly evaluate the involvement of the whole glycoprotein in the immune response against coronaviruses, the MHV-A59 S glycoprotein was purified by affinity chromatography and its immunogenicity ascertained in mice. We show that in addition to the induction of high levels of neutralizing and fusion-inhibiting antibodies, immunization with S alone was able to protect mice from lethal encephalitis induced by experimental MHV-A59 infection, despite the loss of the native protein conformation.

MATERIAL AND METHODS

Virus

The A59 strain of MHV (MHV-A59) was obtained from the American Type Culture Collection (Rockville, MD).
MD), plaque-purified twice, and passaged four times at a multiplicity of infection (m.o.i.) of 0.01 on DRT cells, a murine cell line established from a delayed brain tumor in a CDF1 mouse inoculated intracerebrally with strain Schmidt-Ruppin of Rous sarcoma virus (Hirano et al., 1974; Daniel and Talbot, 1987).

Production and characterization of monoclonal antibodies

Hybridomas secreting monoclonal antibodies (MAbs) against MHV-A59 were obtained as follows: female MHV-seronegative 6-week-old BALB/c mice (Charles River, St-Constant, Québec) were immunized intraperitoneally (i.p.) with 8 \times 10^6 PFU of MHV-A59 in growth medium (after UV inactivation) and boosted 3 weeks later in the same manner. After another 4 days, immune spleen cells were fused with nonsecretor myeloma cells P3-XG3-Ag8.653 at a ratio of 5:1 with polyethylene glycol 1000 (Sigma, St-Louis, MO). The cell suspension was cultured in 96-well plates (2.5 \times 10^5 cells per well) in RPMI medium containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2.5 \mu g/ml fungizone, 100 \mu g/ml penicillin, and 100 \mu g/ml streptomycin (GIBCO Canada, Burlington, Ontario). This medium was supplemented with 100 \mu M hypoxanthine, and 16 \mu M thymidine for the first 4 weeks, and 0.4 \mu M aminopterin (Sigma) for the first week. The medium was aseptically for the protection of virus-specific antibody by enzyme-linked immunosorbent assay (ELISA) (Talbot et al., 1984b). Cells in positive wells were cloned by limiting dilution, expanded, and rechecked. Ascites of positive clones were produced in pristane (2,6,10,14-tetramethylpentadecane)-primed BALB/c mice. The specificity of the MAbs for viral proteins was determined by radioimmunoprecitation of [35S]methionine-labeled virus: after being extensively washed in an ammonium isothiocyanate-phosphate buffer and the mixture was agitated for 16 hr at 4°C.

Affinity chromography

Concentrated virus was solubilized with 2\% (v/v) Nonidet-P40 (NP-40) for 2 hr at room temperature and insoluble debris was sedimented at 100,000 \times g for 45 min. Soluble proteins were mixed with the immunoadsorbent and the mixture was agitated for 16 hr at 4°C on a rotary mixer. The 7-10A-Sepharose gel specificity was determined by immunoabsorption of [35S]methionine-labeled virus; after being extensively washed in an Eppendorf tube, the adsorbed proteins were eluted into electrophoresis sample buffer. For batch chromatography, the gel was poured into a column (1 cm diameter) and washed with 0.1\% (v/v) NP-40 in 0.2 M phosphate buffer, pH 6.2 (containing 0.1 M NaCl and 1 mM EDTA), until the absorbance at 280 nm had dropped to baseline level. The column was then washed with a further 4 gel vol of 0.1\% (v/v) octylglucoside in phosphate buffer. Adsorbed proteins were eluted with 3 M ammonium isothiocyanate in phosphate buffer containing 0.1\% (v/v) octylglucoside. Fractions of 1 ml were collected and dialyzed against 0.05 M ammonium bicarbonate, pH 7.4. A sample of each fraction was lyophilized and resuspended in sample buffer for electrophoresis, which was carried out on a 7–15% linear polyacrylamide gel (Laemmli, 1970) prior to fluo-
Immunization experiments

Eight MHV seronegative female 6-week-old BALB/c mice were inoculated i.p. with approximately 1 μg of purified S emulsified in an equal volume of complete (Day 0) and incomplete (Day 25) Freund's adjuvant. Eight control mice received an equivalent volume of TMEN in adjuvant. On Day 40, immunized and control mice were given an intracerebral (i.c.) challenge with approximately 10 LD₅₀ of MHV-A59 (5 × 1₀⁶ PFU).

Neutralization and fusion inhibition assays

Monoclonal antibodies or pooled plasma samples obtained by bleeding from the retroorbital plexus on Days 0, 25, and 40 were analyzed for their ability to neutralize either 50–100 PFU or 30–300 TCID₅₀ of MHV-A59. Briefly, plasma dilutions made in culture medium were incubated 1 hr at 37°C with appropriate virus dilutions. Residual infectivity was evaluated by a plaque assay (Daniel and Talbot, 1987) or by a CPE assay. For the latter, each virus/plasma sample was distributed into 4 wells of a 96-well plate containing confluent monolayers of DBT cells. After an incubation for 1 hr at 37°C, which allowed virus adsorption, culture medium was added to each well, and plates were incubated for 2 days at 37°C, 5% (v/v) CO₂ in a humidified atmosphere. Neutralizing titers were determined either from the reduction in the number of plaques or by the method of Karber (Hawkes, 1979) for the CPE assay.

The capacity of plasma samples to prevent the formation of syncytia was assayed as follows: nearly confluent DDT cell monolayers in 96-well plates were infected with 50–100 PFU of MHV-A59 per well. After an adsorption period of 1 hr at 37°C, the viral inoculum was removed, culture medium was added, and plates were incubated for 3 hr at 37°C. This medium was then replaced by culture medium containing serially diluted plasma samples and plates were incubated for another 32 hr at 37°C, after which CPE was quantitated by evaluating the percentage of fused cells.

Radioimmunoprecipitation assay (RIPA)

Lysates of [³⁵S]methionine-labeled MHV-A59-infected DBT cells were used as antigen. Cell monolayers showing optimal CPE were washed three times with RIPA buffer (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5 mM MgCl₂) and lysed by incubation on ice for 20 min with RIPA buffer supplemented with 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (w/v) aprotinin (Sigma), and 1 mM phenylmethylsulfonyl fluoride. Cell debris was pelleted by centrifugation at 12,000 g for 15 min and the supernatant was used as antigen for RIPA. Plasma samples were first adsorbed on protein A-Sepharose (50 μl of a 10% (w/v) preparation) for 1 hr at 37°C, after which radiolabeled antigen was added and the mixture was incubated for 2 hr at 37°C. After extensive washing with RIPA buffer containing 0.1% (w/v) Triton X-100, adsorbed proteins were eluted into SDS–PAGE sample buffer. The immunoprecipitated proteins were revealed after electrophoresis and fluorography as described above.

RESULTS

Production of antibody-secreting hybridoma lines

From the fusion described above, we have obtained nine stable cloned hybridoma lines which secrete vi-
TABLE 1

| Hybridoma | Neutralization <sup>a</sup> <sub>in vitro</sub> | Passive protection <sup>c</sup> <sub>in vivo</sub> |
|-----------|---------------------------------|----------------|
|           | Plaque assay | CPE | Fusion inhibition <sup>b</sup> | + |
| 7-10A     | 1-10 x 10<sup>6</sup> | 5-10 x 10<sup>7</sup> | 250 |
|           | (100% up to 10<sup>6</sup>) |     |     |     |
| 4-11G     | 32-64 x 10<sup>3</sup> | 28 x 10<sup>3</sup> | 50 |

<sup>a</sup> Reciprocal of ascites dilution neutralizing 50% of viral infectivity in a plaque assay (50-100 PFU) or in a CPE assay (30-300 TCID<sub>50</sub>).

<sup>b</sup> Reciprocal of ascites dilution reducing CPE by 50% when added 4 hr p.i.

<sup>c</sup> Passive protection against lethal intracerebral challenge with MHV-A59. Ascites fluids (0.2 ml i.p.) given one day prior to challenge with 10 LD<sub>50</sub> (5 x 10<sup>5</sup> PFU i.c.).

Virus-specific MAbs. The protein specificity of each antibody was determined by immunoprecipitation and Western immunoblotting. Two hybridomas (7-10A and 4-11G) were selected for further study, since they secreted MAbs which immunoprecipitated the 180-kDa form of S (Fig. 1). These MAbs were also able to neutralize virus infectivity and virus-induced fusion <em>in vitro</em> and passively protect mice <em>in vivo</em> (Table 1 and Fig. 2). Partial reciprocal competition in an ELISA and the absence of reactivity with the SDS-denatured protein on Western immunoblots (data not shown) or dot blots (Fig. 3) showed that MAbs 7-10A and 4-11G recognize two overlapping conformational determinants. However, the determinant recognized by MAb 4-11G remained reactive after treatment with ammonium isothiocyanate, whereas the one recognized by MAb 7-10A was denatured (Fig. 3).

**Evaluation of the immunoabsorbent specificity**

The 7-10A-Sepharose gel was used to immunoprecipitate <sup>[35S]</sup>methionine-labeled antigen from a concentrated virus preparation. After adsorption of antigen and being extensively washed, proteins adsorbed on the gel were eluted in electrophoresis sample buffer and analyzed by SDS-PAGE. As shown in Fig. 4, this immunoabsorbent rooted specifically with the dimeric form of the S glycoprotein (180 kDa). The purity of this protein, evaluated by densitometry on a Beckman DU-8 spectrophotometer, was estimated to be over 87% (data not shown). Two possible monomeric forms of S (Fig. 4: 96 and 87 kDa) were also reproducibly observed in immunoabsorbent and purification experiments.

**Purification of the S glycoprotein**

Viral antigens, concentrated from 1.8 liters of culture medium from infected DBT cells, were used for purification of S by affinity chromatography. Analysis by SDS-PAGE of fractions eluted after immunoaffinity
VACCINATION WITH PURIFIED MHV-A59 S

Immunoadsorption of [35S]methionine-labeled antigen on 7-10A-Sepharose gel. Lane M, 14C-labeled molecular mass markers (New England Nuclear); lane 1, proteins eluted from the anti-S immunoadsorbent. Molecular masses (in kilodaltons) of markers and S molecules are shown on the left and right, respectively.

Chromatography (Fig. 5) showed that the dimeric and monomeric forms of S were purified without detectable contamination from other viral proteins although a 30-kDa contaminant of probable cellular origin could reproducibly be observed. The loss of reactivity of the purified glycoprotein with the 7-10A MAb (lack of reaction in antigen-capture ELISA and absence of readsoption on affinity column; data not shown) confirmed that it was at least partially denatured by the isothiocyanate elution step. Moreover, immunoaffinity chromatography (Fig. 5) reproducibly yielded more S monomers than dimers, when compared to immunoadsorption (Fig. 4).

Immunogenicity of purified S

BALB/c mice were immunized twice intraperitoneally with 1 μg of purified S or TMEN buffer emulsified in Freund's adjuvant. Plasma samples collected from each group of mice were pooled and assayed for neutralizing and fusion inhibiting antibodies (Table 2). Animals immunized with S showed neutralizing titers of 1/1600 in plaque assay and 1/230 in CPE inhibition assay, whereas control mice showed titers less than 1/15 in both assays. Immune plasma samples were also able to inhibit the formation of syncytia by 50% at a dilution of 1/40 (Table 2). Moreover, immune plasma samples competed with MAb 4-11G for binding to viral antigens (Fig. 6), whereas they did not compete with MAb 7-10A (data not shown).

Both immunized and control mice were challenged i.c. with 10 LD50 of MHV-A59. Control mice died from the MHV-A59 infection within 5 days, whereas all of the S-immunized mice were protected from lethal encephalitis (Fig. 7). However, some clinical signs of infection (ruffled fur, hunched position, hyperirritability)
TABLE 2

PROTECTION FROM LETHAL MHV-A59-INDUCED ENCEPHALITIS BY AFFINITY-PURIFIED S

| Immunization | Time after first immunization (days) | Neutralizing antibodies | Fusion inhibition | Protection (survivors/challenged) |
|--------------|-------------------------------------|-------------------------|------------------|-----------------------------------|
| S            | 0                                    | <15                     | <15              | ND<sup>a</sup>                   | 7/7                               |
|              | 40                                   | 1600                    | 230              | 40                                |
| TMEN         | 0                                    | <15                     | <15              | ND                                | 0/8                               |
|              | 40                                   | 115                     | <15              | 10                                |

<sup>a</sup> BALB/c mice (female, 6 weeks old) were immunized (i.p.) on Day 0 and boosted on Day 25 with 1 μg of purified S or TMEN in Freund's adjuvant.

<sup>b</sup> Reciprocal of plasma dilution neutralizing 50% of viral infectivity in a plaque assay (50–100 PFU) or in a CPE assay (30–300 TCID<sub>50</sub>).

<sup>c</sup> Reciprocal of plasma dilution reducing CPE by 50% when added 4 hr p.i.

<sup>d</sup> Not done.

<sup>e</sup> Ten LD<sub>50</sub> (5 x 10<sup>6</sup> PFU) of MHV-A59 i.c. on Day 40. One mouse immunized with S died prior to virus challenge.

were initially observed in protected mice, which recovered a few days later.

DISCUSSION

In this study, we show that affinity-purified S induced in mice high levels of neutralizing and fusion inhibiting antibodies, which likely were involved in conferring protection against challenge with MHV-A59. The method developed allowed the purification of S glycoprotein from other viral proteins and most other cellular proteins. However, a contaminant (30-kDa doublet in Fig. 5), which was probably of cellular origin since it migrated slower than the 23 kDa viral membrane protein, was reproducibly observed with batch chromatography.

Numerous studies have provided indirect evidence on the importance of the spike (or peplomer) glycoprotein of coronaviruses in their biological activities, including interaction with the immune system. However, few studies have reported direct evidence on its biological importance. Hasony and Macnaughton (1981) showed that surface projections of MHV-3 purified by ultracentrifugation could protect mice against infection with the same virus. In addition, affinity-purified spike glycoproteins of infectious bronchitis virus, another coronavirus, were also able to induce neutralizing antibodies (Mockett, 1985). Our results confirm directly the biological importance of the peplomer protein in the immune response against a neurotropic murine coronavirus.

Fig. 6. Competitive ELISA between biotinylated 4-11G monoclonal antibody and plasma of vaccinated mice. Pooled plasma samples of mice immunized with S (●) or TMEN (○) were assayed for competition with MAb 4-11G as described under Materials and Methods.
VACCINATION WITH PURIFIED MHV-A59 S

FIG. 7. Protection of BALB/c mice against MHV-A59 by vaccination with purified S. Groups of eight mice were immunized with purified S (●) or TMEN (○) and challenged with MHV-A59 as described under Materials and Methods.

interaction is essential to the 7-10A epitope conformation. Moreover, our results on the immunogenicity of purified S show that despite a loss of native conformation, the protein has conserved some critical determinants. This could be expected in view of our previous study where a synthetic peptide was shown to vaccinate against MHV-JHM infection (Talbot et al., 1988). Neutralization-escape variants (Dalziel et al., 1986; Fleming et al., 1986; Wege et al., 1988) and passive protection by MAb (Buchmeier et al., 1984) have shown that some restricted determinants of S are important for pathogenicity. It should be interesting to verify in further experiments how the loss of some determinants on purified S, which has been partially denatured, could modify the pathogenicity of virus after challenge of vaccinated mice.

Sturman et al. (1985) showed that cleavage of S activates cell fusing properties of MHV-A59 virions and that the two subunits, which were isolated by hydroxyapatite chromatography, both had a molecular mass of 90 kDa. On the other hand, we have observed that the purified monomeric forms consistently migrated on SDS–PAGE with apparent molecular masses of 96–97 and 87–88 kDa. These sizes correlate to those estimated from the nucleotide sequence of the MHV-A59 S gene (Luytjes et al., 1987). From this sequence, the molecular mass estimates are 79.8 and 66 kDa for subunits S1 (previously designated 90B) and S2 (previously designated 90A), respectively, both derived from a 146-kDa apoprotein. Assuming that the remaining 34-kDa of carbohydrate residues are added equivalently to each subunit, which both contain 10 potential N-glycosylation sites, the subunits obtained should have molecular masses of 97 and 83 kDa, which is consistent with the sizes reproducibly observed in our experiments. It is possible that the use of gradient gels allows the separation of such closely migrating species. Thus, the use of preparative linear gradient gels should allow the separation of the S monomers, albeit in their denatured forms. It is also possible that the growth of MHV-A59 in DBT cells leads to a different glycosylation pattern which yields two subunits of different molecular masses, unlike virus grown in L2 cells (Sturman et al., 1985).

Finally, we found that the elution by ammonium thiocyanate of S from the immunoaffinity column yielded more of the cleaved S1 and S2 than elution into SDS–PAGE sample buffer. The mechanism underlying this observation remains to be determined.

Our results confirm and extend previous studies that showed the importance of the S glycoprotein in the pathogenicity of coronaviruses. However, it has been recently reported that the N protein (Lecomte et al., 1987) and M glycoprotein (Fleming et al., 1989) could also have some importance in the immune response against the virus and its pathogenicity. Moreover, further studies are needed to evaluate the biological importance of the 65-kDa HE viral protein which has been associated with hemagglutination and esterase activities (Luytjes, 1989), although its presence in the non-hemagglutinating MHV-S strain (Talbot, 1989) is not consistent with a viral hemagglutinin. The relative contributions of these viral proteins and their in vivo interaction will therefore need to be clarified in order to better understand the molecular basis of coronavirus pathogenesis.

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