

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

CLAUDE DANIEL AND PIERRE J. TALBOT¹

Virology Research Center, Institut Armand-Frappier, Université du Québec, Laval, Québec, Canada H7N 4Z3

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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 μ g 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. © 1990 Academic Press, Inc.

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 (Erich 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; Stohlgren *et al.*, 1986) have been related to S. Recently, protective neutralizing antibodies were induced by immunization with a synthetic decapeptide 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,

¹ To whom requests for reprints should be addressed at Virology Research Center, Institut Armand-Frappier, 531 Boulevard des Prairies, Laval, Québec, Canada H7N 4Z3.

MD), plaque-purified twice, and passaged four times at a multiplicity of infection (m.o.i.) of 0.01 on DBT 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 8×10^6 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-X63-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 (2.5×10^5 cells per well) in RPMI medium containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2.5 $\mu\text{g}/\text{ml}$ fungizone, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO Canada, Burlington, Ontario). This medium was supplemented with 100 μM hypoxanthine, and 16 μM thymidine for the first 4 weeks, and 0.4 μM aminopterin (Sigma) for the first week. The medium was screened for the presence 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 radioimmunoprecipitation of [^{35}S]methionine-labeled MHV-A59-infected DBT cell lysates as described below, as well as by Western immunoblotting (Talbot *et al.*, 1984a). Epitopes recognized by some antibodies were topographically mapped by competitive ELISA as described previously (Talbot *et al.*, 1984b), except that biotinylated antibodies (Guesdon *et al.*, 1979) and streptavidin-peroxidase (Sigma) were used. The sensitivity of these epitopes to denaturation by ammonium isothiocyanate (NH_4SCN) and sodium dodecyl sulfate (SDS) was determined by dot immunoblotting (Talbot *et al.*, 1984a), with the modification that phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 was used as blocking and washing buffer.

Antigen preparation

Virus was produced as described previously (Daniel and Talbot, 1987) in culture medium containing 1% (v/

v) FCS. Medium was harvested when optimal cytopathic effect (CPE) was observed (16 hr postinfection) and cell debris was pelleted at 10,000 *g* for 20 min. Virus was concentrated by precipitation with 10% (w/v) polyethylene glycol in 0.5 *M* NaCl, resuspended, and dialyzed against TMEN buffer (0.1 *M* Tris-acid maleate, pH 6.2, 0.1 *M* NaCl, and 1 mM EDTA) and kept at -70° until needed. In some experiments, radiolabeled virus was produced by adding 4 mCi of [^{35}S]methionine (ICN Biomedicals Canada, St-Laurent, Québec) to 30 ml of culture medium (in two 150-cm² flasks of DBT cells) at 6 hr postinfection.

Immunoabsorbent preparation

For the preparation of the S immunoabsorbent, MAb 7-10A was purified from clarified ascites fluid by protein A-Sepharose (Pharmacia, Dorval, Québec) chromatography (Manil *et al.*, 1986). Five milligrams of the purified MAb was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Unreacted groups on the gel were blocked with 0.2 *M* glycine buffer, pH 8.0, and noncovalently bound proteins were removed with three washing cycles using 0.1 *M* acetate buffer, pH 4.0, and 0.1 *M* sodium carbonate-bicarbonate buffer, pH 8.5, each buffer containing 0.5 *M* NaCl. The gel was stored at 4 $^\circ$.

Affinity chromatography

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 *g* for 45 min. Soluble proteins were mixed with the immunoabsorbent and the mixture was agitated for 16 hr at 4 $^\circ$ on a rotary mixer. The 7-10A-Sepharose gel specificity was determined by immunoabsorption of [^{35}S]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% (w/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-

rography with Enlightning (New England Nuclear, Dupont Canada, Lachine, Québec) or silver staining (Oakley *et al.*, 1980) for batch chromatography samples. Fractions containing purified S were pooled and used for immunological studies.

Immunization experiments

Eight MHV seronegative female 6-week-old BALB/c mice were inoculated i.p. with approximately 1 μ g 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 (5×10^5 PFU).

Neutralization and fusion inhibition assays

Monoclonal antibodies or pooled plasma samples obtained by bleeding from the retroorbital plexus with heparinized capillary tubes 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° 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°, which allowed virus adsorption, culture medium was added to each well, and plates were incubated for 2 days at 37°, 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 DBT 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°, the viral inocula were removed, culture medium was added, and plates were incubated for 3 hr at 37°. This medium was then replaced by culture medium containing serially diluted plasma samples and plates were incubated for another 32 hr at 37°, 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

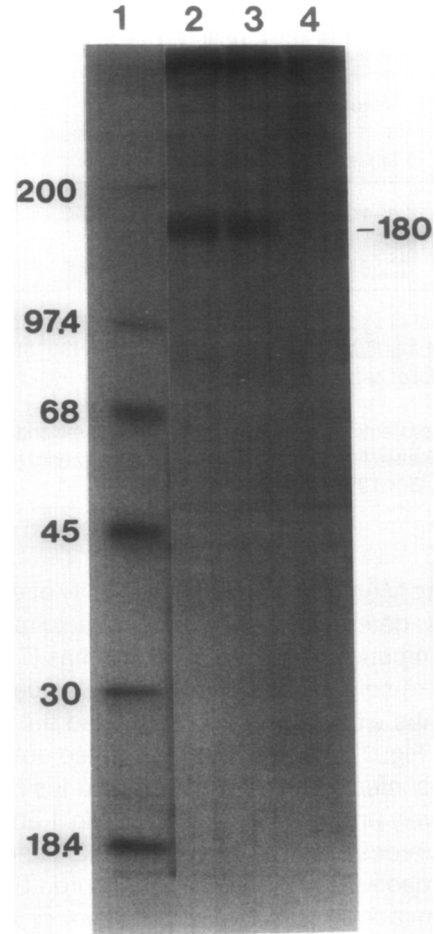


Fig. 1. Immunoprecipitation of [³⁵S]methionine-labeled MHV-A59-infected DBT cells by hybridoma ascites fluids. Lane 1, ¹⁴C-labeled molecular mass markers (New England Nuclear); lane 2, hybridoma 7-10A; lane 3, hybridoma 4-11G; lane 4, myeloma cells. Molecular masses (in Kilodaltons) of markers and of the S dimer are shown on the left and right, respectively.

for 20 min with RIPA buffer supplemented with 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (v/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 μ l of a 10% (w/v) preparation) for 1 hr at 37°, after which radiolabeled antigen was added and the mixture was incubated for 2 hr at 37°. After extensive washing with RIPA buffer containing 0.1% (v/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
BIOLOGICAL PROPERTIES OF ANTI-S MABs

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

^a Reciprocal of ascites dilution neutralizing 50% of viral infectivity in a plaque assay (50–100 PFU) or in a CPE assay (30–300 TCID₅₀).

^b Reciprocal of ascites dilution reducing CPE by 50% when added 4 hr p.i.

^c Passive protection against lethal intracerebral challenge with MHV-A59. Ascites fluids (0.2 ml i.p.) given one day prior to challenge with 10 LD₅₀ (5 × 10⁵ PFU i.c.).

rus-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 *in vitro* and passively protect mice *in vivo* (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 re-

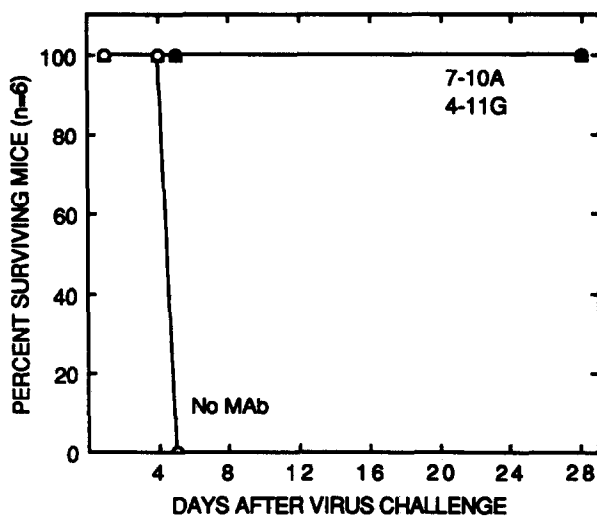


FIG. 2. Passive protection of mice against MHV-A59 by monoclonal antibodies. BALB/c mice received 200 μ l (i.p.) of 7-10A (▲) or 4-11G (●) ascites fluids, or PBS (○), 1 day prior to challenge with 10 LD₅₀ (5 × 10⁵ PFU i.c.) of MHV-A59.

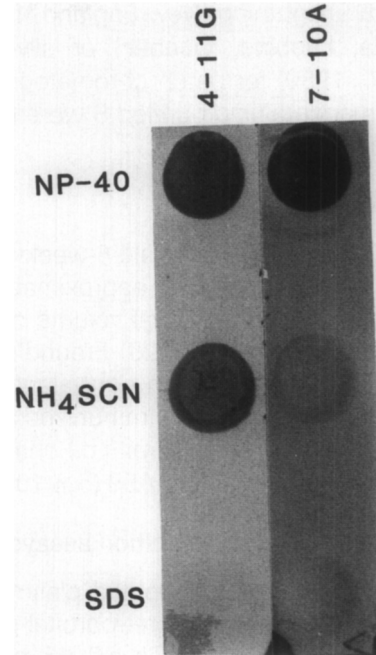


FIG. 3. Sensitivity of two S epitopes to protein denaturation. Concentrated viral antigen solubilized with 2% (v/v) NP-40 was treated with either 4 M NH₄SCN or 1% (w/v) SDS for 5 min at room temperature or 100°, respectively. Dot blot with biotinylated monoclonal antibodies 4-11G or 7-10A was done using 100-fold dilutions of ascites fluids.

mained 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 [³⁵S]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 reacted 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

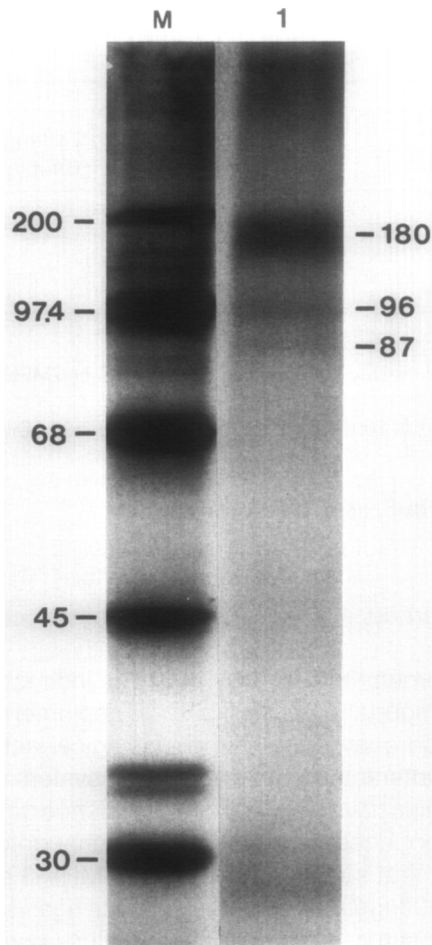


FIG. 4. Immunoadsorption of [^{35}S]methionine-labeled antigen on 7-10A-Sepharose gel. Lane M, ^{14}C -labeled molecular mass markers (New England Nuclear); lane 1, proteins eluted from the anti-S immunoadsorbent. Molecular masses (in Kilodaltons) of markers and S molecules are shown on the left and 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 readsorption 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 LD_{50} 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)

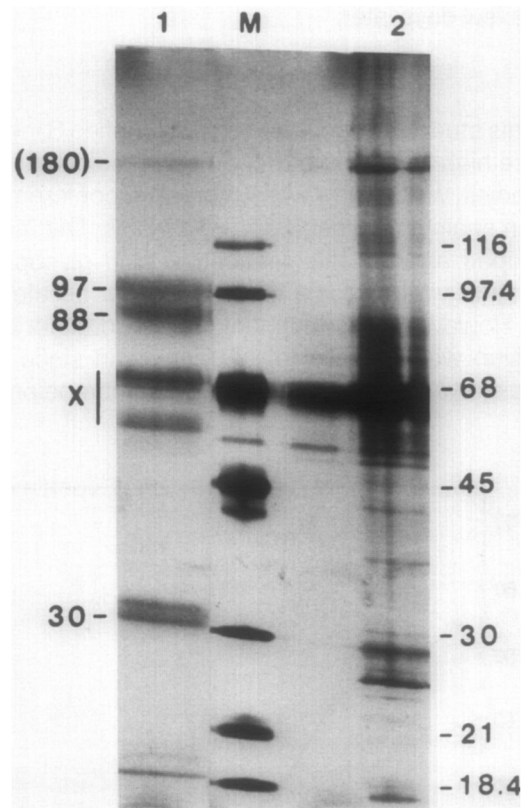


FIG. 5. Silver-stained SDS-PAGE of immunoaffinity-purified S glycoprotein. Lane M, molecular mass markers as indicated on the right side of the figure (in kilodaltons); Lane 1, proteins eluted from the anti-S immunoadsorbent; lane 2, antigen applied on S immunoadsorbent for batch chromatography. The unnumbered lane contains only PAGE sample buffer and shows some artifactual bands (X) reproducibly observed in our silver-stained reducing SDS-PAGE, as also reported by Routledge *et al.* (1988). Molecular masses (in kilodaltons) of proteins eluted from the anti-S immunoadsorbent are shown on the left, with 180 representing an approximation given the fact that the largest molecular mass marker was 116 kDa.

TABLE 2
PROTECTION FROM LETHAL MHV-A59-INDUCED ENCEPHALITIS BY AFFINITY-PURIFIED S

Immunization ^a	Time after first immunization (days)	Neutralizing antibodies ^b		Fusion ^c inhibition	Protection ^e (survivors/challenged)
		Plaque assay	CPE		
S	0	<15	<15	ND ^d	7/7
	40	1600	230	40	
TMEN	0	<15	<15	ND	0/8
	40	<15	<15	10	

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

^b Reciprocal of plasma dilution neutralizing 50% of viral infectivity in a plaque assay (50–100 PFU) or in a CPE assay (30–300 TCID₅₀).

^c Reciprocal of plasma dilution reducing CPE by 50% when added 4 hr p.i.

^d Not done.

^e Ten LD₅₀ (5 \times 10⁵ 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.

Topographical mapping of S antigenic sites have shown that monoclonal antibodies which neutralize virus or inhibit virus-induced fusion bind to structurally different regions (Gilmore *et al.*, 1987), and that both activities could be associated with conformational and nonconformational epitopes (Collins *et al.*, 1982; Talbot *et al.*, 1984a,b). The characterization of the MAbs described in the present study confirms these previous results. In addition, we also show that a conformational antigenic site may be formed of epitopes made of different molecular interactions since epitopes recognized by MAbs 7-10A and 4-11G, which appear to be part of the same SDS-sensitive antigenic site, showed different sensitivities to denaturation by the chaotropic agent ammonium isothiocyanate. These chaotropic agents act mainly on hydrophobic bonds, which are the most significant interactions contributing to structure stability (von Hippel and Wong, 1964; Hatefi and Hanstein, 1969). It seems therefore that this type of

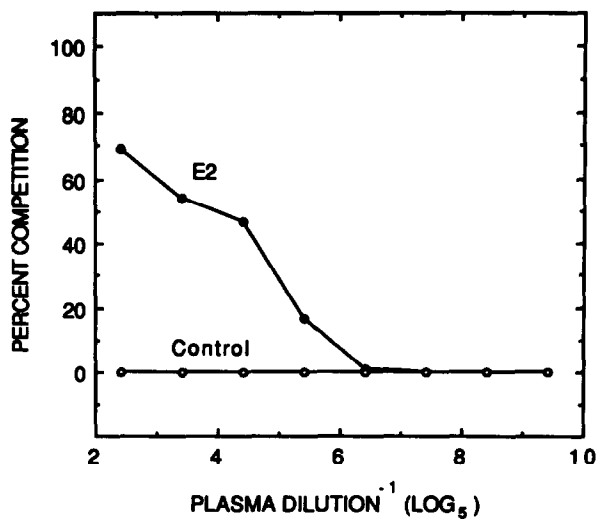


FIG. 6. Competitive ELISA between biotinylated 4-11G monoclonal antibody and plasma of vaccinated mice. Pooled plasma samples of mice immunized with S (●) or TMEN (○) were assayed for competition with MAb 4-11G as described under Materials and Methods.

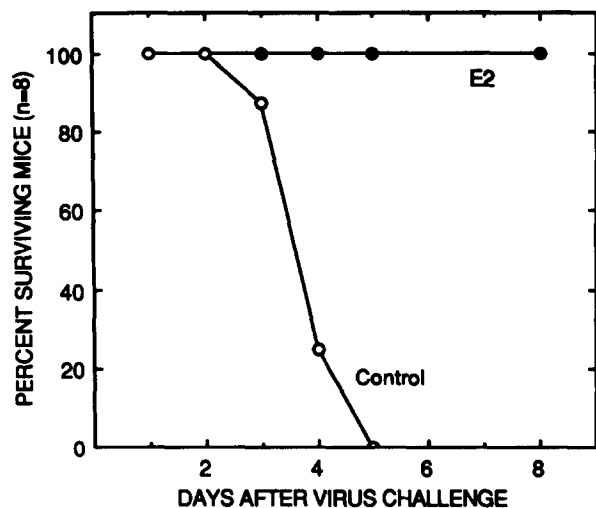


Fig. 7. Protection of BALB/c mice against MHV-A59 by vaccination with purified S. Groups of eight mice were immunized with purified S (●) or TMEN (○) and challenged with MHV-A59 as described under Materials and Methods.

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 MAbs (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-gly-

cosylation 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 isothiocyanate 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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