

## Mapping of Linear Antigenic Sites on the S Glycoprotein of a Neurotropic Murine Coronavirus with Synthetic Peptides: A Combination of Nine Prediction Algorithms Fails To Identify Relevant Epitopes and Peptide Immunogenicity Is Drastically Influenced by the Nature of the Protein Carrier

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The elucidation of the antigenic structure of the S glycoprotein of murine coronaviruses will provide further understanding of the complex pathogenicity of these viruses. In order to identify linear antigenic determinants, the primary structure of the S glycoprotein of murine hepatitis virus strain A59 was analyzed with a combination of nine epitope prediction algorithms. Fifteen potential epitopes were synthesized chemically and injected into BALB/c mice to study their biological relevance. This approach failed to identify novel important epitopes. Furthermore, the algorithms were unable to identify as antigenic the previously mapped immunodominant epitope A [C. Daniel, R. Anderson, M. J. Buchmeier, J. O. Fleming, W. J. M. Spaan, H. Wege, and Talbot, P. J. (1993). *J. Virol.* 67, 1185-1194]. Interestingly, peptide A coupled to KLH induced an immune response that simulated the immune response induced by the corresponding region of the protein much more accurately than when the same peptide was coupled to BSA. This included drastically enhanced competition with monoclonal antibodies and protection from virus challenge. These findings emphasize the shortcomings of amino acid sequence-based epitope prediction algorithms and demonstrate the critical importance of the carrier when synthetic peptides are considered as potential vaccines. © 1994 Academic Press, Inc.

### INTRODUCTION

Coronaviruses are a group of enveloped positive-stranded RNA viruses responsible for a variety of diseases in birds and mammals (Spaan *et al.*, 1990). In humans, strains 229E and OC43 of human coronaviruses (HCV) are recognized as the causative agents of respiratory diseases and may be involved in some diarrhea (Resta *et al.*, 1985). Moreover, their involvement in neurological diseases such as multiple sclerosis (MS) was suggested (Burks *et al.*, 1980; Salmi *et al.*, 1982; Talbot and Jouvence, 1992). Indeed, recent studies have emphasized the possible neurotropism of these human pathogens. Murine-related coronavirus RNA and antigen were detected in postmortem MS-patients brain (Murray *et al.*, 1992a), and primates were shown to be susceptible to infection with murine and murine-related coronaviruses (Murray *et al.*, 1992b). Furthermore, HCV-229E viral RNA was detected in central nervous system (CNS) tissue of some MS patients (Stewart *et al.*, 1992) and a laboratory strain of HCV-229E could infect continuous human cell lines established from human CNS (Talbot *et al.*, 1994).

The A59 and JHM strains of murine hepatitis virus

(MHV) have been studied as animal models of virus-induced disease of the CNS (ter Meulen *et al.*, 1989). These viruses can infect the CNS of mice and cause a biphasic disease, with an acute infection resulting in a mild to severe encephalomyelitis, depending on the strain of virus and the host infected, followed by a chronic demyelinating infection in surviving mice (Lavi *et al.*, 1984; ter Meulen *et al.*, 1989). A chronic infection or a nonlethal demyelinating encephalitis can also be observed in mice infected with point mutation or deletion variants of the spike glycoprotein S or after passive immunization with some monoclonal antibodies (MAbs) specific to this glycoprotein (Buchmeier *et al.*, 1984; Fazakerley *et al.*, 1992; Wang *et al.*, 1992).

In addition to the S glycoprotein (180 kDa), MHV virions contain two or three other structural proteins, which are the membrane glycoprotein M (23 to 26 kDa), the nucleoprotein N (50 to 60 kDa), and the hemagglutinin-esterase HE (65 kDa) (Spaan *et al.*, 1990). Proteolytic cleavage of the S glycoprotein yields the amino-terminal S1 and carboxy-terminal S2 subunits. The S protein is involved in the initial phases of infection by interacting with a cellular receptor and inducing fusion of virion and cell membranes necessary to deliver the viral RNA into the cytoplasm (Gallagher *et al.*, 1991; Vennema *et al.*, 1990; Williams *et al.*, 1991). Hence, it is presumed to bear major

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biological determinants responsible for viral pathogenicity. Indeed, the contribution of the S glycoprotein in the induction of protective immunity was shown directly by vaccination of mice with affinity-purified S glycoprotein (Daniel and Talbot, 1990) and indirectly with the characterization of S-specific MAbs that can protect mice upon passive transfer (Buchmeier *et al.*, 1984).

Recent studies have attempted to localize the antigenic sites of S which are related to important biological determinants. Neutralization-escape variants selected with MAbs have been shown to possess deletions or point mutations in S1 and point mutations in S2 (Parker *et al.*, 1989; Wang *et al.*, 1992). The binding sites of MAbs that have biological activities (neutralization, fusion-inhibition, or protection) have been mapped on the primary structure of the S glycoprotein. It has been suggested that major neutralizing determinants are located in the S1 moiety (Takase-Yoden *et al.*, 1991). However, an epitope recognized by a neutralizing and protecting MAb was localized in S2 (Luytjes *et al.*, 1989). A synthetic peptide encompassing this epitope was shown to induce a protective immune response, despite the absence of neutralizing antibodies (Koolen *et al.*, 1990). This epitope was further shown to be part of an immunodominant linear neutralization domain (Daniel *et al.*, 1993). Somehow, the immunodominant S2 domain could be structurally related to some portion of S1 on the native structure of the protein (Daniel *et al.*, 1993). Another study identified binding sites of neutralizing MAbs on either S1 or S2 (Stühler *et al.*, 1991).

Another approach to locate antigenic determinants involves the use of epitope prediction algorithms and synthetic peptides to identify regions of the protein primary structure which harbor potential epitopes. Such an approach was used successfully to identify a neutralization site in S2 (Talbot *et al.*, 1988). A synthetic decapeptide homologous to this site, coupled to keyhole limpet hemocyanin (KLH), could induce a protective immune response. Methods which have been proposed to predict antibody epitopes usually rely on parameters such as hydrophilicity, surface availability, and flexibility (Hopp, 1986; van Regenmortel, 1989). In the present study, we have analyzed the primary structure of the MHV-A59 S glycoprotein (Luytjes *et al.*, 1987) with nine algorithms in order to identify novel antigenic determinants on this glycoprotein. Fifteen peptides were synthesized chemically and coupled to bovine serum albumin (BSA) to avoid the partial protection previously observed with the KLH carrier protein. The antigenicity and the immunogenicity of these synthetic peptides were evaluated in BALB/c mice. None of these peptides coupled to BSA induced neutralizing antibodies or protected mice against a lethal challenge with MHV-A59. The antigenicity and immunogenicity of peptides coupled to BSA or KLH were shown to be similar in terms of antibody titers. However, anti-

bodies induced by peptides coupled to KLH were much more efficient at competing with peptide-specific MAbs than antibodies induced by peptides coupled to BSA. Thus, peptides coupled with KLH could structurally mimic more accurately epitopes present on the native protein structure and this carrier could also contribute to a more efficient immune response to the viral epitopes.

## MATERIALS AND METHODS

### Virus, cells, and antibodies

The A59 strain of MHV was obtained from the American Type Culture Collection (Rockville, MD), plaque-purified twice, and cultured on DBT cells as described previously (Daniel and Talbot, 1987). MAbs 5B19.2 and E17, specific to the S glycoprotein, were raised against MHV-JHM and characterized as described previously (Buchmeier *et al.*, 1984; Collins *et al.*, 1982; Talbot *et al.*, 1984; Wege *et al.*, 1984). The binding sites of these MAbs were mapped to an immunodominant domain located in S2 (Daniel *et al.*, 1993; Luytjes *et al.*, 1989). An hyperimmune serum against MHV-A59 was obtained after several intraperitoneal inoculations of 50 to 100  $\mu$ g semipurified virus (Talbot, 1989) given at intervals of 2 or 3 weeks, first in complete Freund's adjuvant and then in incomplete Freund's adjuvant.

### Selection and synthesis of peptides

The primary structure of the S polypeptide of MHV-A59 (Luytjes *et al.*, 1987) was analyzed with nine algorithms, each using a window of seven residues. The algorithms used were based on hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Parker *et al.*, 1986), surface probability (Emini *et al.*, 1985; Hopp, 1986; Janin, 1979; Janin *et al.*, 1978; Rose *et al.*, 1985; Welling *et al.*, 1985), and chain flexibility (Karplus and Schulz, 1985). Selected peptides were synthesized as described previously (Talbot *et al.*, 1988) except that the amino-terminal  $^{14}$ C-labeled glycine was omitted. Peptides were coupled to KLH or BSA through a cysteine residue with the coupling agent sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Peptide A was thiolated with 2-iminothiolane (Traut's reagent; Pierce) prior to coupling to the carrier protein. Samples of the conjugated peptides were precipitated with 10% (w/v) trichloroacetic acid and the amount of free peptide which remained soluble was quantified by high-pressure liquid chromatography in order to estimate coupling efficiencies. Conjugated peptides were dialyzed against phosphate-buffered saline (PBS), pH 7.2, and stored at  $-20^{\circ}$ .

### Computer search of a protein sequence

An attempt was made to retrieve the amino acid sequence of KLH for alignment to the sequences of the MHV S proteins to look for conserved sequences. The October 15, 1993 CD-ROM release of the *Entrez*: Sequences databases (GenBank, EMBL, PIR, SWISS-PROT, DDBJ, PDB, PRF, dbEST, U.S. and European Patents) distributed by the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) was used for that purpose.

### Immunization experiments

Groups of 10 MHV-seronegative female 6-week-old BALB/c mice (Charles River, St. Constant, Québec, Canada) were inoculated intraperitoneally with 50  $\mu$ g of conjugated peptides emulsified in an equal volume of complete (first injection) or incomplete (subsequent boosters) Freund's adjuvant. Control mice received an equivalent amount of carrier protein or PBS emulsified in the appropriate Freund's adjuvant.

### Western immunoblotting

Concentrated MHV-A59 antigen was prepared as described previously (Daniel and Talbot, 1990), except that DBT cell monolayers were infected in culture medium adjusted at pH 6.0 instead of pH 7.0 in order to enhance antigenicity (Daniel *et al.*, unpublished observations). Viral proteins were separated by preparative SDS-PAGE (Laemmli, 1970) and electrotransferred to nitrocellulose (Hybond C-Extra, Amersham Searle Corp., Oakville, Ontario, Canada) for 1 hr at 35 V. Strips of nitrocellulose were cut and the reactivity of anti-peptide plasma samples (200-fold dilution) was evaluated by immunoblotting as described previously (Daniel *et al.*, 1993) with the exception that plasma samples were diluted in PBS containing 0.1% (v/v) Tween 20 and 10% (v/v) fetal bovine serum (FBS) in order to adsorb some of the anti-BSA antibodies present in the anti-peptide plasma samples. Culture supernatant of the anti-S 5B19.2 hybridoma (10-fold dilution) was used as positive control in immunoblotting.

### Biotinylation of monoclonal antibodies

Biotin was covalently linked to antibodies with biotinyl-*N*-hydroxysuccinimide (BNHS) (Sigma Chemical Co., St. Louis, MO) as described (Guesdon *et al.*, 1979). Briefly, antibodies at a concentration of 1 mg/ml were dialyzed against PBS, pH 7.4, and mixed with a one-third volume of a 1 mg/ml solution of BNHS in *N,N*-dimethylformamide (Sigma), resulting in an approximately 150:1 molar ratio of BNHS to Ig. The reaction mixture was incubated with agitation at room temperature for 4 hr and then dialyzed

against several changes of PBS and stored at  $-20^{\circ}$  before use.

### ELISA

Synthetic peptides, either unconjugated or conjugated to BSA or KLH, were adsorbed onto wells of 96-well microplates for 16 hr at room temperature at a concentration of 10  $\mu$ g/ml in 0.1 ml per well of 50 mM carbonate buffer (pH 9.6). The reactivity of anti-peptide plasma samples or MAbs against peptides was assayed as described previously (Daniel *et al.*, 1993). The ability of dilutions of anti-peptide plasma samples to inhibit the binding of biotinylated MAbs to peptide A was estimated by competitive ELISA. This assay was performed as described previously (Daniel *et al.*, 1993). In one experiment, KLH (Sigma) was adsorbed onto microplates (10  $\mu$ g/well) and the reactivity of an hyperimmune antiviral antiserum ascertained. Endpoint ELISA titers were determined as the reciprocal of the highest dilution of plasma which gave an absorbance value greater than the cut-off absorbance, a value determined as two standard deviations above the mean absorbance of dilutions of a control plasma (mice inoculated with PBS emulsified in Freund's adjuvant).

## RESULTS AND DISCUSSION

### Selection and immunogenicity of synthetic peptides

Analysis of the primary structure of the MHV-A59 S glycoprotein with epitope prediction algorithms allowed us to identify 15 probable antigenic sites (Fig. 1). A peptide encompassing the previously identified epitope A (Luytjes *et al.*, 1989) was added to this study. The peptide sequences and selection criteria are listed in Table 1. The immunogenicity of peptides coupled to BSA was evaluated in BALB/c mice. The choice of BSA as carrier protein for immunogenicity studies was based on the observation of a partial protection conferred by KLH (Talbot *et al.*, 1988) and which could be explained by a fortuitous cross-reactivity between antisera specific for MHV and KLH (Fig. 2). The molecular basis for this possible molecular mimicry provoked by a sharing of amino acid sequences between B-cell epitopes of KLH and the MHV-A59 S protein could not be ascertained since it appears that the primary structure of KLH has not yet been determined or is not contained within the most recent nucleic acid and protein sequence databases. As shown in Table 2, none of the conjugated peptides induced neutralizing antibodies, as determined by a plaque reduction assay (Daniel and Talbot, 1990), or protected mice against a lethal intracerebral challenge with MHV-A59. However, with the exception of peptides 6, 8, and 12, the conjugated peptides were highly immunogenic since titers of at least 125,000 in ELISA were observed (Table 2).

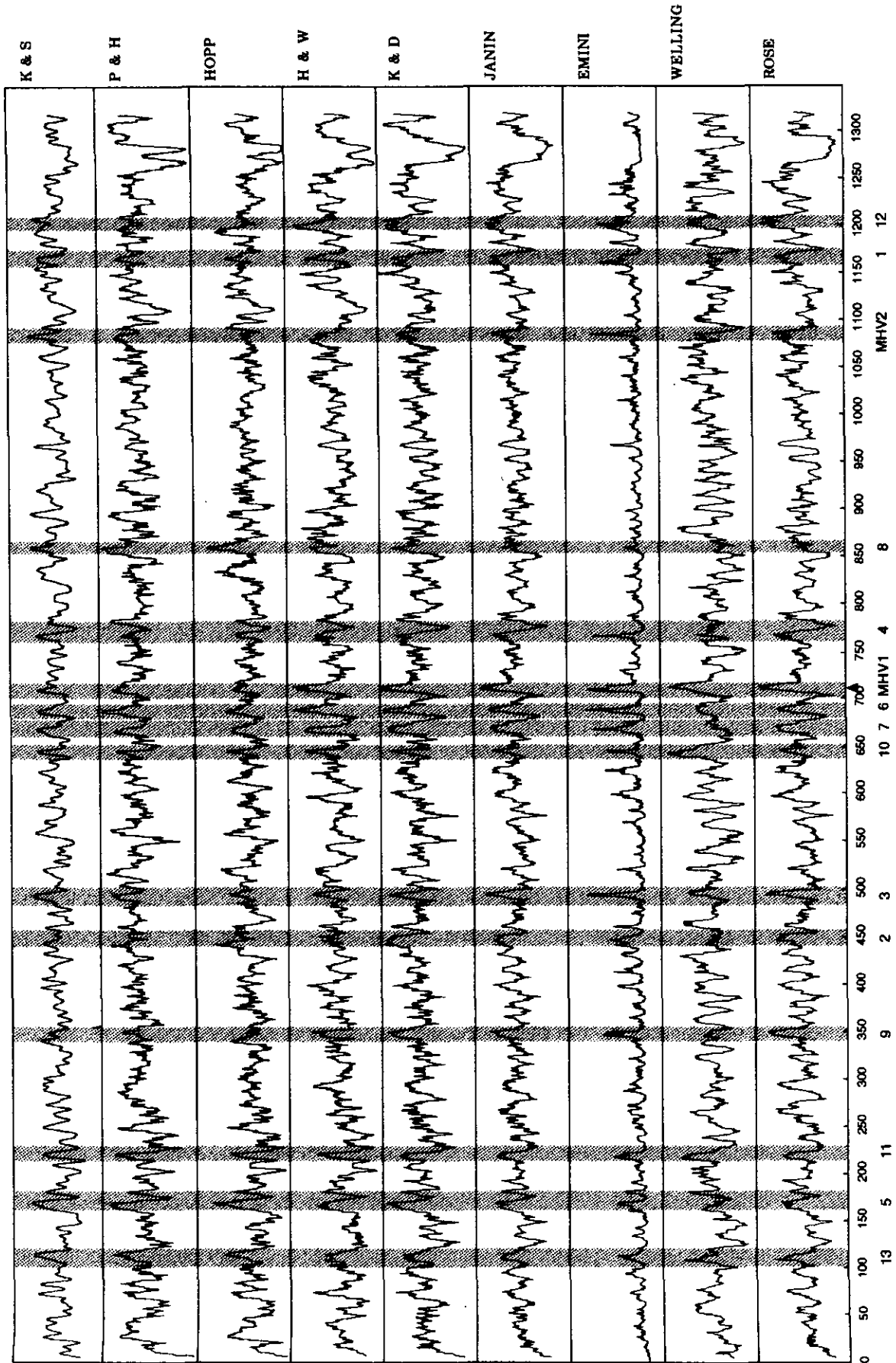


FIG. 1. Analysis of the primary sequence of the MHV-A59 S glycoprotein with nine epitope prediction algorithms. The algorithms used are abbreviated on the right of the figure: K & S (Karpilus and Schulz, 1985), P & H (Parker *et al.*, 1986), Hopp (Hopp, 1986), H & W (Hopp and Woods, 1981), K & D (Kyte and Doolittle, 1982), Janin (Janin, 1978), Janin *et al.*, 1978), Emimi (Emimi *et al.*, 1985), Welling (Welling *et al.*, 1985), and Rose (Rose *et al.*, 1985). The numbers of the peptides synthesized are indicated at the bottom and the arrowhead indicates the proteolytic cleavage site on the S protein.

TABLE 1  
SYNTHETIC PEPTIDES SELECTED

Peptide <sup>a</sup>	Position	Amino acid sequence <sup>b</sup>	Selection criteria
1	1156-1170 (16-mer)	(C)-T-G-S-S-Y-Y-P-E-P-I-T-D-K-N <i>N</i>	Major peak according to Emini.
2	441-458 (18-mer)	Y-N-P-S-S-W-N-R-R-Y-G-F-K-V-N-D-R-C <i>N-D-A-G-V-F</i>	Peptide which overlaps the N-terminal sequence of the deletion observed in MHV-Wb1 isolate. Exceptionally, the residues indicated beneath are those present in the MHV-A59 and MHV-4 S gene sequences.
3	485-499 (15-mer)	D-I-V-S-P-C-T-T-Q-T-K-P-K-S-A <i>A S M</i>	Highest peak according to Emini.
4	761-775 (15-mer)	E-E-F-I-Q-T-R-S-P-K-V-T-I-D-C <i>I A</i>	Major peak according to Emini.
5	165-176 (12-mer)	C-K-P-N-T-N-G-N-K-L-I-G <i>R-V</i>	Highest peak according to Karplus and Schultz; major peak according to Parker and Hodges, Hopp, and Kyte and Doolittle.
6	682-694 (13-mer)	C-V-V-N-A-D-N-R-T-D-E-A-L <i>*</i>	Highest peak according to Parker and Hodges and Hopp and Woods; major peak according to Emini.
7	663-675 (14-mer)	(C)-S-N-N-I-S-R-E-E-N-P-L-N-Y <i>T *</i>	Major peak according to Kyte and Doolittle, Janin, Emini, and Rose.
8	856-866 (11-mer)	C-A-E-D-G-N-G-P-S-A-I	Highest peak according to Hopp; major peak according to Karplus and Schultz, and Parker and Hodges.
9	343-354 (12-mer)	P-L-N-W-E-R-K-T-F-Q-N-C <i>R</i>	Major peak according to Kyte and Doolittle, Janin, Emini, and Rose.
10	633-648 (16-mer)	C-Y-S-G-R-V-S-A-A-F-H-K-D-A-P-E <i>Y E</i>	Highest peak according to Welling; major peak according to Kyte and Doolittle, and Emini.
11	217-227 (12-mer)	Y-A-D-K-P-S-A-T-T-F-L-(C)	Major peak according to Parker and Hodges, and Welling.
12	1198-1208 (12-mer)	D-F-K-E-E-L-D-K-W-F-K-(C)	Major peak according to Hopp and Woods, Kyte and Doolittle, Emini, and Rose.
13	106-120 (16-mer)	(C)-A-K-V-Q-N-L-K-T-S-T-P-S-G-A-T <i>N T</i>	Major peak according to Karplus and Schultz, Parker and Hodges, Welling, and Rose.
MHV1	617-629 (13-mer)	C-V-D-Y-S-K-S-R-R-A-R-R-S <i>H</i>	Highest peak according to Hopp and Woods, Kyte and Doolittle, Janin, Welling, and Rose. The peptide sequence is deduced from MHV-Wb1 and encompasses the putative cleavage site.
MHV2	993-1002 (10-mer)	C-V-K-S-Q-T-T-R-I-N	Major peak according to Karplus and Schultz, and Emini.
A	846-858 (13-mer)	S-P-L-L-G-C-I-G-S-T-C-A-E	Peptide which encompasses the epitope recognized by MA5B19.2 (Luytjes <i>et al.</i> , 1988).

<sup>a</sup> All peptides, except 2, MHV1, and MHV2, are deduced from the gene sequence of the MHV-A59 S glycoprotein and the corresponding numbering (Luytjes *et al.*, 1987). The MHV1 and MHV2 peptides were selected after the analysis of the MHV-JHM (MHV-Wb1) S glycoprotein gene sequence (Schmidt *et al.*, 1987; Talbot *et al.*, 1988).

<sup>b</sup> Amino acid substitutions in the MHV-4 S gene sequence (Parker *et al.*, 1989) are shown in italic beneath the peptide sequence (in A59 for peptide MHV1). The character "(C)" represents an added cysteine used for coupling to a carrier protein and the character "\*" indicates a potential N-glycosylation site.

The reactivity of anti-peptide antibodies with native or denatured S glycoprotein was verified by immunoprecipitation (Daniel and Talbot, 1990) or immunoblotting assays, respectively. None of the plasma samples were able to immunoprecipitate the S glycoprotein. Nevertheless, antibodies raised against peptides 1, 4, and 12 reacted specifically with S2 (96 kDa) on immunoblots (Fig. 3). As expected, the reactivity with BSA present in the antigen used for immunoblotting was also evident (Fig. 3). It is noteworthy that antibodies raised against peptide 8, which was not reactive with the S glycoprotein in immunoblotting (Fig. 3), was previously shown to react specifically with a fragment of the S protein expressed in a prokaryotic vector (Daniel *et al.*, 1993). This suggests an insufficient sensitivity of the

immunoblotting assay with the MHV-A59 antigen, although the use of viral antigen produced at pH 6.0 greatly enhanced the detectability of reactivity against the S glycoprotein (Daniel *et al.*, unpublished observations). The weak reactivity between anti-peptide antibodies and the unfolded S glycoprotein could be explained by a greater flexibility of the peptide on the carrier protein than the corresponding region on the protein. Indeed, unlike internal portions, the amino and carboxy termini of proteins are generally exposed on the surface and they often bear linear epitopes which can be mimicked by synthetic peptides (van Regenmortel *et al.*, 1988). It was impossible to demonstrate a specific reaction of the anti-peptide antibodies against MHV-A59 in ELISA.

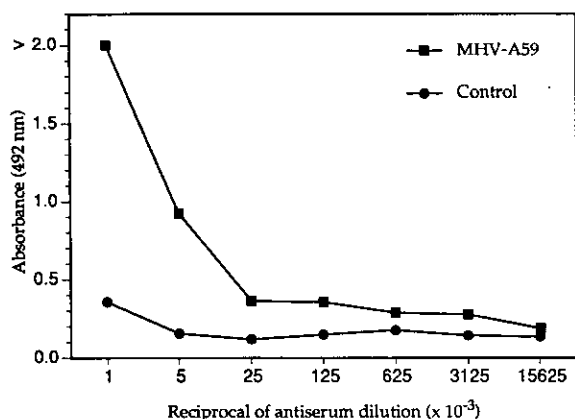


FIG. 2. Cross-reactivity between KLH and MHV. KLH was adsorbed onto 96-well ELISA plates and the reactivity of MHV-A59-specific or preimmune mouse antisera was assayed as described under Materials and Methods.

### Comparison of BSA and KLH as carrier proteins

Peptide MHV2 coupled to KLH (MHV2/KLH) was previously shown to induce neutralizing antibodies and a protective immune response in BALB/c mice (Talbot *et al.*, 1988). However, peptide MHV2 coupled to BSA (MHV2/BSA) neither induced neutralizing antibodies nor protected mice against virus challenge in the current study. Furthermore, the frequency of anti-peptide anti-

TABLE 2

IMMUNOGENICITY OF SYNTHETIC PEPTIDES<sup>a</sup>

Plasma (peptides)	Anti-peptide ELISA titer <sup>b</sup> ( $\times 10^{-3}$ )	Neutralization	Protection
1	625	— <sup>c</sup>	—
2	125	—	—
3	125	—	—
4	625	—	—
5	625	—	—
6	25	—	—
7	625	—	—
8	5	—	—
9	125	—	—
10	625	—	—
11	625	—	—
12	5	—	—
13	125	—	—
MHV1	3125	—	—
MHV2	3125	—	—

<sup>a</sup> Ten BALB/c mice were immunized i.p. on Day 0 with 50  $\mu$ g of synthetic peptides coupled to BSA and emulsified in an equal volume of complete Freund's adjuvant. A second and third injection were given on Days 21 and 40 with the same amount of conjugated peptides emulsified in incomplete Freund's adjuvant. Mice were challenged 2 weeks after the last injection with 10 LD<sub>50</sub> of MHV-A59 ( $5 \times 10^5$  PFU).

<sup>b</sup> Unconjugated peptides were used as antigens in the ELISA.

<sup>c</sup> Neutralization titer (50%) < 1/50.

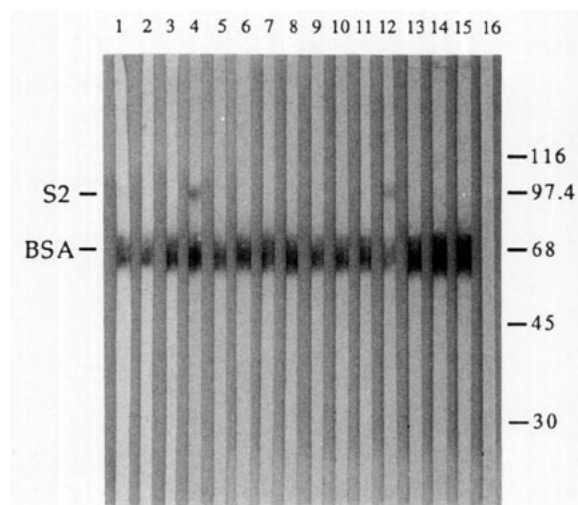


FIG. 3. Immunoblots of MHV-A59 antigen with anti-peptide plasma samples. Lanes 1 to 13, corresponding anti-peptide plasma; 14 and 15, anti-MHV1 and anti-MHV2 plasma samples, respectively; 16, plasma samples of mice inoculated with PBS. The migrations of S2 and BSA are shown on the left and the molecular mass standards (kDa) on the right.

bodies which cross-reacted with the S glycoprotein was deceptively low (3 out of 15). These results prompted us to compare the efficiency of the BSA and KLH molecules as carrier proteins. Mice were immunized with the MHV2 or A peptides coupled to BSA or to KLH. The anti-peptide titers of the plasma raised against MHV2/BSA were 5 times higher than the titer of the plasma raised against MHV2/KLH (Table 3). Inversely, peptide A/KLH was slightly more immunogenic than peptide A/BSA. None of these conjugated peptides induced neutralizing antibodies. Nevertheless, peptide A/KLH could induce an immune response which was protective against a lethal challenge with MHV-A59 (Table 3). Clinical signs of infection (ruffled fur, hunched position, hyperirritability) were

TABLE 3

IMMUNOGENICITY OF PEPTIDES A AND MHV2 COUPLED TO BSA OR KLH<sup>a</sup>

Plasma (peptides)	Anti-peptide ELISA titer <sup>b</sup>	Neutralization	Protection
MHV2/BSA	781,250	— <sup>c</sup>	0/10
MHV2/KLH	156,250	—	0/10
Peptide A/BSA	156,250	—	0/10
Peptide A/KLH	781,250	—	5/5

<sup>a</sup> Ten BALB/c mice were immunized i.p. on Day 0 with 50  $\mu$ g of synthetic peptides coupled to BSA or KLH and emulsified in an equal volume of complete Freund's adjuvant. A booster injection was given on Day 21 with the same amount of conjugated peptides emulsified in incomplete Freund's adjuvant. Mice were challenged 2 weeks after the last injection with 10 LD<sub>50</sub> of MHV-A59 ( $5 \times 10^5$  PFU).

<sup>b</sup> Unconjugated peptides were used as antigens in the ELISA.

<sup>c</sup> Neutralization titer (50%) < 1/50.

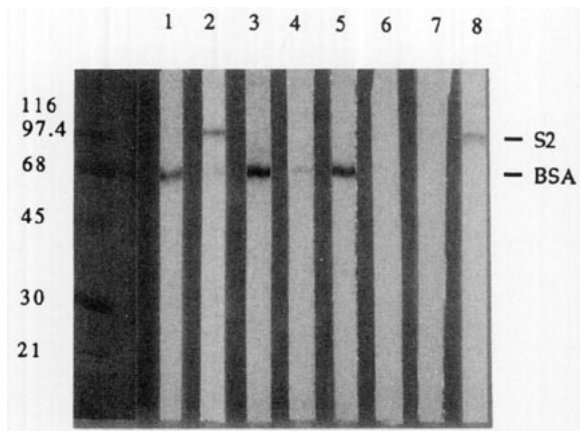


Fig. 4. Immunoblots of MHV-A59 antigen with plasma samples raised against peptides MHV2 and peptide A coupled to BSA or KLH. Lanes 1 and 2, anti-peptide A coupled to BSA or KLH, respectively; 3 and 4, anti-MHV2 coupled to BSA or KLH, respectively; 5 and 6, anti-BSA or anti-KLH, respectively; 7, plasma samples of mice immunized with PBS; 8, 5B19.2 hybridoma supernatant. The migrations of S2 and BSA are shown on the right and the molecular mass standards (kDa) on the left.

less severe in the peptide A/KLH group, confirming protection. Interestingly, the plasma of mice immunized with peptide A/KLH was the only one to react with the S glycoprotein in immunoblotting (Fig. 4). Finally, the ability of antibodies raised against peptide A/KLH or peptide A/BSA to inhibit the binding of MAbs which react with the peptide (5B19.2 and E17; Daniel *et al.*, 1993) was compared by competitive ELISA. As shown in Fig. 5, antibodies raised against peptide coupled to KLH competed much more efficiently with the anti-S MAbs.

The results obtained with peptide MHV2/KLH contrast those of a previous study (Talbot *et al.*, 1988). However, different peptide lots and conjugates were used in each study. It was suggested previously that an anti-peptide titer higher than 65,000 correlated with protection (Talbot *et al.*, 1988). In spite of differences in the ELISA assay used in each study and in the calculation of antibody titers, our results suggest that MHV2/KLH was highly immunogenic. On the other hand, the average peptide/carrier ratio of the MHV2/KLH conjugates was 310 in our study and 36 in the previous study (Talbot *et al.*, 1988). Such a difference between the peptide/carrier ratios, which could influence the presentation of the peptide on the surface of the carrier, may explain the discrepancy between the results of the two studies. In addition, sulfosuccinimidyl 4-(*p*-maleimido-phenyl) butyrate (Sulfo-SMPB) was previously used as coupling agent (Talbot *et al.*, 1988), rather than sulfo-SMCC (this study). Thus, a possible lack of reproducibility of peptide immunization studies performed with different preparations of the same peptides could represent a major problem in correlating experimental results with the antigenic structure of a protein.

We have shown that antibodies raised against peptide

A/KLH inhibit the binding of two MAbs to peptide A in a much more efficient fashion than antibodies raised against peptide A/BSA. We can speculate that both MAbs recognize the native structure of the S glycoprotein since one of them (MAb 5B19.2) neutralizes viral infectivity (Collins *et al.*, 1982) and these MAbs compete with each other (Daniel *et al.*, 1993). The proportion or affinity of antibodies which reacted with a particular conformation of peptide A that is recognized by the MAbs must be greater in the plasma of mice immunized with peptide A/KLH than in the plasma of mice immunized with peptide A/BSA. Thus, our results suggest that peptide A coupled to KLH more efficiently simulated the immune response raised by the corresponding region of the protein on the native S glycoprotein. It was previously reported that peptide A coupled to a T-cell epitope of the influenza virus hemagglutinin (peptide A/HA) protected mice against a lethal challenge with MHV-A59 whereas peptide A/KLH was unable to induce this protection (Koolen *et al.*, 1990). Moreover, protection was conferred without the induction of neutralizing antibodies. These authors suggested a mechanism of antibody-dependent cell cytotoxicity (ADCC) to mediate this protection. They showed a direct correlation between protection and the antibody titer of the anti-peptide sera. In the present study, such a high antibody titer (Table 3) could also explain the protection observed with peptide A coupled to KLH, by a mechanism which may also involve ADCC.

#### Antigenicity of synthetic peptides

To evaluate a possible correlation between the observed drastic differences in the immunogenic potential

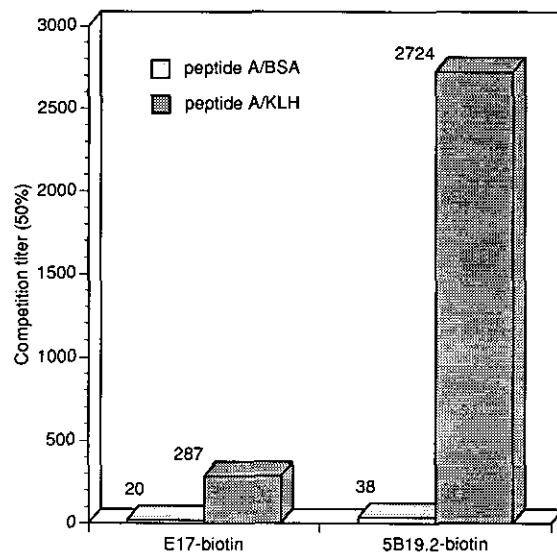


Fig. 5. Inhibition of binding of biotinylated MAbs 5B19.2 and E17 to peptide A by antibodies raised against peptide A coupled to BSA or KLH. The 50% competition titers represent the reciprocal of plasma dilutions which inhibited by 50% the binding of MAbs 5B19.2 or E17 to peptide A in a competitive ELISA. The experimental titers are indicated at the top of each bar.

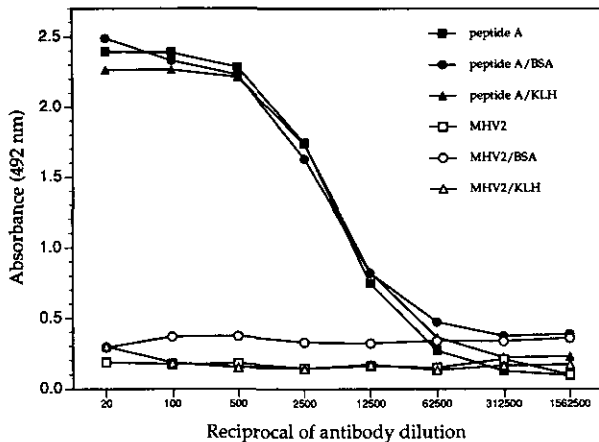


Fig. 6. Reactivity of MAb 5B19.2 against peptides A or MHV2 either free or coupled to BSA or KLH. Peptides were adsorbed onto 96-well ELISA plates and the reactivity of 5B19.2 hybridoma supernatant was evaluated as described under Materials and Methods.

of various peptide preparations with peptide antigenicity, the binding of MAb 5B19.2 to peptide A coupled to KLH or to BSA, or to the uncoupled peptide, was compared by ELISA and results showed that peptide antigenicity was similar, independently of peptide presentation (Fig. 6). Furthermore, we have previously shown that peptide A is recognized by four other different MABs in ELISA (Daniel *et al.*, 1993). The results obtained with peptide A contrast those reported in a previous study where MABs were shown to be more reactive with peptide coupled to a protein carrier (Mäkelä *et al.*, 1989). The possible formation of a disulfide bond in peptide A may constrain the structural mobility of this peptide in a state more representative of its protein counterpart, which may explain the exceptionally high reactivity with MABs, even if the peptide is uncoupled. Finally, in order to identify previously unmapped antigenic determinants, a large library of MABs specific for the S glycoprotein of MHV-A59 or MHV-JHM (Daniel *et al.*, 1993) was assayed for binding to free or BSA-coupled synthetic peptides in ELISA. None of the MABs reacted specifically with any of the 15 peptides (data not shown).

In conclusion, nine B-cell epitope prediction algorithms failed to identify potentially exposed or immunogenic structures on the S glycoprotein. Interestingly, peptide A, which is located within the linear immunodominant domain identified on the primary structure of the S protein (Daniel *et al.*, 1993) and which overlaps the N-terminal extremity of peptide 8, could not be predicted as antigenic by any of the methods and actually corresponds to troughs in the profiles (Fig. 1). Two studies have compared the efficiency of algorithms to predict antigenic determinants (Pellequer *et al.*, 1991; van Regenmortel and Daney de Marcillac, 1988). Generally, the probability of correctly identifying epitopes on the primary structure of proteins does not exceed 50 to 55%. Our

study emphasizes that these estimates may be overly optimistic. Furthermore, the fact that we could not identify new linear epitopes on the primary structure of the S glycoprotein with 15 synthetic peptides selected by an optimized combination of nine epitope prediction algorithms, using both peptide immunization and binding assays with a large library of S-specific MABs, supports our previous conclusions that continuous epitopes are concentrated in the immunodominant domain located in S2 (Daniel *et al.*, 1993). Indeed, more than 90% of B-cell epitopes on globular proteins may be discontinuous (Horsfall *et al.*, 1991) and would be missed by the use of peptides synthesized chemically (this study) or in bacteria (Daniel *et al.*, 1993).

Finally, the observation of a drastic qualitative immunological difference between BSA and KLH peptide conjugates emphasizes the importance of optimizing presentation of synthetic peptides to the immune system when studying immunogenicity of a protein and developing synthetic vaccines. Presumably, the carrier used could influence the nature of the immune response induced via distinctive interactions with cell mediators such as antigen-presenting cells (APC) or different subsets of T helper lymphocytes. For example, BSA and KLH may activate APC by different mechanisms due to their respective sizes and capacities to concentrate in the lymphoid centers (Heyman, 1990; Wiersma, 1992), which could influence the subsets of T-cells triggered and consequently the cytokine responses (Mosmann and Coffman, 1989; Scott and Kaufmann, 1991). T-cell epitopes harbored by each carrier could also influence per se the process of T-cell activation (Lowenadler *et al.*, 1992). This could lead to the preferential production of antibodies bearing immunoglobulin isotypes that are more reactive for antibody-dependent complement- or cell-mediated cytotoxicity, or even the preferential production of higher affinity antibodies or antibodies that more efficiently neutralize the initial stages of viral infection (Kenney *et al.*, 1989). These studies emphasize the importance of evaluating neutralization mechanisms. Such studies are in progress.

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