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Characterization of Murine Coronavirus Neutralization Epitopes with Phage-Displayed Peptides

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Phage-displayed peptide libraries were used to map immunologically relevant epitopes on the surface (S) glycoprotein of a neurotropic murine coronavirus (MHV-A59). Three *in vitro* virus-neutralizing and *in vivo* protective mAbs against either continuous or discontinuous epitopes on the S glycoprotein were used to screen 12 different peptide libraries expressed on the pVIII major coat protein of the fd filamentous bacteriophage. Consensus sequences that matched short sequences within the S glycoprotein were identified. The sequence of a tight-binding, mAb-selected peptide suggested the location of a discontinuous epitope within the N-terminal S1 subunit. Several tightly binding phage were amplified and used directly as immunogens in BALB/c and C57BL/6 mice. Partial protection of C57BL/6 mice against a lethal acute virus infection was achieved with a phage preparation that displayed a linear epitope. Protection correlated with the presence of sufficient levels of specific antiviral antibodies recognizing the same immunodominant domain and 13-mer peptide, located within the C-terminal S2 subunit, as the selecting mAb. Thus, the direct use of phage-displayed peptides to evaluate protective antiviral immune responses complements their use to characterize antibody-binding epitopes. This is the first evaluation of protective immunization induced by mAb-selected phage-displayed peptides.

INTRODUCTION

Coronaviruses are enveloped, positive-stranded RNA viruses of the *Coronaviridae* family (Holmes and Lai, 1996). Among the identified viral structural proteins, the surface (S) glycoprotein forms spike projections from the surface of the virion and is involved in binding to cellular receptors and in membrane fusion (Collins *et al.,* 1982; Sturman *et al.,* 1985; Williams *et al.,* 1991). It is also the target of humoral and cellular immune responses (Collins *et al.,* 1982; Stohlman *et al.,* 1995; Williamson and Stohlman, 1990). Moreover, various reports have emphasized the protective capacity of anti-S glycoprotein antibodies (Buchmeier *et al.,* 1984; Nakanaga *et al.,* 1986). A proteolytic cleavage site divides the S glycoprotein into two subunits, the N-terminal S1 and the C-terminal S2 (Spaan *et al.,* 1988; Sturman *et al.,* 1985). It is believed that S1 forms the bulbous part of the spike and S2 the stalk portion (De Groot *et al.,* 1987). It has also been shown that the N-terminal region of the S1 subunit is involved in binding to the cellular receptor (Kubo *et al.,* 1994). Murine coronaviruses, also called mouse hepatitis viruses (MHV), induce various respiratory, gastrointestinal, and neurological infections in rodents. The neurotropic strains A59 and JHM lead to infections of the central nervous system that result in an acute lethal encephalitis (Cheever *et al.,* 1949; Weiner, 1973). Mice who survive the acute infection develop a chronic demyelinating disease resembling human multiple sclerosis (Siddell *et al.,* 1983).

We have used a panel of specific mAbs to characterize the molecular determinants of the S glycoprotein that are involved in the induction of a protective immune response against MHV-A59-induced lethal encephalitis. MAbs 7-10A, 5B19, and 5B170 were selected for the present study since they neutralize virus infectivity *in vitro* and passively protect BALB/c mice against lethal infection *in vivo* (Buchmeier *et al.,* 1984; Daniel and Talbot, 1990). MAb 7-10A was predicted to recognize a discontinuous epitope on the S1 subunit since it did not bind to the denatured S glycoprotein in Western immunoblots (Daniel and Talbot, 1990) and did bind to purified, native S1 subunit (P. J. Talbot and K. V. Holmes, unpublished data). In contrast, mAbs 5B19 and 5B170 recognize linear epitopes within an immunodominant region of the S2 subunit (Daniel *et al.,* 1993); critical binding residues have been identified for mAb 5B19 (Luytjes *et al.,* 1989). A previous study using papain- and pepsin-derived fragments or recombinant single chain Fv molecules derived from mAb 7-10A showed that the Fc portion of this antibody is not required for neutralization of MHV-A59 *in vitro* and *in vivo* (Lamarre and Talbot, 1995; Lamarre *et al.,* 1997); this mAb can also induce protective anti-idiotypic antibodies (Yu *et al.,* 1996).

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TABLE 1

Yields for Each Round (R) of Panning for All Peptide Libraries

		% Yield (log_{10})				ELISA OD (405–490 nm)	
mAb	Library	R1	R2	R3	R4	R3	R4
5B170	X6	−2.65	< −3.6	< −3.6	< −3.1	0.037	0.035
	LX6B	−3.9	< −3.6	< −3.6	< −3.1	0.032	0.031
	LX8	−3.7	< −3.6	< −3.6	< −3.1	0.032	0.034
	LX10	−3.9	< −3.6	< −3.6	< −3.1	0.031	0.031
	LX4	−3.9	< −3.6	**−0.2**[a]	−0.26	**0.091**[a]	0.153
	X15	−4.5	< −3.6	< −3.6	< −3.1	0.034	0.029
	X8CX8	−4.2	−4.5	−1.2	**−0.6**[a]	0.053	**0.185**[a]
	XCX15/X15CX	−4.1	< −3.6	< −3.6	< −3.1	0.035	0.037
	Cys3	−4.0	< −3.6	< −3.6	< −3.1	0.030	0.032
	Cys4	−1.7	< −3.6	−0.6	−0.1	0.038	0.087
	Cys5/6	−4.5	−2.7	−1.0	**−0.3**[a]	0.287	**0.526**[a]
	f88.4	−5.3	< −3.6	< −3.6	< −3.1	0.037	0.059
5B19	X6	−4.0	−1.6	< −3.6	< −3.1	0.017	0.016
	LX6B	−3.8	−2.7	< −3.6	< −3.1	0.018	0.016
	LX8	−3.8	< −3.6	**−2.2**[a]	< −3.1	**0.015**[a]	0.014
	LX10	−3.8	< −3.6	< −3.6	< −3.1	0.018	0.016
	LX4	−3.9	< −3.6	< −3.6	< −3.1	0.020	0.076
	X15	−4.5	< −3.6	< −3.6	< −3.1	0.015	0.015
	X8CX8	−3.9	< −3.6	**−1.7**[a]	< −3.1	**0.022**[a]	0.035
	XCX15/X15CX	−4.2	< −3.6	< −3.6	< −3.1	0.018	0.018
	Cys3	−4.1	< −3.6	< −3.6	< −3.1	0.013	0.013
	Cys4	−4.1	−2.8	**−0.8**[a]	−1.7	**0.811**[a]	0.690
	Cys5/6	−4.2	< −3.6	< −3.6	< −3.1	0.015	0.016
	f88.4	−5.5	< −3.6	< −3.6	< −3.1	0.018	0.028
7-10A	X6	−4.0	< −3.6	< −3.6	−2.2	0.147	0.068
	LX6B	−3.9	−0.7	−1.0	ND[b]	0.056	0.042
	LX8	−3.6	−2.1	**0.08**[a]	−0.3	**0.127**[a]	0.140
	LX10	−3.9	−2.6	−0.22	−0.9	0.104	0.081
	LX4	−3.7	< −3.6	−1.52	−0.61	0.047	0.047
	X15	−5.1	−1.9	−0.7	−2.2	0.114	0.077
	X8CX8	−3.9	−2.7	−0.5	−0.6	0.123	0.099
	XCX15/X15CX	−4.0	−2.7	−0.5	**−0.9**[a]	0.070	**0.121**[a]
	Cys3	−4.0	< −3.6	−0.7	−0.6	0.090	0.159
	Cys4	−4.7	−2.1	−0.6	**−0.9**[a]	0.086	**0.523**[a]
	Cys5/6	−4.1	−1.8	−0.2	−0.3	0.189	0.305
	f88.4	−4.5	< −3.6	< −3.6	< −3.1	0.041	0.055

[a] Phage pools selected for cloning are indicated in boldface.
[b] ND, not determined.

Phage-displayed peptide libraries offer an attractive approach for the identification of discontinuous (Chen *et al.,* 1996; Hoess *et al.,* 1994) and linear peptide ligands for mAbs (Cwirla *et al.,* 1990; Felici *et al.,* 1991; Hoess *et al.,* 1994; Scott and Smith, 1990; Stephen and Lane, 1992) and for studying the immunogenic properties of the selected sequences (de la Cruz *et al.,* 1988; Demangel *et al.,* 1996; Minenkova *et al.,* 1993; Prezzi *et al.,* 1996). We now report on the characterization of epitopes recognized by protective anti-coronavirus antibodies and the direct use of a selected phage to evaluate protective immune responses in mice. Partial protection correlated with the production of specific antiviral antibodies.

RESULTS

Selection and characterization of phage-displayed peptides by mAbs 7-10A, 5B19, and 5B170

The mAbs 7-10A, 5B19, and 5B170 were used to screen, side by side, different conformationally constrained and unconstrained phage libraries. As indicated in Table 1, phage pools having the highest yields and ELISA signals were selected for further study. From these, phage clones were isolated and sequenced in the nucleotide region encoding the displayed peptides (Table 2). As illustrated in Table 1, the highest yields in rounds 3 and 4 of panning for all three mAbs were from libraries displaying one or two fixed cysteine residues,

TABLE 2

Amino Acid Sequences of Phage-Displayed Peptides Selected with Three mAbs

mAb	Library[a]	Phage clone	Amino acid sequence[b]	ELISA OD (405–490)	Effect of DTT[c]
5B170	Cys 4	9.1,[d] 9.3, 9.4, 9.5, 9.9	SASRS**CIGSQC**STTA	0.847	−
	LX4	7.2, 7.4, 7.6, 7.7, 7.8	G**CIGSYC**V	0.760	−
	X8CX8	8.3	GPALKPRG**CVGSTC**FWA	0.652	−
	X8CX8	8.1, 8.4	AEG**CVGRVC**DSRAMAVM	0.372	±
	X8CX8	8.2, 8.9	SEL**CIGSVC**TFWTTRMR	0.170	ND[e]
	f88.4			0.065	
5B19	Cys 4	33.2, 33.4, 33.5, 33.6, 33.9	ASTTN**CIGSQC**LMTN	1.096	−
	f88.4			0.049	
7-10A	Cys 4	12.4, 12.5, 12.7, 12.8	DPARDCVHNICIFAG	0.647	+
	Cys 4	12.9	ADREYCKYTVCTYPG	0.547	−
	LX8	10.10	RCKDLLTLAKCH	0.392	ND
	LX8	10.1, 10.8	NC**RPR**LELMPCV	0.275	ND
	X15CX	11.1	GMQAFF**RPR**CAKTLSCA	0.216	+
	X15CX	11.5	**TDR**L**DR**CLPAISTDPCF	0.216	+
	X15CX	11.6	MRLQVSPETLFWMECS	0.197	ND
	LX8	10.3	KCTN**TDRP**PFCQ	0.181	BD[f]
	LX8	10.4	QCAPAWRGRNCG	0.150	ND
	X15CX	11.10	SCLVVAG**DR**WMHLPSCE	ND[e]	ND
	f88.4			0.141	

[a] Randomized amino acids in each library are designated by X, and C represents cysteine.
[b] Consensus motifs are shown in boldface.
[c] Data from Fig. 3.
[d] Clone inducing protective antibody responses in some mice.
[e] Not determined.
[f] Below detection level.

and the ELISA data showed further restriction of the tightest binding phage to a single library for each mAb. Analysis of the amino acid sequences of the selected phage allowed the identification of consensus sequences for peptides recognized by the antiviral mAbs 5B19 and 5B170, which recognize continuous epitopes (Table 2). The critical binding sequence for each of these two mAbs was very similar since phage selected by both mAbs cross-reacted: phage selected by mAb 5B170 were recognized strongly by mAb 5B19 and vice versa, albeit at a lower level, whereas no phage reacted with the isotypic control mAb (Fig. 1). For mAb 7-10A, which recognizes a discontinuous epitope (Daniel and Talbot, 1990), consensus amino acid sequences were more difficult to identify; this is typical of antibodies against discontinuous epitopes (e.g., see Felici *et al.,* 1993; Luzzago *et al.,* 1993).

Figure 2 illustrates the amino acid identities between selected phage-displayed peptides and murine coronavirus S glycoprotein sequences. In contrast with other studies (Balass *et al.,* 1993; Felici *et al.,* 1993; Luzzago *et al.,* 1993), amino acid identity was obtained for phage 12.9, selected by mAb 7-10A, and a linear region of the S glycoprotein of several coronavirus strains. The 7-10A

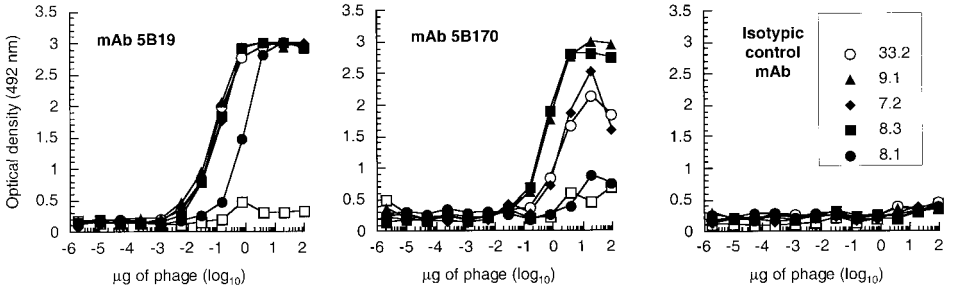

FIG. 1. ELISA for determining cross-reactive phage recognition by mAbs 5B19 and 5B170. Microtiter plates were coated with 1 μg/ml of the indicated mAbs, and binding of varying amounts of mAb 5B19- and 5B170-selected phage was determined using horseradish peroxidase-labeled anti-M13 conjugate. Open symbols represent phage selected by mAb 5B19 (except □, control vector phage f88.4), and filled symbols represent phage selected by mAb 5B170.

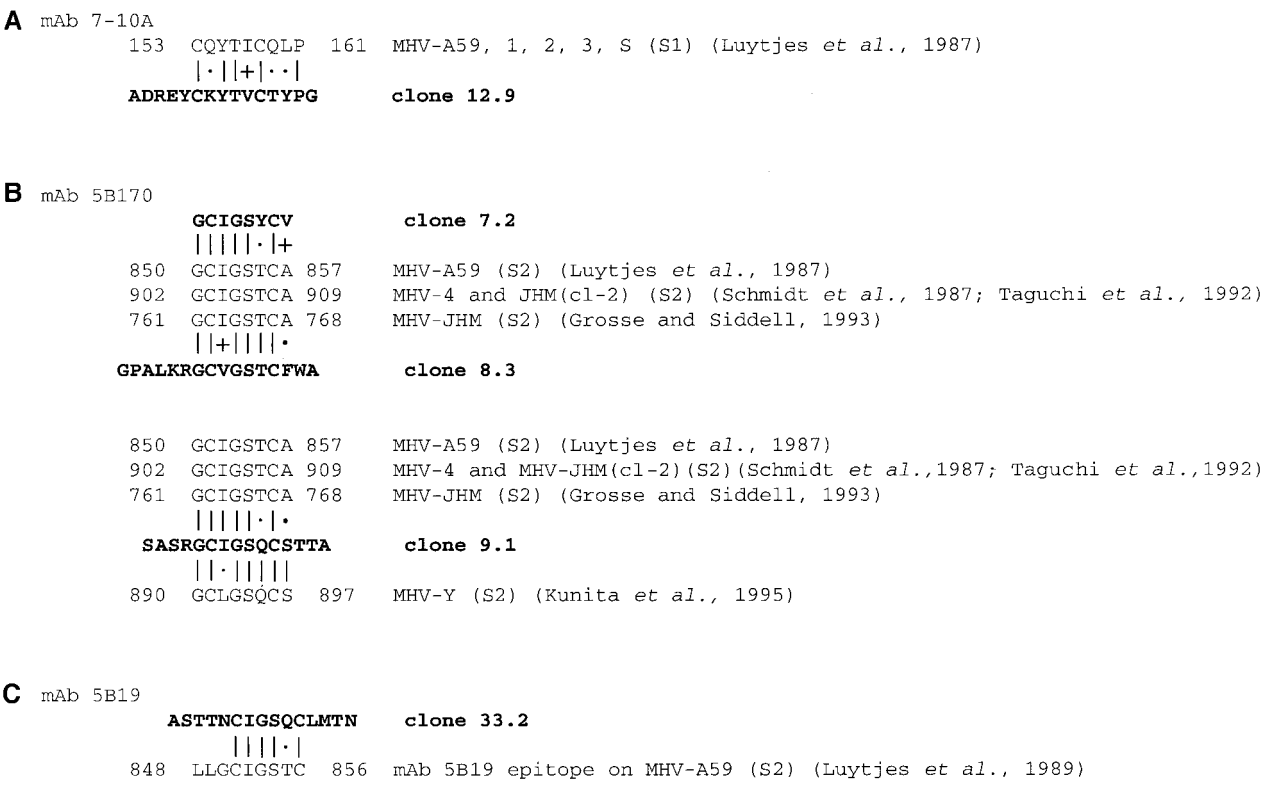

FIG. 2. Alignment of viral protein sequences and peptides selected with mAbs: (A) 7-10A, (B) 5B170, and (C) 5B19. The clones and the amino acid sequences of their phage-displayed peptides are shown in boldface. Amino acid identities are indicated with vertical bars, conservative amino acid differences with plus signs, and dissimilarities with dots. Differences observed in amino acid numbering of different sequences of coronaviruses are due to deletions of various lengths found in the S1 subunit from different strains of MHV (strains A59, JHM, 4).

mAb recognizes a discontinuous epitope on S1, as shown by binding studies on isolated S glycoprotein subunits (P. J. Talbot and K. V. Holmes, unpublished data). Clone 12.9 contains two cysteines and bears similarities to residues 153–161 of the S glycoprotein of MHV. This clone represents the first sequence information obtained on the epitope recognized by mAb 7-10A.

For mAb 5B170, Fig. 2 shows that three different phage clones had amino acid identity with sequences of the S glycoprotein of MHV. Sequences within and including the C(V/I)GSXC consensus of clones 7.2 and 8.3 show a match of 6 amino acids within residues 850–857 of MHV-A59 (Luytjes *et al.,* 1987), 902–909 of MHV-4 (Schmidt *et al.,* 1987) and MHV-JHM (cl-2) (Taguchi *et al.,* 1992), and 761–768 of MHV-JHM (Grosse and Siddell, 1993). Moreover, the matching sequence of clone 8.3 represents an almost exact match with the residues mentioned above since the Ile to Val amino acid change is conservative. Finally, the matching sequence observed for clone 9.1 shares almost complete identity with residues 890–897 of MHV-Y (Kunita *et al.,* 1995); it also shares 6 amino acids with the same residues described above. This is the first detailed information reported on the epitope recognized by mAb 5B170, which was only known to bind to an immunodominant region on the S2 subunit of the S glycoprotein (Daniel *et al.,* 1993). This epitope appears to be conserved among several strains of MHV.

We used mAb 5B19 as a positive control for our experiments since synthetic peptide scanning (PEPSCAN) was previously used to characterize the epitope recognized by this mAb (Luytjes *et al.,* 1989). The peptide sequence that it selected bears the C(V/I)GSXC consensus identified by mAb 5B170, confirming our results. This result is consistent with previous epitope mapping by ELISA (Daniel *et al.,* 1993) showing that mAbs 5B170 and 5B19 compete with each other for binding to virus. The sequences of the binding regions for both antibodies are identical in MHV-JHM and A59, suggesting that the epitopes recognized by mAbs 5B19 and 5B170 are conserved on the S2 subunit of MHV. To exclude the possibility that these two mAbs are the same or a closely related antibody, the V_H regions of both antibodies were sequenced. Table 3 illustrates the very different amino acid sequences of complementarity-determining regions (CDR) of mAbs used in this study.

To evaluate the importance of disulfide bridging in binding to mAbs, an ELISA test was performed in the presence or absence of the reducing agent DTT. The results shown in Fig. 3 indicate that disulfide bridging within the phage-displayed peptides was more important

TABLE 3

Amino Acid Sequences of the H1 and H2 CDRs of mAbs 5B19 and 5B170

mAb	VH family	CDR-H1	CDR-H2	CDR3-H3
7-10A[a]	ND[b]	SGYSWHW	YIHYSGSTTYNPSLKS	YYEYFDY
5B19	VH2	SYGVHW	VIMSDGSTTYNSALKS	EPPTYFAY
5B170	VH6	NYWMS	EIRLKSDNYVT	SAYGLY

[a] Data from Lamarre *et al.,* 1997.
[b] Not determined.

for recognition by mAb 7-10A than for mAbs 5B19 and 5B170, since the binding of mAb 7-10A with peptides was more affected by DTT. As a control, immobilized virus was also treated with DTT; the binding of mAb 7-10A to virus was not affected (Fig. 3B). However, the binding of mAbs 5B19 and 5B170 with the virus was unexpectedly more affected when the virus was treated with DTT. The results suggest that the two cysteines observed in the peptides recognized by mAbs 5B19 and 5B170 may directly interact with the mAb binding site or that disulfide constraints are important but not absolutely required for binding. The alignment analysis shown in Fig. 2C, in which two cysteines form a portion of the epitope, is supported by the fact that several of the clones selected by mAb 5B170 came from the X8CX8 library, in which only one Cys residue is fixed. The second, C-terminal Cys residue in the sequence was selected by mAb from a fully randomized residue.

Immunization and induction of a protective immunity

It was previously shown that the epitope recognized by mAb 5B19 is able to protect BALB/c mice when used as an immunogen (Daniel *et al.,* 1993; Koolen *et al.,* 1990). Having identified peptides that are recognized by three *in vitro* neutralizing and *in vivo* protective mAbs, we examined whether these peptides, displayed on the phage surface, could be used directly as immunogens for the induction of neutralizing and protective antibodies in mice. Selected phage showing relatively strong ELISA signals were injected into both BALB/c and C57BL/6 mice; it has been shown that C57BL/10 mice respond better than BALB/c mice for induction of specific antipeptide antibodies, using the phage itself as a carrier molecule (de la Cruz *et al.,* 1988). Four ip injections were given, followed by viral challenge 10 days after the last injection. The survival of mice was then evaluated. As shown in Fig. 4, of all the different phage selected by the three mAbs, only the mAb 5B170-selected clone 9.1 induced a statistically significant protective immune response against a lethal coronavirus infection. Three of six C57BL/6 mice were protected, whereas no BALB/c mice immunized with the same phage 9.1 survived the viral challenge. Mice immunized with control vector phage or other selected phage died between days 4 and 9 after the viral challenge, a normal outcome of acute viral encephalitis. The observed survival of one of six C57BL/6 mice immunized with clone 12.4 selected by mAb 7-10A was not statistically significant.

Humoral immune response

To evaluate the possible humoral immune mechanisms underlying the observed, statistically significant protection of C57BL/6 mice by the mAb 5B170-selected clone 9.1, postimmunization plasma samples were tested by ELISA for the detection of antiviral antibodies. Antiviral antibodies were specifically induced in protected C57BL/6 mice (Table 4); this was statistically significant with a P value of 0.01 from the Mann–Whitney test (comparing means at a 1/500 plasma dilution). No statistically significant levels of specific antiviral antibodies were detected in mice immunized with other selected phage or the control phage vector, suggesting a strong correlation between the presence of specific antiviral antibodies and protection.

To confirm the specificity of the antiviral antibodies induced by clone 9.1 to the displayed peptide, two complementary experimental approaches were used. First, Western blotting was performed with a bacterially expressed immunodominant S2 subunit region that includes the mAb 5B19 and 5B170 epitopes. As shown in Fig. 5, plasma samples from C57BL/6 mice immunized

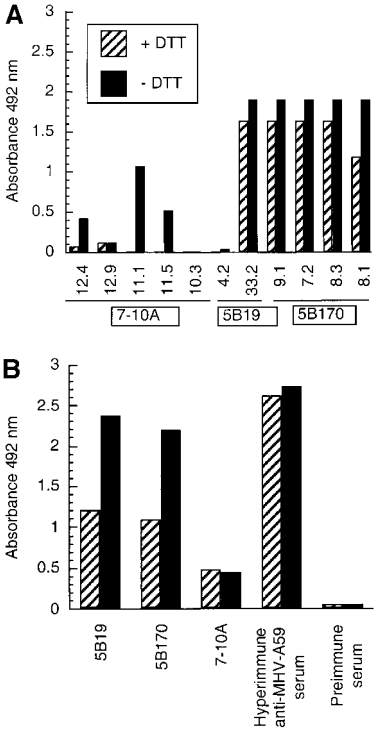

FIG. 3. ELISA of mAb binding to immobilized phage (A) and to immobilized MHV-A59 (B) under reducing and nonreducing conditions. Results are representative of at least two separate experiments.

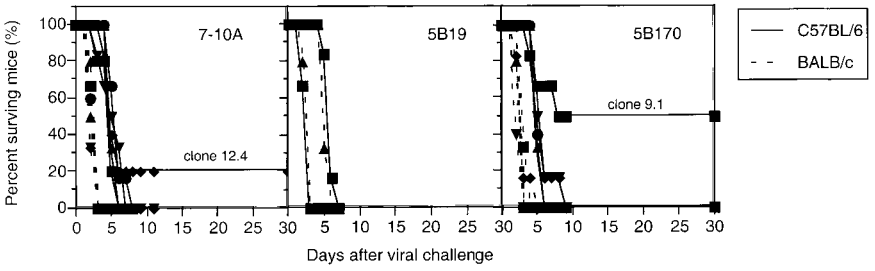

FIG. 4. Survival of BALB/c and C57BL/6 mice immunized with phage-displayed mAb-selected peptides. Groups of six mice were immunized ip with different selected phage and then challenged intracerebrally with 10 LD_{50} of MHV-A59. Phage clones selected by mAb 7-10A: 10.3 (■), 11.1 (●), 11.5 (▲), 12.4 (◆), 12.9 (▼). Clones selected by mAb 5B19: 33.2 (■). Clones selected by mAb 5B170: 9.1 (■), 7.2 (●), 8.3 (▼), 8.1 (◆). Control vector phage, f88.4 (▲). Protection of C57BL/6 mice by clone 9.1 was statistically significant and is representative of two separate experiments.

with clone 9.1 strongly and specifically recognized the immunodominant S2 subunit region, as did mAb 5B170 used for panning. Even BALB/c mice, which were not protected against the viral infection, had raised specific antibodies to the immunodominant S2 subunit region, although the response appeared weaker (fainter bands). The immunoglobulin isotypic control and preimmune plasma, as well as plasma from mice immunized with other phage, did not recognize the immunodominant region, confirming the specificity of antiviral antibodies to the phage-displayed peptides.

Second, a more quantitative and precise assay was used: synthetic peptide ELISA. We initially used synthetic peptide hexamers corresponding to consensus sequences observed on phages that reacted more strongly with virus-specific mAbs (Table 2): CIGSQC (phage clones 9.1, 9.3, 9.4, 9.5, and 9.9 selected with mAb 5B170 and phage clones 33.2, 33.4, 33.5, 33.6, and 33.9 selected with mAb 5B19) and CIGSYC (phage clones 7.2, 7.4, 7.6, 7.7, and 7.8 selected with mAb 5B170). All peptides were tested with plasma of immunized mice and the corresponding mAbs in both linear and conformationally con-

TABLE 4

Induction of Specific Antiviral Antibodies in Mice Immunized with Phage-Displayed Peptides

Mouse strain	Phage clone	No. of mice	Corrected ELISA absorbance at 1/500 plasma dilution	Significance of ELISA absorbance value[a]	Neutralization titer[b]
C57BL/6	9.1	6	1.59 ± 0.50	0.01	<1/50
	7.2	5	0.63 ± 0.13	NS[c]	<1/50
	8.3	6	0.62 ± 0.35	NS	<1/50
	8.1	6	0.56 ± 0.26	NS	<1/50
	10.3	5	0.63 ± 0.48	NS	<1/50
	11.1	6	0.92 ± 0.41	NS	<1/50
	11.5	6	0.51 ± 0.36	NS	<1/50
	33.2	6	0.90 ± 0.72	NS	<1/50
	12.4	5	0.42 ± 0.34	NS	<1/50
	12.9	4	0.31 ± 0.32	NS	<1/50
	f88.4	6	0.57 ± 0.43	ND[d]	<1/50
BALB/c	9.1	6	1.03 ± 0.35	NS	<1/50
	7.2	6	1.19 ± 0.65	NS	<1/50
	8.3	5	0.64 ± 0.67	NS	<1/50
	8.1	6	0.67 ± 0.16	NS	<1/50
	10.3	6	0.44 ± 0.27	NS	<1/50
	11.1	5	0.47 ± 0.40	NS	<1/50
	11.5	6	0.66 ± 0.37	NS	<1/50
	33.2	6	0.59 ± 0.25	NS	<1/50
	12.4	6	0.48 ± 0.35	NS	<1/50
	12.9	5	0.39 ± 0.37	NS	<1/50
	f88.4	5	0.56 ± 0.39	ND	<1/50

[a] *P* values from Mann–Whitney test comparing absorbance means at a 1/500 plasma dilution between mice immunized with phage clones and with control vector phage f88.4

[b] Highest dilution of plasma neutralizing 50% of input virus.

[c] Not significant.

[d] Not determined.

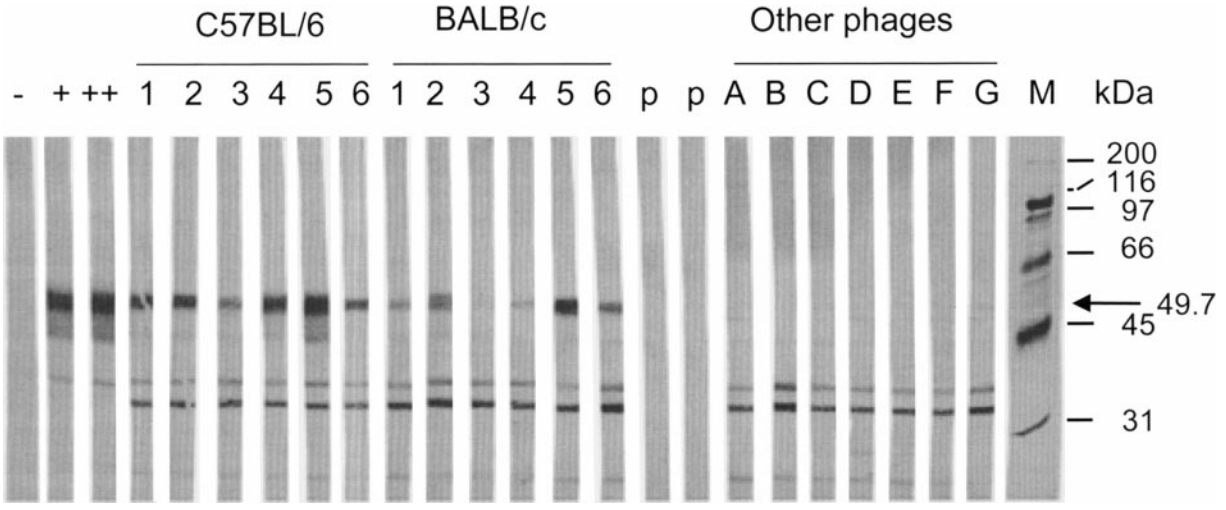

FIG. 5. Western blot analysis of virus-specific antibody in the plasma of mice immunized with selected phage-displayed peptides. The immunodominant region of the S2 subunit of the S glycoprotein of MHV-A59 was loaded into 10% (w/v) polyacrylamide SDS–PAGE gel and electrotransferred onto a nitrocellulose membrane. −, mAb F18 that recognizes an epitope located outside the immunodominant region of the S2 subunit of the S glycoprotein; +, mAb 5B170; ++, mAb 5B19; 1, 2, 3, 4, 5, and 6, individual mouse numbers (clone 9.1-immunized mice); p, C57BL/6 and BALB/c mice preimmune sera; A, phage f88.4-immunized C57BL/6 mouse; B, phage f88.4-immunized BALB/c mouse; C, clone 8.1-immunized C57BL/6 mouse; D, clone 7.2-immunized C57BL/6 mouse; E, clone 8.3-immunized C57BL/6 mouse; F, clone 33.2-immunized C57BL/6 mouse; G, clone 33.2-immunized BALB/c mouse; M, molecular mass standards (Bio-Rad). The molecular mass of 49.7 kDa represents the immunodominant region expressed in *Escherichia coli*.

strained cyclic forms. However, using various amounts of peptides and different buffers, we could barely demonstrate reactivity with mAbs and did not observe reactions with plasma of immunized mice (data not shown). We concluded that the peptides were too short for binding to the plastic of the microtiter plates and instead used the 13-mer peptide A that we had previously identified and used in ELISA (Spaan *et al.,* 1988). This peptide bears a CIGSTC sequence that is very similar to both consensus sequences described above and is flanked on either side by three amino acids from the viral S protein sequence. As shown in Fig. 6, only mice immunized with phage clone 9.1 reacted specifically with peptide A and C57BL/6 mice produced higher titers of peptide-specific antibodies than BALB/c mice. Interestingly, reactivities were seen only with the linear peptide, suggesting that disulfide bridging is not involved in antibody binding, thereby adding strength to data obtained with DTT (Fig. 3).

To evaluate possible mechanisms of protection mediated by specific antiviral antibodies, we evaluated the *in vitro* virus neutralization activity. As shown in Table 4, no *in vitro* neutralization activity was detected at dilutions of plasma samples that could be tested, down to a dilution

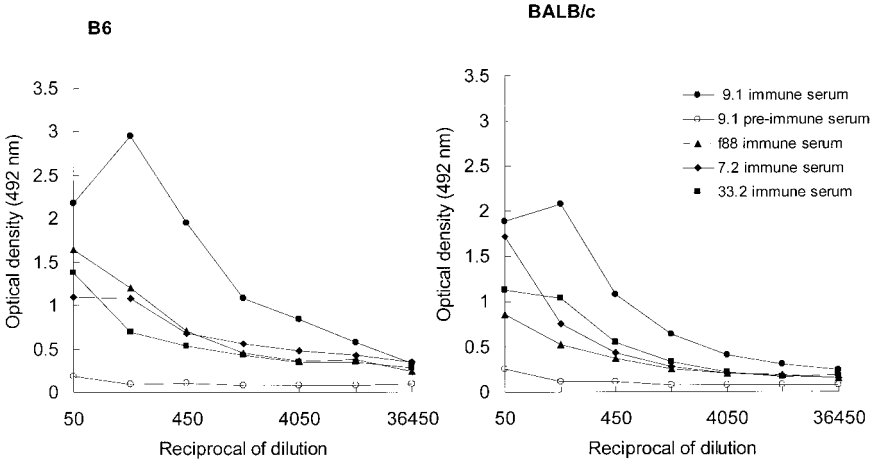

FIG. 6. ELISA analysis of peptide A-specific antibody in the plasma of mice immunized with selected phage-displayed peptides. Microtiter plates were coated with 5 μg/ml peptide A and incubated with serial threefold dilutions of plasma from C57BL/6 (left) or BALB/c (right) mice immunized with phage 9.1, 7.2, or 33.2 or control phage vector f88.

of 1/50. Moreover, antibody-dependent cell-mediated cytolysis (ADCC) and antibody-dependent complement-mediated cytolysis (ADCMC) activities were also evaluated, but such activities were not detected either (down to a dilution 1/50 for ADCC and 1/10 for ADCMC; data not shown). Positive control mAbs were used in all the *in vitro* assays (except for the ADCC assay because, to our knowledge, no positive mAb is available in the coronavirus system). For unknown reasons and despite numerous attempts in our laboratory, ADCC activities could not be demonstrated for various anti-MHV mAbs (data not shown).

In summary, we have used three *in vitro* neutralizing and *in vivo* protective anti-MHV mAbs to select phage-displayed peptides, which allowed further characterization of immunologically relevant epitopes on the S glycoprotein of MHV-A59. Moreover, we were able to demonstrate *in vivo* induction with a phage-displayed peptide of specific antiviral antibodies that were associated with a partial but significant protection.

DISCUSSION

In this study, we used a panel of constrained and unconstrained phage-displayed peptide libraries to define binding motifs for and map the epitopes recognized by three murine coronavirus-specific mAbs. MAb 7-10A was thought to bind a discontinuous epitope (Daniel and Talbot, 1990) on the S1 subunit of the S glycoprotein of murine coronavirus (P. J. Talbot and K. V. Holmes, unpublished data); whereas mAbs 5B19 and 5B170 were known to bind linear epitopes on the S2 subunit of the S glycoprotein (Daniel *et al.,* 1993). Previous studies have shown that peptides can mimic linear, discontinuous, and even nonproteinaceous epitopes (Harris *et al.,* 1997; Oldenburg *et al.,* 1992; Scott *et al.,* 1992; Zhong *et al.,* 1994). All three mAbs selected binding phage from libraries encoding one or two fixed cysteine residues, but not from the unconstrained peptide libraries. Many of the sequences of the mAb-selected phage bore similarity with linear regions on the S glycoprotein of several murine coronaviruses. Immunization studies with a number of the tightest binding phage revealed that one clone, whose displayed peptide was a linear-epitope mimic, was able to induce protective immunity in a significant proportion of mice.

For discontinuous epitopes, only limited identity is usually found between the sequence of a peptide mimic and that of the corresponding epitope on the protein antigen (Felici *et al.,* 1993; Luzzago *et al.,* 1993). Despite the lack of an obvious consensus sequence among the clones selected with mAb 7-10A (Table 2), clone 12.9, which reacted relatively strongly in ELISA, shares five to six homologous amino acids with a linear region between amino acids 153 and 161 of the S1 subunit of the S glycoprotein (Fig. 2). This N-terminal, globular domain is involved in the binding of virus to cells (Kubo *et al.,* 1994); however, for several reasons, the epitope recognized by mAb 7-10A is probably not directly involved in binding to the cellular receptor. First, we have previously shown that mAb 7-10A only weakly inhibits virus binding to the cell, whereas it strongly neutralizes viral infectivity after the virus binds to the cell (Y. Salvas, S. Lemieux, and P. J. Talbot, unpublished data). In contrast, mAb 5B19, whose epitope is located on the C-terminal, stalk-like S2 subunit, is far more potent than mAb 7-10A in inhibiting viral binding to cells (Y. Salvas, S. Lemieux, and P. J. Talbot, unpublished data). Second, clone 12.9 did not inhibit the attachment of MHV-A59 to DBT cells (data not shown), suggesting that the displayed peptide did not bind to the cellular receptor. Third, in a recent study (Saeki *et al.,* 1997), it was shown that amino acid residues 62–65 on S1 are involved in binding to the cellular receptor; the mAb 7-10A epitope mimic displayed by clone 12.9 (Fig. 2) does not localize within this region.

Despite the potential for conformational mimicry, mice immunized with clone 12.9 phage (as well as other phage selected by mAb 7-10A) did not survive a lethal dose of coronavirus (Fig. 4). Presumably, the peptides selected by this mAb did not mimic the complete discontinuous epitope sufficiently and/or did not bind strongly enough to elicit detectable cross-reactivity with the viral antigen. The binding of mAb 7-10A to several clones that did not bear the same peptide sequence as clone 12.9 indicates that further studies using, for example, synthetic peptides bearing amino acid replacements in the sequence of the clone 12.9 peptide are needed to pinpoint the exact nature of this epitope. Such studies will be important given the apparent importance of this epitope in the virus life cycle. Indeed, we have been unable to select viable mAb 7-10A-resistant virus variants (P. J. Talbot, unpublished data) and mAb 7-10A very efficiently protects against acute viral infection (Daniel and Talbot, 1990), without apparent involvement of the Fc portion (Lamarre and Talbot, 1995; Lamarre *et al.,* 1997).

Phage selected by mAbs 5B19 and 5B170 showed consensus sequences that share homology with the S2 subunit of the S glycoprotein. The C(I/V)GSXC motif selected by mAbs 5B19 and 5B170 from different phage libraries highlights the importance of these residues to antibody binding. Most phage selected by both mAbs bear two Cys residues separated by four amino acids. Peptides with this Cys pattern were even selected from libraries in which only one Cys was fixed (i.e., the X8CX8 library), suggesting the importance of the positions of the two Cys in binding mAb. Yet, binding to these peptides was only mildly affected by reducing conditions (Fig. 3), indicating that disulfide-bridge formation is not an absolute requirement for binding and that the Cys residues may be interacting directly with the antibody binding site. This was confirmed by demonstrating that only linear peptide A reacted with antibody.

Previous studies suggest that mAbs 5B19 and 5B170 recognize identical or overlapping linear epitopes located within the immunodominant region of the S2 subunit (Daniel *et al.,* 1993; Luytjes *et al.,* 1989). Our data are consistent with these observations, since phage selected by both mAbs bear consensus sequences that are similar to a portion of the epitope characterized by Luytjes *et al.* (1989). Moreover, phage selected by either mAb were recognized by both mAbs, albeit with weaker binding for mAb 5B170 (Fig. 1). This confirms that both mAbs recognize similar epitopes and that 5B19 has a stronger affinity than 5B170 for peptides selected by either antibody (Fig. 1). The sequences of CDRs H1, H2, and H3 for the two mAbs bore little similarity and the mAbs derive from different V_H families (Table 3). Since the heavy-chain CDRs are likely to be involved in antigen binding (Ward *et al.,* 1989), our results strongly suggest that these mAbs bind similar or identical epitopes on the S2 subunit by different mechanisms.

Tight-binding phage selected by mAbs 7-10A, 5B19, and 5B170 were used as antigens to immunize BALB/c and C57BL/6 mice. Of all the phage tested, only clone 9.1, selected by mAb 5B170, induced a statistically significant protective immune response in C57BL/6 mice (Fig. 4), which correlated with the production of statistically significant levels of antiviral antibodies (Table 4), confirming that the S glycoprotein epitope mimicked by the phage-borne peptide is immunogenic in mice. Surprisingly, biological activities by which antibodies could mediate protection of C57BL/6 mice, such as neutralization, ADCC, and ADCMC, were not detected. However, the presence of antibodies specific to the immunodominant region of the S2 subunit and to the relevant 13-mer peptide A correlated with the protection observed (Figs. 5 and 6, respectively). These results suggest the important role of the specific humoral immune response in clone 9.1-induced protection.

Interestingly, unprotected BALB/c mice immunized with clone 9.1 produced antiviral antibodies that reacted specifically with the immunodominant region of the S2 subunit in Western blots (Fig. 5) and with peptide A in ELISA (Fig. 6); however, as suggested by the intensities of the blotting signals (Fig. 5) and the optical densities observed in ELISA (Fig. 6), the concentration of antibodies was probably too low to protect these animals from a lethal coronavirus infection. Indeed, a recent report suggests that a minimal antibody concentration in serum is necessary for *in vivo* protection against vesicular stomatitis virus infection and that protection is independent of antibody subclass, avidity, and *in vitro* neutralizing activity (Bachmann *et al.,* 1997). Furthermore, we have previously shown that BALB/c mice were protected against MHV infection by immunization with synthetic peptide A, which includes the epitope found by Luytjes *et al.* (1989), when coupled to keyhole limpet hemocyanin. In this case, the protected mice also developed virus-specific antibodies that did not neutralize the virus *in vitro* (Daniel *et al.,* 1994).

Phage from clone 9.1 induced protective immunity, whereas the phage from other clones bearing the C(I/V)GSXC consensus sequence did not (Fig. 4), even though their ELISA signals were similar (Table 2). This apparent discrepancy may be explained by differences between clones in the sequences flanking the consensus sequence. These flanking residues could alter the conformation of the presented peptide (Vijayakrishnan *et al.,* 1997) and, in only some cases, allow it to select virus cross-reactive B cell clones. Thus, by allowing the mAbs to select binding peptides from random-peptide libraries, we were able to identify flanking regions that could not have been anticipated *a priori*. This emphasizes the major contribution that random-peptide libraries can make in identifying immunogenic epitopes.

Our use of the phage vector f88.4 (Bonnycastle *et al.,* 1996) was in part based on the work of Greenwood *et al.* (1991), who showed that peptides expressed on the pVIII major coat protein are more immunogenic than those expressed on the pIII minor coat protein; presumably, this is due to the higher copy number of the displayed peptide. Moreover, it has also been shown that peptides displayed on phage induce a stronger immune response than a peptide coupled to another carrier molecule (de la Cruz *et al.,* 1988; Lenstra *et al.,* 1992; Meola *et al.,* 1995). However, other studies have demonstrated that phage-displayed peptides selected by mAbs are not necessarily immunogenic. For example, a study by Felici *et al.* (1993) reported that phage clones selected for their ability to bind a mAb against the *Bordetella pertussis* toxin failed to elicit a specific immune response against the toxin, whereas similar work by the same group on the human hepatitis B virus surface antigen (Meola *et al.,* 1995) resulted in cross-reactive antibody production. Thus, the ability of an antibody-selected peptide mimic to elicit antibodies that will recognize a target antigen depends on a number of variables, including (i) its ability to mimic the structure of the epitope, (ii) its ability to bind specifically to the selecting antibody, and (iii) the structure and biological properties of the selecting antibody. Also, direct immunization of the selected peptide displayed by a phage could be optimized by combining T helper and B epitopes.

The genetics of the mouse could also play a role in vaccination; various studies have shown both successful vaccination in different strains of mice (Lenstra *et al.,* 1992; Motti *et al.,* 1994; Willis *et al.,* 1993) and unsuccessful vaccination (Demangel *et al.,* 1996). In our work, successful immunization with peptides displayed on phage depended on the strain of mice used, which is consistent with the work reported by de la Cruz *et al.* (1988). Moreover, Meola *et al.* (1995) reported no significant differences between the two strains of mice used,

although C57BL mice tended to respond better, as we have also shown.

To our knowledge, this study shows for the first time an evaluation of a protective immune response produced by direct immunization with a phage-displayed peptide that was specifically selected by a pathogen-specific mAb. Several studies have reported mAb-selected peptides that can induce antibodies that, in turn, recognize the mAb's cognate antigen; these experimental systems, however, were not appropriate to evaluate protection (Folgori *et al.,* 1994; Motti *et al.,* 1994; Willis *et al.,* 1993). Recently, Bastien *et al.* (1997) reported the induction of a protective immune response by immunizing BALB/c mice with a recombinant phage expressing a protective epitope of human respiratory syncytial virus that had been previously identified with synthetic peptides. Also, other authors have reported the induction of protection by immunization with synthetic peptides that are homologous to the peptides borne by the selected phage (Chargelegue *et al.,* 1998; Steward *et al.,* 1995; Stoute *et al.,* 1995). Our study represents an example of the successful use of phage-displayed peptide libraries for epitope characterization and emphasizes for the first time the possibility of a direct evaluation of protective immune responses induced by the selected peptide displayed by a phage carrier.

MATERIALS AND METHODS

Animals

Four- to five-week-old MHV-seronegative BALB/c and C57BL/6 mice were purchased from Charles River (St-Constant, Québec).

Virus, cells, and viral production

The A59 strain of MHV was initially obtained from the American Type Culture Collection (ATCC; Rockville, MD), plaque-purified twice, and passaged four times at a multiplicity of infection of 0.01 on DBT astrocytoma cells (Kumanishi, 1967) as described previously (Daniel and Talbot, 1987). The DBT cells were a gift from Dr. Michael J. Buchmeier (The Scripps Research Institute, La Jolla, CA). N-11 immortalized mouse microglial cells (Lutz *et al.,* 1994) were provided by Drs. Yves Lombard and Jacques Borg (Université Louis Pasteur de Strasbourg, Illkirch). Persistently infected N-11 cells were obtained by infecting the cells with MHV-A59 at a multiplicity of infection of 0.9. After a 60-min incubation at 37°C with 5% (v/v) CO_2, the cells were washed twice with 10 ml of PBS, pH 7.4, then 10 ml of RPMI medium supplemented with 10% (v/v) FCS was added. The infected cells were incubated at 37°C with 5% (v/v) CO_2 for 2 days before being passaged. Cells were tested for production of infectious virus at each passage by plaque assay as described previously (Daniel and Talbot, 1987). Virus production levels varied between 3×10^4 and 2×10^7 PFU/ml.

Monoclonal antibodies

We have previously reported the production and characterization of a mouse hybridoma secreting the neutralizing mAb 7-10A, which is specific for a discontinuous epitope on the S glycoprotein of MHV-A59 (Daniel and Talbot, 1990). The mAbs 5B170 and 5B19, which are specific for continuous epitopes on the S glycoprotein (Collins *et al.,* 1982; Daniel *et al.,* 1993; Luytjes *et al.,* 1989) of MHV-JHM (conserved in MHV-A59), were a kind gift from Dr. Michael J. Buchmeier (The Scripps Research Institute). A mAb against murine ICAM-1 (YN1/1.7.4, CRL 1818; ATCC) was used in some tests: it was a kind gift from Dr. Yves St-Pierre (INRS–Institut Armand–Frappier). An isotype control mAb for ELISA was specific for anti-c-myc (CRL 1729; ATCC). All these antibodies were purified by standard protein A–Sepharose chromatography (Manil *et al.,* 1986).

Nucleotide sequencing of mAb cDNAs

Isolation of total cellular RNA from hybridoma cells, RT-PCR, and nucleotide sequencing were performed as described previously (Lamarre *et al.,* 1997). Briefly, total cellular RNA from 5B19 and 5B170 hybridomas was isolated by cesium chloride density gradient centrifugation, and 2.5 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Pharmacia Canada, Inc., Baie-d'Urfé, Québec) using the MOCG12FOR primer (Lamarre *et al.,* 1997). Then, PCR amplification of the V_H cDNA was performed using *Taq* DNA polymerase (BIO/CAN Scientific, Mississauga, Ontario) with the VH1FOR and VH1BACK primers (Lamarre *et al.,* 1997). The PCR products were then cloned into the pCRII TA cloning vector (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. Nucleotide sequencing was performed on both strands by the dideoxynucleotide chain termination method, using T7 DNA polymerase (Pharmacia) and [α-^{35}S]dATP (ICN Pharmaceuticals Canada Ltd., Montréal, Québec) according to the manufacturer's instructions. Nucleotide sequences were analyzed with GeneWorks 2.5.1 software (Oxford Molecular, Oxford), BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/), and DNAPLOT analysis (http://www.genetik.uni-koeln.de/dnaplot).

Peptide libraries

The 12 phage-displayed peptide libraries used in this study were X_6, X_{15}, X_8CX_8, $X_{15}CX$, XCX_{15}, XCX_4CX (LX4), XCX_6CX (LX6), $XCX_{10}CX$ (LX10), $X_5CX_3CX_5$ (Cys3), $X_5CX_4CX_5$ (Cys4), $X_5CX_5CX_4$ (Cys5), and $X_4CX_6CX_4$ (Cys6), where X represents a randomized amino acid and C represents a fixed cysteine residue. The Cys3–6 libraries were kindly provided by Dr. G. P. Smith (University of Missouri at Columbia); the remaining libraries were constructed as described (Bonnycastle *et al.,* 1996). Peptides

are expressed at the N-terminus of the major coat protein (pVIII) of the phage vector f88.4 (Zhong *et al.,* 1994).

Affinity selection of phage libraries

Screening of the peptide libraries with antibodies is described in detail elsewhere (Bonnycastle *et al.,* 1996). Briefly, microtiter wells were coated with streptavidin and then biotinylated mAb. Phage libraries (10^{11} particles/library) were added to each of 11 wells, with f88.4 phage being used in a 12th well as control. Phage were allowed to bind for 4 h at 4°C, then the plates were washed using an automated plate washer. Bound phage were eluted in acid, and the eluates were neutralized and used to infect K91 *Escherichia coli* cells. The cells were grown in microtiter wells in LB medium containing tetracycline. The phage produced were used in subsequent rounds of panning, or infected clones were chosen at random from selected phage pools, and used for ELISA and DNA sequencing.

Nucleotide sequencing of phage DNA

Nucleic acid sequencing protocols are described in detail elsewhere (Bonnycastle *et al.,* 1996; Smith and Scott, 1993). Briefly, the DNA from $\sim 10^{11}$ PEG-purified virions was released by treatment in 180 mM NaOH containing sequencing primer. The solutions were neutralized, and dideoxynucleotide sequencing was performed using Sequenase and 5′[α-^{35}S]dATP. Labeled reactions were analyzed by polyacrylamide gel electrophoresis under denaturing conditions.

ELISA under reducing conditions

Ninety-six-well flat-bottomed microtiter plates (ICN) were coated with 1 μg/well of caprylic acid-purified, rabbit anti-phage antibody (Bonnycastle *et al.,* 1996) or with MHV-A59-infected DBT cell lysate (60 ng/well in PBS, pH 7.4) and incubated for 16 h at room temperature. The plates were blocked with TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 2% (w/v) BSA for 30 min at 37°C and then washed three times with TBS containing 0.1% (v/v) Tween 20 (TBS-T). Approximately 10^{10} phage particles, purified by precipitation with PEG and NaCl (Bonnycastle *et al.,* 1996), were added in 50-μl aliquots to wells containing immobilized anti-phage antibody and incubated for 2 h at 4°C. The plates were then washed six times with TBS-T. Fifty microliters of 15 mM DTT (Sigma Chemical Co., St. Louis, MO) was added to some wells and incubated for 30 min at 4°C. These wells were then washed once with 75 μl of 50 μM DTT. One hundred microliters of 60 nM mAb in TBS-T containing 50 μM DTT was then added and incubated for 2 h at 4°C. For non-DTT-treated wells, TBS-T was used instead. Plates were washed six times with TBS-T, then 100 μl peroxidase-labeled goat anti-mouse Fc antibody (ICN) diluted 1/2000 in TBS-T was added and incubated for 90 min at room temperature. The plates were washed six times with TBS-T, and specific mAb binding was revealed by the addition of 100 μl of 2.2 mM *O*-phenylenediamine and 3 mM hydrogen peroxide. The enzymatic reaction was stopped by the addition of 100 μl of 1 N HCl, and the absorbance was read at 492 nm using an SLT EAR 400 AT plate reader (SLT-Labinstruments, Austria).

ELISA for determining cross-reactive phage recognition by mAb

Ninety-six-well flat-bottomed microtiter plates (ICN) were coated with 0.1 μg/well of purified 5B170 and 5B19 mAb, as well as an irrelevant anti-c-myc mAb used as an isotypic control. The plates were incubated for 16 h at room temperature. Then, the plates were blocked with 150 μl of ELISA blocking solution [PBS, pH 7.4, containing 10% (v/v) FCS and 0.2% (v/v) Tween 20] for 2 h at room temperature. Serial, fivefold dilutions of PEG/NaCl-precipitated phage (10 mg/ml) diluted 1/10 were added to the wells and incubated for 90 min at room temperature. Wells were washed five times with PBS-T [PBS, pH 7.4, containing 0.1% (v/v) Tween 20], and 100 μl peroxidase-labeled anti-M13 conjugate (Pharmacia Biotech, Inc., Baie d'Urfé, Québec) diluted 1/5000 in ELISA blocking solution was added. Plates were incubated for 90 min at room temperature and washed five times with PBS-T; the remaining steps were performed as described above.

Phage immunization and protection assay

Phage were purified by two consecutive PEG/NaCl precipitation steps and quantitated, assuming 1 mg/ml = 1 OD_{260} (Harris *et al.,* 1997). Groups of six BALB/c or C57BL/6 mice were immunized four times, at 14-day intervals, by ip injection of 1 mg of phage emulsified in complete Freund's adjuvant [1:1 (v/v); VWR/Canlab Inc., Ville Mont-Royal, Québec] for the first injection and in incomplete Freund's adjuvant for the three subsequent booster doses. Seven days after the last injection, blood samples were drawn from the retro-orbital plexus. An intracerebral viral challenge was given 3 days later with 10 LD_{50} (Lenstra *et al.,* 1992) of MHV-A59, a dose that corresponds to 5×10^5 PFU for BALB/c mice and 6×10^4 PFU for C57BL/6 mice. This dose reproducibly yielded 100% mortality by 5 days after infection (Talbot *et al.,* 1988; Yu *et al.,* 1996). The statistical significance of survival of mice was examined with Cox's proportional hazards model (Armitage and Berry, 1987).

Neutralization assay

Serial dilutions of mouse plasma were incubated in tubes with approximately 80 PFU of MHV-A59 for 60 min at 37°C. The mixtures were transferred in duplicate onto 12-well plates (ICN) containing confluent monolayers of DBT cells. After a 60-min incubation at 37°C with 5% (v/v) CO_2, the virus–antibody mixtures were removed and the

cells were overlaid with 1.5% (w/v) Bacto-Agar (Difco Laboratories, Detroit, MI) in Earle's minimal essential media/Hanks' M199, 1:1 (v/v) (Canadian Life Technologies, Inc., Burlington, Ontario), supplemented with 5% (v/v) FCS and 50 μg/ml gentamicin (Canadian Life Technologies, Inc.). The plates were incubated for 48 h at 37°C with 5% (v/v) CO_2. The cells were then fixed with 25% (v/v) formalin and stained with 0.1% (w/v) crystal violet. Plaques were counted visually and viral neutralizing titers were expressed as the reciprocal of the dilution of plasma that neutralized 50% of viral input. A known virus-neutralizing mAb (5B170) was used as positive control.

Western immunoblotting

Ten micrograms of the previously described immunodominant region of the S2 subunit of the MHV S glycoprotein expressed in *E. coli* (Daniel *et al.,* 1993) was electrophoresed on 10% (w/v) polyacrylamide gels in SDS buffer (Laemmli, 1970) and transferred to a 0.45-μm nitrocellulose membrane (Amersham Life Science, Inc., Oakville, Ontario). Following transfer, the nitrocellulose membrane was blocked with PBS-T for 30 min at room temperature and cut into strips. Strips were incubated for 90 min at room temperature with 1.5 ml of mouse plasma diluted 1/500 in PBS-T and then washed five times with PBS-T. The strips were then incubated for 90 min at room temperature with 1.5 ml horseradish peroxidase-labeled goat anti-mouse IgG (H+L) (Kirkegaard and Perry, Gaithersburg, MD) diluted 1/500 in PBS-T. The strips were washed five times with PBS-T, and specific antibody binding was revealed with 0.5 mg/ml 4-chloro-1-naphtol (Bio-Rad, Mississauga, Ontario) and 0.01% (w/v) H_2O_2. The reaction was stopped after 30 min by washing in deionized water.

Peptide synthesis, purification, and use in ELISA

Peptides corresponding to consensus sequences observed on some phage (CIGSQC and CIGSYC), as well as peptide A, with the sequence SPLLGCIGSTCAE (Daniel *et al.,* 1994) were prepared in both linear and cyclic forms, as follows. The various peptides were synthesized with a homemade manual multireactor solid-phage synthesizer according to protocols described previously (Forest and Fournier, 1990; Forget *et al.,* 1990). A benzhydrylamine resin was used as solid support. The following side chain-protected Boc-amino acids were used in the synthesis: 4-methylbenzyl cysteine, benzyl-glutamic acid, benzyl ether serine, and 2-bromobenzyl-oxycarbonyl tyrosine. Boc-amino acid couplings were performed in dimethylformamide with BOP reagent (Fournier *et al.,* 1988) in the presence of diisopropylethylamine (Forest and Fournier, 1990; Le-Nguyen *et al.,* 1987). Every coupling was monitored with the use of the ninhydrin test. Peptide–resins were cleaved from the solid support with liquid hydrofluoric acid (10 ml/g) in the presence of *m*-cresol (1 ml/g) and dimethyl sulfide (1 ml/g) as scavengers. Crude peptides were purified through preparative reverse-phase high-performance liquid chromatography (HPLC) and the material was eluted with a linear gradient of trifluoroacetic acid/H_2O (0.06%) and acetonitrile. The fractions corresponding to the purified peptide were pooled and lyophilized. To cyclize the peptides, they were dissolved in degassed aqueous acetic acid (80%), and a solution of iodine (50 Eq) in methanol was added dropwise. The reaction was monitored with the use of analytical HPLC and it was stopped by the addition of ascorbic acid until complete discoloration. Most of the acetic acid was evaporated and the remaining solution was diluted with water. The solution was injected onto a preparative HPLC column, and the peptide was purified as described above. Each preparation was characterized through analytical HPLC and electrospray mass spectrometry. For determination of peptide-specific antibodies by ELISA, synthetic peptides were used as described previously (Daniel *et al.,* 1994), except that the optimal amount used was determined to be 5 μg/ml.

Antibody-dependent complement-mediated cytolysis

Approximately 1.5×10^7 N-11 cells persistently infected with MHV-A59, as described above, were labeled with 600 μCi of sodium chromate ($Na_2{}^{51}CrO_4$; ICN Pharmaceuticals Canada Ltd.) in flat-bottomed microwells for 18 h at 37°C with 5% (v/v) CO_2. Labeled target cells were then washed four times with Hanks' balanced sodium salts (Canadian Life Technologies) and detached by adding 0.4 mM EDTA (Anachemia, Montréal, Québec) for 5 min. Flat-bottomed microtiter wells were seeded with 10^4 target cells per well and the cells were allowed to bind to the plastic for 2 h at 37°C. The medium was aspirated and mouse plasma was serially diluted in RPMI medium containing 10% (v/v) FCS and added in triplicate to the target cells, in a final volume of 100 μl per well. After 1-h incubation at 37°C, the antibodies were aspirated and 100 μl/well of rabbit complement (Pel-Freez, Rogers, Arkansas), diluted 1/15 in RPMI medium containing 10% (v/v) FCS, was added and allowed to react for 45 min at 37°C and 5% (v/v) CO_2. For determination of spontaneous and maximum release, medium or 5% (v/v) Triton X-100 was added to the wells, followed by 100 μl/well of RPMI medium without FCS. The cells were then centrifuged at 200 *g* for 8 min, and 150 μl of supernatant was collected from each well and counted for radioactivity in a Beckman Gamma 7000 counter [Beckman Instruments (Canada) Inc., Mississauga, Ontario]. A mAb against murine ICAM-1 was used as a positive control.

Results are expressed as percentage specific release:

$$100 \times \frac{(\text{cpm experimental} - \text{cpm spontaneous})}{(\text{cpm maximum} - \text{cpm spontaneous})}.$$

Antibody-dependent cell-mediated cytolysis

Mouse plasma was serially diluted and added in quadruplicate to flat-bottomed microwells, with a final volume of 50 μl per well. Ten thousand target cells/well (50 μl), prepared as described above, were added to the diluted plasma and the mixture was incubated for 1 h at 37°C. One million naive BALB/c mouse splenocytes per well (100 μl) were added as effector cells to the mixture of target cells and sera. For determination of spontaneous and maximum release, medium or 5% (v/v) Triton X-100 was added to the wells. Plates were incubated for 6 h at 37°C with 5% (v/v) CO_2. At the end of the incubation period, plates were centrifuged, the supernatant was collected, and the results were expressed as described above.

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