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$CD8^+$ T-Cell Epitopes within the Surface Glycoprotein of a Neurotropic Coronavirus and Correlation with Pathogenicity

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$CD8^+$ T cells with cytotoxic activity against the surface glycoprotein (S) of mouse hepatitis virus, strain JHM, have been identified in the central nervous system (CNS) of both acutely and chronically infected C57BL/6 mice. In this report, two specific epitopes recognized by these CNS-derived cells were identified, using a panel of peptides chosen because they conformed to the allele-specific binding motif for MHC class I H-$2K^b$ and H-$2D^b$. The active peptides encompassed residues 510 to 518 (CSLWNGPHL, H-$2D^b$) and 598 to 605 (RCQIFANI, H-$2K^b$). Both epitopes are located within the region of the S protein previously shown to be prone to deletion after passage in animals. These deleted strains are generally less neurovirulent than the wild-type virus but still are able to cause demyelination. Since C57BL/6 mice become persistently infected more commonly than many other strains of mice, these data are consistent with a role for $CD8^+$ T-cell escape mutants in the pathogenesis of the demyelinating disease. This is the first report of $CD8^+$ T-cell epitope localization within the S protein, the protein most strongly implicated thus far in pathogenesis in the host.

Mouse hepatitis virus strain JHM (MHV-JHM) is a neurotropic coronavirus which causes acute and chronic neurological infections in susceptible mice and rats (4, 19, 23, 27, 30, 36). MHV-JHM causes an invariably fatal acute encephalitis in susceptible mice, but this infection can be modified if mice are protected by passive infusion of antiviral antibody or T cells or if they are infected with an attenuated strain of virus (2, 6, 9, 12, 15, 20, 24, 31, 33, 39). Analysis of some of these attenuated viruses reveals point mutations or deletions in the surface (S) glycoprotein (18, 26, 34, 35), a protein which is involved in the binding to receptor on susceptible cells and in the formation of syncytia (17). These deletions all occur in the same region of the amino-terminal portion of the S protein and are overlapping, although the size of the deletion is variable. Mice infected with these variants do not develop acute encephalitis, but in most cases demyelination still occurs. The demyelinating disease induced by MHV-JHM has been studied extensively as an animal model for such human diseases as multiple sclerosis.

We have shown that suckling C57BL/6 mice (*H-2^b*) nursed by dams previously immunized to MHV-JHM are protected from the acute encephalitis, but 40 to 90% develop hindlimb paralysis with histological evidence of demyelination (27). An S-specific $CD8^+$ T-cell response can be demonstrated by direct ex vivo cytotoxicity, using cells isolated from the brains and spinal cords of mice acutely and chronically infected with the virus. Since these virus-specific cytolytic cells can also be isolated from mice with hindlimb paralysis, we concluded that the S-specific $CD8^+$ T-cell response does not protect mice from the chronic demyelinating disease. In contrast, suckling BALB/c mice (*H-2^d*) infected with MHV-JHM do not develop hindlimb paralysis although they are equally susceptible to the acute encephalitis. A $CD8^+$ T-cell response to the nucleocapsid (N) protein can be demonstrated in the central nervous system (CNS) of BALB/c mice with acute disease (32), but in a study using C57BL/10 mice recombinant in the major histocompatibility complex (MHC) locus [B10.A(18R)-H-$2K^b$, D^d, L^d), it was shown that the presence of this response is by itself unable to prevent the development of hindlimb paralysis in the *H-2^b* mouse (3).

Although these results show that neither anti-S nor anti-N $CD8^+$ T cells protect mice from developing hindlimb paralysis, the possibility still remains that the $CD8^+$ T-cell response is involved in the process of virus persistence and consequent demyelination. Thus, $CD8^+$ T cells could facilitate persistence by incompletely clearing virus during the early stages of the infection and could also contribute to the demyelinating process by destroying virus-infected cells in the CNS during the persistent stage. Recent data from other viral infections have correlated naturally occurring mutations in $CD8^+$ T-cell epitopes with virus persistence (1, 5, 14, 28) and have shown that cytotoxic T-lymphocyte (CTL) escape mutants generated in tissue culture cells are more likely than the parental strain to persist in infected animals (22).

The S glycoprotein is the only structural protein recognized by $CD8^+$ T lymphocytes derived from the CNS of C57BL/6 mice infected either acutely or chronically with MHV-JHM (3). To determine the epitopes recognized by these cells, the sequence of this protein was visually inspected for peptides conforming to the MHC class I H-$2K^b$ (XXXX[Y,F]XX[L,M, I,V]) and H-$2D^b$ (XXXXNXXX[M,I,L]) consensus binding motifs (8). This approach was taken because the large size of the S protein precluded use of a set of overlapping peptides to identify epitopes. Of the 47 peptides identified, 28 peptides matched the H-$2K^b$ consensus binding motif and 19 matched the H-$2D^b$ motif. Peptides were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia), using the Multipin peptide synthesis system. Only 46 peptides were synthesized because the peptide encompassing amino acids 893 to 900 contained both an H-$2D^b$ and an H-$2K^b$ epitope.

All of these peptides were tested in pools and individually for the ability to sensitize EL-4 cells for lysis by lymphocytes isolated from the CNS of mice with acute encephalitis. As shown in Fig. 1, two peptides, located at amino acids 510 to 518 (CSLWNGPHL, H-$2D^b$) and 598 to 605 (RCQIFANI, H-$2K^b$), conferred sensitization in this assay. A third peptide, encompassing residues 1143 to 1151 (NFCGNGNHI, H-$2D^b$), also sensitized cells for lysis but at 5,000 times the concentration

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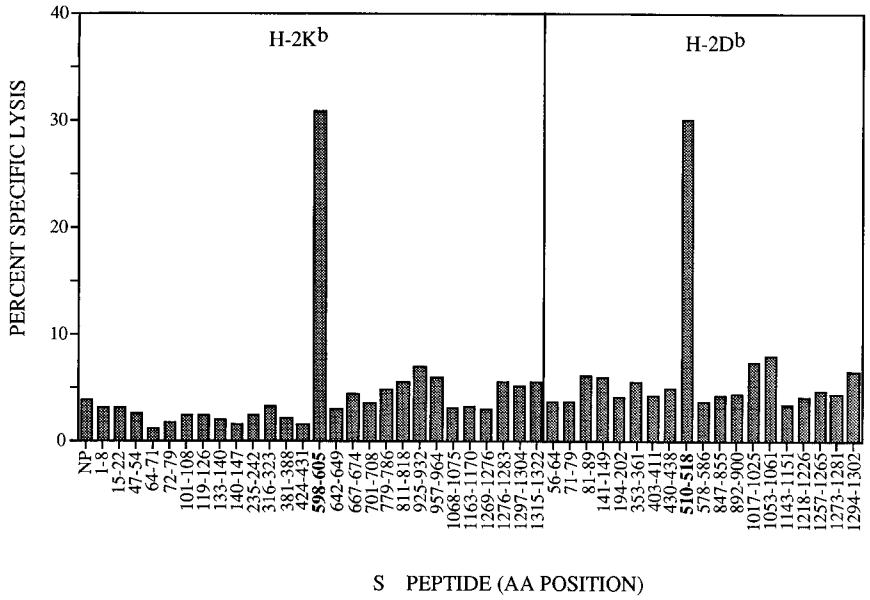

FIG. 1. Identification of S-specific peptides recognized by CNS-derived T lymphocytes. Six-week-old C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, Ind.) developed acute encephalitis after intranasal inoculation with 5×10^4 PFU of MHV-JHM. The variant used in these studies is very neurovirulent and causes death in 100% of susceptible mice. Cells were harvested from the CNS of these mice at 6 to 7 days postinfection and analyzed in CTL assays as described previously (3). Targets were EL-4 cells coated with the peptides indicated. Peptides were used at a final concentration of 1 μM. The E:T ratio was 50:1. NP corresponds to EL-4 cells used as targets without any peptide added. The average spontaneous release was <12%. aa, amino acid.

required for the other H-2D^b epitope (data not shown). Because this peptide was needed at such a high concentration for sensitization of targets, it was resynthesized independently by the Protein Structure Facility at the University of Iowa, using manual solid-phase synthesis with protection of the α-amino groups by 9-fluorenylmethoxycarbonyl and eventual activation using HBTU/HoBt. The peptide was purified by high-pressure liquid chromatography using a C_{18} reverse phase column. This purified peptide was inactive in our assays and was not further analyzed.

To confirm the specificity of the recognition of the H-2D^b and H-2K^b epitopes, CTL activity was tested by using a second set of target cells, P815 cells (*H-2^d*) stably transfected with either the MHC class I H-2K^b or H-2D^b gene. Peptide 598–605 (H-2K^b) was able to sensitize P815 cells transfected with the H-2K^b gene but not those transfected with the H-2D^b gene (Fig. 2A). In contrast, peptide 510–518 (H-2D^b) sensitized P815 cells transfected with the H-2D^b gene but not the H-2K^b gene (Fig. 2A). Next, the concentration of each immunogenic peptide sufficient to sensitize EL-4 targets for CTL recognition was determined (Fig. 2B). The epitopes encompassing residues 510 to 518 (H-2D^b) and 598 to 605 (H-2K^b) sensitized targets with half-maximal lysis at 20 pM and 5 nM, respectively.

As an alternative approach to identifying CTL epitopes within the S protein, recombinant vaccinia virus (VV) constructs encoding segments of the protein were made as shown in Fig. 3A. A recombinant VV encoding the S gene of MHV-4 but lacking the transmembrane domain [S(Sol)] was obtained from M. Buchmeier (The Scripps Research Institute) and T. Gallagher (Loyola University School of Medicine), and a second recombinant VV, encoding a deleted form of the gene, was constructed from cDNA clones provided by S. Siddell, University of Würzburg, as previously described (21). The numbering of the amino acids used in the truncations is based on the sequence of MHV-4 (26). S(1-302) and S(1-453, 595-1102) were constructed by truncating the deleted form of the S gene at nucleotides 906 (*Sca*I) and 3308 (*Hin*cII), respectively. S(285-706) and S(700-1055) were constructed by using PCR. The 5′ and 3′ primers used to construct S(285-706) were GCC CATGGCCAGCAGCTACATTAGTG and GCCTGCAGTT AACGATAGAGCAGAGCCGGTTC, and those used to construct S(700-1055) were GCGCCATGGAACCGGCTCTGCT CTATCG and GCGGGATCCGCTAATTAACAACGGACT GG. All of the S gene constructs were subcloned into the VV shuttle vector pTM3 and then recombined into VV as described previously (21). Each construct was shown to express an S-specific protein of the appropriate size by using immunoprecipitation with a polyclonal anti-MHV-JHM antibody and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (21) (data not shown).

When MC57 cells coinfected with these constructs and vTF7.3 (a recombinant VV which expresses T7 RNA polymerase, kindly provided by B. Moss, National Institutes of Health) were used as targets in primary CTL assays, the results shown in Fig. 3B were obtained. As expected, if the bulk of the CTL activity was directed against the two peptides identified above, constructs S(1-302) and S(700-1055) were not lysed in these assays. The targets expressing S(1-453, 595-1102), a deleted form of the S protein (29) containing a single epitope (H-2K^b), were lysed about 50% as efficiently as the entire S protein. Finally, the targets expressing S(285-706), which contained the two epitopes, had approximately the same activity as the entire protein. These data are consistent with the results shown in Fig. 1.

As further evidence that these epitopes were recognized by CNS-derived lymphocytes, a recombinant VV expressing the nine amino acids of the H-2D^b epitope encompassing residues 510 to 518 was constructed and analyzed in primary CTL assays. As shown in Fig. 3C, target cells infected with this recombinant VV were recognized nearly as efficiently as were cells infected with recombinant VV expressing the entire S(Sol) protein.

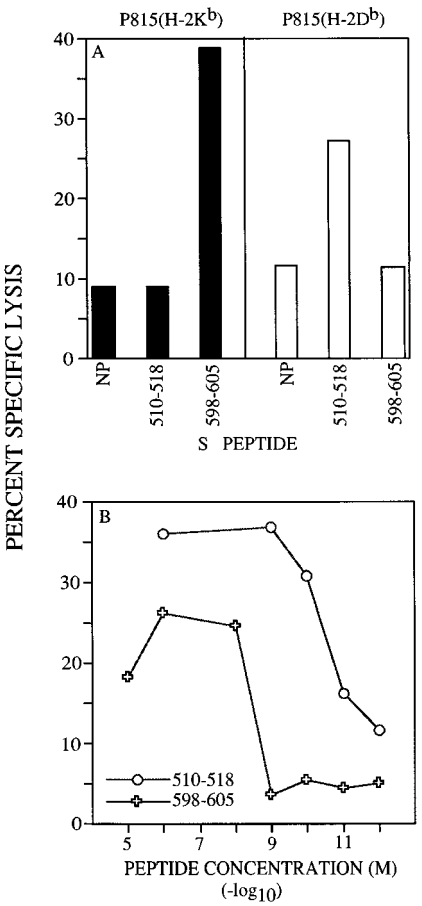

FIG. 2. MHC restriction of S-specific peptide epitopes and concentration of S-derived peptide required for sensitization. (A) P815 cells (*H-2*^{d}) stably transfected with either the MHC class I H-2K^b or H-2D^b gene (provided by J. Harty, University of Iowa) were labeled with ^{51}Cr for 1 h, mixed with each indicated peptide (1 μM, final concentration), and used as CTL targets. The percentage of specific release was determined after a 4-h incubation with lymphocytes derived from the CNS of acute encephalitic C57BL/6 mice at an E:T ratio of 50:1. NP corresponds to P815 cells used as targets without any peptide added. (B) EL4 cells (*H-2*^{b}), labeled with ^{51}Cr for 1 h and then mixed with each indicated peptide at the specified concentrations for an additional 30 min at 37°C, were used as CTL targets in a ^{51}Cr release assay. The percentage of specific release was determined after a 4-h incubation with lymphocytes derived from the CNS of acute encephalitic C57BL/6 mice at an E:T ratio of 50:1.

In some viral infections, the CTL response to a single epitope is sufficient to protect the host from acute disease. To determine if this was also true for C57BL/6 mice infected with MHV-JHM, mice were immunized with the recombinant VV expressing the H-2D^b epitope (Fig. 3C), using a protocol shown to elicit a protective CD8$^+$ T-cell response in other animal models of viral infection (13, 16, 37). Three mice were infected intraperitoneally and three mice were infected intravenously with this recombinant VV (2×10^7 PFU) 3 weeks prior to challenge with our standard intranasal dose (5×10^4 PFU) of MHV-JHM. The same number of mice were also infected at 6 weeks prior to challenge with MHV-JHM. With none of these regimens were mice protected against the acute encephalitis caused by the virus.

To determine the efficiency of the vaccination, spleens were harvested 8 days postinfection from three additional mice and stimulated with an EL-4 cell clone expressing the S glycoprotein (EL4-S). EL4-S was constructed by electroporation of EL4 cells with pHβApr-1-neo (11) containing the S gene inserted into the *Hin*dIII-*Bam*HI sites. G418-resistant clones were selected and monitored for expression of the S glycoprotein in primary CTL assays using CNS-derived lymphocytes. The characterization of this cell line will be further described elsewhere (3a). For the purpose of stimulation, 35×10^6 to 50×10^6 splenocytes were incubated with 4×10^6 EL4-S cells in 20 ml of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M β-mercaptoethanol, and antibiotics. After 5 days in vitro, these cells were analyzed in CTL assays using MC57 cells infected with either wild-type VV or VV expressing the S protein as targets. A moderate amount of S-specific ^{51}Cr release was observed in these experiments (17, 15, and 6% specific release at effector-to-target [E:T] ratios of 100:1, 50:1, and 20:1, respectively).

These results are consistent with those of Stohlman et al. (31), who found that immunization with a single N-specific CTL epitope did not protect BALB/c mice against a lethal infection with MHV-JHM. This lack of protection may be due to several factors. In previous studies in which protective and nonprotective CTL epitopes were analyzed, the amount of CD8$^+$ T-cell priming observed after immunization with recombinant VV was greater with the former than the latter (13). The 510–518 (H-2D^b) epitope expressed as a minigene is recognized as well as the entire S(Sol) protein by CNS-derived CD8$^+$ T cells (Fig. 3C), but the amount of CD8$^+$ cytotoxic T-cell priming which we observed was only moderate and suggests that efficient priming of CD8$^+$ T cells specific against MHV-JHM in vivo may require other factors such as a CD4$^+$ T-helper-cell epitope. This quantitative deficiency may have contributed to the lack of protection which we observed. In other studies, the requirement for both CD4$^+$ and CD8$^+$ T cells for virus clearance has been demonstrated (38), although the precise requirement for each cell type in the process is not known.

The objective of this study was to identify CD8$^+$ T-cell epitopes within the MHV-JHM S protein by using lymphocytes derived from the CNS of a diseased mouse. This study is different from previous studies because cells were analyzed directly without stimulation in vitro. Use of CNS-derived CD8$^+$ T cells in primary CTL assays complements results obtained in studies using CNS-derived CD8$^+$ T-cell clones (32). The results show that the allele-specific binding motifs defined for many MHC molecules from analyses of cells harvested from spleens and lymph nodes can be used to predict CD8$^+$ T-cell epitopes utilized within the CNS. Two epitopes were identified with the CNS-derived cells, and while finding multiple CD8$^+$ T-cell epitopes within a protein is not unique, most proteins analyzed thus far contain only one such epitope (7). The increased number of epitopes recognized by CNS cells in this study may reflect the large size of the S protein (1,376 amino acids). Alternatively, this result may be a function of using a primary CTL assay since it is possible that in vitro stimulation, used in most studies, causes a selective amplification of only a subset of virus-specific CD8$^+$ T cells.

Of note, both of the epitopes are deleted in many attenuated variants of MHV-JHM (Table 1). The list of affected viruses includes variants which were isolated from rats with chronic demyelination (18, 34) as well as many mildly neurotropic viruses such as MHV-A59 and MHV-X (26). Attenuated virus deleted in this region has also been selected in vitro by using neutralizing monoclonal antibodies (6, 35). A common feature of these viruses is that they all infect neurons less efficiently than MHV-JHM but still infect glial cells. The result is that they cause milder neurological diseases, although demyelina-

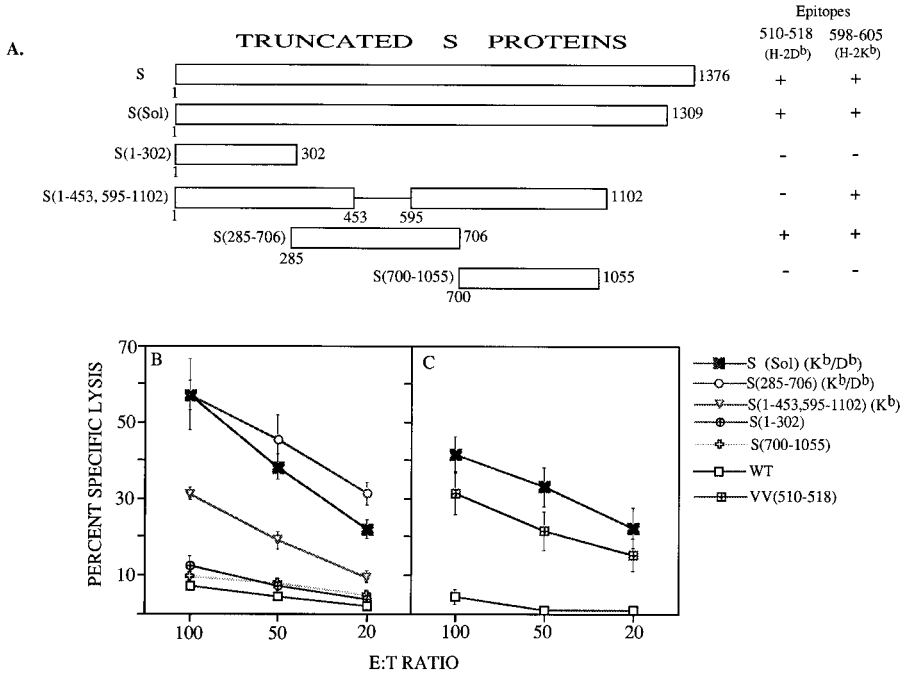

FIG. 3. CTL recognition of VV expressing S glycoprotein fragments. (A) Recombinant VV constructs encoding fragments of the S protein were made as described in the text. The presence or absence of the two epitopes is shown for each construct. (B) To assay these recombinant VV constructs, MC57 cells were dually infected with each recombinant VV and vTF7.3. At 6 h postinfection, cells were harvested, labeled with ^{51}Cr, and analyzed in primary CTL assays. The effector population was CNS-derived lymphocytes from acute encephalitic C57BL/6 mice at 6 to 7 days postinfection. Each point represents an average of at least three experiments, and bars show the standard error for each point. WT corresponds to MC57 cells infected with wild-type VV. The average spontaneous release was <23%. (C) A recombinant VV expressing the H-2D^b epitope encompassing residues 510 to 518 was constructed by using two complementary oligonucleotides which contained the DNA sequence of the identified epitope, an amino-terminal methionine, and appropriate restriction enzyme sequences: TCGACAGGATGTGTTCTCTTTGGAATGGGCCCCATT TGGGTAC and CCAAATGGGGCCCATTCCAAAGAGAACACATCCTG. The oligonucleotides were annealed and ligated into pSC65 (a VV shuttle vector with a strong synthetic VV early/late promoter for expressing the gene of interest, kindly provided by B. Moss, National Institutes of Health). MC57 cells infected with the recombinant VV were used as CTL targets in ^{51}Cr release assays. The effector population was lymphocytes derived from the CNS of C57BL/6 mice with acute encephalitis. Each point represents an average of at least four experiments, and bars show the standard error for each point. WT corresponds to MC57 cells infected with wild-type VV.

tion is unchanged or even increased in animals infected with these viruses (6, 10). However, the diminished infection observed with these viruses is not solely a function of escape from CTL immune recognition, since these variants cause a less severe infection in strains of mice, such as BALB/c mice (6), which lack a CD8$^+$ T-cell response to the S protein (3, 32). However, selection of viruses with a deletion of the H-2D^b or H-2K^b epitope may facilitate viral escape from immune surveillance and increase the likelihood of persistence in the infected C57BL/6 mouse.

TABLE 1. Amino acid residues deleted within the S glycoprotein of MHV variants and the corresponding deletion of the identified MHC class I H-2K^b or H-2D^b epitope

Variant (reference)	Amino acid deletion	Epitope deletion
MHV-A59 (26)	501–552	H-2D^b
JHM-X (26)	446–598	H-2D^b, H-2K^b
V5A13.1 (26)	434–575	H-2D^b
V4B11.3 (26)	429–586	H-2D^b
V5A13 (26)	436–585	H-2D^b
JHM-At11f (18)	442–589	H-2D^b
JHM-MM56 (34)	449–602	H-2D^b, H-2K^b
JHM-MM85 (34)	444–595	H-2D^b
JHM-Würzburg (29)	453–596	H-2D^b
2.2/7.2-V-2 (35)[a]	508–542	H-2D^b

[a] Also has a point mutation at nucleotide 3340 and is nonpathogenic.

In other chronic infections, such as those caused by hepatitis B and the human immunodeficiency virus, mutations in CTL epitopes are believed to contribute to virus persistence (1, 5, 14, 25, 28). In both of these cases, changes in immunogenic epitopes which not only prevent recognition by CTLs but also antagonize recognition of the wild-type sequence have also been reported (1, 5, 14). Since at least one other CTL epitope is still present in most variant strains of MHV-JHM, deletion or change in a single CTL epitope is unlikely to eliminate completely the cytotoxic response to the virus. On the other hand, in situations in which the host has difficulty clearing a viral infection, generation of these escape mutants may facilitate the process of virus persistence.

A feature of the H-2D^b epitope is that the concentration of peptide required for half-maximal lysis is substantially less than for the H-2K^b epitope. Recognition of a particular peptide epitope depends on the affinity of binding to the MHC class I molecule as well as the affinity of the T-cell receptor for the peptide-MHC complex. Since the measurement was made with bulk populations of CNS-derived CD8$^+$ T cells, it includes cells with differing affinities for the peptide-MHC molecule complex. Nonetheless, the relatively low concentration required for activity is consistent with lysis of cells presenting this epitope being most important under conditions of limiting antigen presentation. This might occur early during the infection in the mouse and might contribute to selection of viruses lacking this epitope.

The identification of $CD8^+$ T-cell epitopes within the S protein as described in this report is being used to develop reagents to answer questions about the role of MHV-specific $CD8^+$ T cells and MHV CTL escape variants in both virus clearance and the demyelinating process.

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