

## Identification of a CD4<sup>+</sup> T Cell Epitope within the M Protein of a Neurotropic Coronavirus

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A significant CD4<sup>+</sup> T cell response against the transmembrane (M) protein can be detected in the spleens of C57Bl/6 mice infected intraperitoneally with a sublethal injection of the neurotropic JHM strain of mouse hepatitis virus (MHV-JHM), but not in those of mice with the chronic demyelinating encephalomyelitis caused by this virus. With the ultimate goal of determining the role of the M-specific response in the pathogenesis of MHV-JHM-induced neurological diseases, CD4<sup>+</sup> T cell epitopes within the M protein were identified using vaccinia virus recombinants expressing truncated forms of the protein and peptides spanning most of the M protein in cell proliferation assays. Peptides covering residues 128-147 contain at least one CD4<sup>+</sup> T cell epitope for MHV-JHM. Within this region is a sequence (residues 135-143) which matches the recently described MHC class II I-A<sup>b</sup> binding motif. Delineation of this epitope should facilitate analysis of the role of the M-specific CD4<sup>+</sup> T cell response in the development of acute and chronic neurological infections caused by MHV-JHM.

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### INTRODUCTION

Mouse hepatitis virus (MHV), a coronavirus, causes hepatitis, gastroenteritis, and encephalomyelitis in susceptible mice and rats (Compton *et al.*, 1993). Most strains of mice infected with the JHM strain of MHV (MHV-JHM) develop an acute, fatal encephalitis characterized by infection of neurons (Weiner, 1973). This fatal disease can be prevented if mice are infected with attenuated virus or if they are passively immunized with antiviral antibody or T cells. Use of attenuated virus or passive immunization results in protection of neurons, but not glial cells, and mice develop a demyelinating encephalomyelitis (Haspel *et al.*, 1978; Buchmeier *et al.*, 1984; Stohlman *et al.*, 1986; Perlman *et al.*, 1987; Körner *et al.*, 1991; Yamaguchi *et al.*, 1991). The latter disease serves as an animal model for the human disease, multiple sclerosis.

While viral lysis of neurons is recognized as the predominant pathological feature in mice dying of the acute encephalitis, the etiology of the demyelinating disease is more controversial. Mice with acute demyelination have been investigated in most reports. Earlier studies suggested that direct virus destruction of oligodendrocytes caused the acute demyelinating disease while more recent studies implicated the immune response to virus-infected cells as crucial to the development of demyelination (Lampert *et al.*, 1973; Weiner, 1973; Watanabe *et al.*, 1983; Kyuwa and Stohlman, 1990; Wang *et al.*, 1990). Less is known about chronic demyelination caused by

MHV-JHM, although infection of astrocytes has been hypothesized to be an important component of the pathological process (Perlman and Ries, 1987; Kyuwa and Stohlman, 1990).

The immune response to MHV-JHM has been partially delineated. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are required for virus clearance from the infected rodent brain. CD8<sup>+</sup> T lymphocytes with cytotoxic activity (CTL) to the nucleocapsid (N) protein have been identified in the brains of infected BALB/c mice (Stohlman *et al.*, 1993). A CD8<sup>+</sup> T cell response to the surface glycoprotein (S) has been identified in the brains and spinal cords of C57Bl/6 mice acutely and persistently infected with MHV-JHM (Castro *et al.*, 1994). In neither case can the CTL response be detected in the spleens of infected mice, although an anti-N CTL response can be measured in the spleens of BALB/c mice immunized to the virus. Both CD4<sup>+</sup> T cells directed against the N and S proteins and anti-S CD8<sup>+</sup> T cells have been shown to protect Lewis rats from the MHV-JHM-induced acute encephalomyelitis (Körner *et al.*, 1991; Flory *et al.*, 1993).

We have described a CD4<sup>+</sup> T cell response to the S and transmembrane (M) proteins in the spleens of mice immunized by a sublethal intraperitoneal injection of live MHV-JHM (Mobley *et al.*, 1992). The M-specific response cannot be detected in the spleens of mice with chronic MHV-induced neurological disease, although splenic CD4<sup>+</sup> T cells which respond to the S protein can be found in these mice. We hypothesized that these CD4<sup>+</sup> T cells have a major role in the chronic demyelinating disease caused by MHV-JHM and that, like anti-MHV CD8<sup>+</sup> T cells, the anti-M CD4<sup>+</sup> T cells were sequestered in the central nervous system (CNS). CD4<sup>+</sup> T cells are

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more prominent than CD8<sup>+</sup> T cells in mice persistently infected with MHV-JHM (Castro *et al.*, 1994) and in at least one other model of virus-induced demyelination, mice infected with Theiler's murine encephalomyelitis virus, the anti-viral CD4<sup>+</sup> T cell response plays a major role in the resulting pathological changes observed in the CNS (Friedmann *et al.*, 1987; Gerety *et al.*, 1994). We have been unable to measure directly the CD4<sup>+</sup> T cell proliferative response in the CNS of persistently infected C57Bl/6 mice, so it is not known if M-responsive lymphocytes are present at this site. An alternative approach to determining the role of M-specific CD4<sup>+</sup> T cells in the infected brain is to develop T cell clones responsive to this protein. As part of this process, we have used bulk populations of spleen cells to identify a T cell immunodominant region within the M protein of MHV-JHM.

## MATERIALS AND METHODS

### Animals

MHV-negative 6-week-old C57Bl/6 mice were purchased from Sasco Laboratories (Omaha, NE) or Jackson Laboratories (Bar Harbor, ME).

### Cells

DBT cells, derived from a murine astrocytoma cell line, were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. MC57 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics.

### Viruses

MHV-JHM, originally obtained from Dr. S. Weiss, University of Pennsylvania, was grown and titered as previously described (Perlman *et al.*, 1987). Vaccinia virus (VV), strain WR, was obtained from the ATCC.

### Construction of M truncation clones and of VV recombinants

The VV recombinants used in this study are detailed in Fig. 1. First, recombinant VV encoding the M protein truncated at amino acids 133, 164, and 193 downstream from a T7 promoter were constructed. M(1-133), containing the first 133 amino acids of the M protein, was constructed by treatment of the M clone (Mobley *et al.*, 1992) with *KpnI* and religation. Clones containing the first 164 and 193 amino acids [M(1-164) and M(1-193)] were constructed from the M clone using PCR. The 5' primer for each reaction was TATGAGTAGTACTACTC and the 3' primers were GGTGCCTAGCTTAACAC [M(1-164)] and CCTTGTCTAAGAATGC [M(1-193)]. A *Bam*HI restriction site was added to the 5' primer and a *Pst*I site to the 3' primers to facilitate cloning. Second, a clone containing amino acids 108-164 [M(108-164)] was

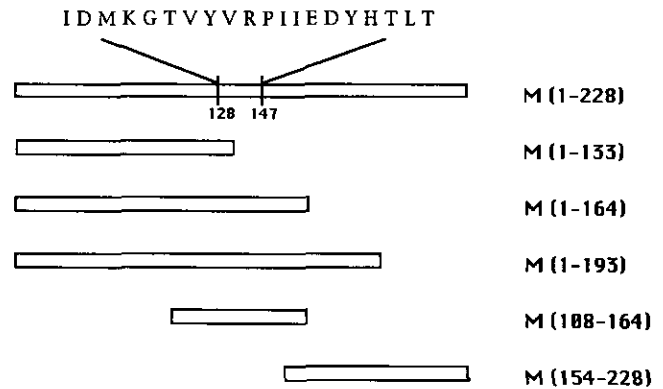


FIG. 1. Recombinant VV expressing parts of the M protein. Recombinant VV expressing regions of the M protein indicated were constructed as described under Materials and Methods. The sequence of the immunogenic region, residues 128-147, is also shown.

constructed using a 5' primer nearly identical to nucleotides 316-334 (AcCATGgTGTTTATCAGGA) with changes made to introduce an initiator ATG (underlined) and to improve the context of this codon (lowercase). The 3' primer used in this construction was the same as that used in clone M(1-164). Third, a C-terminal clone was constructed using a primer nearly identical to nucleotides 527-545 (CACTTCaATATGCAAGGTG) with a single change made to improve the context of the initiator codon. This primer included a *Nco*I site for purposes of cloning. The AUG from the *Nco*I site was in frame with the methionine encoded by the M gene. Two AUGs were included since we have occasionally lost the initiator codon from the *Nco*I site during the process of cloning. The 3' primer used in this construction was the same one we used originally to produce the full-length M protein (Mobley *et al.*, 1992) and contains a termination codon. The five clones were subcloned into the VV shuttle plasmid pTM3 and recombined into VV as described previously (Mobley *et al.*, 1992).

### Immunoprecipitation

Expression of the truncated M proteins was assayed by infecting MC57 cells with vTF7.3 (a recombinant VV expressing T7 RNA polymerase, kindly provided by Dr. B. Moss, N.I.H.) and the appropriate VV recombinants or with vTF7.3 alone. Cells were labeled with [<sup>35</sup>S]-methionine (Translabel, ICN, Costa Mesa, CA) from 10 to 12 hr p.i. As a control, DBT cells were infected with MHV-JHM and also labeled with [<sup>35</sup>S]methionine. Lysates were prepared and labeled M protein was immunoprecipitated using an anti-M monoclonal mouse antibody, J1.3 (kindly provided by Dr. J. Fleming, University of Wisconsin), as previously described (Mobley *et al.*, 1992). Samples were analyzed on 12% polyacrylamide-SDS gels.

## Peptides

Peptides were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia) using the Multipin peptide synthesis system with two exceptions. M(129–144) was synthesized by the Protein Structure Facility at the University of Iowa using manual solid-phase synthesis with FMOC-protected amino acids and HBTU/HOBt activation. M(205–228) was synthesized on an Applied Biosystems 4000A automated peptide synthesizer by the solid-phase method using t-BOC chemistry and was kindly provided by Dr. M. Buchmeier, The Scripps Research Institute. The latter two peptides were purified by C-18 reverse-phase HPLC (Beckman Instruments, Fullerton, CA). Peptides were resuspended in 40% acetonitrile (HPLC grade; Fisher Scientific, Fairlawn, NJ) and extensively sonicated. In preliminary experiments, we found that adding acetonitrile at the concentration used in these experiments (<0.1%) did not affect the proliferative response. Peptides M(34–47), M(50–63), M(55–68), M(61–74), and M(67–80) were very hydrophobic and only partially soluble in the 40% acetonitrile.

## Proliferation assays

Proliferation assays were performed using either peptides or lysates from cells expressing part of the M protein. For preparation of lysates, DBT cells were dually infected with vTF7.3 and one of the VV recombinants each at an m.o.i. of 10 or with vTF7.3 alone. After 12–14 hr, antigen was prepared as previously described (Mobley *et al.*, 1992). For proliferation assays, C57Bl/6 mice were infected with a sublethal intraperitoneal injection of  $6-10 \times 10^4$  PFU of MHV-JHM in 0.5 ml phosphate-buffered saline. Eight days later, a single cell suspension of spleen cells was prepared in RPMI 1640 medium containing  $5 \times 10^{-5}$  M 2-mercaptoethanol, 2 mM glutamine, 10% fetal calf serum, and antibiotics. Cells ( $2 \times 10^5$ ) were cultured in 0.2 ml in the presence of 1:200 recombinant antigen or peptide at concentrations indicated in the figure captions in 96-well flat-bottom culture plates (Corning Inc., Corning, NY). Cells were also incubated in the absence of antigen (negative control). After 4 days in culture at 37°, the cells were pulsed for 4 hr with 1  $\mu$ C/well [methyl-<sup>3</sup>H]thymidine (Amersham Corp., Arlington Hts., IL) and then harvested onto glass fiber filters with a cell harvester. Incorporated radioactivity was measured in a liquid scintillation counter. Each antigen was tested in 6 replicate wells.

To analyze the data, a mean proliferative response and standard error for each antigen or peptide was calculated for each group of mice. The proliferative index was calculated as the ratio of stimulation either by lysate from cells dually infected with vTF7.3 and VV expressing M protein to lysate from cells infected with vTF7.3 alone or by peptide to no added antigen. A proliferation index greater than 3 was considered significant (Wahren *et al.*, 1981).

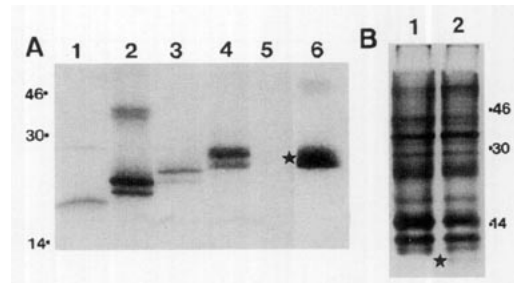


FIG. 2. M-truncated proteins expressed by VV recombinants. MC57 cells were infected with VV recombinants encoding the M constructs and vTF7.3 or vTF7.3 alone. As a positive control, DBT cells (grown in DMEM with 10% fetal calf serum) were infected with MHV-JHM. (A) <sup>35</sup>S-labeled M protein was immunoprecipitated using an anti-M monoclonal mouse antibody and analyzed as described under Materials and Methods. Lane 1, M(1–133); lane 2, M(1–164); lane 3, M(1–193); lane 4, M; lane 5, vTF7.3 alone; lane 6, MHV-JHM-infected DBT cells. Higher molecular weight proteins related to the M protein (most prominent in lane 2) were inconsistently detected in these analyses and most likely were readthrough products of the M gene. \*, M protein. (B) Lysates infected with vTF7.3 (lane 1) or M(154–228) and vTF7.3 (lane 2) were directly analyzed. \*, protein with appropriate molecular weight for M(154–228) product.

## RESULTS

### Analysis of M proteins expressed by recombinant VV

For the analyses described below, recombinant VV expressing segments of the M protein and a set of overlapping peptides which covered most of the M protein (Pfleiderer *et al.*, 1986) were used. In the first set of experiments, the approximate location of CD4<sup>+</sup> T cell epitopes was determined using the VV recombinants. Initially, each of the recombinant VV was assayed for protein expression using a monoclonal antibody which recognized the N-terminal region of the M protein. As shown in Fig. 2, each of the C-terminal truncated constructs [M(1–133), M(1–164), and M(1–193)] expressed an appropriately sized protein when dually infected cells were labeled with [<sup>35</sup>S]methionine and analyzed by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. Two proteins with similar electrophoretic mobility were reproducibly expressed by three of the VV recombinants [M, M(1–164), and M(1–193)]. The exact relationship between the two forms of the protein was not further investigated.

Since our preliminary data suggested that at least one epitope was present in the sequence between amino acids 108 and 164, we also constructed a recombinant VV virus encoding these amino acids behind a T7 promoter. We also constructed a recombinant VV virus encoding the C-terminal region of the M protein [M(154–228)]. Using polyclonal murine anti-MHV-JHM antibodies or a panel of four anti-M monoclonal antibodies (provided by Dr. J. Fleming), we could not detect any protein of the proper size in cells infected with either of these recombinant VV (data not shown). However, direct sequence analysis of the VV shuttle vector confirmed the accuracy

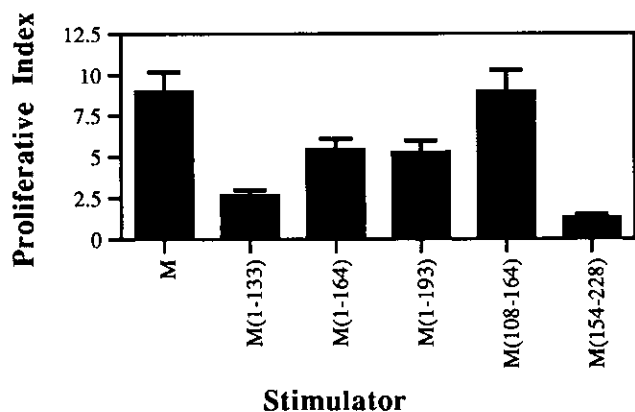


FIG. 3. Cell proliferative response to VV recombinants expressing truncated M proteins. Twenty-two mice were infected intraperitoneally with  $1 \times 10^6$  PFU MHV-JHM. Splenocytes were harvested 8 days later and analyzed in cell proliferation assays as described under Materials and Methods. Proliferative indices are shown, along with standard errors for each index. Mean incorporation in response to vTF7.3 lysates was 4095 cpm. Each lysate was analyzed in 5–22 mice.

of the subcloning procedures in both cases (data not shown). In addition, the synthesis of a protein with the predicted molecular weight was observed when cells were infected with VV recombinant M(154–228) and vTF7.3 (Fig. 2B). Also, as shown below, lymphocytes from infected mice proliferated in response to lysates from cells infected with the recombinant virus M(108–164), consistent with the presence of M(108–164) protein in these lysates.

#### Proliferative response to recombinant VV and M-specific peptides

Next, antigen was prepared from DBT cells co-infected with the recombinant VV and vTF7.3 as previously described (Mobley *et al.*, 1992) and tested in cell proliferation assays using splenocytes from mice infected with a sublethal intraperitoneal injection of MHV-JHM. These mice remained completely asymptomatic, but, as shown previously, mount a CD4<sup>+</sup> T cell response to the virus (Mobley *et al.*, 1992). As shown in Fig. 3, lysates expressing M(1–164) and M(1–193) exhibited mean proliferative indices of 5.5 and 5.3, respectively, whereas the M(1–133) lysate caused only a minimal amount of proliferation (2.7). These results suggested that one site of proliferative activity was located between amino acids 133 and 164. Since the amount of recombinant protein present in each of the lysates infected with the VV was not easily quantitated, it was not possible to conclude from these experiments whether any immunogenic peptides were present in the C-terminal part of the M protein. To assay this directly, cells expressing M(154–228) were also assayed in proliferation assays. As shown in Fig. 3, no stimulatory activity was present in this part of the M protein. To confirm and extend these results, spleen cells were also stimulated with M(108–164), which includes

the putative immunogenic region. As shown in Fig. 3, this protein fragment elicited a strong proliferative response, again suggesting that an immunogenic epitope was present in this region of the protein.

To delineate further the CD4<sup>+</sup> T cell epitope, a series of overlapping peptides based on the published sequence of the M protein (Pfleiderer *et al.*, 1986) was analyzed in proliferation assays. Although the results using VV recombinants suggested that most of the activity resided between residues 133 and 164, two panels of overlapping synthetic peptides corresponding to the first 164 amino acids were synthesized. The first was a panel of 14-mer peptides covering the region from amino acids 2 to 141 and offset by 5 or 6 amino acids (Fig. 4A). In the second panel (Fig. 4B), the peptides were also 14 amino acids long, offset by 1, and spanned the region from residue 134 to 166 since the data shown in Fig. 3 suggested that an immunogenic epitope was present in this region. As shown in Figs. 4A and 4B, the peptide encompassing amino acids 134–147 (VYVRPIIEDYHTLT) was the most active in these assays. The peptides containing residues 128–141 (IDMKGTVYVRPIIE) and 135–148 (YVRPIIEDYHTLTA) retained partial activity, although removal of residue 135 led to a complete loss of activity.

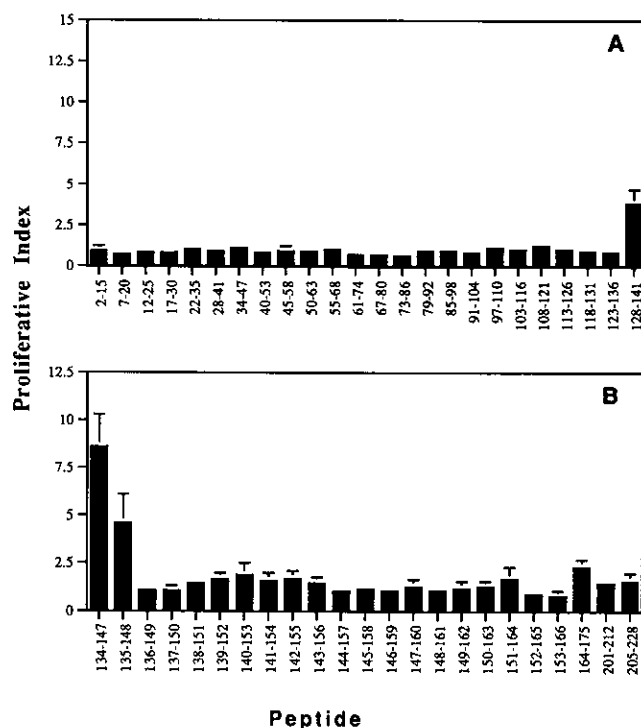


FIG. 4. Cell proliferative response to M-specific peptides. Twenty-two mice were infected intraperitoneally with MHV-JHM and splenocytes were analyzed 8 days later in cell proliferation assays using peptides at a final concentration of 1.5–2.6  $\mu$ M. The proliferative index is the ratio of incorporated counts for cells stimulated with peptide to those stimulated by medium alone. Each peptide was tested in 3–15 mice and the means and standard errors are shown. Mean incorporation in the absence of peptide was 5275 cpm.

A minimal consensus motif for binding to the I-A<sup>b</sup> allele, the only I-A allele present in C57Bl/6 mice, was recently defined (Wall *et al.*, 1994). Although the recombinant VV expressing the C-terminal part of the M protein had minimal activity, three additional peptides, M(164–175), M(201–212), and M(205–228), containing sequences which conformed to this consensus motif were synthesized and tested in proliferation assays. None of these peptides caused significant proliferation in our assays (Fig. 4B).

The overlap between peptides M(128–141) and M(135–148) is only seven amino acids. Since nearly all previously identified CD4<sup>+</sup> T cell epitopes are longer than seven amino acids (Rudensky *et al.*, 1992; Wall *et al.*, 1994), these results are consistent with the presence of two contiguous or overlapping epitopes. Alternatively, one epitope might be present with reduced stimulatory activity demonstrated by peptides containing part of that sequence. With the goal of determining which of these alternatives was correct, proliferation assays were performed with 10-fold dilutions of the three stimulatory peptides M(128–141), M(134–147), and M(135–148). Five-fold more peptide than used in Fig. 4 was also added to aliquots of cells in this experiment. As shown in Fig. 5, maximal stimulation was obtained with the concentrations of peptide used in Fig. 4 with the possible exception of M(128–141). As also shown in Fig. 5, M(134–147) was most active in this analysis, with an approximately 10-fold lower concentration of peptide required for the same proliferative response. These results suggest that if a second epitope is present in M(128–141) it has significantly less stimulatory activity than the one identified in M(134–147).

## DISCUSSION

The basis for demyelination in mice infected with MHV-JHM is not known with certainty. However, in mice with acute demyelination, most of the evidence is consistent with a model in which both viral lysis of oligodendrocytes and the immune response to infected oligodendrocytes contribute to the disease process (Kyuwa and Stohlman, 1990). While CD8<sup>+</sup> T cells are more common than CD4<sup>+</sup> T cells in the brains of mice with the early stages of MHV-JHM-induced encephalitis, CD4<sup>+</sup> T cells predominate in the CNS of mice with later stages of acute encephalitis and in those with chronic demyelination (Williamson *et al.*, 1991; Castro *et al.*, 1994). CD4<sup>+</sup> T cells appear to be critical for complete clearance of MHV during the acute stage of the infectious process (Pearce *et al.*, 1994). Inadequate viral clearance may set the stage for virus persistence and the subsequent development of demyelination. In addition, if the pathogenesis of MHV-induced chronic demyelination is similar to that induced by Theiler's virus (Friedmann *et al.*, 1987; Gerety *et al.*, 1994), CD4<sup>+</sup> T cells may contribute to the disease

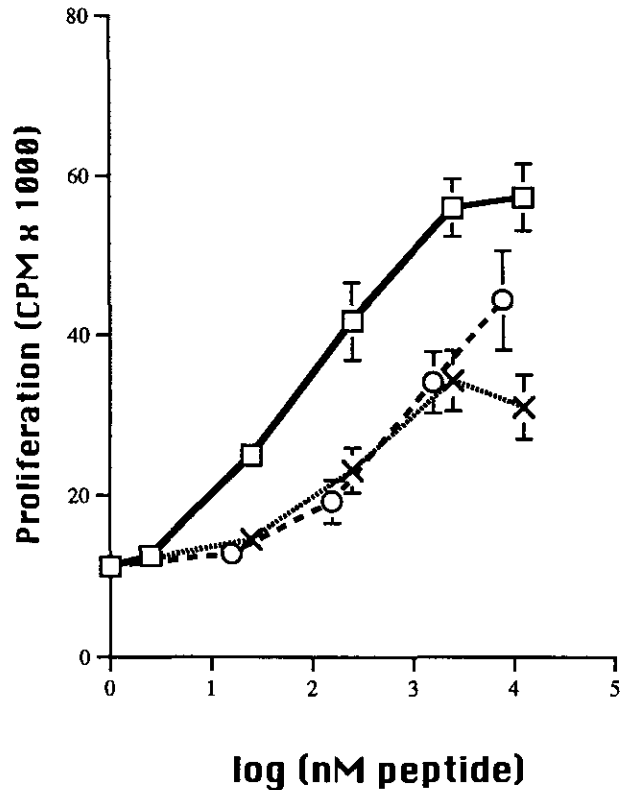


FIG. 5. Cell proliferative response to different concentrations of three stimulatory peptides. Six mice were infected intraperitoneally with MHV-JHM and splenocytes analyzed 8 days later in cell proliferation assays. Three stimulatory peptides, M(128–141), M(134–147), and M(135–148), were used at the concentrations shown. The data shown are the mean number of counts and standard error for each concentration of peptide. 0, M(128–141); □, M(134–147); X, M(135–148).

process. The antigen specificity of the CD4<sup>+</sup> T cells in the brains of chronically infected mice is not known, but is likely to include cells with anti-viral activity. We and others (Imrich *et al.*, 1994) have been unable to demonstrate a virus-specific T cell proliferative response using cells isolated from the central nervous system. This inability may reflect the presence of inhibitory factors in the preparation of CNS-derived lymphocytes (Stohlman *et al.*, 1993). Alternatively, these lymphocytes may be fully activated and no longer able to proliferate in response to exogenous antigen (Imrich *et al.*, 1994). Generation and characterization of T cell lines and clones from the CNS and spleen should circumvent these difficulties. In support of this notion, we have recently developed CD4<sup>+</sup> T cell lines responsive to the immunogenic region of the M protein and, in preliminary experiments, have found that they have protective activity against the acute encephalitis caused by MHV-JHM (unpublished observations).

In this report, we showed that three overlapping peptides exhibited significant proliferative activity, with one peptide, M(134–147) being more active (Fig. 5). Since peptide M(128–141) also has significant but lesser activ-

ity, our results can be interpreted in one of two ways. The M protein may contain one immunodominant sequence, only part of which is present in M(128–141). The only class II MHC molecule expressed in C57Bl/6 mice is the I-A<sup>b</sup> allele. The structural requirements for peptide binding to this MHC class II molecule have been defined using two different approaches. In the first, the motif for peptide binding was studied using peptide sequence alignment of class II-bound peptides (Rudensky *et al.*, 1992). In these analyses, the minimal consensus motif was N(D,Q)XXXXXP(I,S). This motif is not present in residues 128–147. In the second approach, full-length, truncated, and substituted synthetic peptide analogs were analyzed in binding and proliferation assays (Wall *et al.*, 1994). A consensus motif for I-A<sup>b</sup> binding was defined and consisted of an aromatic or hydrophobic residue, preferentially Y or F, at position 1, an uncharged residue at position 6, and an uncharged or possibly negatively charged residue at position 9. A sequence containing this I-A<sup>b</sup> binding motif is present between residues 135 and 143 since a tyrosine is at residue 135, an isoleucine at residue 140, and a tyrosine at 143. Only part of this sequence is included within M(128–141), but the study of Wall *et al.* showed that peptides containing only part of the consensus motif retained some stimulatory activity. Thus, it is possible that there is a single stimulatory sequence located at residues 135–143. Alternatively, a second epitope with less stimulatory activity may be present in M(128–141), although no sequence matching either I-A<sup>b</sup> binding motif is present in this peptide. Apparent overlapping or contiguous CD4<sup>+</sup> T cell epitopes have been identified on several other proteins (Kono *et al.*, 1988; Gammon *et al.*, 1991). Whether one or more epitopes in fact exists in this region will be definitively established when cloned populations of M-specific CD4<sup>+</sup> T cells are developed and analyzed.

Of note, M(135–148) has significantly less activity than M(134–147). Previous results showed that peptides eluted from MHC class II molecules, as opposed to those eluted from class I molecules, were in general longer and contain variable N- and C-termini (Rudensky *et al.*, 1992), and also that the presence of extra amino acids beyond the minimal stimulatory sequence increased the proliferative activity of T cells in response to the peptide (Wall *et al.*, 1994). Therefore, the requirement for additional N-terminal amino acids beyond the tyrosine at residue 135 for optimal activity is not surprising.

The data presented above do not eliminate the possibility that other regions of this protein also elicit a CD4<sup>+</sup> T cell response, albeit lesser in magnitude. The response to the epitope defined in this report not only may help protect mice from acute encephalitis but also may contribute to the disease process in mice with the chronic demyelinating disease. These data identify reagents which are useful in the generation of M-specific CD4<sup>+</sup> T-cell lines and clones. These cells will be used, in turn,

to analyze the role of the M-specific response in the pathogenesis of MHV-JHM-induced neurological diseases.

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