The JHM strain of mouse hepatitis virus induces a spike protein-specific D\textsuperscript{b}-restricted cytotoxic T cell response

Cornelia C. Bergmann, \textsuperscript{1*,2} Qin Yao, \textsuperscript{1} Mark Lin \textsuperscript{3} and Stephen A. Stohlman \textsuperscript{1,2}

Departments of \textsuperscript{1} Neurology, \textsuperscript{2} Microbiology and \textsuperscript{3} Pathology, University of Southern California School of Medicine, 1333 San Pablo, MCH 142, Los Angeles, California 90033, USA.

Cytotoxic T lymphocyte (CTL) activity specific for mouse hepatitis virus (MHV) JHM strain (JHMV or MHV-4) was examined using \textit{in vitro} stimulated spleen cells derived from immunized C57BL/6 (H-2\textsuperscript{b}) mice. Target cells infected with JHMV were specifically recognized; however, analysis of target cells expressing the virus structural proteins via recombinant vaccinia viruses showed no recognition of the viral nucleocapsid (N), membrane (M), small membrane (sM) or haemagglutinin–esterase (HE) proteins. Only target cells expressing the virus spike (S) protein were recognized. Furthermore, the majority of CTL activity was restricted to target cells expressing the MHC class I D\textsuperscript{b} molecules. Analysis of truncations and deletions of the S protein expressed by recombinant vaccinia viruses and peptide coated targets identified a single antigenic epitope, aa 510–518, conforming to the D\textsuperscript{b} binding motif. These amino acids are contained within a domain deleted from a number of strains of mouse hepatitis virus, suggesting a role for immune pressure. To determine the potential for CTL specific for an epitope (s) within a non-structural protein, 24 CTL lines were established and characterized. No evidence for the induction of non-specific CTL activity or virus-specific CTL restricted to a single epitope in a non-structural protein was obtained. These data indicate that the predominant CTL activity in JHMV-infected C57BL/6 mice is D\textsuperscript{b} restricted and specific for a single epitope contained within aa 510–518 of the S protein.

Introduction

Infection with the neurotropic mouse hepatitis virus (MHV) JHM strain (JHMV or MHV-4) induces an acute encephalomyelitis associated with primary central nervous system (CNS) demyelination in both rodents and primates (Fazakerley & Buchmeier, 1993; Kersting & Pette, 1956; Kyuwa & Stohlman, 1990; Murray \textit{et al.}, 1992a). Chronic demyelination is found in survivors of acute infection with wild-type virus, following infection with a variety of virus variants especially those with alterations in the spike (S) protein, or following infection of animals partially protected by a variety of immunotherapeutic approaches (Kyuwa & Stohlman, 1990). Chronic JHMV-induced disease is studied as a model of multiple sclerosis, the most common human demyelinating disease. Human coronaviruses have been implicated in multiple sclerosis (Murray \textit{et al.}, 1992b; Steward \textit{et al.}, 1992), although direct association with this group of human respiratory pathogens is far from assured. Most immune effector mechanisms participate in the immune response to JHMV infection. Analysis of JHMV infection in unmanipulated hosts, recipients of specific immunotherapy, or hosts depleted of specific immune effectors has confirmed the concept that the immune response is critical to both survival and the incidence of chronic disease (Kyuwa & Stohlman, 1990). However, the relative contributions of each effector mechanism are not clear. For example, passive transfer of both neutralizing monoclonal antibodies (MAb) specific for the S protein (Buchmeier \textit{et al.}, 1984) and non-neutralizing MAb specific for the matrix (M), nucleocapsid (N) and haemagglutinin–esterase (HE) proteins provides protection from acute disease (Fleming \textit{et al.}, 1988; Lecomte \textit{et al.}, 1987; Nakanaga \textit{et al.}, 1986; Yokomori \textit{et al.}, 1992). Whereas neutralizing antibodies appear to protect via a direct reduction in virus replication, protection by non-neutralizing MAb is not associated with a dramatic decrease in virus replication. Although the mechanism of protection provided by the non-neutralizing antibodies remains unclear, it has been speculated that the expression of peptides derived from virus structural proteins present on the surface of infected cells may provide targets for immune recognition (Kyuwa \textit{et al.}, 1994). JHMV-specific CD4\textsuperscript{+} T cells of the Th1 phenotype also provide protection (Korner \textit{et al.}, 1991; Stohlman \textit{et al.}, 1986; Yamaguchi \textit{et al.}, 1991). In some models CD4\textsuperscript{+}
T cell-mediated protection was not associated with decreased virus replication (Fleming et al., 1988; Stohlman et al., 1986); however, limited reductions by CD4+ T cells have also been described (Korner et al., 1991; Yamaguchi et al., 1991). It is not clear if the ability of these cells to limit replication is mediated by soluble factors or by direct recognition and lysis of infected cells expressing MHC class II molecules. Th1 populations not specific for viral components, even those specifically recruited to the CNS, are unable to provide protection (Stohlman et al., 1995b). The majority of the CD8+ CTL within the CNS of infected BALB/c (H-2b) mice are specific for a single epitope (aa 318–326) within the N protein (Bergmann et al., 1993a). However, CTL clones specific for an unidentified epitope, believed to be contained within a non-structural protein, were also identified (Stohlman et al., 1993). In contrast to BALB/c mice (Bergmann et al., 1993a; Stohlman et al., 1993), few JHMV-specific CTL were detected following peripheral infection of C57BL/6 (H-2b) mice with JHMV (Castro et al., 1994). In addition, it has been suggested that CD4+ CTL predominate in this haplotype following infection with the related MHV-A59 strain due to reduced expression of MHC class I following infection (Heemskerk et al., 1995). Although infection with MHV decreases host mRNA levels (Kyuwa et al., 1994), translation of host mRNAs (Tahara et al., 1994) and MHC class I cell surface expression (Kyuwa et al., 1994), sufficient class I molecules are expressed for CTL recognition of target cells infected with a variety of MHV strains, including MHV-A59 (Bergmann et al., 1993a). CD8+ CTL activity can be detected in the CNS during both acute and chronic JHMV infection of C57BL/6 mice (Castro et al., 1994). Specificity of these CTL was determined using target cells expressing the S, N and M proteins. Only CTL specific for the S protein were identified. Interestingly, little CTL activity was detected in spleen cells during acute infection and no activity was detected during chronic infection (Castro et al., 1994). This observation is consistent with previous analysis of the CTL response following CNS infection of BALB/c mice, which demonstrated no CTL specific for the N protein in splenic or cervical lymph node populations during acute CNS infection (Stohlman et al., 1993).

In this report we confirm that CTL specific for the JHMV S protein are induced in H-2b mice and that no CTL specific for either the M or N proteins are induced (Castro et al., 1994). In addition, no HE-specific or small membrane (sM)-specific CTL were detected. The CTL response was restricted to the H-2 Db MHC class I molecules. Analysis of the S protein sequence suggested the presence of at least 19 potential Db-specific binding motifs. Using truncation and deletion variants of the S protein expressed by recombinant vaccinia virus (rVV), the CTL response was found to be restricted to a single epitope within the amino-terminal third of the protein (aa 510–518) containing the Db binding motif. All 24 CD8+ CTL lines analysed recognized the S protein in the context of Db, consistent with the previous suggestion of a dominant CTL response in H-2b mice (Castro et al., 1994). These data demonstrate that peripheral infection with JHMV induces a CTL response in H-2b mice which appears to be restricted to a single Db-restricted epitope within the S protein. This contrasts to the induction of JHMV-specific CTL in mice of the H-2a haplotype which recognize at least two distinct epitopes in heterologous viral proteins (Stohlman et al., 1993).

Methods

Mouse strains and cell lines. C57BL/6 (H-2b) mice were purchased from the Jackson Laboratories (Mass., USA) at 6 weeks of age. Mice were housed in microisolation cages and immunized by infection with 1 × 10^5–2 × 10^6 p.f.u. of the DM isolate of JHMV (Stohlman et al., 1993) injected into the peritoneal cavity. The (H-2b)-derived MC57G and IC-21 cell lines were obtained from the ATCC. The CV-1, 143TK- and BSC-1 cell lines were obtained from B. Moss (Laboratory of Viral Diseases, NIAID, Md., USA). L929 cell (H-2b) derivatives designated 2-5 (expressing the Db gene) and 1-4 (expressing the Kb gene) were obtained from H. Allen (Biogen Research, Mass., USA).

Viruses. The derivation of clonal JHMV has been described previously (Stohlman et al., 1982). The wild-type WR strain of vaccinia virus (vv) was obtained from the ATCC. The derivation of rVV expressing the JHMV N protein (vIN), the JHMV S protein (vJS), the JHMV HE protein (vJHE) and the JHMV M protein (vJM) and the control rVV, expressing the Escherichia coli lacZ gene (vSC8), has been described previously (Stohlman et al., 1992). The rVV expressing the S protein derived from MHV-A59 was provided by W. Spaan (University of Leiden, The Netherlands). The rVV expressing a separate JHMV S protein, designated wMHV-4 S, was supplied by M. Buchmeier (Scripps Institute, Calif., USA). The rVV expressing the JHMV 5b gene product (sM protein) (Yu et al., 1994) was supplied by J. Leibowitz (University of Texas, Houston, USA). Truncated JHMV S genes (Fig. 1) were derived by subcloning of the 5', middle and 3' gene fragments used to assemble the full-length S protein cDNA (Stohlman et al., 1993). The three JHMV S gene
fragments designed to encode approximately one-third of the JHMV S protein (vJS3-1, vJS3-2, vJS3-3) were generated by PCR amplification of cDNA using three pairs of S-gene-specific oligonucleotide primers SS13/TA72, TA76/TA73 and TA74/TA125 described previously (Stohlman et al., 1993). Nucleotide and amino acid numbering correspond to the JHMV S protein sequence described by Parker et al. (1989). Briefly, construct JS3-1 encoding aa 1-518 was subcloned into the SalI and StuI sites of plasmid pSC11ss using primers SS13 (nt 1-29) and TA72 (nt 1535-1554) resulting in plasmid pSC1372. Terminal restriction sites were introduced with the respective primers. Digestion of the amino-terminal gene fragment in pSC1372 with SpeI deleted nt83~1471, encoding aa 279-490. The resulting deletion mutant encoding aa 1-278 and 491-518 was designated vJS3-1Δs.

The middle gene fragment extending from ApaI (nt 1543) to NruI (nt 2856) was amplified using primers TA76 (nt 1543-1566) and TA73 (nt 2841-2861) and subcloned into the ApaI and StuI sites of plasmid pSC11ss to yield construct vJS73 encoding aa 1-953. The 2440 bp NcoI/EcoRI S gene fragment was subcloned into compatible sites of plasmid pTM1 (Elroy-Stein et al., 1989). Construct vJS73e encoding aa 1-815 was generated by inserting the blunt ended pTM1-derived NcoI/StuI fragment into the StuI site of pSC11ss. Construct vJS73s encoding an 1-278 was derived by SpeI and StuI digestion of the latter plasmid followed by religation of the blunt ended sites.

Construct vJS3-2, encoding the middle domain of the S protein spanning aa 515-953, was generated by subcloning the TA76/TA73 PCR product into the NcoI and StuI sites of a modified pSC11ss plasmid, designated pK (Bergmann et al., 1993b). Finally, the 3' gene fragment extending from the NruI site (position 2856) to the end of the coding region (position 4128) was generated by PCR amplification using primers TA74 (5' CCC CCA TGG TTC AGG TGG GGT CTT GCG ACC CTC; nt 2845-2866) and TA125 (nt 4110-4130). The NcoI and StuI digested 3' fragment was inserted into compatible sites of plasmid pK yielding construct JS3-3 encoding the carboxy-terminal aa 949-1376. Minigene JS510, encoding aa 510-518 was cloned into the NcoI/StuI sites of plasmid pK using complementary oligonucleotides CB101 (5' CAT GTG TTC TCT TTG GAA TGG GCC CCA TTT GTA AG) and CB102 (5' CCT CAC AAT GGG GCC CAT TCC AAA GAG AAC A). 5' and 3' termini of all constructions were verified by sequence analysis as described previously (Stohlman et al., 1992).

JHMV sequences were recombined into the WR strain of vv by lipofectin-mediated transfection of plasmid DNA into WR-infected CV-1 cells. The rVV were plaque purified at least three times as described previously (Stohlman et al., 1992). Virus pools used throughout were derived from crude HeLa cell lysates.

Induction of bulk effector CTL. Spleen cell suspensions were prepared from immune mice 3–8 weeks after infection with the DM isolate of JHMV. Spleen cells (1 × 10⁸) from immune mice were cultured for 6
days at 37 °C with 5 × 10^7 irradiated (25 Gy) syngeneic spleen cells from naïve mice infected with JHMV at an m.o.i. of 1.0-0.1 in 30 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (Gemini Bioproducts), 2 mM-glutamine, 25 μg/ml gentamicin, 1 mM-sodium pyruvate, 5 × 10^-4 M-2-mercaptoethanol and non-essential amino acids (RPMI complete).

Complement-mediated depletion. Effectors were washed twice by centrifugation in RPMI, resuspended in 20 ml RPMI at 2 × 10^6 viable cells/ml and incubated for 1 h at 4 °C with rat MAb 31M specific for CD8 (Sarimento et al., 1980), rat MAb RL172.4 specific for CD4 (Ceredig et al., 1985) or without antibody. The cells were washed twice in RPMI, resuspended in 1.8 ml RPMI containing 25 mM-HEPES pH 7.2 and 0.3% bovine serum albumin. Low-Tox rabbit complement (C'; Accurate Chemicals) was added at 0.2 ml and the mixture incubated for 45 min at 37 °C. Following two additional washes, the cells were resuspended and tested for residual CTL activity.

Isolation of T cell lines. JHMV-specific T cell lines were isolated from in vitro stimulated spleen cells following depletion of CD4^+ T cells using MAb RL172.4 plus C' (Ceredig et al., 1985) by limiting dilution in 96-well plates containing 5 × 10^4 JHMV-infected syngeneic feeder cells per well. Lines were expanded and maintained in RPMI complete supplemented with 10% FCS and 10% Con A supernatant by weekly transfer of approximately 2 × 10^5 T cells per well of a 24-well plate containing 4 × 10^4 JHMV-infected irradiated syngeneic spleen cells. T cell lines were examined for cell surface expression using rat anti-CD4 (MAb RL172.4), rat anti-CD8 (MAb 31M) and rat anti-T cell receptor (TCR) vfl8 (MAb F23.1) followed by goat anti-rat FITC-(Fab')2 antibody (Caltag) as described previously (Stohlman et al., 1992). Cells were labelled, washed, and resuspended in PBS containing 0.1% formaldehyde and analysed on a FACStar (Becton Dickinson).

Cytotoxicity assay. Cytolytic activity was measured in a 4 h ^51Cr release assay as described previously (Stohlman et al., 1992). In vitro stimulated spleen cells and T cell lines were used as effectors. IC-21 target cells were infected with JHMV at an m.o.i. of 1:1; MC57G cells, the 1-4 and 2-5 cell lines were infected with rVV at an m.o.i. of 5-10. Following incubation for either 5 h (IC-21) or 14 h (MC57G) at 37 °C target cells were washed and labelled with 100 μCi Na^24CrO_4 (New England Nuclear). Washed target cells at a concentration of 1 × 10^5 in 100 μl volume were added to the effector cells. In some experiments target cells were pre-incubated for 15 min at 37 °C with 0.5-1.0 μm-peptides prior to addition to the assay. Data are expressed as percentage specific release defined as: [experimental release] - [spontaneous release]/[total detergent release] - [spontaneous release]. Maximum spontaneous release values were ≤ 20% of total release values.

Synthetic peptides and oligonucleotides. JS-derived peptides containing the D^t binding motif (XXXXXXXMX,M,I,L; Rammensee et al., 1993) were purchased from Chiron Mimotopes (Clayton, Australia). Purity, as well as quantity, estimated to be 18 mg per well, were assessed by the supplier for two control peptides by HPLC and amino acid analysis. Peptides were dissolved in 50% DMSO in sterile distilled water at 1 mg/ml and stored under nitrogen gas at -20 °C to minimize oxidation. Oligonucleotides were synthesized on a model 394 DNA synthesizer (Applied Biosystems). The sequences of JS-specific primers SS13, TA72, TA76, TA73 and TA125 were described previously (Stohlman et al., 1993).

**Results**

Characterization of the CTL response in C57BL/6 mice

CTL specific for the S protein have been detected within the CNS during both acute and chronic infection of H-2^d mice (Castro et al., 1994). However, only low levels of S protein-specific CTL were detected within the spleen during acute infection and none was detected during persistent infection. No CTL specific for the N or M proteins were detected in the CNS. By contrast, CTL specific for at least two distinct epitopes have been described in H-2^b mice (Stohlman et al., 1993). To examine the potential diversity of CTL in the H-2^b haplotype, C57BL/6 mice were infected intraperitoneally (i.p.) with 1 × 10^6 - 2 × 10^6 p.f.u. of JHMV. CTL were derived by *in vitro* expansion of memory cells using JHMV-infected spleen cells as stimulators as described previously (Stohlman et al., 1992). CTL activity was analysed using JHMV-infected IC-21 target cells or MC57G target cells infected with rVV expressing the JHMV S (vJS), M (vJM), N (vJN), HE (vJHE) and sM (vJsM) structural proteins. JHMV-induced CTL lysed infected IC-21 cells but not uninfected cells (Fig. 2a), clearly demonstrating the presence of virus-specific CTL. Analysis of MC57G target cells infected with rVV expressing JHMV structural proteins showed that only cells expressing the S protein were recognized, consistent with the report of Castro et al. (1994). In addition, these CTL recognized MC57G cells infected with rVV expressing an independently constructed full-length JHMV S protein designated wtMHV-4 S protein but not target cells expressing the MHV-A59-derived S protein (data not shown). No CTL activity was detected using target cells expressing the HE, N or M proteins (Fig. 2b). Furthermore, target cells expressing the gene 5b sM protein (vJsM) were not recognized by the JHMV-specific CTL (Fig. 2b). These data demonstrate that JHMV induces CTL specific for the S protein but not other structural proteins.

To rule out the possibility of B cell (Wysocka et al., 1989) or CD4^+ T cell-mediated lysis of target cells (Stohlman et al., 1993) expressing the S protein, activated splenocytes were examined for activity following CD8 MAb plus C'-mediated depletion. Treatment of the bulk CTL population with anti-CD8 MAb plus C' dramatically reduced the CTL activity while treatment with C' only or with anti-CD4 MAb plus C' had little or no effect on specific lysis (Fig. 3). Consistent with these data no lysis was detected on vJS-infected target cells which do not express the appropriate MHC restriction element (see below).

**CTL are restricted to MHC class I D^b**

To determine whether both H-2^b MHC class I molecules provided restriction elements for S protein-specific CTL L929 (H-2^d) target cells expressing either the K^b or D^b class I molecules and MC57G (K^dD^b) cells were tested for recognition. JHMV-induced CTL efficiently recog-
Fig. 2. Specificity of JHMV-induced CTL in H-2b mice. (a) CTL recognition of IC-21 target cells infected with JHMV at an m.o.i. of 1 for 5 h. (b) CTL recognition of MC57G target cells infected with rVV expressing the JHMV structural S (vJS), N (vJN), HE (vJHE) and sM (vJsM) proteins at an m.o.i. of 5 for 14 h. VSC8-infected target cells served as a negative control. JHMV-specific CTL were derived from spleen cells of immunized C57BL/6 mice and restimulated in vitro with irradiated syngeneic splenocytes infected with JHMV.

Fig. 3. Cytolytic activity is mediated by CD8+ T cells. JHMV-immune splenocytes were treated with complement (C') only, anti-CD4 MAb plus C' or anti-CD8 MAb plus C'. Untreated and treated effector populations were tested for cytolytic activity on MC57G target cells infected with rVV vJS.

Epitope mapping using rVV

The S protein contains 19 peptides corresponding to the predicted Db binding motif equally distributed throughout the entire protein. Target cells infected with three rVV expressing sequential carboxy-truncated JHMV S proteins encompassing aa 1–953 (vJS73), 1–815 (vJS73e) or 1–518 (vJS3-1) were recognized with equal efficiency compared to vJS-infected target cells (Fig. 5). No lysis was detected in cells infected with the rVV expressing amino-terminal aa 1–278 (vJS73s), excluding this domain containing five potential Db motif peptides. Although a similar approach using carboxy-terminal truncations was effective at defining the H-2d-restricted epitope within the N protein (Bergmann et al., 1993a), from these data it is not possible to distinguish between single or multiple epitopes contained within aa 278–1376. Therefore, rVV were constructed expressing aa 515–953 (vJS3-2), aa 949–1376 (vJS3-3) and aa 1–518 comprising a 213 aa deletion extending from residue 279 to 490 (vJS3-1As) (Fig. 1). No CTL activity was detected using target cells expressing aa 515–953 (vJS3-2) or 949–1376 (vJS3-3). By contrast, JHMV-specific CTL efficiently recognized target cells expressing the amino-terminal aa 1–278 plus 491–518, suggesting at least one immunodominant CTL epitope is contained within the amino acids expressed by rVV vJS3-1As. Recognition of target cells expressing aa 1–278 plus 491–518 (vJS3-1As) versus the absence of recognition of target cells expressing aa 1–278 (vJS73s) alone eliminates five of nine potential Db binding epitopes within aa 1–518 and suggests the
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Fig. 4. S protein-specific CTL are dominantly D\(^b\) restricted. CTL activity was measured using MC57G (H-2\(^b\)), 2-5 (H-2\(^b\), D\(^b\)) or 1-4 (H-2\(^b\), K\(^b\)) target cells infected with vJS at an m.o.i of 5 for 14 h as indicated in each panel. VSC8-infected target cells were negative controls.

Fig. 5. Recognition of targets expressing the JHMV S protein truncations. MC57G target cells expressing wild-type S protein (vJS), aa 1–953 (vJS73), aa 1–815 (vJS73e), aa 1–518 (vJS3-1) and aa 1–278 (vJS73s) (a); or aa 1–278 plus 491–518 (vJS3-1As), aa 515–953 (vJS3-2) and aa 949–1376 (vJS3-3) (b) were tested for recognition by JHMV-immune CTL. VSC8-infected target cells were used as a negative control.

presence of an antigenic determinant within aa 491–518. This region contains a single sequence corresponding to the D\(^b\) motif; i.e. aa 510–518. The CTL response to the entire S protein is therefore limited to one epitope within aa 491–518 and to three other candidates contained within the domain deleted (aa 279–490) in construct vJS3-1As.

**CTL are specific for aa 510–518**

Identity of the epitope(s) within this region was examined using four peptides containing potential D\(^b\) motifs. Target cells were treated with peptides encompassing aa 353–361 (NCNFNLSSL), 403–411 (LQIGNSGF), 430–438 (SLPKNNVTI) and 510–518 (CSLWNGPHL). Only target cells coated with peptides 510–518 were recognized by JHMV-induced CTL expanded \textit{in vitro} on JHMV-infected stimulators (Fig. 6a). These data indicate that aa 510–518 represent the immunodominant D\(^b\)-restricted epitope within the S protein. To confirm recognition of this epitope, aa 510–518 were expressed from a minigene using rVV (vJS510; see Fig. 1). Fig. 6(b) shows that MC57G target cells infected with vJS510 were recognized as efficiently as target cells expressing the full-length S protein.

**JHMV-specific CTL lines**

These data indicate that JHMV induces CTL specific for a single epitope (aa 510–518) contained within the amino-terminal portion of the S protein in C57BL/6 mice. To address the possibility of epitopes within the
MHV-specific CTL

Fig. 6. The immunodominant epitope within the S protein maps to aa 510–518. (a) Four potential 9-mer peptides located within aa 278–518 containing potential D\(^{\alpha}\) binding motifs were tested for CTL recognition on MC57G target cells at 0.5 \(\mu M\). Cytotoxicity was assayed at an effector: target ratio of 40:1 and 20:1. Peptides are designated by amino acid numbers corresponding to their location within the S protein. (b) MC57G target cells infected with rVV expressing wild-type S protein (vJS) or the peptide comprising aa 510–518 (vJS510) were compared for CTL recognition.

Table 1. Characteristics of H-2\(^{\alpha}\)-derived JHMV-specific CTL lines

<table>
<thead>
<tr>
<th>Line</th>
<th>CD8(^{+})</th>
<th>TCR(^{+})</th>
<th>Restriction element†</th>
<th>Target recognition</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>vJS vJS3-1 vJS3-2 vJS3-3 vJS3-1(^{\alpha})</td>
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<tr>
<td>Y1</td>
<td>+</td>
<td>D(^{\beta})</td>
<td>–</td>
<td>+ + – – + + + + + +</td>
</tr>
<tr>
<td>Y2</td>
<td>+</td>
<td>D(^{\beta})</td>
<td>–</td>
<td>+ + – – + + + + + +</td>
</tr>
<tr>
<td>Y5</td>
<td>+</td>
<td>D(^{\beta})</td>
<td>+</td>
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</tr>
<tr>
<td>Y6</td>
<td>+</td>
<td>D(^{\beta})</td>
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<tr>
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* Determined by FACS.
† Determined by analysis of D\(^{\alpha}\)- and K\(^{\alpha}\)-transfected L-929 cells infected with vJS3-1.

non-structural proteins, 24 JHMV-specific CD8\(^{+}\) lines were isolated and characterized using JHMV-infected feeder cells to minimize selective pressure (see Methods). Previous analysis had shown the presence of CTL lines specific for both the JHMV N protein and for an epitope(s) encoded within a non-structural protein(s) in both the CNS (Stohlman et al., 1993) and spleens (unpublished data) of BALB/c (H-2\(^{\alpha}\)) mice. C57BL/6-derived lines were initially tested for recognition of target cells expressing the S protein (vJS) compared to target cells infected with the control rVV (vSC8). All 24 lines expressed CD8 and specifically recognized target cells expressing the S protein (data not shown) (Table 1). The absence of T cell lines exhibiting non-specific CTL activity contrasts with the report of self-reactive T cell induction following infection of BALB/c mice with JHMV (Kyuwa et al., 1991) and the analysis of H-2\(^{\alpha}\)-derived clones (Stohlman et al., 1993).

Eight CD8\(^{+}\) T cell lines were examined for recognition of target cells infected with rVV expressing the truncated S protein-derived peptides. Table 1 shows a summary of these results and Fig. 7 shows an example of the activity of two of these clones, one TCR v\(\beta\)^8\(^{-}\) (Y5) and one v\(\beta\)^8\(^{-}\) (Y18). All eight lines recognized target cells expressing the entire S protein (vJS), target cells expressing aa 1–518 (vJS3-1) and aa 1–278 plus 491–518 (vJS3-1\(^{\alpha}\)). No recognition of target cells expressing aa 515–953 (vJS3-2), aa 949–1376 (vJS3-3) or control rVV were
detected. These data indicate that all of the clones recognize target cells expressing the deletion mutant (vJS3-1As) encoding aa 1–278 plus 491–518 in the context of the class I D\(^b\) molecules, suggesting that the dominant CTL response in the H-2\(^b\) haplotype is restricted to these residues within the JHMV S protein.

Discussion

Both host and viral components are involved in the complex demyelinating encephalomyelitis induced by JHMV infection of the CNS (Kyuwa & Stohlman, 1990). One critical host response to JHMV infection is the induction of CD8\(^+\) CTL which contribute significantly to the clearance of virus from the CNS (Stohlman et al., 1995b; Sussman et al., 1989; Williamson & Stohlman, 1990). Preliminary limiting dilution analysis suggests a CTL precursor frequency of approximately 1/3500 spleen cells in H-2\(^a\) mice (unpublished data), indicating that JHMV is very efficient at inducing CTL. CTL specific for the S protein but not the M or N proteins, have previously been described within the CNS of acutely infected C57BL/6 mice (Castro et al., 1994). However, only low CTL activity specific for the S protein was detected in the spleens of these mice. In addition, CTL specific for the S protein could be demonstrated in the CNS during persistent infection; but not in the spleen of these mice (Castro et al., 1994). Interestingly, CTL specific for the L\(^a\)-restricted N protein epitope (Bergmann et al., 1993a) were detected in the CNS, but not the spleen, of both BALB/c- and B10.A (18R) (K\(^b\), D\(^4\), L\(^a\))-infected mice (Castro et al., 1994; Stohlman et al., 1993). The present study was undertaken using in vitro stimulated CTL derived from immune donors; a more sensitive assay system than direct analysis of primary CTL derived from infected mice (Castelmur et al., 1993). Analysis of CTL derived from the spleens of immune donors demonstrated the presence of CTL which specifically recognized target cells infected with JHMV or expressing the S protein, but neither the M nor N proteins, consistent with previous data (Castro et al., 1994). In addition, no CTL capable of recognizing the HE or sM proteins were detected. Furthermore, the majority of the CTL were D\(^b\) restricted, although occasional low S protein-specific CTL activity was detected on K\(^b\)-expressing target cells. These data may suggest the presence of a minor K\(^b\)-restricted epitope within the S protein (Castro & Perlman, 1995).

Analysis of CTL in H-2\(^a\) mice demonstrated that, in addition to a major epitope within the N protein, an additional epitope(s) was present within the non-structural proteins (Stohlman et al., 1993). The presence of CTL specific for this epitope(s) were only detected by analysis of CTL lines which recognized JHMV-infected target cells but were unable to recognize target cells expressing any of the viral structural proteins (Stohlman et al., 1993). To determine if a similar population of CTL specific for non-structural proteins was present in H-2\(^b\) mice, 24 CD8\(^+\) CTL lines were tested for recognition of target cells expressing the S protein. All lines recognized target cells expressing the S protein. In addition, none lysed target cells infected with a control rVV, a property exhibited by 10% of lines derived from BALB/c mice (Stohlman et al., 1993). These data suggest that in contrast to the BALB/c mice, JHMV-induced CTL in C57BL/6 mice recognize only a single viral protein, the S protein.

Differential recognition of target cells infected with vJS73s and vJS3-1As suggested that the S protein contained a single epitope in the region 491–518. To
confirm these data target cells were coated with peptides encompassing potential Db epitopes within aa 288–518 encompassing both the deleted and recognized amino acids in vJS3-1As. Analysis of these peptide coated target cells identified the presence of a single epitope encompassing aa 510–518. Similar analysis of CTL derived directly from the CNS of JHMV-infected C57BL/6 mice suggests that indeed these amino acids constitute a Db-restricted epitope; however, an additional Kk-restricted epitope encompassing aa 598–605 was also detected in primary CNS-derived populations (Castro & Perlman, 1995). This epitope is present within the amino acid sequence of both JHMV and A59 (Luytjes et al., 1987; Parker et al., 1989); however, target cells infected with rVV expressing the MHV-A59 S protein were not recognized by our CTL population (data not shown). This observation confirmed data obtained from analysis of target cells expressing truncated forms of the JHMV S protein and the absence of Kk-restricted S protein-specific splenic CTL lines. The reason(s) for this discrepancy is not clear, although it may reflect the differences in CTL populations analysed. Differential expansion or induction of JHMV-specific CTL within the CNS has previously been suggested to account for the absence of N protein-specific CTL in the peripheral organs of H-2d mice during acute infection (Stohlman et al., 1993). Alternatively, the non-selective stimulation of the immune CTL in the present report may have underestimated the potential for subdominant epitopes.

The definition of an immunodominant CTL epitope within the amino-terminal segment of the S protein has a number of interesting biological consequences for the analysis of MHV infection. Parental JHMV expresses an S protein of 1376 aa (Parker et al., 1987); however, some clonal populations of JHMV have naturally occurring deletions encompassing this region of the S protein (Schmidt et al., 1987; Parker et al., 1989). Furthermore, a number of MAb-derived variants with reduced neurovirulence or altered neuropathogenicity in BALB/c mice have deletions within this region (Gallagher et al., 1990; Parker et al., 1989). Viruses recovered during persistent CNS infections also have deletions within this region (La Monica et al., 1991; Morris et al., 1989). These data have been interpreted to suggest that alterations in the structure of the S protein affect neuropathogenicity (Gallagher et al., 1990); however, the contribution of the loss of a major CTL epitope has not been appreciated previously. Although MHV infection induces increased MHC mRNA levels in vivo (Gombold & Weiss, 1992) and MHC class I expression on oligodendroglia in vitro (Suzumura et al., 1986), activated JHMV-specific CTL were relatively inefficient at reducing virus replication in oligodendroglia (Stohlman et al., 1995). The hepatotropic MHV-A59 strain produces a relatively avirulent infection of the CNS accompanied by demyelination (Lavi et al., 1984) and has a 52 aa deletion in the S protein relative to the JHMV S protein, encompassing the immunodominant Db-restricted epitope (Luytjes et al., 1987; Parker et al., 1989). It has been suggested that MHV-A59 infection decreases MHC class I expression in vivo resulting in a predominant MHC class II-restricted CTL activity (Heemskerk et al., 1995). This suggestion is consistent with decreased host mRNA and protein synthesis in MHV-infected cells (Hilton et al., 1986; Kuywa et al., 1994). However, H-2Lk-restricted CTL specific for the JHMV N protein recognize cells infected with MHV-A59 (Bergmann et al., 1993a). The data in this report suggest that the MHV-A59 S protein lacks the immunodominant CD8+ CTL epitope, which may account for the predominance of CD4+ CTL in MHV-A59-infected C57BL/6 mice (Heemskerk et al., 1995) and the lack of virus clearance in C57BL/6 mice genetically deleted for the expression of β2-microglobulin (Gombold et al., 1995). The absence of this epitope in MAAb-derived variants of JHMV (Gallagher et al., 1990; Parker et al., 1989), viruses recovered during persistent infection (La Monica et al., 1991; Morris et al., 1992), clonal populations of JHMV isolated in the absence of selective pressure (Schmidt et al., 1987; Parker et al., 1989) and the MHV-A59 strain (Luytjes et al., 1987) may contribute to altered neuropathogenicity by abolishing the potential CTL responses in these mice. However, this aspect of the immune response cannot completely account for the alterations observed since MAAb-derived variants with altered neuropathogenicity express this epitope (Wang et al., 1992). It is not clear whether alterations in the S protein or preferential infection of oligodendroglia, thereby escaping CTL-mediated clearance from the CNS, or both of these contribute to the ability to produce acute and chronic demyelination (Fleming et al., 1986, 1987; Stohlman et al., 1995a).

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References


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