Characterization of the L^d -Restricted Cytotoxic T-Lymphocyte Epitope in the Mouse Hepatitis Virus Nucleocapsid Protein

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The mouse hepatitis virus (MHV) JHM strain (JHNV) produces primary demyelination in the central nervous system associated with acute encephalomyelitis. Humoral and cellular immune responses both participate in controlling the development of chronic MHV-induced demyelination. A subset of the CD8⁺ cytotoxic T lymphocytes (CTL) induced by immunization of BALB/c ($H-2^d$) mice with JHNV is specific for the viral nucleocapsid protein. This CTL population recognizes an epitope located within the carboxy-terminal 149 amino acids in association with the L^d class I molecule (S. A. Stohlman, S. Kyuwa, M. Cohen, C. Bergmann, J. P. Polo, J. Yeh, R. Anthony, and J. G. Keck, *Virology* 189:217–224, 1992). Using a panel of vaccinia virus recombinants expressing truncated forms of the nucleocapsid protein and a series of overlapping synthetic peptides, we mapped the response to 15 amino acids. This sequence, encompassing the MHV epitope, contains the L^d -specific binding motif. The predicted 9-mer peptide (residues 318 to 326: APTAGAFFF) was sufficient and highly active in sensitizing target cells for CTL recognition when either added exogenously or synthesized intracellularly. Cross-reactivity of JHNV nucleocapsid protein-specific CTL with six other MHV strains indicated that natural sequence variations within the 9-mer epitope are tolerated in positions 4 and 5, whereas all other amino acids are conserved. These data define a novel 9-mer L^d -restricted CTL epitope which represents the first MHV CTL epitope. Characterization of this epitope provides a molecular basis to study the role of nucleocapsid protein-specific CTL in the clearance of JHNV from the central nervous system.

Mouse hepatitis viruses (MHV) are natural pathogens of mice which cause a variety of diseases, including hepatitis and encephalitis (5, 15). Infection of susceptible strains of mice with the neurotropic JHM strain of MHV (JHNV) produces a panencephalitis accompanied by primary demyelination with little or no evidence of hepatitis. This infection is studied as a model of virus-induced central nervous system (CNS) demyelination (5, 9, 15, 33). Mice which survive the acute phase of JHNV infection show evidence of a chronic form of CNS disease characterized by the persistence of viral antigen and ongoing demyelination (9, 15, 33). Primary demyelination is associated with the ability of JHNV to infect oligodendroglial cells and appears to be a function of the virion spike protein (5, 12, 15), while persistence appears to be associated with chronic viral infection of astrocytes (22). By contrast, less is understood about the role of the host immune response to JHNV during the acute infection. The immune response during the acute phase of the disease appears to play a pivotal role in determining the eventual outcome of the infection, i.e., either viral clearance resulting in the absence of chronic disease or partial clearance resulting in chronic disease (5, 15, 38). The major components of the immune system, including antiviral antibody, natural killer cells, monocytes, and CD4⁺ and CD8⁺ T cells, are all present within the CNS during the acute infection (15, 39). However, their respective roles in protection from the acute infection and in the chronic disease are unclear. The passive transfer of nonneutralizing monoclonal antibody or the adoptive transfer of CD4⁺ T helper cells specific for the virion structural components both result in protection of mice from a lethal infection but have minimal or no effect on virus replication (5, 15, 32). By contrast, the adoptive transfer of virus-

specific CD8⁺ cytotoxic T-lymphocyte (CTL) clones not only protects mice from a lethal JHNV infection but also reduces virus replication within the CNS, thereby providing some measure of protection from chronic disease (40). To understand the component of the immune system that appears to reduce the incidence of chronic infection within the CNS, our laboratory has focused on the role of the CD8⁺ CTL populations in this disease process.

CTLs recognize peptides derived from immunogenic proteins in association with major histocompatibility complex (MHC) class I molecules (1, 34). We have previously demonstrated that immunization of BALB/c ($H-2^d$) mice with JHNV induces CTLs that are restricted to the L^d class I molecule and are specific for the nucleocapsid (N) protein (31). The N protein is a basic, highly phosphorylated internal virus structural protein that forms a helical nucleocapsid in association with the 32-kb genomic RNA (16). It also has a specific affinity for sequences found at the 5' ends of all of the viral mRNAs and is believed to play a role in regulation of viral transcription and translation (30). Using recombinant vaccinia viruses (VV) expressing native JHNV N protein (vJN) and carboxy-terminal deletions, the epitope recognized by N protein-specific CTLs was mapped to the relatively acidic 149 carboxy (C)-terminal amino acids (aa). In this paper we report the first definition of an MHV-specific CTL epitope. Additional VV recombinants encoding C-terminal peptides of the N protein and a panel of synthetic peptides were used to precisely define the L^d -restricted epitope. A 9-mer peptide containing the predicted L^d -specific binding motif was sufficient for recognition and represents only the third L^d -restricted viral epitope described (6). JHNV-induced N protein-specific CTLs cross-reacted with targets infected with six other MHV strains. Sequence analysis indicates that natural variations within the 9-mer epitope are well tolerated.

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MATERIALS AND METHODS

Mice. BALB/cBy ($H-2^d$) mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age. Mice were immunized within 1 week of arrival. Mice were housed in an accredited animal facility at the University of Southern California and were used between 8 and 14 weeks of age. No group was found to have preexisting anti-MHV antibody.

Cell lines. The derivation and propagation of the J774.1 ($H-2^d$) cell line and the L929 cell ($H-2^k$) derivative K2a7 (expressing the L^d gene) have been described (31). The $T2/L^d$ cell line (7) was kindly provided by P. Cresswell (Section of Immunobiology, Howard Hughes Medical Institute, Yale University, New Haven, Conn.).

Viruses. The derivation of the clonal JHMV, the A59 strain of MHV (MHV-A59), VV recombinants vSC8 (encoding the *Escherichia coli lacZ* gene) and vJN (encoding the JHMV N nucleocapsid protein), and the wild-type WR strain have been described previously (31). VV recombinant vTF7.3 (encoding the bacteriophage T7 polymerase) (13) was obtained from B. Moss (National Institutes of Health, Bethesda, Md.). MHV-1, MHV-2, and MHV-S virus strains were plaque purified from stocks obtained from M. Collins (Microbiological Associates, Bethesda, Md.) and K. Fujiwara (University of Tokyo, Tokyo, Japan). The MHV-K, MHV-D, and MHV-Nuu viruses were obtained as cloned stocks from K. Fujiwara. The derivation of these MHV strains has been previously described (11). The plaque-purified MHV-3 strain was obtained from J. Leibowitz (University of Texas, Houston, Tex.), and a plaque-purified MHV-A59 strain (designated MHV-HA59) was obtained from K. Holmes (Uniformed Services University of the Health Services, Bethesda, Md.).

VV recombinants vJNb, vJN82, vJN155, vJN51, and vJN20 were generated by homologous recombination into the wild-type WR strain as previously described (31). The pSC11ss-derived plasmids containing truncated JHM N genes were constructed by using standard cloning procedures. Plasmid pSC11-JN encoding the entire N protein served as the template for the 3' *BsmI* deletion mutant, designated JNb, and for polymerase chain reaction (PCR) products encoding internal protein fragments (see Fig. 1). In analogy to the truncations described previously (31), the 280-bp 3' deletion (JNb) was constructed by digesting plasmid pSC11-JN with *BsmI* (at position 1133) and *StuI* (in the pSC11ss polylinker), blunt ending with Klenow polymerase in the absence of deoxynucleoside triphosphates, and then religation. The 3' gene fragments encoding JN82 (aa 374 to 455) and JN155 (aa 301 to 455) were generated by PCR amplification from the JHMV N gene by using synthetic oligonucleotide primers ON1203 (5'-TTT CCA TGG AGA ATT TGA ATG CCT ACC AGA AT, nucleotides [nt] 1203 to 1226), ON984 (5'-CCC GCT AGC CAT GGT AAA GCT TGG AAC TAG TGA TCC, nt 984 to 1009), and ON1427 (5'-TTA CCC GGG CAC ATT AGA GTC ATC TTC TAA C, nt 1427 to 1447). Numbering of nucleotides includes 84 nt of untranslated leader sequence. *NcoI* and *SmaI* sites were incorporated into the primers to introduce an ATG start codon and allow unidirectional cloning. Minigenes encoding JN51 (aa 301 to 351) and JN20 (aa 301 to 320) were derived from the PCR product encoding JN155 by digestion with either *NcoI*-*SspI* (at position 1135) or *NcoI*-*PvuII* (at position 1042). All four PCR-derived fragments were subcloned into the *NcoI* and *SmaI* sites of plasmid pK, a modified version of pSC11ss (3). The JN9 minigene encoding aa 318 to 326 with an additional amino (N)-terminal methionine was generated by annealing complementary oligonucleotides 5'-CAT GGC CCC AAC AGC TGG TGC CTT CTT

CTT TTA GG and 5'-CCT AAA AGA AGA AGG CAC CAG CTG TTG GGG C. The resulting fragment, containing start and stop codons, was inserted into the *NcoI* and *StuI* sites of plasmid pTM1 for use in a T7 polymerase-driven transient VV-based expression system (8). Sequences were confirmed by using either the Sequenase 2.0 kit (U.S. Biochemicals Corp., Cleveland, Ohio) or the Amplitaq sequencing kit (Perkin Elmer, Norwalk, Conn.) with pSC11ss-specific primers as previously described (31).

Synthetic peptides and oligonucleotides. Peptides pN318-335 and pN318-326 were synthesized on an automated model 430A synthesizer (Applied Biosystems, Foster City, Calif.), chromatographed on Sephadex G-10, and purity assessed by high-pressure liquid chromatography. Peptides for epitope scanning were synthesized on pins (19) according to the supplier's instructions (Cambridge Research Biochemicals, Wilmington, Del.). Peptides were cleaved from the pins by overnight incubation at room temperature in 150 μ l of phosphate-buffered saline (PBS). Purity, as well as quantity, estimated to be 5 to 7 μ g per pin, was assessed by capillary electrophoresis with a P/ACE system 2060 (Beckman, Fullerton, Calif.). Oligonucleotides were synthesized on a model 394 DNA synthesizer (Applied Biosystems). Peptides and oligonucleotides were synthesized by the USC Norris Cancer Center Microchemistry Core Laboratory.

Sequencing of viral RNA. Monolayers of DBT cells in 150-mm-diameter plates were infected with the MHV strains JHMV (DL), MHV-1, MHV-2, MHV-3, MHV-A59, MHV-HA59, MHV-K, MHV-D, MHV-Nuu, and MHV-S at a multiplicity of infection (MOI) of 1 to 10. Total mRNA was extracted with guanidinium isothiocyanate at 6 to 18 h postinfection (p.i.) and purified by centrifugation at $40,000 \times g$ for 18 h through 5.7 M CsCl_2 . RNA was resuspended in sterile water, and 20- μ g aliquots were stored in ethanol at -20°C until use. N-protein-specific cDNA was prepared by using avian myeloblastosis virus reverse transcriptase and primer ON1427 specific for the 3' end of the N gene (see above). Primers ON1427 and ON984 (see above) were used to amplify a 463-bp fragment of the 3' coding region for sequence analysis. Automated sequencing of PCR products was carried out by using the N-specific 5' primer ON984 and fluorescent dideoxynucleotides on a model 373 DNA sequencer (Applied Biosystems).

Generation of CTLs. BALB/c mice were immunized by intraperitoneal injection of 2×10^6 PFU of JHMV and spleen cells obtained 2 to 6 weeks postimmunization. JHMV-specific CTL populations were generated by restimulation with JHMV-infected, irradiated syngeneic spleen cells as previously described (31). JN-protein-specific CTLs were induced by restimulation on vJN-infected, irradiated syngeneic spleen cells or, where indicated, in the presence of 1 μM pN318-335 peptide and 10% supernatant from concanavalin A-stimulated rat spleen cells containing 25 mM methyl- α -D-mannopyranoside as a source of growth factors without added feeder cells. The JN-specific CTL populations recognize target cells infected with JHMV and vJN (VV recombinant expressing the JHMV N protein) but not VV recombinants expressing the JHMV spike, matrix, or hemagglutinin-esterase proteins. The N protein-specific CTL clone, designated B8.5, was derived by a second limiting dilution from an established CTL line (29). The JN-specific CTL line, designated B8, was isolated by limiting dilution from CD4-depleted cultures of mononuclear cells isolated from the brain tissue of acutely infected BALB/c mice by using Percoll gradients as previously described (39). Briefly, mononuclear cells were cultured in the presence of JHMV-infected, irradiated syngeneic spleen cells and depleted of CD4 $^+$ T cells by monoclonal antibody plus complement

FIG. 1. Schematic representation of the wild-type and truncated JHMV N proteins expressed by using VV recombinants. The designations of the proteins and the amino acids comprising each truncation are indicated on the left. The positions of individual amino acids are shown in relation to corresponding restriction sites used for subcloning of the gene fragments. Restriction sites in brackets were not regenerated following subcloning into VV shuttle vectors.

treatment prior to isolation by limiting dilution (31). Lines were isolated and expanded by using irradiated (2,000 rads), JHMV-infected syngeneic spleen cells in Iscove's modified Dulbecco's modified Eagle medium (IDMEM) supplemented with 2 mM L-glutamine, 25 µg of gentamicin per ml, 1 mM sodium pyruvate, 5×10^{-5} M β-2-mercaptoethanol and nonessential amino acids (complete IDMEM), 10% fetal calf serum (Gemini Bioproducts), and 10% supernatant from concanavalin A-stimulated rat spleen cells.

CTL assay. Cytolytic activity was measured in a 4-h ^{51}Cr -release assay as previously described (31). MHV-susceptible J774.1 cells (*H-2^d*) were infected at an MOI of 1 to 5 and incubated for 6 h prior to labeling. K2a7 (*H-2^k/L^d*) or J774.1 cells were infected with VV recombinants at an MOI of 5 to 10 and incubated for either 6 or 12 h at 37°C prior to labeling. A transient expression system based on a recombinant VV-expressing T7 polymerase (8) was used to assay CTL recognition of an endogenously expressed 9-mer peptide (2). Briefly, target cells were infected with vTF7.3 at an MOI of 10. Following adsorption for 1 h, cells were washed once and transfected with 5 µg of pTM1-derived plasmid containing the JN9 minigene by using the DOTAP transfection reagent (Boehringer Mannheim, Indianapolis, Ind.) and labeled at 6 to 10 h p.i. The viability of transfected cells was >90% at 10 h p.i., and transfection efficiency was >50% (2). For epitope-mapping assays, labeled J774.1 cells were resuspended at 2×10^5 /ml in complete IDMEM supplemented with 5% fetal calf serum and added to duplicate wells containing 20 µl of peptide in PBS. Targets were incubated with peptide for 1 h at 37°C before effector cells were added at the indicated effector-to-target (E/T) ratios. Dose-response titrations were carried out as described for the mapping assays, using triplicate wells with peptide present throughout the 4-h assay. Data

are expressed as percent specific lysis defined as [(experimental lysis) – (spontaneous lysis)]/[total (detergent lysis) – (spontaneous lysis)]. Maximum spontaneous release values were always <15% of total release values.

RESULTS

Construction and expression of N protein fragments. Previous data indicated that JHMV induces *L^d*-restricted CTLs specific for a region in the C-terminal 149 aa of the N protein (31). To more precisely locate the amino acids involved in N protein-specific recognition, both C- and N-terminal truncated forms of the N protein were constructed and tested for recognition by polyclonal JHMV-specific CTLs by using VV recombinants. Figure 1 presents a schematic of the full-length N protein and the deletion mutants. Unique restriction sites found in the wild-type gene were used to facilitate subcloning of the truncated genes into the VV shuttle vectors pSC11ss and pK (see Materials and Methods); their locations are shown in relationship to individual amino acid positions within the protein. The three constructs representing nested deletions in the 155 C-terminal residues (vJN155, vJN51, and vJN20) utilized the methionine found at position 301 as the initiating amino acid to include amino acids encoded by the *SpeI* site. Genes encoding deletions starting at internal aa 301, 318, and 374 were preceded by an initiation start codon introduced via an *NcoI* site in the PCR primer and terminated by stop codons located in the vector. To exclude expression of additional codons in the JN9 construction, the coding sequence for the 9-mer peptide terminated with an additional in-frame stop codon adjacent to the 3' restriction site.

Mapping of the N-specific response by using VV recombinants. Our previous report demonstrated that only the VV

FIG. 2. Recognition of targets expressing the JHMV N protein truncations. (A) CTL activity measured by using J774.1 ($H-2^d$) target cells infected with VV recombinants expressing wild-type N protein (vJN) or aa 1 to 378 (vJNb), aa 374 to 455 (vJN82), aa 301 to 455 (vJN155), aa 301 to 351 (vJN51), and aa 301 to 320 (vJN20). CTLs were derived from JHMV-immune spleen cells stimulated in vitro with irradiated syngeneic spleen cells infected with JHMV. (B) CTL activity measured against L929 target cells expressing L^d (K2a7) infected with VV recombinants expressing wild-type N protein (vJN) or aa 301 to 351 (vJN51). CTLs were derived from JHMV-immune spleen cells stimulated in vitro with irradiated syngeneic spleen cells infected with vJN. VSC8-infected target cells were used as a negative control.

recombinant expressing full-length N protein (vJN) but none of the C-terminal deletions corresponding to the *ApaI* (vJNa: aa 1 to 134), *NaeI* (vJNb: aa 1 to 250), or *SpeI* (vJNc: aa 1 to 306) sites resulted in recognition by JHMV-specific CTLs (31). We therefore initially tested two constructs encoding either the N-terminal protein extending to the *BsmI* site (vJNb: aa 1 to 378) or the complementary C-terminal 82 aa (vJN82: aa 374 to 455) for CTL recognition. Figure 2A shows that JHMV-specific CTLs from JHMV-immunized mice restimulated on JHMV-infected syngeneic spleen cells lysed $H-2^d$ (J774.1) target cells infected with vJNb more efficiently than those infected with vJN. In contrast, the protein fragment containing the C-terminal 82 aa (vJN82) was not recognized, indicating that the epitope is located between aa 301 and 378 (corresponding to the gene fragment flanked by the *SpeI* and *BsmI*

restriction sites). This conclusion was confirmed by analysis of three constructs representing a panel of deletions with common N termini beginning at aa 301 (vJN155, vJN51, and vJN20). As demonstrated in Fig. 2A, both vJN155 (aa 301 to 455)- and vJN51 (aa 301 to 350)-infected target cells were lysed at levels comparable to vJNb. However, further deletion to a peptide comprising residues 301 to 319 (vJN20) resulted in the loss of recognition. The L^d -restricted nature of the response to aa 301 to 351 is confirmed in Fig. 2B. N protein-specific CTLs from JHMV-immunized mice recognize vJN51 (aa 301 to 351) as effectively as vJN (full-length N protein) on L^d -expressing $H-2^k$ fibroblasts (K2a7). These data demonstrate that the JHMV-induced N protein-specific CTL response maps to aa 301 to 351 by using two different targets. The findings that naturally processed CTL epitopes generally comprise 8 to 10 aa (10, 24, 35) and that N protein aa 301 to 320 (vJN20) are not recognized suggested that the L^d -restricted N protein-specific epitope is located within residues 310 to 351.

Identification of the L^d -restricted epitope by peptide scanning. To more precisely map the amino acid sequence required for recognition, a panel of 15-mer peptides spanning aa 305 to 375 of the N protein and overlapping by five residues was synthesized by using the pin technique (19). Recognition was assessed by the ability of each peptide to sensitize J774.1 ($H-2^d$) target cells for lysis by either polyclonal CTLs derived from JHMV-immunized mice and restimulated on irradiated, vJN-infected syngeneic spleen cells (Fig. 3A) or an N protein-specific clone designated B8.5 (Fig. 3B). The results from two separate experiments (Fig. 3A) demonstrate that the cytotoxic activity detected by using the polyclonal CTL population was variable and inconsistent. In addition to peptide 315 to 330, which was recognized in both experiments, the more C-terminal peptide 345 to 360 was recognized to a variable extent. Recognition of peptides 320 to 335 and 325 to 340 observed in experiment 1 could not be reproduced in the second assay. In contrast, the N protein-specific CTL clone B8.5 recognized a single peptide comprising aa 315 to 330 (Fig. 3B). The limited quantity of peptide synthesized per pin (5 to 7 μ g) restricted further analysis; however, the finding that peptide 315 to 330 was the only peptide recognized efficiently by both an N protein-specific CTL clone and a polyclonal CTL population suggested strongly that at least one N protein-specific CTL epitope was contained within aa 315 to 330.

To confirm aa residues 315 to 330 as an L^d -restricted epitope, a peptide comprising aa 318 to 335 (pN318-335) was synthesized and analyzed for its ability to sensitize targets. Additional basic C-terminal residues, derived from the wild-type sequence, were added to enhance solubility. The potency of pN318-335 to sensitize J774.1 targets is demonstrated by the peptide titration shown in Fig. 4. Half-maximal lysis was observed at a concentration of approximately 5×10^{-10} M peptide, using N protein-specific CTL stimulated in the presence of pN318-335 as the effector population (Fig. 4A) or N protein-specific CTL stimulated by vJN-infected syngeneic spleen cells (data not shown). Similar results were obtained when the peptide activity was tested on $T2/L^d$ target cells by using the N protein-specific CTL clone B8.5 (data not shown).

During the course of these studies a peptide binding motif for L^d MHC class I molecules was proposed by comparison of known L^d -restricted T-cell epitopes and sequence analysis of in vivo-processed peptides eluted from a soluble analog of the L^d molecule (6). The preferred peptide motif consists of proline at position 2 and a C-terminal hydrophobic residue, predominantly leucine, phenylalanine, or methionine, at position 9. Positions 318 to 326 in pN318-335 are indeed consistent with this motif. Previous findings for a number of human and

FIG. 3. Epitope mapping with synthetic peptides. A series of 12 overlapping 15-mer peptides spanning aa 305 to 375 were synthesized on pins and tested for CTL recognition on J774.1 (*H-2^d*) target cells. Cytotoxicity was assayed at an E/T ratio of 30:1 with a heterogeneous CTL population derived from JHMV-immune spleen cells stimulated in vitro with irradiated syngeneic spleen cells infected with vJN (A) and an N-protein-specific clone, designated B8.5, at an E/T ratio of 5:1 (B). The results of two separate experiments are shown in panel A. The peptide recognized by both effector CTL populations is boxed.

murine MHC class I molecules demonstrate that 8-mer and 9-mer peptides are preferentially bound and are optimal at target sensitization compared with longer derivatives (24, 27, 35). This observation has also been documented for two other viral, *L^d*-restricted epitopes: pIE89 168-176 (YPHFMPNTL), derived from the murine cytomegalovirus (MCMV) pp89 early regulatory protein (23), and pNP118-126 (RPQASGVYM), derived from the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) (26). Therefore, we determined whether truncation to a 9-mer peptide, predicted to contain the *L^d* motif, would enhance N-peptide immunogenicity as a target antigen. As demonstrated in Fig. 4B, sensitization of target cells with the 9-mer peptide was 50- to 100-fold more efficient than sensitization with the 18-mer; half-maximal lysis was detected at a peptide concentration of approximately 10^{-11} M on both J774.1 target cells (Fig. 4B) and T2/*L^d* target cells (data not shown). These titration data are compatible with those obtained with the *L^d*-restricted CTL peptides from MCMV (23) and LCMV (26) and demonstrate the predictive value of peptide binding motifs for the fine mapping of optimal CTL epitopes (6, 17, 20, 26).

To determine whether the predictive value of the optimal peptide in target sensitization correlates with presentation of an endogenously synthesized epitope, the 9-mer peptide was expressed by using an assay system based on the VV recombinant vTF7.3, which encodes the bacteriophage T7 polymerase (8). Following infection of both J774.1 and K2a7 cells with vTF7.3, cells were transfected with pTM1 derivatives containing either the 9-mer peptide coding sequence or the entire N gene downstream of a T7 promoter and tested for recognition by N-protein-specific CTLs (Fig. 4C). Control transfections were carried out with pTM1 containing no insert. Target cells infected with VV recombinant vNb were used as a positive control for CTL recognition. Recognition of the endogenously synthesized 9-mer in transfected target cells demonstrates the correlation between peptide sensitization and endogenous processing and, furthermore, suggests that this epitope is identical to the one naturally presented during viral infection.

Cross-reactivity of JHMV N protein-specific CTLs. The precise mapping of the N protein epitope to aa 318 to 326 allowed us to analyze the ability of JHMV CTL specific for the N protein to recognize targets infected with eight other MHV strains. Comparisons of the amino acid sequences of the N proteins from five different strains revealed two clustered regions of divergence which do not coincide with the CTL epitope (14, 21, 28). However, despite the overall highly conserved nature of the amino acid sequence, the 9-mer JHMV N protein epitope is identical to only two other published sequences, i.e., those of MHV-S and MHV-Nu67. The other sequences diverge at either aa position 4 or at both aa 4 and aa 5. To analyze which natural sequence variations within the 9-mer epitope are either tolerated or result in the loss of recognition, target cells infected with eight MHV strains were tested for recognition by JHMV N protein-specific CTL. Figure 5 demonstrates that targets infected with six of the eight strains were recognized, with a minimum value of $\geq 20\%$ specific release as indicative of recognition. The N protein-specific amino acid sequence from each strain was determined by sequence analysis of mRNA isolated from virus-infected cells. The sequence data obtained from the strains used in these experiments are consistent with the published sequences for JHMV, MHV-A59, MHV-1, and MHV-2; however, variations were found in positions 4 and 5 for MHV-3 (VG→PS [21]) and in position 5 for MHV-Nuu (A→G [14]). The corresponding epitopes from the strains examined are conserved at most positions. Sequence divergence is found only at position 4 and/or position 5 (Fig. 5), and both conservative and nonconservative amino acid changes are tolerated in these positions for recognition by JN protein-specific CTLs.

Surprisingly, sequence analysis revealed that the amino acids comprising the epitope in strains MHV-1 and MHV-2 are identical to those of strain MHV-3. Although MHV-3-infected targets were recognized efficiently, neither MHV-1- nor MHV-2-infected targets were recognized in repeated experiments. MHV-2 and MHV-3 are relatively pathogenic for adult mice, while MHV-1 is less virulent. This, however, appears to play no

FIG. 4. Target sensitization by exogenous and endogenous peptides. The synthetic 18-mer peptide pN318-335 (A) and the 9-mer peptide pN318-326 (B) were tested for the ability to sensitize target cells at various concentrations. (C) Recognition of the endogenously synthesized 9-mer epitope. J774.1 cells were infected with vTF7.3 expressing the T7 polymerase and transfected with a plasmid containing the 9-mer coding sequence (pTM-JN9), the full-length N gene (pTM-JN) downstream of the T7 promoter, or a control plasmid (pTM1), as described in Materials and Methods. CTLs were derived by stimulation of spleen cells from JHMV-immune mice for 6 days in vitro with 1 μ M pN318-335 peptide.

role in target recognition, since there was no difference in recognition between the relatively nonpathogenic MHV-A59 and the pathogenic MHV-HA59 strains. Furthermore, lack of recognition cannot be attributed to antigen processing, since the sequence analysis revealed that the 12 residues flanking both termini of the epitope were identical in all strains. To further rule out that lack of recognition reflects differential quantities of antigen, the relative expression of N protein in MHV-1- and MHV-2-infected J774.1 cells was compared with that of cells infected with MHV-3 and JHMV 10 h p.i. by

FIG. 5. Recognition of target cells infected with six other MHV strains by JHMV N protein-specific CTL. J774.1 target cells were infected with eight other strains of MHV, and the cytotoxicity was compared with that of JHMV-infected and pN318-335-coated target cells. CTLs were derived from JHMV-immune spleen cells stimulated for 6 days in vitro with 1 μ M pN318-335 peptide and used at E/T ratios of 30:1, 15:1, and 7.5:1. The sequence corresponding to aa 301 to 455 of the JHMV N protein was determined for each strain by RNA sequence analysis, as described in Materials and Methods. The 9-mer sequence containing the L^d binding motif is shown on the left for each strain.

enzyme-linked immunosorbent assay. Cell lysates were adjusted to equal protein concentrations and N protein detected by using monoclonal antibody J.3.14 (11), which cross-reacts with all four strains. Comparable levels of N protein in all infected cell lysates indicated that the antigen concentration is not the limiting factor for the loss of CTL recognition (data not shown).

The cell surface expression of MHC class I molecules is reduced following JHMV infection (37), suggesting that the inability of the N-specific CTLs to recognize MHV-1- and MHV-2-infected target cells could be due to decreased class I expression. To analyze whether MHV-1- and MHV-2-infected targets are capable of presenting exogenous antigen as a measure of class I expression, infected targets were coated with pN318-335 and compared for recognition by JN protein-specific CTLs. Figure 6 shows the representative lysis of MHV-1-, MHV-2-, MHV-3-, and JHMV-infected and uninfected targets in the presence and absence of 100 nM pN318-335 at two E/T ratios. Whereas peptide-coated targets infected with MHV-3 and JHMV are lysed at levels comparable to those of uninfected targets (>60%), lower lysis was detected for peptide-coated MHV-1 (38%)- and MHV-2 (18%)-infected cells at an E/T ratio of 20:1. This effect was most dramatic at the lowest E/T (5:1) ratio: only 15 and 5% lysis were observed for MHV-1- and MHV-2-infected pN318-335 peptide-coated targets, respectively, compared with approximately 40% lysis found for the other three targets. The reduced levels of lysis of peptide-coated MHV-1- and MHV-2-infected targets may indeed reflect lower levels of L^d class I surface molecules. The low levels of lysis above background may be attributed to few residual class I molecules on the cell surface, as only 200 class I molecules per cell is sufficient for

FIG. 6. Influence of MHV infection on exogenous antigen presentation. J774.1 target cells were infected with MHV-1, MHV-2, MHV-3, and JHMV at an MOI of 4 and tested for lysis in the presence and absence of 100 nM pN318-335. Uninfected target cells were used as a control. CTLs were derived from JHMV-immune spleen cells stimulated 6 days in vitro with 1 μ M pN318-335 peptide. Cytotoxicity is shown for E/T ratios of 20:1 and 5:1.

CTL recognition (4, 24). Although the basis for the lack of recognition of MHV-1- and MHV-2-infected cells is not clear, the phenomenon may be attributed, at least in part, to virus-mediated downregulation of class I expression; however, an unknown virus-mediated processing defect in the infected target cells may also contribute to a defect in CTL recognition.

DISCUSSION

To investigate the role of JHMV-specific CTLs in both the acute and chronic demyelinating diseases of the murine CNS, we have mapped the N protein-specific response in BALB/c (*H-2^d*) mice. Although adoptive transfer of JHMV-specific CTL clones protects infected recipients and reduces virus replication in recipient mice (40), the epitopes involved in CTL recognition have not yet been characterized. This report defines the *L^d*-restricted CTL response to the N protein by using a combination of truncated forms of the N protein expressed by VV recombinants and a series of overlapping peptides. The N protein-specific response was mapped to a single epitope comprising residues 315 to 330, which contains the recently described *L^d* binding motif (6). Target sensitization with the predicted optimal 9-mer, pN318-326 (APTAGAFF), was 50- to 100-fold more efficient than sensitization with the longer 18-mer, pN318-335 (APTAGAFFFG SKLELVKK), originally synthesized to confirm the epitope. The pN 9-mer was recognized at 10^{-11} to 10^{-12} M, which is comparable to the activities reported for the two other *L^d*-restricted viral peptides derived from MCMV and LCMV (23, 26, 36). Half-maximal lysis with the latter 9-mer peptides was achieved at an approximate concentration of 10^{-10} to 10^{-12} M

on L929 *L^d*-expressing target cells. These viral epitopes share the *L^d* motif and are most potent as 9-mer peptides, although recognition could still be demonstrated following truncation to minimal sequences of 4 and 5 aa for MCMV and LCMV, respectively. In this context it is interesting to note that N peptide 310 to 325 contains eight of the nine residues comprising the predicted epitope but did not sensitize target cells (Fig. 3). Similar results have been described following deletion of the hydrophobic residue in position 9 in the LCMV NP peptide or alanine replacement in the MCMV peptide, both of which completely destroy antigenicity (23, 26). These data confirm the predictive value of the *L^d* motif for fine mapping of optimal epitopes and indicate that the C-terminal residue in position 9 is crucial for peptide binding and/or recognition.

Several epitopes have recently been identified by utilizing MHC class I binding motifs (17, 20, 25). Scanning of the protein sequence for the minimal peptide consensus motifs revealed 5 potential epitopes in ovalbumin (17) and 11 in listeriolysin (20). Although three of five potential ovalbumin peptides bind to its restriction element (*K^b*), only two were antigenic and elicited a CTL response. A single epitope in listeriolysin was identified by using a single CTL clone; however, no further analysis was carried out to determine the antigenicity of the other potential peptides. In this context, it is interesting to note that the N protein contains other regions that fit the predicted *L^d* binding motif. One of these (GPTK DVEYL) is found in the peptides 335 to 350 and 340 to 355 used in the epitope-scanning analysis (Fig. 3). However, our data from using polyclonal CTL in combination with N protein truncations and peptide scanning indicate that only a single peptide with this motif is recognized. Although the data presented in this report cannot exclude further potential epitopes within residues 345 to 360, previous studies demonstrated that N protein-specific CTL activity was only detected by using L929-derived fibroblasts expressing the *L^d* class I molecule, not *K^d* or *D^d* (31). Any other potential epitopes contained within aa 345 to 360 must therefore be restricted to *L^d*. However, there are no amino acids within this peptide which conform to the recently described *L^d* motif (6), suggesting that residues 315 to 330 comprise the only N-specific epitope in BALB/c mice. In addition, six CTL lines specific for the N protein all recognize target cells in association with the pN318-335 peptide.

Recognition of only one of a number of potential motifs has been described for other proteins (17, 20) and demonstrates that while the predictive value of peptide binding motifs is helpful, it is not sufficient for epitope mapping. Antigen processing and the induction of tolerance may provide further mechanisms that govern the selection of antigenic CTL epitopes. Furthermore, epitopes not containing the predicted motif may easily be overlooked. For example, an exception to the *L^d* consensus motif is found in the *L^d*-restricted tumor antigen tum⁻ epitope (ISTQNHRALVA), which is optimal in sensitizing target cells as an 11-mer at 10^{-9} M peptide (18). Although sequence analysis of class I-eluted peptides indicates that consensus binding motifs play a crucial role in class I presentation, additional rules are necessary to reliably predict epitopes.

Analysis of the cross-reactivity of JHMV N protein-specific CTLs with target cells infected with eight other MHV strains indicates that residues at positions 4 and 5 are not critical for CTL recognition (Fig. 5). Position 4 can be occupied by alanine, valine, and proline, while position 5 may contain either glycine or serine. All other residues are conserved and confirm the *L^d* binding motif, consisting of a proline at position 2 and a hydrophobic amino acid at position 9 (6). Four of the

MHV strains (MHV-3, MHV-D, MHV-Nuu, and MHV-K) recognized by JHMV N protein-specific CTLs contain a proline in position 4, supporting a preference for a second proline found in other L^d -restricted peptides. An unexpected result is the recognition of MHV-3- but not MHV-1- or MHV-2-infected target cells by the JHMV N protein-specific CTLs despite comparable levels of N protein in the infected cells. A possible explanation may reside in differential kinetics of MHC class I downregulation mediated by different MHV strains. A dramatic decrease of class I molecules on the surface of J774.1 cells has been reported between 8 and 12 h p.i. with JHMV (37). The data in this report demonstrate that targets infected with JHMV and MHV-3 retain sufficient class I expression to efficiently present both endogenous and exogenous peptide to JHMV N protein-specific CTL. However, dramatically decreased recognition was found for peptide-coated target cells infected with both MHV-1 and MHV-2, suggesting that decreased expression of class I could account, at least partially, for the absence of recognition following infection with viruses containing identical epitopes.

The data in this report have defined a unique L^d -restricted CTL epitope within the N protein of JHMV. This is the first epitope defined for MHV, and its amino acid sequence is consistent with the recently defined L^d peptide motif (6) along with two other previously defined viral L^d -restricted epitopes (23, 26). Definition of this epitope will facilitate determination of the role of JHMV-specific CTLs in both the acute and the chronic forms of JHMV-induced demyelination in mice.

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