Specificity of the H-2 L^d-Restricted Cytotoxic T-Lymphocyte Response to the Mouse Hepatitis Virus Nucleocapsid Protein

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Cytotoxic T lymphocytes provide protection against persistent infection of the central nervous system by the JHM strain of mouse hepatitis virus. In BALB/c (*H-2^d*) mice, the dominant response is directed against an L^d-restricted peptide in the nucleocapsid protein (APTAGAFF). Characterization of the fine specificity of this response revealed that the predicted anchor residues at positions 2 and 9 were the most critical for class I binding. Amino acids at positions 7 and 8 were identified as T-cell receptor contact residues. Virus-induced cytotoxic T lymphocytes to other L^d motif-containing nucleocapsid peptides were not detected, despite the identification of two epitopes with reduced L^d affinity. These data suggest that mutations within four residues of the dominant epitope could contribute to the persistence of the JHM strain of mouse hepatitis virus.

Infection of mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis associated with primary central nervous system (CNS) demyelination (16, 20). Although this infection induces a wide variety of immune effectors including virus-specific antibodies, CD4⁺ T cells, and cytotoxic T lymphocytes (CTL), JHMV is able to establish a persistent CNS infection associated with chronic ongoing demyelination (10, 16, 20, 37). In BALB/c mice, JHMV infection induces an immunodominant CTL response to the nucleocapsid (N) protein (36). During acute infection, N-specific CTL could be isolated only from the CNS and could not be detected in the spleen or cervical lymph nodes (36). However, despite the local CTL response, CTL are unable to completely clear virus, resulting in persistent CNS infection. By contrast, adoptive transfer of N-specific CTL coincident with infection provides protective immunity, concomitant with decreased virus titer (35). An early CTL response, therefore, appears to be critical in providing anti-viral activity sufficient to prevent persistent infection. Both the inability of CTL induced during JHMV infection to effectively clear virus (35) and the rapid accumulation of viral mRNA quasispecies during infection (1) implicate a possible role for viral escape from CTL recognition (8, 19, 21, 22, 24).

Recognition of infected cells by CTL is mediated by presentation of peptides derived from immunogenic proteins in association with class I molecules (2, 17). Individual class I alleles present a unique array of peptides characterized by allele-specific consensus motifs consisting of 8 to 10 amino acids with two to four conserved anchor residues (13, 23, 26). Mutations in residues affecting either class I binding or T cell receptor (TcR) recognition provide a mechanism for immune evasion and are believed to contribute to viral persistence (8, 19, 21, 22, 24). Such CTL escape variants have been demonstrated during infection with human immunodeficiency virus type 1, hepatitis B virus, and lymphocytic choriomeningitis virus (8, 19, 21, 22, 24). To gain a more detailed understanding of the mechanisms involved in CTL epitope selection and to identify residues potentially important in the escape from CTL-mediated clear-

ance, this report characterizes the specificity of the CTL response to the JHMV N protein (5). The role of individual residues within the dominant L^d-restricted N epitope consisting of residues 318 to 326 in class I binding and TcR recognition was evaluated by using truncated and mutated peptides. Furthermore, the potential for subdominant epitopes was explored by analyzing additional L^d motif peptides within the N protein. N-derived peptides were purchased from Chiron Mimotopes (Clayton, Australia), solubilized at approximately 1 mM in dimethyl sulfoxide, and diluted in phosphate-buffered saline for CTL assays. Purity, as well as quantity, was assessed by the supplier for three control peptides by high-performance liquid chromatography and amino acid analysis.

The majority of peptides eluted from L^d consist of nine residues characterized by a motif containing P in position 2 and a hydrophobic residue in position 9; however, octamers and longer peptides have also been identified (13, 38). To determine the core sequence required for CTL recognition of the N epitope, peptides with truncations from either the amino or carboxy terminus were compared with the 9-mer for their ability to sensitize T2/L^d targets (5) at concentrations ranging from 1 nM to 1 μ M (Fig. 1). ⁵¹Cr-labeled target cells were incubated with peptide for 15 to 30 min at 37°C prior to the addition of CTL. Cytolytic activity was measured in a 4-h ⁵¹Cr release assay in Iscove's complete Dulbecco modified Eagle medium supplemented with 2% fetal calf serum (6). All truncated peptides were recognized less efficiently than the 9-mer or were incapable of sensitizing targets for recognition by polyclonal N-specific CTL (Fig. 1). The recognition patterns of two N-specific CTL clones, B6.1 and B21.3 (36), were identical to the results obtained with polyclonal CTL (data not shown). These data demonstrate that the 9-mer constitutes the optimal epitope; however, six residues (TAGAFF) provide a minimal sequence for N-epitope specific CTL recognition. The recognition of minimal core epitopes in the absence of anchor residues is consistent with results obtained from mapping L^d-restricted murine cytomegalovirus and lymphocytic choriomeningitis virus epitopes (27, 28, 31). These data imply that binding of core sequences to class I results in a conformation similar to that of native peptide. However, recognition of minimal peptides may be limited to exogenous peptides, since core sequences lacking major histocompatibility complex (MHC) anchors are not presented from endogenously synthesized pep-

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FIG. 1. Recognition of pN318-326 peptides containing amino- and carboxy-terminal truncations by N-specific CTL. Polyclonal N-specific CTL from mice immunized with JHMV (5) were assayed for recognition of peptides at concentrations ranging from 1 nM to 1 μ M on T2/L^d target cells (5) at an E-to-T ratio of 30:1. Peptide sequences are shown in the single-letter code, and L^d-specific anchor residues in positions 2 and 9 are underlined. Data are expressed as percent specific lysis, which is defined as (experimental lysis - spontaneous lysis)/(Total (detergent lysis) - (spontaneous lysis)).

tides (15). Furthermore, deletion of the carboxy-terminal anchor residue did not effect recognition as severely as deletion of the amino-terminal residue. The marked effect of this deletion most likely reflects the inability of the truncated peptide to form interactions between the class I side chains and main chain atoms of the peptide amino terminus, which contribute significantly to the stability of peptide-class I complexes (9, 23, 34).

The contribution of individual residues to L^d binding and TcR interaction was assessed by analyzing peptides containing A or G substitutions at each position for both CTL recognition and L^d binding. Modeling of peptide-L^d complexes indicates that residues 1, 2, and 9 have minimal exposed surface area, leaving residues 3 to 8 accessible for TcR interaction (13). Whereas residues in position 9 are generally conserved and strictly involved in class I contact, residues in position 1 are variable and, in addition to interacting with class I via main chain atoms, may also participate in TcR interactions via bulky side chains (12, 26, 33). Cross-reactivity of JHMV N-specific CTL with six other mouse hepatitis virus strains indicated that natural sequence variations in positions 4 and 5 are tolerated (5). Thus, residues in positions 6, 7, and 8 are candidates for TcR contact. Substituted peptides were initially compared for their ability to sensitize T2/L^d targets at 1 nM to 1 μ M for lysis by polyclonal N-specific CTL. As expected, substitutions at the two anchor residues (positions 2 and 9) significantly reduced recognition (Fig. 2A). Although at least 10⁴-fold-higher concentrations relative to native peptide were required for maximal lysis, these substitutions did not completely abrogate recognition. The A→G substitution in position 1 resulted only in decreased lysis at the lowest peptide concentration tested (1 nM). Substitutions at positions 3, 4, 5, and 6 did not affect recognition, even at the lowest peptide concentration tested. By contrast, the A substitution at position 7 dramatically reduced lysis, even at the highest concentration tested. Significant reduction in lysis was also observed following substitution at position 8. These data suggest that F in position 7 and F at

position 8 constitute the dominant and a subdominant TcR contact residue, respectively. Analysis of these peptides with two N-specific clones (B6.1 and B21.3) revealed the same recognition patterns (data not shown), and no difference in fine specificity was detected.

To differentiate between reduced class I binding affinity or loss of TcR contact sites, substituted peptides were tested for L^d binding by competition assays as described elsewhere (6). Target cells were mixed with a suboptimal dose of a heterologous L^d-restricted antigenic peptide, pM18-8 (6). N-derived competitor peptides were compared for their abilities to inhibit target lysis by pM18-8-specific CTL (Fig. 2B). All peptides with substitutions not affecting recognition (positions 1, 3, 4, 5, and 6) inhibited lysis by approximately 50% at 0.1 μ M, similar to the wild-type peptide. By contrast, peptides with substitutions in the anchor residues (positions 2 and 9) inhibited lysis by only 30% at 1 μ M, which was the highest concentration tested. Peptides with substitutions in position 7 and 8, which were only poorly recognized by N-specific CTL (Fig. 2A), effectively competed at 0.1 μ M with slightly reduced efficiency compared with

FIG. 2. Effect of A/G-substitutions on CTL recognition (A) and on class I L^d binding (B). Peptides are shown with substituted residues in the pN318-326 sequence underlined. (A) N-specific polyclonal CTL were tested for recognition of T2/L^d targets preincubated with increasing peptide concentrations at an E-to-T ratio of 30:1. (B) L^d binding was assessed by testing the substituted peptides as inhibitors for recognition of the heterologous L^d-restricted stimulator peptide pM18-8 (MPGRAFTVI) on T2/L^d targets by vM18-8 induced CTL at an E-to-T ratio of 30:1 (6). Stimulator peptide (0.2 pM) and competitor peptides at the indicated concentrations were present throughout the assay. The pMCMV (YPHFMPNTNL) peptide was used as a positive competitor. Lysis in the absence of competitor was 25% and in the absence of stimulator peptide was 1%. The results shown are from a single experiment and are representative of two separate assays. Data are expressed as percent inhibition, defined as $[1 - (\% \text{ specific lysis with competitor}) / (\% \text{ specific lysis without competitor})] \times 100$.

FIG. 3. Effect of carboxy-terminal substitutions on CTL recognition (A) and class I L^d binding (B). (A) Peptides with carboxy-terminal substitutions were tested at the indicated concentrations for recognition by N-specific polyclonal CTL as described in the legend to Fig. 1. (B) L^d binding was assessed by competition assays as described in the legend to Fig. 2B.

that of wild-type peptide (Fig. 2B). These data confirmed that residues at positions 2 and 9 are crucial for MHC binding, whereas the F residues at positions 7 and 8 are primarily involved in TcR contact.

MHC class I molecules generally show a preference for peptides with hydrophobic residues in position 9 (13, 26); however, binding at both the amino and carboxy termini is largely sequence independent because of the stability provided by hydrogen bonding between the peptide backbone and conserved class I residues (9). For L^d -binding peptides, the anchor residue at position 2 is generally P and infrequently S. Position 9 shows increased variability in that it can accommodate hydrophobic residues I, F, L, and M (13). A total of 13 substitutions at position 9 were tested to determine which amino acids influenced recognition. As expected, both L and I substituted for F, with the L replacement resulting in reactivity higher than that of the wild-type peptide (Fig. 3A). The V substitution was tolerated as well as I, although V is not contained within any known L^d -restricted peptides (3, 4, 13, 38, 39). Competition assays demonstrate that the peptides that are efficiently recognized are also the most efficient at binding (Fig. 3B), suggesting that recognition coincides with efficient binding. Substitutions of position 9 with small (A, G, S), more bulky (W, Y), or charged side chains (E, K) all resulted in significant loss of recognition due to suboptimal L^d binding. These data demonstrate that three substitutions could be tolerated at position 9 without significantly influencing CTL recognition.

Although peptide motifs have facilitated the prediction of CTL epitopes (3, 4), only a limited number of potential epitopes induce CTL in vivo (3, 4, 11, 18, 29). A dominant response frequently correlates directly with high peptide-class I affinity (12, 18, 33), which is determined by the presence of anchor residues as well as residues in nonanchor positions (12, 18, 25, 29). To probe for the possible existence of additional L^d -restricted CTL epitopes within the 455-amino-acid N protein, 10 other L^d motif peptides were tested for recognition by JHMV-specific CTL that were expanded with JHMV-infected spleen cells (Table 1). Although the known L^d -restricted peptides contain hydrophobic residues at the carboxy terminus (3, 4, 13, 39), peptides ending with residues having small nonpolar side chains were also included. Only the previously identified pN318-326 peptide was recognized at a concentration of less than 10 μ M (data not shown). One peptide, pN429-437 (TPEDRSLLA), was recognized by pN318-326-specific CTL at high concentrations (10 μ M) in multiple independent assays, indicating cross-reactivity between these epitopes (data not

TABLE 1. JHMV N peptides conforming to the H-2 L^d binding motif^a

N peptide	Sequence	Heterologous viral epitope (reference)	Sequence
pN56-64	123456789 QPN SGSVVP	HIV pM18-8 (6)	123456789 MPGRA FVTI
pN89-97	VPIANGIPA	MCMV pp89 (28)	YPHFMP TNL
pN114-122	TPDGQKQL	LCMV NP (32, 41)	RPQASGVYM
pN123-131	LPRWYFYL	MV NP 281-289 (4)	YPALGLHEF
pN168-176	DPSSHEAIP	MV HA 343-351 (3)	DPVIDRLYL
pN230-338	KPDMAEEIA	MV HA 544-552 (3)	SPGRSFSYF
pN291-299	GPNQNFGGP	HBV S 21-40 ^b (30)	LLTIRLTIPQSLDSWW ^c TSLN
pN311-319	FPILAE LAP		
pN318-326	APTAGAFFF		
pN342-350	GPTKDVYEL		
pN429-437	TPEDRSLLA		
L^d motif	XPXXXXXXL		
	S M		
	F		

^a Sequences are presented in the single-letter code. N peptides are designated according to their locations within the N protein. The antigenic peptide is highlighted. The L^d peptide consensus sequence is indicated at the bottom. Heterologous antigenic L^d -restricted viral epitopes are included for comparison. MCMV, murine cytomegalovirus; LCMV, lymphocytic choriomeningitis virus; MV, measles virus; HBV, hepatitis B virus; HA, hemagglutinin.

^b The hepatitis B virus peptide is aligned to match the L^d motif.

FIG. 4. Competition of L^d motif peptides within the N protein for class I L^d binding. Binding was assessed by testing N-derived L^d motif peptides as inhibitors for recognition of heterologous peptides as described in the legend to Fig. 2. (A) Peptides were tested for blocking recognition of stimulator peptide pN318-335 (1 nM) on T2/ L^d targets by polyclonal N-specific CTL at an E-to-T ratio of 30:1. The modified human immunodeficiency virus peptide (MPGRAFTVI) was used as a positive competitor (6). Lysis in the absence of competitor was 30% and in the absence of stimulator peptide was 1%. (B) L^d binding competitors pN89-97 and pN123-131 identified in panel A were compared with the antigenic pN318-326 peptide as inhibitors for recognition of stimulator peptide pM18-8 (5 pM) on J774.1 targets by vM18-8-induced CTL at an E-to-T ratio of 30:1. Lysis in the absence of competitor was 25% and in the absence of stimulator peptide was 5%. The results shown in both panels are from a single experiment and are representative of three independent assays.

shown). This may be explained by the hydrophobic L residues at the TcR contact sites in positions 7 and 8. However, because of the high peptide concentration required to detect this cross-reactivity, this observation was not pursued.

To analyze the role of peptide class I affinity in the immunodominant pN318-326-specific CTL response, the relative levels of binding of the motif peptides to L^d were assessed by competition assays. Target cells were mixed with a limiting concentration of antigenic peptide pN318-326, and competitor peptides were compared with the control peptide pM18-8 for inhibition of target lysis by pN318-326-specific CTL (Fig. 4A). Only peptides pN89-97 and pN123-131 competed with the antigenic peptide. However, inhibition of lysis comparable to that obtained with pM18-8 (>70% at 0.1 μ M) required 100-fold-higher concentrations, suggesting weak L^d binding affinity. This was confirmed by a direct comparison of binding affinities among pN89-97, pN123-131, and the immunogenic pN318-326 peptide by using CTL specific for the heterologous pM18-8 peptide (Fig. 4B). Whereas pN318-326 competed effectively at 0.1 μ M, approximately 100-fold-higher concentrations of pN89-97 and pN123-131 were required for similar levels of lysis inhibition. These data suggest that pN318-326 exhibits optimal class I binding affinity among N peptides bearing the L^d consensus motif. The fact that pN114-122 and pN342-350 did not show significant binding despite optimal anchor residues supports a critical role for nonanchor residues in class I binding (12, 19, 25). Furthermore, the observation that pN89-97, containing a small amino acid side chain in position 9, binds as efficiently as pN123-131 suggests that optimal interactions of nonanchor residues with class I may overcome the suboptimal interaction observed following A substitutions in carboxy-terminal peptide positions (18, 27, 31). It was not possible to assess the relative affinity of pN429-437 in either competition assay because of recognition of 10 μ M peptide by both CTL populations; however, recognition of this peptide is consistent with the ability of peptides with A at position 9 to bind L^d .

Numerous viruses induce L^d -restricted CTL responses to epitopes derived from the nucleocapsid and envelope proteins

(3, 4, 30, 32, 41) but also to epitopes in regulatory proteins (28). These responses are generally limited to a single epitope (4, 31, 32) or infrequently to two epitopes (3). The limited response of CTL to a single peptide of 11 candidates within the JHMV N protein extends numerous reports describing limitations of the predictive value of binding motifs (3, 4, 11, 18, 29). Although peptide competition studies confirmed high peptide class I affinity as a major factor for this limited response, additional factors, such as antigen concentration, turnover rate, and flanking residues may also play critical roles in epitope selection (14, 29, 40). Structural analysis of class I molecules reveals that peptides are most exposed at the center of the cleft, suggesting that the primary antigenic character of the peptide-class I complex can be attributed to the central peptide region (23). Our study of the structure-function relationship of JHMV peptide residues interacting with the L^d molecule and the TcR confirms anchor positions 2 and 9 as crucial MHC contact residues and identifies the F residues in positions 7 and 8 as primary TcR contact residues. The carboxy-terminal location of JHMV peptide antigenicity may be explained by the lack of prominent side chains pointing out at the center of the peptide binding cleft. Functional analysis of residues within L^d -restricted peptides suggests a high degree of flexibility for the role of individual peptide positions (27, 31, 39, 41). The major TcR contact residue in the murine cytomegalovirus epitope was F at position 4; Y at position 1 interacted with both MHC and TcR, while residues at positions 4 to 8 were identified as spacers (27). By contrast, positions 8 and 9 are critical for recognition in the lymphocytic choriomeningitis virus epitope (31). Interestingly, two distinct dipeptidyl TcR recognition sites, YL at positions 3 and 4 and LV at positions 7 and 8, respectively, were found within a single tumor antigen-derived peptide (39). Recognition of both F residues in positions 7 and 8 in the N epitope confirms a tendency for preferred TcR interaction with hydrophobic side chains, independently of their position (Table 1). Our data confirm that the interaction of each residue with only MHC, TcR, or both is dependent on the individual peptide sequence,

in which a cooperative contribution of all residues determines conformation and TcR contact (18, 23, 27, 28, 31, 34, 39).

JHMV infection elicits a variety of immune effectors which can protect mice from a lethal challenge and prevent chronic infection (10, 16, 20, 35, 37). However, in the case of sublethal infections, virus evades these effectors to produce a chronic infection of the CNS. Whether the ability to evade the immune response is due to immune-mediated mechanisms which facilitate persistence without completely eliminating virus or to virus-specific mechanisms is not clear. Immune-mediated mechanisms are suggested from the ability of the virus to persist following the adoptive transfer of anti-viral antibodies and CD4⁺ virus-specific T cells (10, 37). Support for a virus-specific mechanism(s) has recently been suggested on the basis of the rapid accumulation of mutations associated with JHMV in vivo replication (1). Sequence analysis of viral RNA from the CNS of acutely and persistently infected C57BL/6 (*H-2^b*) mice suggests a high mutation rate in both the N protein- and spike protein-encoding RNAs, with 65% of mutations giving rise to amino acid changes (1). Amino acids critical for class I and TcR contact are thus likely targets for immune escape. Mutations within the L^d-restricted N epitope have not been identified during persistence in C57BL/6 mice (1). This finding is not surprising, since these mice mount a CTL response only to the spike protein (7, 11); however, three variants in a dominant epitope of the spike protein (11), including one deletion and two amino acid changes, have been detected (1), suggesting a role for immune pressure. The potential emergence of escape mutants under CTL pressure in H-2^d mice responding to the N protein, therefore, remains to be evaluated. Furthermore, the identification of two additional peptides with weaker L^d binding affinities suggests the possibility that CTL specific for these epitopes may be generated in the presence of mutations affecting the dominant epitope (22). The CTL response to the N protein, therefore, provides an excellent model to study the role of CTL in chronic demyelinating disease of the murine CNS.

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