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Diversity of T-Cell Receptors in Virus-Specific Cytotoxic T Lymphocytes Recognizing Three Distinct Viral Epitopes Restricted by a Single Major Histocompatibility Complex Molecule†

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Cytotoxic T lymphocytes (CTL) recognize virus peptide fragments complexed with class I major histocompatibility complex (MHC) molecules on the surface of virus-infected cells. Recognition is mediated by a membrane-bound T-cell receptor (TCR) composed of α and β chains. Studies of the CTL response to lymphocytic choriomeningitis virus (LCMV) in *H-2^b^* mice have revealed that three distinct viral epitopes are recognized by CTL of the *H-2^b^* haplotype and that all of the three epitopes are restricted by the *D^b^* MHC molecule. The immunodominant *D^b^*-restricted CTL epitope, located at LCMV glycoprotein amino acids 278 to 286, was earlier noted to be recognized by TCRs that consistently contained Vα4 segments but had heterogeneous Vβ segments. Here we show that CTL clones recognizing the other two *H-2D^b^*-restricted epitopes, LCMV glycoprotein amino acids 34 to 40 and nucleoprotein amino acids 397 to 407 (defined in this study), utilize TCR α chains which do not belong to the Vα4 subfamily. Hence, usage of Vα and Vβ in the TCRs recognizing peptide fragments from one virus restricted by a single MHC molecule is not sufficiently homogeneous to allow manipulation of the anti-viral CTL response at the level of TCRs. The diversity of anti-viral CTL likely provides the host with a wider option for attacking virus-infected cells and prevents the emergence of virus escape mutants that might arise if TCRs specific for the virus were homogeneous.

Cytotoxic T lymphocytes (CTL) play an important role in the control and elimination of many viral infections (7, 8, 18, 19, 22, 26, 39, 41). CTL recognize proteolytic fragments of viral proteins, usually 8 to 9 amino acid (aa) residues (11, 27, 32), complexed with major histocompatibility complex (MHC) glycoprotein (GP) molecules on the cell surface of infected cells (30, 41). This CTL recognition is mediated by a membrane-bound T-cell receptor (TCR) composed of α and β chains (10). The interaction of the TCR with a viral fragment-MHC molecule complex initiates a series of processes leading to the destruction of a virus-infected cell that would otherwise serve as a factory producing progeny virus. The TCR α and β chains have variable (V) and constant regions. Noncontiguous V, diversity (D), and joining (J) gene segments undergo somatic rearrangements during T-cell development, generating unique V regions for each T cell (10).

To delineate and eventually manipulate the CTL response in viral infections, we began a systematic study of those molecules involved in CTL recognition in mice infected with lymphocytic choriomeningitis virus (LCMV) (5, 17). They include viral epitopes (16, 23, 33–35), restricting MHC molecules (16, 23, 35), and TCRs recognizing viral fragment-MHC molecule complexes (38). LCMV, a member of the family *Arenaviridae*, has a bisegmented RNA genome consisting of a large (L) and a small (S) segment (3). The S RNA encodes the GP precursor, GP-C, and the nucleoprotein (NP). The GP-C is posttranslationally cleaved into the mature proteins GP-1 and GP-2 (4). Using vaccinia virus (VV) recombinants expressing the GP and NP molecules of the LCMV Armstrong strain (LCMV ARM), we have shown that there is a vigorous bulk CTL response to both GP and NP in mice of the *H-2^b^* haplotype (*H-2* is murine MHC) (34). GP epitopes mapped to GP-1 and GP-2 were defined as GP aa 34 to 40 and GP aa 278 to 286, respectively, and were shown to be restricted by a class I MHC molecule, *D^b^* (16, 23). The majority (over 85%) of LCMV-specific CTL clones generated in vitro in C57BL/6 mice (*H-2^b^*) react to the GP-2 epitope (23, 33). Our analysis of TCRs showed that all of the six *D^b^*-restricted, GP-2 epitope-specific CTL clones examined utilized Vα4 in their TCR α chains whereas they used at least three different Vβs (38). Using a different strain of LCMV (the WE strain), Aebischer et al. also showed that all of the five *D^b^*-restricted GP-2 epitope-specific CTL clones used Vα4 and Vβ10 (2). These results indicated the homogeneous nature of the LCMV-specific CTL response to the *D^b^*-restricted epitope GP aa 278 to 286. Restricted usage of TCR V segments has also been observed in the CTL responses to the influenza A matrix peptide (21) and to the simian immunodeficiency virus gag peptide (9).

In this report, we define the minimal sequence of another CTL epitope (in the NP) of LCMV ARM, describe two CTL clones specific for this epitope, and then examine the TCRs in CTL clones specific for each of the three epitopes of LCMV recognized by *H-2^b^* mice.

Using VV recombinants expressing truncated LCMV ARM NP molecules, we found that the NP epitope of LCMV resided within NP aa 322 to 558, because target cells infected with the VV recombinant expressing NP aa 1 to 558 (the full-length NP), but not with VV NP aa 1 to 201 or VV NP aa 1 to 321, were efficiently lysed by spleen cells obtained from C57BL/6 mice infected 7 days previously with LCMV (anti-LCMV splenocytes) by in vitro ^{51}Cr release assay (data not shown). To further define the epitope, synthetic peptides

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TABLE 1. CTL response against LCMV GP and NP epitopes[a]

CTL[b]	% Specific ^{51}Cr released from:					
	H-2^b target					*H-2^d* target LCMV ARM
	LCMV ARM	GP aa 28-42[c]	GP aa 272-286[c]	NP aa 116-127[c]	NP aa 397-408[c]	
C57BL/6 (*H-2^b*) anti-LCMV splenocytes	19	22	12	0	10	3
BALB/c (*H-2^d*) anti-LCMV splenotypes	1	1	1	0	0	58
CTL clones						
N18	64	0	3	1	47	0
N20	56	0	4	0	10	1

[a] CTL response was examined by an in vitro cytotoxicity assay as described previously (35). MC57 (*H-2^b*) cells and BALB CL 7 (*H-2^d*) fibroblasts were used as target cells. The percent specific ^{51}Cr released was calculated as 100 × (cpm release by CTL − cpm spontaneous release)/(cpm total release − cpm spontaneous release).

[b] Primary anti-LCMV splenotypes were prepared by inoculating mice with 2 × 10^5 PFU of LCMV ARM intraperitoneally. Seven days later, the spleens were harvested from the mice and cleared of erythrocytes. Splenocytes were used at effector/target ratios of 50:1. CTL clones were maintained in culture as described previously (6) and used at effector/target ratios of 5:1.

[c] Peptides were synthesized either by the tea bag method (13) or by using an automated peptide synthesizer (Model 430A; Applied Biosystems, Inc., Foster City, Calif.). Peptide, solubilized in tissue culture medium, was incubated at a final concentration of 200 μg/ml with uninfected target cells throughout the cytotoxicity assay. A 10-fold dilution of the peptides gave similar results. NP aa 116-127 is an epitope restricted by the *L^d* MHC molecule (35).

(LCMV NP aa 116 to 127, aa 350 to 366, aa 365 to 381, aa 382 to 399, aa 397 to 408, aa 400 to 419, aa 420 to 437, and aa 438 to 456, as well as LCMV GP aa 28 to 42 and aa 272 to 286) were used to coat *H-2^b* and *H-2^d* target cells, which were then reacted with LCMV-specific CTL. The NP synthetic peptides were arbitrarily selected from LCMV NP aa 322 to 558 without reference to prediction schemes of T-cell epitopes (10a, 26a). *H-2^b* anti-LCMV splenocytes killed *H-2^b* target cells coated with NP aa 397 to 408 as well as targets coated with peptides containing previously defined epitopes (GP aa 28 to 42 and GP aa 272 to 286) (Table 1). Targets coated with other NP peptides used were not lysed by anti-LCMV splenocytes (data not shown). LCMV-specific CTL clones were generated from spleen cells of immunized C57BL/6 mice according to the protocol described in detail previously (6). Two clones, N18 and N20, which killed targets infected with VV NP aa 1 to 558 were selected. These CTL clones were also found to kill target cells coated with NP aa 397 to 408 (Table 1). Killing with these CTL clones was confirmed in three additional experiments, with a specific ^{51}Cr release ranging from 44 to 28% and from 32 to 18% for N18 and N20, respectively.

In order to define the minimal sequence of this NP epitope, a series of truncated peptides were synthesized and used to coat targets, which were then reacted with the CTL clone N18. Results indicated that NP aa 397 to 407 (QPQNGQFIHFY) were the minimal epitope sequence needed for CTL recognition and lysis (Table 2). Further deletion of the peptide at the amino or carboxy terminal abolished the lysis of target cells by the CTL.

The GP-1 and GP-2 epitopes have been shown to be restricted by the *D^b* MHC molecule (16, 23). The use of MHC recombinant mice has suggested that the NP epitope was also *D^b* restricted (12, 28). In order to determine this restriction directly, L cells (*H-2^k*) transfected with the *D^b* gene (15) were coated with NP peptide aa 397 to 408. L-cell targets expressing the *D^b* molecule were killed by anti-LCMV splenocytes or the NP-specific CTL clones, but L cells lacking the *D^b* molecule were not lysed (Table 3), confirming that this NP epitope is restricted by the *D^b* molecule.

In order to examine the overall TCR diversity of LCMV-specific CTL in *H-2^b* mice, we analyzed the TCR of CTL clones specific for each of the three epitopes by using Northern blot (RNA) analysis (Fig. 1). Clone N20, described in this report, is specific for the NP epitope. Clone 45 has been shown to be specific for the GP-1 epitope (16). Clones 228 (23) and 7 (22a) are specific for the GP-2 epitope. The TCR of clone 228 was characterized by DNA sequencing and found to use Vα4 and Vβ9 (38). Since all of the GP-2 epitope-specific CTL clones thus far examined used Vα4, we determined whether CTL clones specific for the other epitopes also use this Vα gene. As expected, clones 228 and 7 expressed Vα4 gene, but neither clone 45 (GP-1 specific) nor clone N20 (NP specific) used Vα4 (Fig. 1). Interestingly, two clones (N20 and 228) specific for different viral peptides shared the same Vβ (Vβ9).

In this report, we show that the LCMV ARM NP epitope recognized by virus-specific CTL in *H-2^b* mice was minimally identified by LCMV NP aa 397 to 407 and that this epitope was restricted by the *D^b* class I MHC molecule. By a similar analysis using the WE strain of LCMV, Schulz et al. showed that target cells coated with NP aa 391 to 405 could be lysed by LCMV-specific splenocytes from *H-2^b* mice (28). Our results indicated that F (aa 406) and/or Y (aa 407), but not NP aa 391 to 396, were also essential for the recognition by *D^b*-restricted CTL clone N18. The reason for

TABLE 2. Identification of the minimal sequence of the LCMV NP epitope[a]

LCMV ARM NP sequence[b]	% Specific ^{51}Cr released from:	
	H-2^b target	*H-2^d* target
397 408		
QPQNGQFIHFYR	55	ND[c]
QPQNGQFIHFY	59	2
QPQNGQFIHF	3	ND
QPQNGQFIH	1	ND
PQNGQFIHFY	2	ND
QNGQFIHFY	8	ND

[a] Nested sets of synthetic peptides (LCMV ARM sequences) were used in an in vitro cytotoxicity assay to define the minimal sequence of the NP epitope for CTL clone N18. N18 was used at effector/target ratios of 5:1.

[b] Numbers indicate amino acids positions.

[c] ND, not determined.

TABLE 3. D^b restriction of the LCMV NP epitope[a]

CTL	% Specific ^{51}Cr released from:			
	L-cell target (H-2^k)		L cell transfected with the D^b gene	
	LCMV ARM	NP aa 397-408	LCMV ARM	NP aa 397-408
C57BL/6 anti-LCMV splenocytes	0	0	13	11
CTL clones				
N18	0	1	16	17
N20	0	1	11	ND

[a] The restricting MHC molecule of the LCMV NP epitope was determined by an in vitro cytotoxicity assay in which L(TK⁻) cells (H-2^k) and the same cells stably transfected with pCMU-D^b containing a full-length cDNA for the D^b gene (15) were used as targets. Splenocytes were used at effector/target ratios of 50:1, and CTL clones were used at effector/target ratios of 5:1. ND, not determined. Similar results were obtained in an additional experiment.

the discrepancy is not known, as there is no difference in aa sequences within this region between the two strains of LCMV (29). The presence of the amino-terminal 6 aa (aa 391 to 396) may have influenced processing or recognition of NP aa 391 to 405 so that the peptide was recognized by CTL without F (aa 406) and Y (aa 407). Alternatively, some of the D^b-restricted, NP-specific CTL may have recognized the smaller fragment. Our analysis of L^d-restricted, NP-specific CTL showed that some of the CTL clones could lyse targets coated with the peptide comprising only 5 aa residues (35). We are currently generating additional D^b-restricted, NP-specific CTL clones to address this issue.

Recently, Falk et al. showed that pooled peptides of 8 or 9 aa, eluted from a purified MHC class I molecule, had dominant aa residues at one or more positions and that peptides from different MHC class I molecules yielded distinct aa motifs (11). These MHC allele-specific motifs of naturally processed peptides were found to fit well with many of known T-cell epitopes. When the asparagine residue (aa 400) is aligned with position 5 of the D^b-restricted peptide motif described by Falk et al. (11), LCMV NP aa 397 to 407, which include a hydrophobic residue at position 3, an asparagine residue at position 5, and a hydrophobic residue at position 9, agree well with this motif. At present, we do not know whether the deviation of LCMV NP aa 397 to 407 from the nonamer (aa 396 to 404) predicted from the D^b-specific motif is real or artifactual because of the use of exogenously added synthetic peptides to define the epitope (11, 27).

We and others have shown that all of the LCMV GP-2-specific CTL clones currently studied (the total number is 12) in H-2^b mice use Vα4 in their TCR α chain (references 2 and 38 and this paper). Restricted V-segment usage in the TCR is of interest in view of the potential manipulation of the T-cell response to viral infections by using anti-TCR antibodies. Where the main clinical manifestations are a result of the virus-specific CTL response and are not directly caused by the virus (e.g., choriomeningitis following intracerebral injection of LCMV [5, 17] and liver injury by hepatitis B virus infection [20]), such an immunological manipulation may be beneficial to the host. In fact, immune intervention using anti-TCR antibody has been successfully used to treat experimental allergic encephalomyelitis (1, 31). However, for this strategy to work in virus-induced immunopathology, the majority of virus-specific CTL should use similar TCR V segments. Our results showed that unlike GP-2-specific clones, CTL clones recognizing the GP-1 and the NP epitope do not use Vα4, indicating that the CTL response against a particular epitope can be relatively homogeneous but the overall response against the virus is likely to be diverse. Pircher et al. also reported that a D^b-restricted CTL clone (P14) specific for an unknown GP epitope other than the GP-2 epitope used Vα2, not Vα4 (24). Unlike experimental allergic encephalomyelitis induced by myelin basic protein peptides (40), the T-cell response to a virus on a single MHC background may be directed against multiple epitopes (33, 37). The overall T-cell response to the virus may then be heterogeneous, as our example illustrates. While T-cell manipulation using anti-TCR antibodies may prove difficult, the diverse TCRs specific for a virus should provide the host with a better chance to fight against the virus and prevent the emergency of T-cell-resistant virus variants which could occur when only homogeneous TCRs exist in the host to recognize the virus (25).

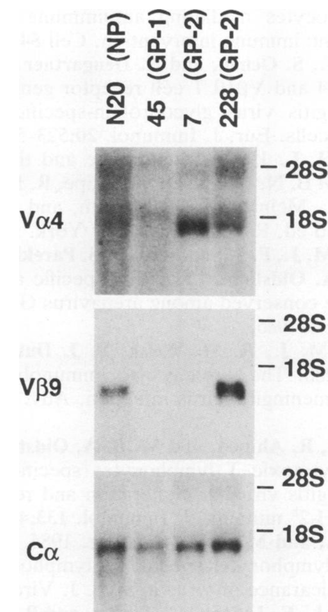

FIG. 1. Northern blot analysis of the TCR gene in LCMV-specific CTL clones. Total RNA isolated from LCMV-specific CTL clones (10 μg each) was analyzed by Northern blot analysis (38) using Vα4, Vβ9, and Cα probes. The Vα4 probe used was a 350-bp *Pst*I-*Rsa*I fragment of Vα4.4 (36) (a generous gift from Ed Palmer, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). The Vβ9 probe was a 350-bp *Bgl*II-*Pst*I fragment derived from the TCR β chain cDNA of CTL clone 228 (38). The Cα probe was a 350-bp *Dde*I fragment derived from the TCR α chain cDNA of CTL clone 5/10-20D (14). The same filter was successively hybridized to these probes.

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