Molecular Analyses of a Five-Amino-Acid Cytotoxic T-Lymphocyte (CTL) Epitope: an Immunodominant Region Which Induces Nonreciprocal CTL Cross-Reactivity†

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The cytotoxic T-lymphocyte (CTL) response to lymphocytic choriomeningitis virus infection determines the outcome of infection. Here we show that this response in BALB/c mice (*H-2^d*), when analyzed both at the primary CTL level and using CTL clones, is predominantly monospecific. The vast majority of CTL have a common specificity for a single epitope in the virus nucleoprotein, which can be minimally identified by amino acids GVYMG. This epitope is presented by the *L^d* class I glycoprotein. We used these data to design a subunit CTL vaccine, whose effectiveness is demonstrated in the accompanying report (L. S. Klavinskis, J. L. Whitton, and M. B. A. Oldstone, *J. Virol.* 63:4311-4316, 1989). Further analysis indicates that, while CTL clones share a common minimal epitope, they differ in their ability to recognize cells infected with a related but distinct strain of lymphocytic choriomeningitis virus. Studies on the molecular nature of CTL cross-reactivity indicate that CTL induced by similar sequences may cross-react in a unidirectional manner. These novel observations suggest that CTL vaccines, to achieve optimal effectiveness, should not simply include virus sequences which will yield a CTL response; the immunizing sequences should also be selected to ensure that the fine specificities of the induced CTL are such that they maximize the chance of recognizing serotypically diverse strains.

The cytotoxic T-lymphocyte (CTL) response is a cardinal factor in the control of many virus infections (38) and may play a critical role in combating the establishment and dissemination of tumors (3). Furthermore, simplicity of the system in a molecular sense allows a clear definition of signals needed for cell-cell (CTL target) recognition. Thus, an understanding of the interactions between CTL and their potential target cells is both of broad biological and medical relevance. To date, most of the data concerning CTL recognition have derived from virus model systems. It has been known for some time that CTL recognize virus antigen not as an isolated entity but, rather, when associated with a host glycoprotein encoded by (usually) class I genes of the major histocompatibility complex (MHC) (37). Recent crystallographic resolution of a human class I MHC molecule has shown two immunoglobulin-like domains supporting a membrane-distal structure comprising two helices flanking a narrow groove (7). Although the precise mechanisms involved are unknown, it appears that the class I MHC proteins present virus antigens as short peptides (32, 33), carrying them to the cell surface in the groove and displaying them to the cellular immune system (8). Antigen specificity is conferred upon the T cell by its heterodimeric membrane-bound T-cell receptor (TcR). The recognition complex is, therefore, at least trivalent, comprising the MHC protein, peptide antigen, and TcR. We have set out to study the second of these components, asking several questions. What are the minimal virus sequence requirements needed for both induction of and target cell recognition by CTL? What are the effects of slight modifications of the epitope sequences? Can potential epitope sequences be predicted from

the primary or secondary structure of a protein? Can such data be used to define a specific and successful CTL vaccine that will provide in vivo protection?

To address these issues, we studied the primary CTL response to a natural virus infection and chose to analyze in detail the response to lymphocytic choriomeningitis virus (LCMV) infection of its natural murine host. This model was selected as the prime example of a natural virus-host interaction, the outcome of which is determined predominantly by the ability of the host to mount a rapid and effective CTL response. A brisk CTL response eradicates the infection and confers subsequent protective immunity; a failed response allows the microbe to establish persistent infection in most tissues (for a review, see reference 22).

LCMV is the prototype arenavirus and has a bisegmented, single-stranded RNA genome (4). The larger segment (L) encodes at least two proteins, a 200,000 to 250,000-dalton viral polymerase and a 10,000 to 12,000-dalton zinc-binding protein (30, 30a). The small (S) segment encodes a 558-amino-acid nucleoprotein (NP) and a 498-residue glycoprotein precursor (GP-C) (5, 27) which undergoes posttranslational cleavage to generate the mature virion glycoproteins GP-1 (residues 1 to 262) and GP-2 (residues 263 to 498) (6). We have constructed recombinant vaccinia viruses (VV) expressing the LCMV NP and GP moieties (35) and have used them to delineate the CTL responses on a variety of *H-2* (murine MHC) backgrounds. We have shown that, on the *H-2^b* haplotype, a vigorous bulk CTL response occurs to both the NP and GP molecules, and we have identified a 9-residue CTL epitope restricted by the *D^b* molecule (24, 34). In contrast, on the *H-2^d* background, a vigorous response is seen only to the viral NP; the response to the GP is undetectable (35).

In this study, we used recombinant VV and synthetic peptides to molecularly define the CTL response on the *H-2^d*

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background. We show that not only is the anti-LCMV response confined to the NP, but it is directed in an almost monospecific fashion to a short region of this virus protein. This epitope is presented by the L^d molecule and thus represents the target sequence detected in earlier work (25). These data form the foundation for the accompanying report (20), in which a successful CTL vaccine, devoid of a biologically relevant B-cell (antibody) response, was developed and used to provide complete protection against an ordinarily lethal viral challenge. Further, in this report we address the question of CTL cross-reactivity between different strains of virus, an important topic since its frequent occurrence is a major conceptual advantage of CTL vaccines, which in theory may protect against many distinct virus serotypes. On the $H-2^d$ background, LCMV strain Pasteur (PAST) induces CTL which cross-react with (kill) cells infected with the LCMV Armstrong (ARM) strain. Conversely, CTL induced by LCMV ARM fail to efficiently lyse LCMV PAST-infected cells (1). Here we analyze the reason for this nonreciprocal cross-reactivity and show that, despite local sequence divergence, the same general region is used in both strains to induce a CTL response. However, the CTL induced by the two viruses, although directed toward the same overall site, show marked differences in target cell recognition.

MATERIALS AND METHODS

Viruses. LCMV ARM and LCMV PAST are biologically and serotypically distinct and have been previously characterized and cloned (14).

Mouse strains and tissue culture cell lines. The mouse strains used are of standard genotype and were provided by the breeding facility at the Research Institute of Scripps Clinic. The strains used were C57BL/6 ($H-2^b$), BALB/c ($H-2^d$), and B10.D2 ($H-2^d$). The cell lines used were free from mycoplasma contamination (by culture and DNA staining). These included MC57 ($H-2^b$), BALB C1.7 ($H-2^d$), and 143TK⁻ (human osteosarcoma). Additional cell lines, LTK⁻ ($H-2^b$) and K2A10 (LTK⁻ expressing the L^d gene) (15), were obtained from L. Hood, Department of Biology, California Institute of Technology, Pasadena. All cell lines were maintained in minimal essential medium containing penicillin, streptomycin, and amphotericin B (Fungizone).

Construction of recombinant VV. Recombinant VV were constructed by standard methods as described previously (34, 35), using the transfer plasmid pSC11 (10). C-terminal truncations of the LCMV NP gene were generated by restriction enzyme cleavage of a full-length NP gene; the enzyme *Cla*I generated a fragment of 321 residues, and the enzyme *Spy*I generated a 201-amino-acid fragment. All plasmid constructs were sequenced at the cloning junctions by using the chemical degradation procedure.

Analysis of LCMV-specific RNAs expressed by recombinant VV. 143TK⁻ (human osteosarcoma) cells were infected with recombinant VV at a multiplicity of infection of 3 and were harvested 4 h postinfection; cytoplasmic RNA was prepared as previously described (34, 35), and a sample was electrophoresed on a 1% agarose-6% formaldehyde gel, transferred by capillary blotting to a nitrocellulose filter, and probed with a ³²P-labeled fragment of LCMV NP cDNA. For the purposes of one experiment (see Fig. 1), four recombinants were used. Three encoded complete or partial copies of the LCMV NP gene (NP, VVNPaa1-321, and VVNPaa1-201), and the fourth was a recombinant lacking LCMV sequences (VVSC11).

Cloning of CTL. We have previously described the generation of CTL clones on the $H-2^b$ background (34). By using the same limiting-dilution technique, CTL clones were obtained from B10.D2 ($H-2^d$) mice. Fifty clones were isolated and maintained in culture as described previously (34).

Preparation of effector cells for in vitro cytotoxicity assay. Effector cells for this assay (see below) were of three types. (i) Primary anti-LCMV splenocytes were prepared by inoculation of 2×10^5 PFU of LCMV intraperitoneally; 7 days later (9 days for mice infected with the LCMV PAST strain) the mice were killed, and the spleens were harvested, disrupted, and cleared of erythrocytes. Cells were then counted to determine effector:target ratios. (ii) Primary anti-VV CTL were prepared in a similar manner, but the spleen was harvested 6 days after intraperitoneal inoculation of 2×10^6 PFU of VVSC11. (iii) Many assays involved the use of CTL clones. The isolation and maintenance of these cells are described above. They were harvested and counted immediately prior to assay.

In vitro cytotoxicity assay. This procedure has been described elsewhere (34). In brief, target cells were incubated for 1 h with 100 μ Ci of ⁵¹Cr, washed three times, and plated into flat-bottom 96-well plates at 2×10^4 cells per well. Effector cells with or without peptide were added to a total final volume of 200 μ l per well. After a 5-h incubation, 100 μ l of supernatant was removed from each well, and radioactivity was measured in a gamma counter. The percent specific ⁵¹Cr released was taken as a measure of CTL lytic activity and was calculated by the following formula: (experimental - spontaneous) $\times$ 100/(total - spontaneous). Peptides, when used, were weighed and suspended in medium immediately before addition to the assay and remained in the well at the stated concentration throughout the assay. Peptides were synthesized either by the "tea bag" method (17) or by using a model 430A (Applied Biosystems) and were assayed for purity by high-pressure liquid chromatography.

Sequence determination of LCMV PAST. The sequence of the S segment of LCMV ARM strain has been published elsewhere (31). The sequence of LCMV PAST strain has

FIG. 1. Production of LCMV NP-specific RNA by recombinant VV. Cells were infected at a multiplicity of infection of 3 with VVNP (lane A), VVNPaa1-201 (lane B), VVNPaa1-321 (lane C), or VVSC11 (lane D), as described in Materials and Methods. Four hours postinfection total cytoplasmic RNA was isolated, electrophoresed in a denaturing gel, transferred to nitrocellulose, and probed with NP-specific ³²P-labeled cDNA. The position of 18S rRNA is shown.

TABLE 1. CTL response against epitope contained within NP residues 1 to 201

CTL	E:T ^a	% ⁵¹ Cr released from targets infected with ^b :							
		<i>H</i> -2 ^d						<i>H</i> -2 ^b	
		Nothing	ARM	VVNPaa1-558	VVNPaa1-321	VVNPaa1-201	VVSC11	Nothing	ARM
Anti-ARM	50:1	0	59	62	55	48	10	0	0
<i>H</i> -2 ^d	25:1	0	45	38	40	48	0	0	0
Anti-VV	50:1	1	0	21	22	30	30	0	0
<i>H</i> -2 ^d	25:1	0	0	23	25	17	15	0	0
Anti-ARM	50:1							0	45
<i>H</i> -2 ^b									

^a Effector cells were incubated at the indicated effector:target (E:T) ratios.

^b Percent specific ⁵¹Cr release is shown, from targets infected with recombinant VV as described in the text. The recombinant VV express full-length NP (residues 1 to 558) and truncations (residues 1 to 321 and 1 to 201); VVSC11 has no LCMV sequences. Boxed areas show levels of ⁵¹Cr release significantly above background.

been determined by the primer-directed chain termination sequencing method, using virus RNA as a template (Salvato and Oldstone, unpublished results).

RESULTS

Recombinant VV expressing full-length or truncated LCMV NP. Using recombinant VV expressing the NP and GP of LCMV ARM, we previously established that bulk splenic CTL from BALB/c (*H*-2^d) mice are directed almost solely to the virus NP (35). To define more precisely the region of NP involved in target recognition, recombinant VV expressing truncated copies of the gene were made, as previously described for the GP molecule (34). The truncated NP open reading frames encoded residues 1 to 321 or 1 to 201. These truncated open reading frames (apposed to a translation termination codon to prevent the formation of fusion proteins) were introduced into VV. Figure 1 shows a Northern (RNA) blot of RNAs produced from the VV recombinants. VVSC11 is a recombinant VV described elsewhere (35) that encodes no LCMV sequences. The expected size of each of the NP-specific RNAs can be estimated as the size of the open reading frame plus 650 bases; the RNA bands in Fig. 1 correspond to these predictions.

Primary anti-LCMV CTL response directed to VVNPaa1-201. The recombinants described above were used to infect

cells in an in vitro cytotoxicity assay (see Materials and Methods), and the results are shown in Table 1. The data show that cells infected with each of the three NP constructs were lysed to a similar level by *H*-2^d anti-LCMV primary CTL. While the data cannot exclude the presence of an epitope between residues 202 and 558, they indicate that a major activity in the population of primary CTL is concentrated on residues 1 to 201. Table 1 also documents the virus specificity and *H*-2 restriction.

CTL clones (96%) recognize NP. Fifty LCMV-specific CTL clones were generated on the *H*-2^d background, and their specificities were analyzed against LCMV and recombinant VV expressing GP, NP, and the NP truncations. The results for five representative clones are shown in Table 2. Of the 50 clones obtained, 48 (96%) were specific for NP. A single clone (HD10) was specific for GP (Table 2), and the remaining clone (HD38) recognized LCMV but neither GP nor NP. Its target is unclear, although it may be directed to a protein encoded by the L segment (data not shown). Of the 48 anti-NP clones, 12 were randomly selected and tested against VVNPaa1-201; all 12 recognized this moiety, and Table 2 shows results for four representative clones (HD3, HD8, HD31, and HD32). The pattern of specificity exhibited by the CTL clones thus reflects that established by using bulk CTL, suggesting that the dominant *H*-2^d epitope lies in

TABLE 2. Recognition of LCMV GP and NP by representative CTL clones^a

CTL	E:T ^b	% ⁵¹ Cr released from targets infected with:							
		<i>H</i> -2 ^d							<i>H</i> -2 ^b
		Nothing	ARM	GP	NP1-558	NP1-321	NP1-201	SC11	ARM
Anti-ARM	50:1	5	47	5	40	34	42	0	2
<i>H</i> -2 ^d	25:1	0	38	1	33	31	37	0	0
HD3	5:1	0	40	0	65	67	59	0	0
HD8	5:1	0	46	0	69	68	64	0	13
HD10	5:1	0	77	72	0	0	0	0	0
HD31	5:1	0	34	0	61	65	68	0	0
HD32	5:1	2	56	0	70	68	70	5	1
Anti-ARM	50:1								76
<i>H</i> -2 ^b									

^a In vitro cytotoxicity assay showing percent specific release for five independent CTL clones against targets. See Table 1, footnote *b* for details. Boxed areas show levels of ⁵¹Cr release significantly above background.

^b E:T, Effector:target ratio.

FIG. 2. Comparison of the NP sequences for residues 119 to 127 of LCMV ARM and PAST. The locations of sequence divergence are shown (■), along with the protein sequences of the amino acid 119 to 127 area in single-letter code.

VVNPaal-201. It is possible that some degree of inadvertent selection has occurred during the cloning procedure, giving a skewed representation of target specificities. In our analyses of 18 clones generated on the *H-2^b* background, we found that the GP:NP ratio was 17:1 (compared with approximately 1:1 in the primary response) (34); thus, some selection in favor of GP clones had apparently occurred. Nevertheless, when these clones were analyzed at the level of fine specificities (24) or TcR rearrangement (18), they were found to be discrete; thus, the selection appeared to operate not at the level of expansion of a single clone, but rather at the level of expansion of a population of independent clones of similar (but not identical) specificities. At present we cannot exclude the possibility that some degree of selection has occurred during the cloning procedure on the *H-2^d* background; however, the similarity between the GP:NP ratio for bulk splenocytes (undetectable:overwhelming) and for CTL clones (1:48) suggests that selection alone is unlikely to explain our results.

Sequence comparison allows identification of the CTL epitope. There are several biologically distinct strains of LCMV (14), whose cross-reactivities at the CTL level have been described (1). On the *H-2^d* haplotype, CTL from LCMV ARM are ineffectual in lysing target cells infected with LCMV PAST. One explanation for this phenomenon is that LCMV PAST lacks a CTL recognition sequence present in LCMV ARM. The complete sequence of the S segment of LCMV PAST has been obtained (Salvato and Oldstone, unpublished results), and comparison of the NP amino acid sequence with that of LCMV ARM (31) revealed only two regions of divergence, between residues 116 and 129 and residues 336 and 349 (Fig. 2). Given the foregoing results which showed that the LCMV ARM site lay within residues 1 to 201, the former sequence was considered a likely candidate for the LCMV ARM CTL epitope. Synthetic peptides corresponding to both regions of divergence were synthesized and used to sensitize uninfected target cells to lysis by either bulk CTL or a variety of CTL clones. The bulk CTL recognized cells coated with NPaa116-129, confirming the presence of an epitope, and all of the anti-VVNPaal-201 CTL clones tested also lysed peptide-coated targets, while the anti-GP clone HD10 failed to do so (Table 3). *H-2^d* CTL did not lyse *H-2^d* targets coated with LCMV ARM peptide spanning amino acids 336 to 349 (data not shown). To date, we have found no *H-2^d* clone (aside from HD10 and HD38, described above) which fails to recognize the peptide spanning amino acids 116 to 129; thus, most of the overwhelming anti-NP response in *H-2^d* mice appears to be centered on this short region. Note also (Table 3) that the anti-ARM primary splenocytes did not lyse PAST-infected target cells efficiently, although these targets were very efficiently lysed by the only anti-GP clone, HD10. In contrast, the four anti-NP clones shown here mirrored the

TABLE 3. CTL clone recognition of NP 116-129^a

CTL	E:T ^b	⁵¹ Cr released ^c from <i>H-2^d</i> targets treated with:			
		Nothing	ARM	PEP	PAST
Anti-ARM <i>H-2^d</i>	50:1	0	70	50	15
HD3	5:1	0	83	76	2
HD8	5:1	20	83	69	1
HD10	5:1	0	69	3	71
HD31	5:1	0	72	68	0
HD32	5:1	6	83	70	12

^a Primary anti-LCMV ARM splenic CTL and various CTL clones were incubated with *H-2^d* targets which were either uninfected, coated with peptide (PEP; LCMV ARM sequence residues 116 to 129, 40 µg per well), or infected with LCMV ARM or PAST strains.

^b E:T, Effector:target ratio.

^c Boxed areas show levels of ⁵¹Cr release significantly above background.

behavior of the bulk splenocytes in their failure to lyse PAST-infected cells. Thus, LCMV ARM infection of *H-2^d* mice induces an overwhelming anti-NP response, which is generally unable to lyse PAST targets (i.e., is not cross-reactive), and a small anti-GP response (detected only by the appearance of clone HD10) which is cross-reactive.

Identification of a 5-residue minimal epitope. We (24) and others (13) have found that T-cell epitopes can be represented by peptides as short as 7 or 8 amino acids. In order to more exactly define the location of the epitope within the 14-mer, nested sets of peptides were synthesized and analyzed for their ability to sensitize uninfected cells to lysis both by bulk CTL and CTL clones. These studies revealed (Table 4) that a remarkably short peptide, GVYMG, was sufficient to fulfill this function for one of the clones, HD9, while the hexamer SGVYMG could direct lysis by all of the CTL clones tested and by primary CTL. The five CTL clones show subtle differences in their recognition of peptide-coated targets. For instance, HD27 and HD47 did not recognize cells coated with PQASGVYMG, setting this pair apart from HD8, HD9, HD31 and the primary CTL, all of which recognized this peptide. Clones HD8, HD9, and HD31

TABLE 4. Identification of minimal recognizable epitopes^a

LCMV ARM NP sequence ^b	% ⁵¹ Cr released by <i>H-2^d</i> CTL ^c					
	Clone					Primary
	HD8	HD9	HD27	HD31	HD47	
116						
SERPQASGVYMGNL	50	55	62	58	43	47
PQASGVYMGNL	41	48	79	46	ND	44
ASGVYMGNL	27	30	43	44	30	24
SGVYMGNL	33	34	58	31	22	29
PQASGVYMG	36	39	3	45	0	56
SGVYMG	44	18	28	ND	38	21
GVYMG	12	60	14	ND	18	0
VYMG	14	9	7	ND	5	8
SERPQASGV	4	9	20	14	7	8
336						
SEKPAVNSPRPAPG	<1	5	ND	3	ND	<1

^a Nested sets of synthetic peptides (LCMV ARM sequences) were used to define the minimal recognizable epitopes for four CTL clones and for primary anti-ARM CTL. Results show patterns established in at least three experiments for each CTL-peptide combination.

^b Numbers indicate amino acid positions.

^c Released at effector-to-target cell ratios of 5:1. ND, Not detected.

TABLE 5. *H-2^d* epitope restriction

<i>H-2^d</i> CTL source	E:T ^a	% ⁵¹ Cr released from <i>H-2^k</i> targets by <i>H-2^d</i> CTL ^b									
		LTK ⁻ (<i>K^kI^kD^kL^k</i>)					K2A10 (<i>K^kI^kD^kL^d</i>)				
		UN	ARM	PEP	VVNPaa1-201	VVSC11	UN	ARM	PEP	VVNPaa1-201	VVSC11
Anti-ARM <i>H-2^d</i>	50:1	0	0	0	0	0	0	41	14	40	0
HD8	5:1	0	0	0	0	0	0	54	35	33	0
HD9	5:1	0	0	0	0	0	0	56	41	40	0
HD27	5:1	0	4	0	0	0	0	52	30	40	0

^a E:T, Effector:target ratio.^b Primary CTL and three CTL clones were incubated with two groups of target cells. The groups differ in that the K2A10 cell line expresses the *L^d* gene, in contrast to the parental LTK⁻ line (*H-2^k*). In each group, cells were uninfected (UN), coated with peptide (PEP; ARM NP sequence residues 116 to 129), or infected with LCMV ARM, VVNPaa1-201 or VVSC11 (no LCMV sequences). Boxed areas show levels of ⁵¹Cr release significantly above background.

can, however, be distinguished since HD9 recognized GVYMG, while the other two failed to do so. These findings mirror our earlier results with *H-2^b* clones, in which similar differences in fine specificity (24) were reflected by novel TcR rearrangements in each clone (18).

***H-2^d* epitope SGVYMG restricted by the *L^d* molecule.** Previous studies have shown that a major portion of the *H-2^d* CTL response is determined by the *L^d* glycoprotein (25). Since we have shown that the great majority of the *H-2^d* response is directed toward a single NP epitope, we anticipated that the epitope must be presented by *L^d*. To confirm this prediction, L cells (*H-2^k*) expressing the *L^d* gene (cell line K2A10, kindly provided by L. Hood) (15) were infected with LCMV or VVNPaa1-201 or coated with peptide as described above. The *L^d* molecule was required for anti-LCMV CTL to lyse LCMV-infected cells (thus confirming earlier work) (Table 5). Additionally, CTL lysed VVNPaa1-201-infected or peptide-treated cells, thus showing that the *L^d* molecule presents the epitope under study.

CTLs recognizing the same minimal epitope nevertheless have distinct patterns of cross-reactivity among LCMV strains. As described previously (1) and confirmed here (Table 3), anti-LCMV ARM *H-2^d* restricted CTL do not efficiently lyse cells infected with LCMV PAST. In fact, advantage was taken of this observation (Fig. 2) to successfully identify the LCMV ARM CTL epitope. However, what is the reason for this failure? We considered two possible explanations in the light of the local sequence divergence between the ARM and PAST strains in this area. First, the CTL epitope region may not be presented during PAST infection, and PAST-infected targets would therefore not be recognized and lysed by anti-ARM CTL. Alternatively, the epitope region could be presented during PAST infection, but its sequence or structure would differ from that of the ARM epitope, so that the anti-ARM CTL would have a reduced affinity for it. We addressed these possibilities first by screening all of the NP-specific anti-ARM CTL clones for the ability to lyse PAST-infected cells and second by assessing the specificities of primary CTL induced by PAST infection. Certain anti-ARM clones (for example, HD27), although specific for the NPaa116-129 epitope, did lyse PAST-infected cells (Table 6), indicating that the amino acid 116 to 129 region is presented during PAST infection. Furthermore, CTL induced by PAST infection were able to lyse cells coated with the ARM peptide, indicating that the PAST virus must have expressed this region of NP on the cell surface in order to induce CTL of this specificity.

However, most of the anti-LCMV ARM CTL clones (Table 6, CTL clones HD8 and HD32 and Table 3, clones

HD3 and HD31, as examples) and primary anti-ARM failed to lyse PAST-infected cells. We argue (above) that this failure of CTL lysis cannot be attributed to complete failure of PAST-infected cells to present the NP epitope region, although it remains possible that PAST-infected cells present less of this region than do ARM-infected cells. Alternatively, the inability to lyse PAST-infected cells may reflect the fine specificity of the TcR on the anti-ARM CTL. If this is the case, the difference must be quantitative rather than qualitative, since the anti-ARM CTL, even when unable to lyse PAST-infected cells, lysed cells coated with a peptide comprising the PAST NP amino acid 116 to 129 sequence (Table 6). To better address these issues, we carried out a dose-response measurement of the lytic activity of primary CTL and CTL clones against cells coated with decreasing amounts of either ARM or PAST peptide (Fig. 3). Anti-ARM primary CTL and clones HD8, HD9, and HD32 all recognized both peptides at high concentrations but continued to recognize the ARM peptide at much lower concentrations than the PAST peptide. There are at least two possible interpretations of these data. First, the *L^d* molecule may differ in its affinity for ARM and PAST peptides, such that administration of equal quantities of peptide results in greater numbers of MHC-peptide complexes for ARM than for PAST. Alternatively, the two peptides may bind equally well to MHC, but target cell recognition and lysis by the anti-ARM CTL may require more PAST-MHC target com-

TABLE 6. Comparison of LCMV ARM and LCMV PAST epitopes^a

<i>H-2^d</i> CTL source	E:T ^b	% ⁵¹ Cr released ^c from <i>H-2^d</i> targets				
		UN	Infected with LCMV		Coated with NPaa116-129 of:	
			ARM	PAST	ARM	PAST
Anti-ARM	50:1	0	73	10	67	42
Anti-PAST	50:1	0	75	50	52	ND
HD27	5:1	0	65	48	66	33
HD8	5:1	3	85	5	75	40
HD32	5:1	8	78	2	46	42

^a Primary anti-ARM and anti-PAST CTL or CTL clones (all anti-ARM) were incubated with *H-2^d* cells that were uninfected (UN), coated with either ARM or PAST peptide (40 µg per well of NP sequence residues 116 to 129 to both cases), or infected with either LCMV ARM or LCMV PAST.^b E:T, Effector:target ratio.^c Boxed areas show levels of specific ⁵¹Cr release significantly above background. ND, Not determined.

FIG. 3. Dose-response of CTL against ARM and PAST NPaa116-129. Anti-ARM CTL clones HD8, HD9, HD27, and HD32 and primary anti-ARM CTL were incubated with uninfected cells and various concentrations of ARM (—) or PAST (---) peptides (both peptides being residues 116 to 129 of their respective NP sequences). Peptide amounts are shown in nanograms (total peptide added to each 200- μ l well volume). ⁵¹Cr release was measured as described in Materials and Methods.

plexes than ARM-MHC target complexes (i.e., the anti-ARM CTL TcRs differ in their affinities for ARM-MHC and PAST-MHC). These two possibilities could be distinguished by direct studies of affinities of *L^d* for these two peptide molecules. We reason that the peptides probably have comparable affinities for MHC, because clone HD27, which is unusual in that it lyses PAST-infected cells (Table 6), recognizes ARM and PAST peptides with almost equal efficiency. Thus the inability of primary anti-ARM CTL (and of most anti-ARM CTL clones) to lyse PAST-infected targets more probably reflects the lower affinity of the CTL for the PAST-MHC complex. These anti-ARM CTL, in order to be triggered by PAST-infected cells, may therefore require higher levels of presentation by *L^d* of the PAST sequence than are attained by the usual infectious mechanisms; administration of PAST peptide in large amounts (e.g., 200 μ g/ml = approximately 10^{12} peptides per target cell) can overcome this block.

Our results clearly demonstrate, however, a novel observation on the nature of CTL cross-reactivity. We show that CTL induced by PAST strain (LKTSGVYMG) can recognize the ARM peptide and ARM-infected cells, while CTL induced by the same region of ARM (PQASGVYMG) are

generally far less able to recognize the PAST peptide or PAST-infected cells; i.e., we demonstrate unidirectional cross-reactivity between the same region of two virus strains.

DISCUSSION

CTL recognition of virus-infected cells (and, implicitly, their recognition of other novel antigens, e.g., tumor recognition) has been a topic of burgeoning and fruitful research. Workers using various model systems have established that CTL can recognize exogenously applied peptides in an MHC-restricted manner, an observation we have used in this study and earlier studies to accurately delimit the minimal sequences required to allow recognition. In this article, we show that infection of mice of the *H-2^d* haplotype induces a CTL response which is almost monospecific in its nature. The primary CTL response constitutes an overwhelming anti-NP activity, with anti-GP specificity being undetectable in our assay conditions, and when clonal analysis was carried out, 48 of 50 clones were NP specific. Finer analysis of these clones reveals that they are strikingly similar in their specificities. All of the clones randomly chosen for further analysis were specific for an epitope contained in NP residues 1 to 201, and peptidic analysis of a random selection of this group showed them all to be directed to the same region, circumscribed by residues 116 to 129. This contrasts with our findings on the *H-2^b* background, in which three separate epitopes were identified (34), all entirely distinct from this *H-2^d* epitope. This underlines again the profound effect exerted by the MHC glycoproteins in selecting short regions of virus product for presentation to and perusal by the cellular immune system. Thus, on the *H-2^d* background, the CTL response is limited to a single region of the LCMV genome. This restrictiveness of the CTL response contrasts with the relative frequency with which B-cell epitopes can be found on most proteins. Accumulating results of CTL epitope mapping on three different MHC backgrounds (*H-2^b*, *H-2^d*, and *H-2^k*), we found a total of only six epitopes, all of which lie on the virus S segment. If these figures accurately reflect the normal frequency of epitopes, then they suggest that an inbred mouse strain, which expresses three distinct class I glycoproteins, will on average respond to only two epitopes from 1,056 (498 plus 558) residues of foreign protein. Our current figures therefore suggest that an MHC class I glycoprotein may need to screen up to 1,500 amino acids of protein in order to find one immunodominant CTL epitope sequence.

This relative paucity of CTL epitopes and the decisive effect of MHC serve to highlight considerations necessary for the design and use of subunit vaccines. We have previously pointed out (34, 35) that the pivotal role of MHC in selecting virus epitopes counsels caution in administration of subunit vaccines, since a vaccine recipient would remain unprotected if his or her MHC could not present any of the subunit sequences. Our data from this study and the figures presented above underline this risk, and in the accompanying report (20), we demonstrate its reality; a subunit vaccine fully protects one mouse strain (*H-2^d*) but is wholly ineffective in protecting another strain (*H-2^b*).

Although schemes have been proposed to identify T-cell epitopes from primary sequence (29) or secondary structure (12), neither of the T-cell epitopes which we have analyzed in detail would have been predicted by either scheme. The epitopes SGVYMG (this article) and VENPGGYCL (24, 34) do not contain a discernible amphipathic helix, nor the

suggested primary sequence motif. Our results indicate that current schemes relying on such motifs, while showing some degree of success, nevertheless are liable to pass over epitopes which are of proven biological relevance. This is especially noteworthy with the epitope mapped here (SGVYMG) which is the immunodominant region for LCMV CTL responses in BALB mice and is of proven biological relevance, since immunization with a minigene expressing this region protects mice from a lethal virus challenge (20). We are unable to discern a predictable pattern in the epitopes we have identified to date. Recent speculative analyses of the possible structure of the TcR (relying on the assumption that it folds in a manner analogous to that of an antibody Fab) indicates that, in the TcR-peptide-MHC complex, the peptide may in fact not be helical but may assume an extended and unpredictable conformation (11). Our results are consistent with this hypothesis.

Despite the attendant difficulties, however, the possible use of CTL epitopes in eliciting protective immunity has several attractive advantages. First, while antibodies in general function only when directed toward virus structural components, CTL may function almost regardless of the nature of the target protein, and CTL have been detected against almost all virus proteins for which they have been sought. Interestingly, in the herpesviruses, which exhibit a cascade of virus protein expression, most CTL activity is directed toward proteins which appear immediately upon infection before the making of structural proteins or infectious progeny virus, e.g., human (28) and murine (26) cytomegalovirus, varicella-zoster virus (16), and herpes simplex virus (21). Further, immunization using such proteins has been achieved (19, 20). Second, in certain infections (e.g., human immunodeficiency virus), antibody responses appear ineffectual in combating disease; CTL responses may offer a better prospect, although the balance between CTL-directed immunopathology and viral clearance is delicate and immune responses may in fact be harmful to the host (23, 36). Third, while antibody responses to virus infection most often are strain specific, CTL responses are commonly cross-reactive, and therefore a single CTL vaccine might induce protection against many serologically distinct strains of virus.

This third potential advantage of CTL vaccines had led us to analyze the nature of cross-reactivity between two strains of LCMV. Our data suggest that many CTL induced by LCMV PAST infection are specific for the NP amino acid 116 to 129 region and can recognize ARM- and PAST-infected cells very well. In contrast, CTL induced by ARM infection (sequence PQASGVYMG) have TcRs with the same minimal epitope but lyse PAST-infected cells much less efficiently than ARM-infected cells. Thus, the CTL induced by this NP region during ARM or PAST infection share a common minimal epitope but appear to differ in their fine specificities. Similar findings have emerged from our earlier studies, in which we found that *H-2^b* CTL that recognize an identical nonameric minimal epitope VENPGGYCL nevertheless can do so with at least six different TcR rearrangements (18). Furthermore, although they share the same minimal epitope, these TcRs can be functionally distinguished by their target specificities against epitopes with single amino acid substitutions (24). The apparent dichotomy (same minimal epitope but functionally distinct TcR) encountered on both *H-2^d* and *H-2^b* backgrounds suggests that these MHC class I-restricted CTL carry TcRs which differ not in the minimal epitope they recognize but more likely in the way they recognize it or in their relative affinities or both.

Thus, the TcRs of anti-PAST CTL may view the epitope in such a way that they can equally well recognize the ARM target sequence, while in contrast the anti-ARM TcRs are less able to see (have a lower affinity for) the PAST target sequence. In this light, the structure of the TcR of clone HD27 will be of particular interest, since this LCMV ARM clone sees PAST-infected cells and also recognizes ARM and PAST peptides at equivalent concentrations (Table 6 and Fig. 3). We hypothesize that the TcR structure of HD27 may differ from that of the other anti-ARM *H-2^d* clones and may be more like that of the anti-PAST CTL. The idea that class I-restricted CTL could view the same epitope in a variety of ways is analogous to a multiview suggestion proposed for class II-restricted T helper cells (2, 9).

Regardless of the exact mechanism involved, we clearly show that in two virus strains slightly divergent sequences are able to induce CTL but that these CTL differ in their ability to cross-react with the other strain. The concept is potentially important, as it would predict a hierarchy of epitope sequences, all of which might induce CTL but which would vary in their ability to induce cross-reactive CTL. Thus, not only would it be important to choose the correct region of a virus protein (so that it would be presented by MHC), but it would be necessary to select the optimal sequence to induce cross-reactivity with other virus strains. We have constructed recombinant viruses to further examine this possibility.

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LITERATURE CITED

1. Ahmed, R., J. A. Byrne, and M. B. A. Oldstone. 1984. Virus specificity of cytotoxic T lymphocytes generated during acute lymphocytic choriomeningitis virus infection: role of the *H-2* region in determining cross-reactivity for different lymphocytic choriomeningitis virus strains. *J. Virol.* 51:34-41.
2. Allen, P. M., G. R. Matsueda, E. Haber, and E. R. Unanue. 1985. Specificity of the T-cell receptor: two different determinants are generated by the same peptide and the *I-A^k* molecule. *J. Immunol.* 135:368-373.
3. Anichini, A., G. Fossati, and G. Parmiani. 1987. Clonal analysis of the cytotoxic T cell response to human tumors. *Immunol. Today* 8:385-389.
4. Bishop, D. H., and D. D. Auperin. 1987. Arenavirus gene structure and organization. *Curr. Top. Microbiol. Immunol.* 133:5-17.
5. Buchmeier, M. J., and M. B. A. Oldstone. 1979. Protein structure of lymphocytic choriomeningitis virus: evidence for a cell associated precursor of the virion glycoproteins. *Virology* 99:111-120.
6. Buchmeier, M. J., P. J. Southern, B. S. Parekh, M. K. Wooddell, and M. B. A. Oldstone. 1987. Site-specific antibodies define a cleavage site conserved among arenavirus GP-C glycoproteins. *J. Virol.* 61:982-985.
7. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (London)* 329:506-508.
8. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (London)* 329:512-515.
9. Cease, K. B., I. Berkower, J. York-Jolley, and J. A. Berzofsky.

1986. T cell clones specific for an amphipathic α -helical region of sperm whale myoglobin show differing fine specificities for synthetic peptides. *J. Exp. Med.* **164**:1779-1784.
10. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5**:3403-3409.
11. Claverie, J.-M., A. Prochnicka-Chalufour, and L. Bougueleret. 1988. Implications of a Fab-like structure for the T cell receptor. *Immunol. Today* **10**:10-14.
12. Delisi, C., and J. A. Berzofsky. 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA* **82**:7048-7052.
13. Del Val, M., H. Volkmer, J. B. Rothbard, S. Jonjić, M. Messerle, J. Schickedanz, M. J. Reddehase, and U. Koszinowski. 1988. Molecular basis for cytolytic T-lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp89. *J. Virol.* **62**:3965-3972.
14. Dutko, F. J., and M. B. A. Oldstone. 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J. Gen. Virol.* **64**:1689-1698.
15. Goodenow, R. S., M. McMillan, M. Nicolson, B. T. Sher, K. Eakle, N. Davidson, and L. Hood. 1982. Identification of the class I genes of the mouse major histocompatibility complex by DNA-mediated gene transfer. *Nature (London)* **300**:231-237.
16. Hickling, J. K., L. Borysiewicz, and J. G. P. Sissons. 1987. Varicella-zoster virus-specific cytotoxic T lymphocytes (Tc): detection and frequency analysis of HLA class I-restricted Tc in human peripheral blood. *J. Virol.* **61**:3463-3469.
17. Houghten, R. A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* **82**:5131-5135.
18. Joly, E., M. Salvato, J. L. Whitton, and M. B. A. Oldstone. 1989. Polymorphism of distinctive lymphocytic choriomeningitis virus H-2D^d-restricted cytotoxic T-lymphocyte clones that recognize a defined nine-amino-acid immunodominant domain of viral glycoprotein. *J. Virol.* **63**:1845-1851.
19. Jonjić, S., M. Del Val, G. M. Kell, M. J. Reddehase, and U. H. Koszinowski. 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *J. Virol.* **62**:1653-1658.
20. Klavinskis, L. S., J. L. Whitton, and M. B. A. Oldstone. 1989. Molecularly engineered vaccine which expresses an immunodominant T-cell epitope induces cytotoxic T lymphocytes that confer protection from lethal virus infection. *J. Virol.* **63**:4311-4316.
21. Martin, S., R. J. Courtney, G. Fowler, and B. T. Rouse. 1988. Herpes simplex virus type 1-specific cytotoxic T lymphocytes recognize virus nonstructural proteins. *J. Virol.* **62**:2265-2273.
22. Oldstone, M. B. A., R. Ahmed, J. Byrne, M. J. Buchmeier, Y. Riviere, and P. Southern. 1985. Virus and immune responses: lymphocytic choriomeningitis virus as a prototype model of viral pathogenesis. *Br. Med. Bull.* **41**:70-74.
23. Oldstone, M. B. A., and F. J. Dixon. 1970. Tissue injury in lymphocytic choriomeningitis viral infection: virus-induced immunologically specific release of a cytotoxic factor from immune lymphoid cells. *Virology* **42**:805-813.
24. Oldstone, M. B. A., J. L. Whitton, H. Lewicki, and A. Tishon. 1988. Fine dissection of a nine amino acid glycoprotein epitope, a major determinant recognized by lymphocytic choriomeningitis virus-specific class I-restricted H-2D^b cytotoxic T lymphocytes. *J. Exp. Med.* **168**:559-570.
25. Orn, A., R. S. Goodenow, L. Hood, P. R. Brayton, J. G. Woodward, R. C. Harmon, and J. A. Frelinger. 1982. Product of a transferred H-2L^d gene acts as a restriction element for LCMV-specific killer T cells. *Nature (London)* **297**:415-417.
26. Reddehase, M. J., W. Mütter, K. Münch, H.-J. Bühring, and U. H. Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J. Virol.* **61**:3102-3108.
27. Riviere, Y., R. Ahmed, P. J. Southern, M. J. Buchmeier, F. J. Dutko, and M. B. A. Oldstone. 1985. The S RNA segment of lymphocytic choriomeningitis virus codes for the nucleoprotein and glycoproteins 1 and 2. *J. Virol.* **53**:966-968.
28. Rodgers, B., L. Borysiewicz, J. Mündin, S. Graham, and P. Sissons. 1987. Immunoaffinity purification of a 72K early antigen of human cytomegalovirus: analysis of humoral and cell-mediated immunity to the purified polypeptide. *J. Gen. Virol.* **68**:2371-2378.
29. Rothbard, J. B., and W. R. Taylor. 1988. A sequence pattern common to T cell epitopes. *EMBO J.* **7**:93-100.
30. Salvato, M., E. Shimomaye, and M. B. A. Oldstone. 1989. The primary structure of the lymphocytic choriomeningitis virus L gene encodes a putative RNA polymerase. *Virology* **169**:377-384.
- 30a. Salvato, M. S. 1989. The ambisense nature of the L RNA of lymphocytic choriomeningitis virus, p. 168-173. *In* B. Mahy and D. Kolakofsky (ed.), *Genetics and pathogenicity of negative strand viruses*. Elsevier Biomedical Press, New York.
31. Southern, P. J., M. K. Singh, Y. Riviere, D. R. Jacoby, M. J. Buchmeier, and M. B. A. Oldstone. 1987. Molecular characterization of the genomic S RNA segment from lymphocytic choriomeningitis virus. *Virology* **157**:145-155.
32. Townsend, A. R. M., J. Bastin, K. Gould, and G. G. Brownlee. 1986. Cytotoxic T lymphocytes recognize influenza hemagglutinin that lacks a signal sequence. *Nature (London)* **324**:575-577.
33. Townsend, A. R. M., F. M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* **42**:457-467.
34. Whitton, J. L., J. R. Gebhard, H. Lewicki, A. Tishon, and M. B. A. Oldstone. 1988. Molecular definition of a major cytotoxic T-lymphocyte epitope in the glycoprotein of lymphocytic choriomeningitis virus. *J. Virol.* **62**:687-695.
35. Whitton, J. L., P. J. Southern, and M. B. A. Oldstone. 1988. Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. *Virology* **162**:321-327.
36. Zinkernagel, R. M. 1988. Virus-triggered AIDS: a T-cell-mediated immunopathology? *Immunol. Today* **9**:370-371.
37. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of *in vitro* T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (London)* **248**:701-702.
38. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determine T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* **27**:51-178.