*Brief Definitive Report*

# CLASS I MHC CAN PRESENT AN ENDOGENOUS PEPTIDE TO CYTOTOXIC T LYMPHOCYTES

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Cytotoxic T lymphocytes (CTL) recognize viral and cellular antigens in association with class I glycoproteins encoded in the host MHC (1). The recognition event results from a cell surface interaction between the CTL and the target cell, but it has been conclusively demonstrated in a wide variety of systems that many virus proteins that are not detectable by standard methods on the cell membrane are, nevertheless, detected by CTL (2, 3). These observations have spawned many experiments to characterize the precise nature of the MHC/antigen complex with which CTL interact. It has been demonstrated that short peptides, some comprising only seven amino acids, can, when applied exogenously to uninfected target cells, sensitize them to CTL recognition and lysis; such sensitization, however, usually has required a vast molar excess of peptide to target cells (often used at $10^{-5}$ M, which may represent $\sim 10^{11}$ peptide molecules per target cell) (4-6). Since it is likely that class I MHC generally presents antigens that are synthesized within the presenting cell, and not antigens applied in gross excess to the cell surface (7), we have attempted to ascertain the ability of class I MHC to detect and present a known CTL epitope when it is synthesized within a cell in the form of an extremely short polypeptide molecule that we have chosen to term an "endogenous peptide." We show that a nine amino acid epitope, when flanked by a minimal number of neighboring residues, is sufficient to allow virus-specific MHC-restricted recognition by bulk and cloned CTL.

We have used as a model system the infection by lymphocytic choriomeningitis virus (LCMV) of its natural murine host. Acute LCMV infection induces a brisk primary CTL response, readily detectable by in vitro assay (8); the response is biologically relevant, being instrumental in effecting viral clearance from the host (9). Early attempts to assign CTL recognition relied upon the use of segmental reassortants of this bisegmented RNA virus, and identified the viral S segment as encoding the target molecules (10). This segment encodes two proteins, a 558 amino acid nucleoprotein and a 498 glycoprotein (11, 12) that undergoes posttranslational modification to yield the two virion glycoproteins GP-1 and GP-2 (residues 1-262 and 263-498, respectively) (13). We have expressed these proteins in vaccinia virus (14), and have generated families of recombinant viruses expressing serially truncated LCMV genes (5). These recombinants have been used to map CTL epitopes

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on several different MHC backgrounds, and we have shown that epitopes differ in a manner dictated by the class I molecules themselves (14). On the H2b haplotype (in C57BL/6 mice) we identified three discrete epitopes, two on the viral glycoprotein and one on the nucleoprotein. One of the glycoprotein epitopes was further characterized using nested sets of synthetic peptides, which allowed the delineation of a nine amino acid core sequence comprising the minimal CTL epitope (6). The sequence, VENPGGYCL, lies near the $NH_2$ terminus of GP-2, at residues 278–286. Its sequence suggests that it is neither amphipathic nor an $\alpha$-helix, and it lacks a motif proposed as predictive for T cell epitopes; thus, simple sequence analysis would have failed to highlight this region as a potential epitope. The nonameric peptide, when applied exogenously to H2b target cells, sensitizes it to lysis both by cloned CTL and primary CTL from an acutely infected mouse (6). This lysis is maximal at peptide concentrations of 5 $\mu$M, and half-maximal at 0.1 $\mu$M. This latter concentration represents $\sim 10^{13}$ molecules in each 200-$\mu$l in vitro assay, each of which contains $2 \times 10^4$ target cells. Thus, $5 \times 10^8$ peptide molecules per target cell are required to achieve half-maximal lysis. These peptide concentrations are similar to those used by other groups to sensitize target cells (4).

The ability of extremely short peptides to sensitize targets to CTL lysis in vitro is often cited as evidence that in vivo presentation of virus antigen by class I MHC occurs by an identical mechanism. We would argue that the fact that CTL *can* see targets sensitized using a great molar excess of peptide does not constitute evidence that peptide is the form normally encountered, and certainly does not demonstrate the ability of target cells to process/present such molecules under normal circumstances. Since it is hypothesized that MHC may function in vivo by "screening" short peptides that result from degradation of endogenously synthesized proteins, it is critical to determine whether MHC can in fact detect such short molecules. The purpose of our experiment is, therefore, to establish whether very short sequences made *within* a virus-infected target cell can be presented to CTL.

## Materials and Methods

*Cell Lines, Viruses, and Mouse Strains.* BALB C17 ($H2^{dd}$) and MC57 ($H2^b$) cells were grown as described (14). LCMV (Armstrong strain) and recombinant vaccinia viruses were made by standard techniques, as described (5). All recombinant plasmids were subjected to DNA sequencing to ensure that their structure was as intended. The mouse strains used were BALB/c ($H2^{dd}$) and C57BL/6 ($H2^{bb}$).

*Analysis of LCMV-specific RNAs Produced by Recombinant Vaccinia.* 143 $TK^-$ (human osteosarcoma) cells were infected with recombinant vaccinia at a multiplicity of infection (moi) of 3, and 4 h after infection total cytoplasmic RNA was prepared as previously described (5), subjected to Northern blot transfer, and probed with a $^{32}$P-labeled fragment of the LCMV GP cDNA. After washing (final stringency 0.2 × SSC, 0.5% SDS, 55°C) the filter was exposed to photographic film.

*Preparation of Effector Cells for In Vitro Cytotoxicity Assay.* Effector cells for this assay were: (*a*) Primary anti-LCMV splenocytes prepared by inoculation of $2 \times 10^5$ PFU of LCMV intraperitoneally; 7 d later (9 d for mice infected with the LCMV PAST strain) the mouse was killed, and splenocytes were harvested. (*b*) Primary anti-vaccinia CTL spleens were harvested 6 d after inoculation of $2 \times 10^6$ PFU i.p. of $VVSC_{11}$. (*c*) Many assays involved the use of CTL clones. The isolation and maintenance of these cells is as described (5).

*In Vitro Cytotoxicity Assay.* This procedure has been described elsewhere (5). Peptides, where 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.

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## Results

*Construction of Recombinant Viruses Expressing an LCMV Minigene.* Using serially deleted LCMV GP molecules we have shown that the VENPGGYCL epitope can be recognized in a vaccinia construct in which several non-LCMV residues are covalently attached to the COOH terminus of a truncated GP encoding residues 1–293 (5). We have now made a recombinant virus encoding only 22 amino acids (272–293) of the LCMV GP-2 molecule, followed by termination codons in all three reading frames and lacking the non-LCMV COOH-terminal residues (Fig. 1 *A*). This recombinant virus, named VVg, directs the transcription of a single LCMV-specific RNA species of the appropriate size, as detected by Northern blot analysis (Fig. 1 *B*). We have analyzed five independent isolates of the recombinant, and all behave identically.

*CTL Efficiently Recognize the Minigene Protein Product.* Fig. 2 shows that target cells infected with VVg are lysed both by a CTL clone (HL 2-2-8) specific for this epitope, and by bulk CTL. Lysis is epitope specific, since HL 2-2-8 fails to lyse cells infected with VVf (a recombinant expressing a different LCMV epitope contained in GP residues 1-173 and which is recognized by bulk CTL), and is H2 restricted.

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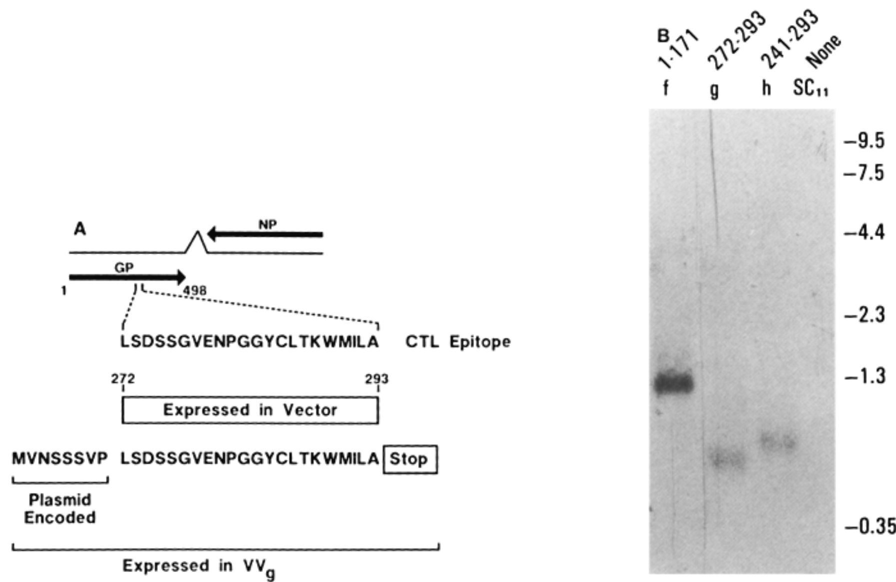


FIGURE 1. (*A*) The LCMV CTL epitope sequences expressed from recombinant vaccinia virus VVg. The S RNA segment of LCMV, which encodes GP and NP, is shown, along with the amino acid sequence of the CTL epitope under analysis. The DNA sequence encoding these residues was directly apposed to a translation termination cartridge containing stop codons in all three reading frames, to prevent the synthesis of a 3' fusion protein, and thereafter cloned into a pUC derivative to place the plasmid ATG in frame with the LCMV sequences. This minigene was cloned into the vaccinia transfer plasmid pSC11, and at this stage (immediately before introduction into vaccinia virus) the minigene DNA sequence was confirmed by the chemical degradation technique. (*B*) A Northern blot analysis of RNA produced by VVg. Total cytoplasmic RNA was prepared as previously described (5) from cells infected with either of four recombinant vaccinia. VVf and VVh contain fragments of LCMV GP encoding residues 1–173 and 241–293, respectively. VVg is described in *A*. VVSC11 is a control recombinant that contains no LCMV sequences. The blot was hybridized with a strand-specific probe from the LCMV GP, washed, and exposed to photographic film. The sizes of marker RNA molecules are shown (kb).

Thus the LCMV amino acid sequences encoded in VVg are expressed in recognizable form. That they are being expressed in the form of the short peptide encoded by the sequence-confirmed open reading frame, rather than as part of a longer fusion protein, is suggested by a series of facts. First, the mRNA is of the correct size, indicating that the cap and termination sites are as anticipated. Second, there is only one ATG signal between the cap site and the epitope sequence, indicating that an erroneous start (and hence a potential 5′ fusion) is very unlikely. Third, inadvertent production of a 3′ fusion protein could occur if there were a specific rearrangement beginning precisely at the last base of the ORF epitope sequence and deleting the translation termination codons that lie in all frames immediately downstream. This unlikely possibility is rendered vanishingly improbable because all five independent isolates of the recombinant (isolated in separate experiments) are recognized by CTL. We therefore conclude that the short ORF most probably is translated to produce an endogenous peptide, which can be presented by class I MHC.

VVg contains the immunodominant LCMV epitope VENPGGYCL recognized by H-2b-restricted CTL (6). Note that recognition by CTL clones of VVg-infected cells is approximately equivalent to that of LCMV-infected cells (Figs. 2 and 3). Hence the foreshortened molecule is processed/presented as satisfactorily as the native, by the criterion of CTL lysis.

*The Minigene Product Is Presented by the "Endogenous" Route.* The formal possibility remained that target cells were sensitized not by MHC presentation of the endogenous peptide, but rather by secretion of the short molecule, with subsequent external attachment to MHC. We therefore incubated unlabeled VVg-infected H2b cells along with $^{51}$Cr-labeled uninfected H2b cells. CTL lysis of labeled, uninfected, cells did not occur (Fig. 3, column 4), indicating that the labeled cells were not coated with peptide secreted from neighboring VVg-infected cells. It is, therefore, very likely that recognition/lysis of VVg infected cells (Fig. 2, and Fig. 3, column 3) results from CTL recognition of virus antigen presented by the "endogenous" pathway usually used by class I glycoproteins.

## Discussion

Together these results show that a CTL epitope made as a short peptide and lacking extensive neighboring residues can be properly processed and presented by class I MHC. This attests to the sensitivity and flexibility of the MHC screening system, and represents the first direct evidence that class I MHC molecules are in fact capable

| | E:T | H-2$^b$ UN | LCMV | VV$_g$ | VV$_f$ | VV$_{SC11}$ |
|---|---|---|---|---|---|---|
| H2$^b$ αLCMV Splenocytes | 50 | 3 | 65 | 16 | 47 | 3 |
| | 25 | 1 | 62 | 9 | 25 | 4 |
| HL 2-2-8 αGP$_{278-286}$ | 5 | 7 | 72 | 46 | 2 | 0 |
| | 2.5 | 3 | 73 | 44 | 0 | 0 |
| H2$^d$ αLCMV Splenocytes | 50 | 0 | 4 | 1 | 2 | 1 |
| | 25 | 1 | 1 | 1 | 2 | 1 |

FIGURE 2. VVg is recognized by bulk CTL and by CTL clones. An in vitro cytotoxicity assay was carried out as previously described (5). In brief, H2b (MC57) target cells were infected with either LCMV, VVg, VVf (containing LCMV GP 1-173, which harbors a second CTL epitope), or VVsc11 (which contains no LCMV sequences). UN, uninfected cells. The $^{51}$Cr-labeled target cells were incubated with an H2-matched CTL clone (HL 2-2-8) that is specific for the GP 272-283 epitope (7) or with H2-matched (C57BL/6) bulk splenocytes, or with H2-mismatched (BALB/c) bulk splenocytes. After a 5-h incubation, supernatants were harvested and their radioactivity was counted. Levels of Cr release significantly above background are boxed.

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| | | E:T | H2b UN | H2b LCMV ARM | H2b VVg | H2b VVg + UN | H2b $VV_{SC_{11}}$ | H2d LCMV ARM | H2d VVg |
|---|---|---|---|---|---|---|---|---|---|
| H2b αGP Clones | HL 2-2-8 | 5 | 5 | 67 | 55 | 10 | 4 | 0 | 1 |
| | HL 2-32 | 5 | 21 | 55 | 50 | 20 | 16 | 0 | 10 |
| H2b Splenocytes | α LCMV | 50 | 2 | 60 | 22 | 4 | 3 | 0 | 4 |
| | | 25 | 2 | 42 | 13 | 3 | 3 | 0 | 0 |
| | α VV | 50 | 0 | 3 | 58 | 4 | 52 | 0 | 0 |
| | | 25 | 3 | 2 | 34 | 4 | 36 | 0 | 15 |
| H2d Splenocytes | α LCMV | 50 | 2 | 1 | 1 | 1 | 3 | 64 | 6 |
| | | 25 | 0 | 4 | 2 | 2 | 4 | 41 | 0 |

FIGURE 3. VVg sensitizes cells by the endogenous (natural) route. An in vitro cytotoxicity assay was carried out, as described in the legend to Fig 2. In this case, however, target cells were used (VVg infected + uninfected) that comprised a 1:1 mixture of $^{51}Cr$-labeled uninfected H2b cells with unlabeled VVg-infected H2b cells. These cells were co-incubated for 1 h before the 5-h assay. CTL clones 2-2-8 and 2-3-2 are specific for GP 272–293 epitopes and are restricted by $D^b$ (6).

of such a feat. We (5) and others (15) have previously shown that imcomplete genes can generate recognizable CTL epitopes, but in all of these constructs the epitope was translated in a protein significantly larger than the "minigene" reported here. Our results further suggest that unresolved density found in the antigen binding groove of the recently crystallized HLA-A2 molecule (16) may indeed reflect the presence of the actual peptide bound and presented by MHC, rather than merely the result of proteolysis of a larger molecule (which could have occurred during the purification of HLA-A2, a procedure that itself involved proteolytic digestion).

Where the class I MHC molecule, a glycoprotein synthesized on membrane-bound ribosomes of the rough endoplasmic reticulum, meets with and binds the endogenous peptide, which lacks any signal sequence and presumably is translated by cytoplasmic ribosomes, remains an open question, but one that is being explored with vigor.

Our results, therefore, further delimit the minimal sequences needed for target cell recognition by CTL. However, evidence to clarify the virus sequence requirements for *induction* of CTL presently remains elusive, in part because it has proven difficult to induce class I-restricted CTL using soluble protein or peptide; hence these reagents are of limited value in assessing induction requirements. CTL are best induced by a replicating agent, and materials such as VVg, which express very limited viral sequences but remain recognizable by CTL, will be invaluable in assessing the viral sequence requirements for the induction of CTL responses.

## Summary

Since class I MHC glycoproteins may function by "screening and selecting" degraded proteins, we wished to determine whether very short peptides made within a cell were detected and bound by MHC, and presented for T cell perusal. We show that a 22 amino acid viral sequence containing a $D^b$-restricted nonameric CTL epitope is sufficient to direct CTL recognition/lysis of $H2^b$ target cells. The mechanism of epitope presentation is by the "natural" endogenous route, and appears to direct lysis as effectively as wild-type virus infection, in which the epitope is part of a 236 residue glycoprotein.

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