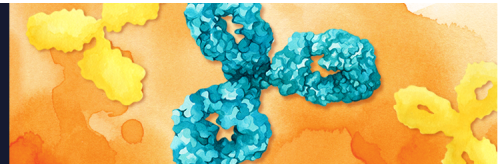


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## Class I-Restricted Alloreactive Cytotoxic T Lymphocytes Recognize a Complex Array of Specific MHC-Associated Peptides

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# Class I-Restricted Alloreactive Cytotoxic T Lymphocytes Recognize a Complex Array of Specific MHC-Associated Peptides<sup>1</sup>

Wei Wang,\* Stephen Man,<sup>2\*</sup> Pamela H. Gulden,<sup>‡</sup> Donald F. Hunt,<sup>†‡</sup> and Victor H. Engelhard<sup>3\*</sup>

A major issue in understanding alloreactive T cell responses is whether the Ags recognized reside in allogeneic MHC proteins themselves regardless of the structure of the associated peptides or whether specific peptides presented by allogeneic MHC proteins determine each epitope. We developed HLA-A\*0201-specific alloreactive human CD8<sup>+</sup> CTL lines and clones to address this issue. Acid treatment of HLA-A\*0201<sup>+</sup> target cells resulted in the loss of Ab-defined epitopes as well as recognition by all alloreactive CTL. In the presence of brefeldin A, no class I molecules were re-expressed at the surface of the acid-treated cells. Addition of a mixture of synthetic peptides corresponding to known, naturally processed, HLA-A\*0201-associated peptides together with exogenous human  $\beta_2m$  restored binding by specific Ab but not recognition by alloreactive CTL. However, addition of a more complex mixture of peptides directly extracted from HLA-A\*0201 reconstituted CTL recognition. This demonstrates that these alloreactive CTL recognize specific peptides and not a common peptide-dependent conformation of HLA-A\*0201. Reverse phase HPLC fractionation of the extracted peptides resulted in the loss of recognition by CTL lines from three individuals. This was not due to the loss of specific peptide species because repooling of the HPLC fractions led to a recovery of recognition. Furthermore, three HLA-A\*0201-alloreactive CTL clones recognized single distinct peptide peaks from the same HPLC fractionation. These data suggest that the epitopes recognized in allogeneic responses to HLA-A\*0201 are complex, and the response is a result of recognition of multiple unique peptide-MHC complexes. *The Journal of Immunology*, 1998, 160: 1091–1097.

The role of peptides in the recognition of non-self MHC molecules by alloreactive CTLs has been the focus of extensive studies. In contrast to CTLs that recognize antigenic peptides in association with self-MHC molecules, alloreactive CTLs recognize non-self MHC molecules and are present at high precursor frequency, even in naive animals (1). One model to explain this type of recognition suggested that the epitopes are not dependent on the structure of bound peptides, but instead reflect the direct recognition of allelically polymorphic residues on the MHC molecule (2). Until recently, the principal data directly supporting this model consisted of a study in which it was demonstrated that immobilized class I molecules apparently free of bound peptide could stimulate an alloreactive T cell line (3). However, it has been buttressed by the observation that a significant fraction of H-2K<sup>b</sup>-alloreactive CTL are able to recognize class I molecules on acid-treated target cells or soluble class I molecules expressed in *Drosophila* cells (4–6). A second model, in which alloreactive T

cells recognize epitopes that are dependent on both the MHC molecule and on specifically bound peptides (7), has received wider experimental support. However, whether peptides contribute to specific epitopes or simply stabilize a particular conformation of MHC molecules for direct recognition by alloreactive T cells remains controversial. On the one hand, several studies have demonstrated that recognition by alloreactive CTL clones was dependent upon the addition of specific fractions from naturally processed peptide extracts (8–12) or cytoplasmic proteins cleaved using cyanogen bromide (13). On the other hand, based on the observations that different peptides induce distinct conformational changes in class I MHC molecules (14) and that the epitopes recognized by some alloreactive T cells could be reconstituted by more than one peptide (15, 16), it was suggested that alloreactive T cells detect structural features of the MHC molecule that are influenced by the peptide, rather than precise structural features of the peptide itself.

Most of these studies examined limited numbers of CTL clones that may not be representative of the bulk alloreactive response. Analysis of large numbers of short term alloreactive CTL specific for either HLA-A\*0201 or H-2D<sup>d</sup> demonstrated that 80 to 90% recognized epitopes dependent upon the presence of endogenous peptides that are absent or significantly reduced on the Ag-processing mutant T2 (17, 18). Since the HLA-A\*0201 molecules on T2 cells are occupied by peptides produced directly in the endoplasmic reticulum (19, 20), this suggests that this fraction of alloreactive CTL directed against this Ag recognizes one or more specific peptides whose expression depends upon the normal Ag-processing pathway. Although such endoplasmic reticulum-derived peptides may not be presented by other MHC molecules (20), the ability of CTL to discriminate murine and human target

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Table I. Previously identified naturally processed class I MHC-associated peptides<sup>a</sup>

Associated with HLA-A*0201	Associated with HLA-B*0702
SLLPAIVEL	AASKERSGVSL
YLLPAIVHI	APRAFXPXPV
KXNEPVXLL	APRQPGLMA
LLDVPTAAV	APRXPXTGX
LLLDVPTAAV	LVMAPRTVL
LLLDVPTAAVQA	MPRGVVVTL
MLLSVPLLLG	RPSGGPPEX
VLFRGGPRGLLAVA	RVMAPRALL
VLFRGGPRGLLAV	A[Abu]RTVALTA <sup>b</sup>
GILGFVFTL	
YMDGTMSQV	
ALWGFFPVL	
YIEPGPVTA	
LLFGYPVYV	
SLFGGSVKL	
ALDYATYET	
SLPSNLGV	
ALAKAAAV <sup>b</sup>	

<sup>a</sup> Sequences were identified as described in reference 34 and original references therein. X refers to Leu or Ile and indicates that an equimolar mixture of these two residues was used during synthesis. Abu is amino butyric acid.

<sup>b</sup> These peptides bind to the relevant MHC molecule, but are not known to be naturally processed.

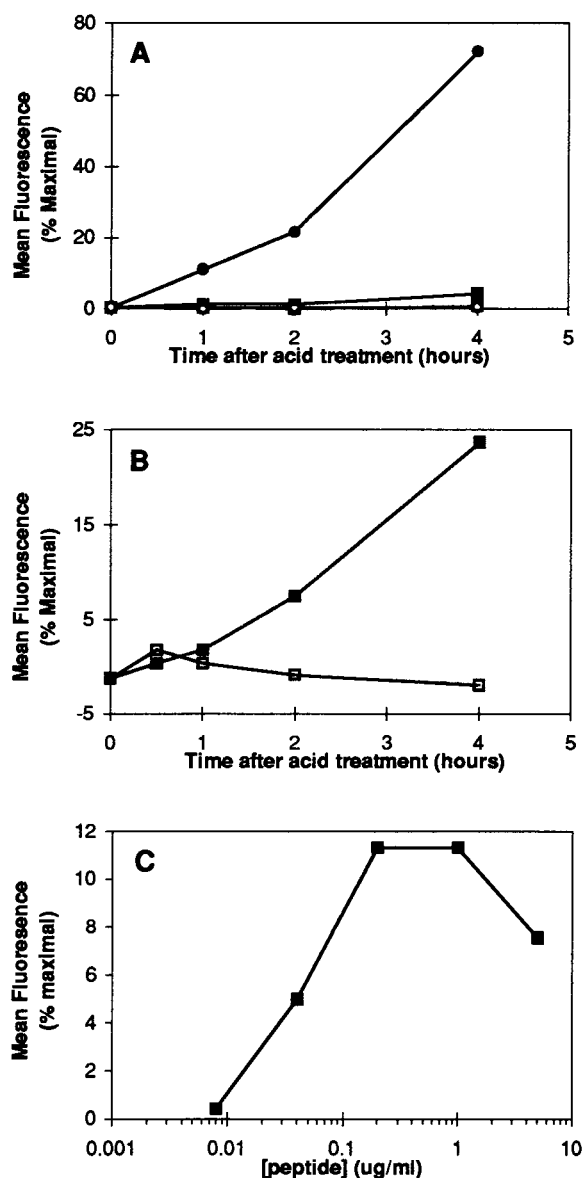
cells expressing H-2D<sup>d</sup> suggests that the majority of alloreactive CTL directed against this Ag are also peptide specific.

In addition to the controversy concerning the recognition of specific peptides, there is also uncertainty regarding how many different peptides are recognized, and whether a small number might be immunodominant. Some studies have suggested that T cells stimulated by a single alloantigen are directed at different peptides presented by that Ag, and among the clones evaluated there is no evidence for two with identical peptide specificity. Other studies have shown that single peptide species were recognized by large fractions of the alloreactive CTL clones that responded to the foreign MHC molecule used as a stimulator (10, 21). In an effort to address some of these discrepancies, we have now evaluated the role of peptides in Ag recognition by short term bulk HLA-A\*0201- and HLA-B\*0702-reactive CTL lines, which are more representative of the alloreactive T cell repertoire than selected clones. We demonstrate that these alloreactive lines are not only peptide dependent, but are also peptide specific, and that the number of different peptide Ags recognized is very high. These data indicate that individual alloreactive T cells are exquisitely specific for individual peptides presented by MHC molecules, but the collective response is directed at a wide array of different peptides.

## Materials and Methods

### Generation of alloreactive T cell lines

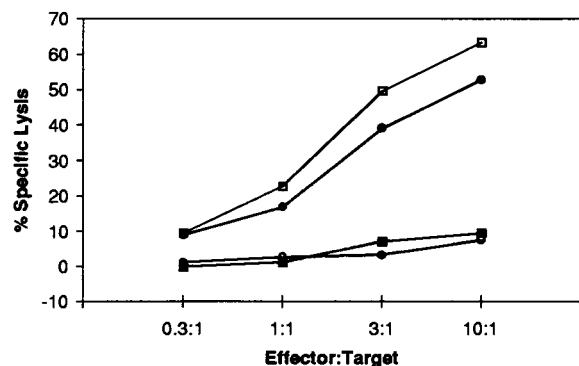
Bulk alloreactive T cell lines were established by culturing  $2 \times 10^6$  HLA-A\*0201-negative, Ficoll-Hypaque-separated PBLs with  $1 \times 10^5$  irradiated JY cells (homozygous for HLA-A\*0201, HLA-B\*0702) in 2 ml of RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine in 24-well Linbro plates (Flow Laboratories, McLean, VA). After 1 wk, viable cells were harvested and restimulated with fresh irradiated JY cells at a ratio of 20:1 in medium containing 20 U/ml of human rIL-2; 20 U/ml of rIL-2 was also added on day 4 following each restimulation. This protocol was repeated weekly for 3 to 4 wk until HLA-A\*0201 specificity was established. Alloreactive CTL lines were also initiated by stimulating  $2 \times 10^6$  HLA-A\*0201<sup>-</sup> PBLs with  $1 \times 10^6$  irradiated HLA-A\*0201<sup>+</sup> PBLs, followed by three or four weekly restimulations using irradiated PBLs from different HLA-A\*0201<sup>+</sup> donors. All bulk lines were used in assay between 4 and 10 wk.



**FIGURE 1.** A, Inhibition of HLA-A\*0201 re-expression on acid-treated T2 cells in the presence of brefeldin A. T2 cells were acid treated as described in *Materials and Methods*, and re-expression of HLA-A\*0201 was examined at room temperature (squares) or 37°C (circles) in the absence (closed symbols) or the presence (open symbols) of 5  $\mu$ g/ml brefeldin A. After the indicated time, cells were stained with the Ab BB7.2 and quantitated by FACS. B, Peptides reconstitute the expression of a class I Ab epitope on acid-treated cells. Acid-treated T2 cells were incubated at room temperature in the presence of 5  $\mu$ g/ml brefeldin A and 3  $\mu$ g/ml  $\beta_2$ m, either with (■) or without (□) a mixture of HLA-A\*0201 binding synthetic peptides, as listed in Table I, at a 1  $\mu$ g/ml final concentration. After the indicated time, cells were stained with the Ab BB7.2 and quantitated by FACS. C, Acid-treated T2 cells were incubated at room temperature for 4 h in the presence of 5  $\mu$ g/ml brefeldin A, 3  $\mu$ g/ml  $\beta_2$ m, and the indicated final concentration of the synthetic peptide mixture. Cells were stained with the Ab BB7.2 and quantitated by FACS.

### Generation of alloreactive CTL clones

HLA-A\*0201-specific alloreactive bulk CTL lines, 3 to 4 wk of age, were plated in graded numbers (1–1,000 cells/well) in a 96-well U-bottom plates (Flow Laboratories) containing 2,000 irradiated JY cells and 50,000 irradiated PBLs/well, 20 U/ml rIL-2, and 1  $\mu$ g/ml PHA. On day 7, another 20 U/ml rIL-2 was added. On day 14, cultures were screened for positive



**FIGURE 2.** Reconstitution of CTL epitope on acid-treated target cells. T2 cells were labeled with  $^{51}\text{Cr}$ , acid stripped to remove peptide, and then incubated for 2 h at room temperature in the presence of 5  $\mu\text{g}/\text{ml}$  brefeldin A alone (■), with influenza A M1<sub>58-66</sub> peptide (GILGFVFTL; ○), or with the xenogenic peptide epitope 1049 (ALWGFFPVL; ●). The peptide 1049-specific CTL clone AHIII12-2 was added, and the plate was further incubated at 37°C for an additional 4 h before assessing  $^{51}\text{Cr}$  release. Untreated T2 cells (□) that endogenously present the peptide 1049 were used as the positive control.

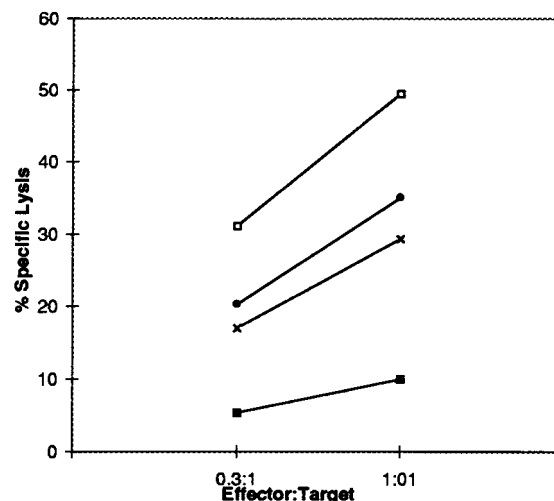
growth and tested on HLA-A\*0201-positive and -negative targets. HLA-A\*0201-specific alloreactive clones were expanded by stimulating  $2 \times 10^6$  CTLs with  $5 \times 10^6$  EBV-lymphoblastoid cell lines and  $1 \times 10^7$  PBLs in 80 ml of RPMI 1640 supplemented with 15% FCS and 20 U/ml rIL-2. On day 5, another 40 ml of medium with the same concentrations of FCS and rIL-2 was added. CTLs were harvested on day 7 and either used immediately or frozen in aliquots for later use.

#### Extraction of endogenous peptides

HLA-A\*0201 molecules were immunoaffinity purified from  $2 \times 10^{10}$  JY cells using mAb BB7.2 as previously described (22), except that 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was substituted for Nonidet P-40 in all preparative steps. Peptides were eluted from HLA-A\*0201 molecules with 10% acetic acid, pH 2.1, and separated using a 5000-dalton cut-off filter. HLA-B7-associated peptides were extracted using the same protocol, except an HLA-B7-specific Ab ME1-1.2 was used in the immune purification. A portion of the extract corresponding to  $5 \times 10^7$  cell equivalents was concentrated in a Speed-Vac (Savant Instruments, Farmingdale, NY) to near dryness and resuspended in 100  $\mu\text{l}$  of RPMI 1640 (Life Technologies, Grand Island, NY) containing 5% FCS and 2 mM glutamine (RPMI medium) for *in vitro* reconstitution assays. In some cases, peptide extracts were fractionated by HPLC on a reverse phase C<sub>18</sub> column (7- $\mu\text{m}$  particles, 300-Å pore size, 2.1-mm inner diameter, 3-cm length; Brownlee, Rainin Instruments, Braintree, MA), using an Applied Biosystems (Foster City, CA) model 130a Separations System with a gradient of acetonitrile and 0.1% trifluoroacetic acid. The concentration of acetonitrile was increased from 0 to 9% (0–5 min), 9 to 36% (5–55 min), and 36 to 60% (55–62 min; v/v), and 200- $\mu\text{l}$  fractions were collected every minute. One-half microliter of each fraction, corresponding to  $1 \times 10^8$  cell equivalents, was diluted into RPMI medium, as described above, for use in *in vitro* reconstitution assays.

#### Synthetic peptides

Peptides were synthesized by solid phase F-moc methodology using Applied Biosystems or Gilson Medical Electronics (Middleton, WI) AMS422 peptide synthesizers. Peptides were purified to >90% homogeneity by reverse phase HPLC, and their identities were confirmed by mass spectrometric analysis. For *in vitro* reconstitution assays, equal amounts of 18 synthetic peptides corresponding to naturally processed or other peptides known to bind to HLA-A\*0201 (22, 23) or nine synthetic peptides corresponding to naturally processed or other peptides known to bind to HLA-B\*0702 (24) (Table I) were mixed in DMSO, and 1  $\mu\text{l}$  was diluted into 125  $\mu\text{l}$  of RPMI 1640 medium. Assuming the average m.w. of these peptides to be 1000, the final concentrations of these synthetic peptide mixtures were 1 and 0.5  $\mu\text{M}$ , respectively, and were equivalent to the molar quantities of endogenous HLA-A\*0201- or HLA-B\*0702-associated peptides extracted from  $5 \times 10^7$  and  $2.5 \times 10^7$  JY cells, respectively.



**FIGURE 3.** Brefeldin A is required to prevent re-expression of epitopes recognized by CTL.  $^{51}\text{Cr}$ -labeled T2 cells were treated with acid to remove peptides on the cell surface and were subsequently incubated for 2 h at room temperature alone (x), with 5  $\mu\text{g}/\text{ml}$  brefeldin A (■), or with 5  $\mu\text{g}/\text{ml}$  brefeldin A and the xenogenic peptide epitope 1049 (ALWGFFPVL; ●). The peptide 1049-specific CTL clone AHIII12-2 was added, and the plate was incubated at 37°C for an additional 4 h before assessing  $^{51}\text{Cr}$  release. Untreated T2 cells (□) that endogenously present peptide 1049 were included as the positive control.

#### Acid treatment of cells used as targets in the $^{51}\text{Cr}$ release assay

HLA-A\*0201-positive T2 cells or T2-B7 cells that also express transfected HLA-B\*0702 were labeled with  $^{51}\text{Cr}$  for 2 h, washed, and then incubated with 250  $\mu\text{l}$  of 300 mM glycine/1% BSA, pH 2.5, for 3 min at room temperature (25). The acid was immediately neutralized with 15 ml of RPMI medium, and the cells were centrifuged at  $700 \times g$  for 3 min and resuspended in 1 ml of RPMI medium containing 5  $\mu\text{g}/\text{ml}$  brefeldin A to prevent expression of newly synthesized class I molecules. One thousand cells in a final volume of 100  $\mu\text{l}$  of RPMI medium supplemented with 3  $\mu\text{g}/\text{ml}$  of human  $\beta_2\text{m}$  were pulsed with peptides for 2 h at room temperature. CTLs were added at an E:T ratio of 10, and incubation was continued for 4 h at 37°C before measuring  $^{51}\text{Cr}$  release into the supernatant.

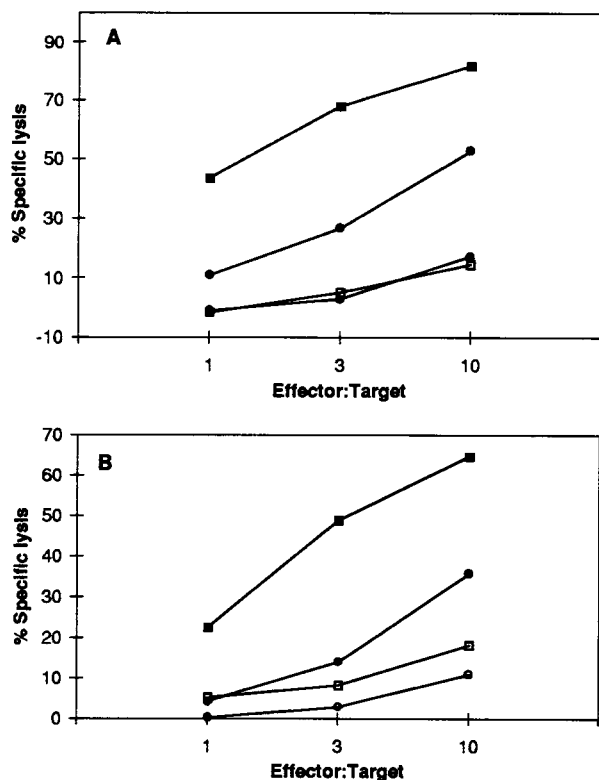
#### Cell surface expression of HLA-A\*0201

Untreated or acid-treated T2 cells were incubated with saturating amounts of an HLA-A\*0201-specific mAb BB7.2 and fluorescein-conjugated F(ab')<sub>2</sub> of sheep anti-mouse IgG. All buffers contained 0.02% azide to prevent new synthesis and expression of HLA-A\*0201. The mean log fluorescence was determined by FACS analysis. The value for cells without acid treatment was considered the maximum, while the value for cells stained with secondary Ab only was used as the minimum: % maximal = [(mean fluorescence – minimum)/(maximum – minimum)]  $\times 100$ .

## Results

#### Cell surface class I MHC molecules are denatured by acid treatment, but refold in the presence of peptide

To assess the role of specific class I MHC-associated peptides in the formation of epitopes recognized by class I MHC-alloreactive CTL, we developed a system in which the presence and the composition of such peptides at the cell surface could be regulated. HLA-A\*0201<sup>+</sup> T2 cells were briefly incubated at pH 2.5 to denature the MHC complexes. As shown in Figure 1A, acid-treated T2 cells were no longer recognized by the HLA-A\*0201-specific Ab BB7.2. However, HLA-A\*0201 was re-expressed to about 80% of pretreatment levels within 4 h at 37°C. This re-expression did not occur if the cells were incubated at 25°C or if they were incubated at 37°C in the presence of brefeldin A. This indicates that re-expression was dependent upon newly synthesized HLA-A\*0201 molecules, while those expressed on the surface at the

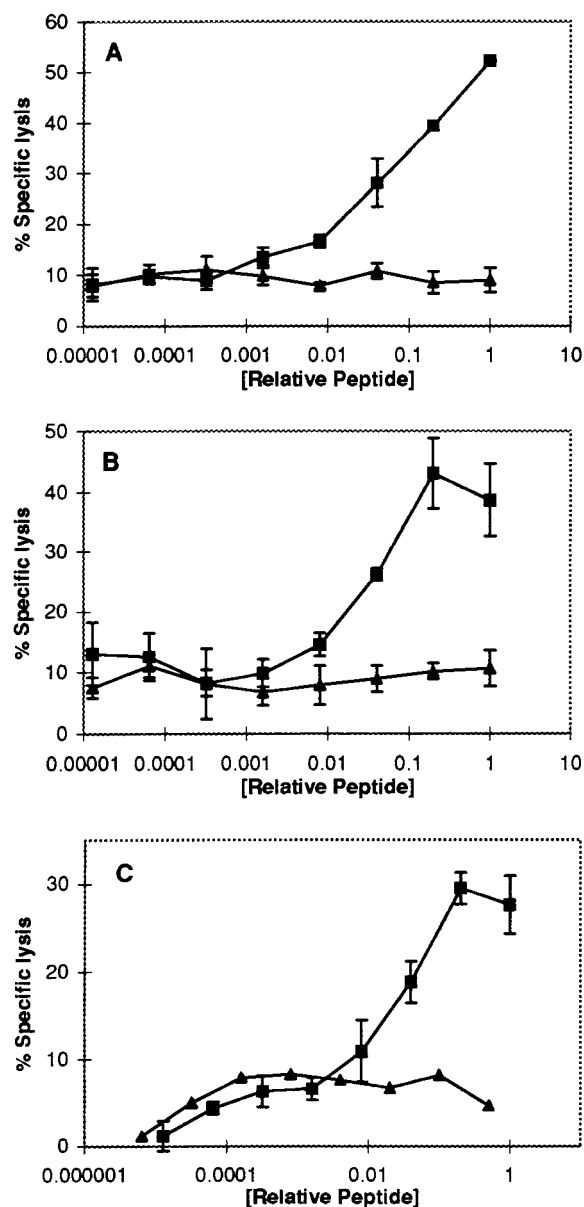


**FIGURE 4.** Specificity of bulk HLA-A\*0201-reactive CTL lines. MHN anti-JY (A) and MHN anti-PBL (B) CTL lines were assayed on  $^{51}\text{Cr}$ -labeled C1R-A2.1 (■), C1R (□), T2 (●), and acid-treated T2 (○).

time of acid treatment remained denatured, even if incubated at a temperature that confers stability on peptide-free class I molecules (6).

We attempted to refold these denatured HLA-A\*0201 molecules by incubation of these cells with a mixture of 18 synthetic peptides that either correspond to species that are known from mass spectrometric identification to be naturally processed and presented by HLA-A\*0201 or bind to this MHC molecule with relatively high affinity (Table I). In the presence of brefeldin A, peptides, and  $\beta_2\text{m}$ , up to 25% of the denatured HLA-A\*0201 molecules refolded in 4 h at room temperature into a conformation recognizable by BB7.2 (Fig. 1B). The incubation at room temperature was critical for the refolding; if incubated at  $37^\circ\text{C}$  immediately after acid treatment, no increase in HLA-A\*0201 expression was observed. However, molecules that were expressed after refolding at room temperature remained stable upon shifting the temperature to  $37^\circ\text{C}$ , consistent with their occupancy by added peptide (data not shown). The refolding of HLA-A\*0201 molecules was also dependent on the peptide concentration and was maximal when the cells were incubated with  $0.2\ \mu\text{g}/\text{ml}$  of total peptide (Fig. 1C).

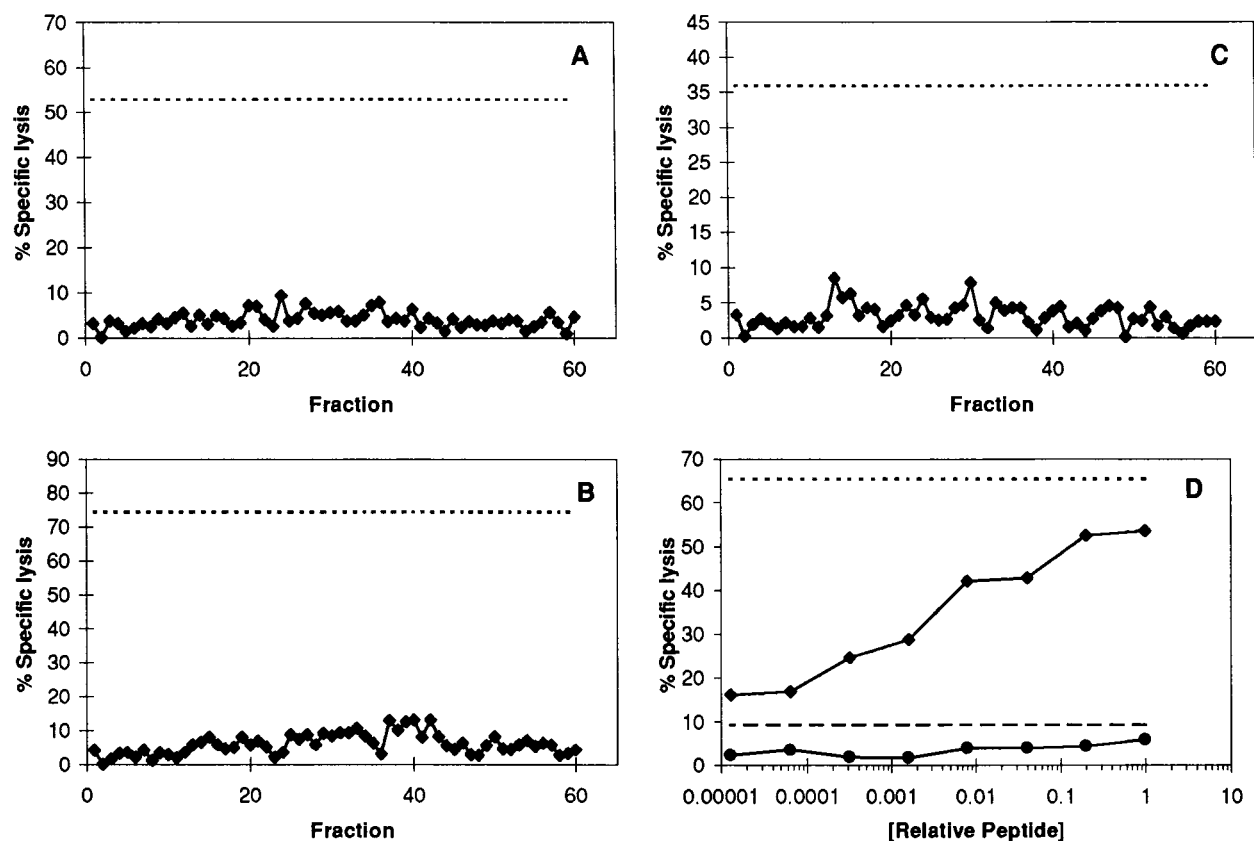
Acid treatment of T2 cells also abolished expression of HLA-A\*0201-dependent epitopes recognized by T cells. We previously identified a peptide called 1049 that is recognized by a HLA-A\*0201-specific xenogeneic murine CTL clone AHIII12-2 and is expressed on T2 cells despite their Ag processing defect (26). The AHIII12-2 clone no longer recognized T2 cells after acid treatment and incubation in the presence of brefeldin A (Fig. 2). However, when peptide 1049 was incubated with acid-treated T2 cells at room temperature for 2 h in the presence of brefeldin A and  $\beta_2\text{m}$ , recognition by AHIII12-2 was restored to normal levels. We obtained similar results using other defined HLA-A\*0201-restricted



**FIGURE 5.** Reconstitution of alloantigen recognition by bulk CTL lines with MHC binding peptides. T2 cells were labeled with  $^{51}\text{Cr}$ , acid stripped to remove peptides, and then incubated with either synthetic peptide mixtures (▲) or endogenous peptides (■) for 2 h at the room temperature in the presence of  $5\ \mu\text{g}/\text{ml}$  brefeldin A and  $3\ \mu\text{g}/\text{ml}$   $\beta_2\text{m}$ . A relative peptide value of 1 corresponds to the amount of endogenous peptides extracted from  $5 \times 10^7$  JY cells and to a final concentration of synthetic peptide mix (Table I) of  $1\ \mu\text{g}/\text{ml}$ . Peptide-pulsed targets were examined using bulk CTL lines, 4 to 6 wk of age: A, MHN anti-JY, HLA-A\*0201 specific; B, MHN anti-PBL, HLA-A\*0201 specific; and C, ST anti-PBL, HLA-B\*0702 specific. Background lysis without CTLs was  $<5\%$  across the concentrations for both endogenous and synthetic peptides. Error bars represent SDs of triplicate wells. The E:T ratio was 10:1.

peptides that were recognized by a melanoma-specific CTL line (data not shown) or by CTL clones directed at three other epitopes.<sup>4</sup> It is important to note that in the absence of brefeldin A, re-expression of these epitopes to  $>50\%$  of pre-acid treatment

<sup>4</sup> C. J. Luckey, G. M. King, B. F. Maier, V. L. Crotzer, J. Shabanowitz, D. F. Hunt, and V. H. Engelhard. Proteasomes can either generate or destroy MHC class I epitopes: evidence for non-proteasomal epitope generation in the cytosol. *Submitted for publication.*



**FIGURE 6.** Specific endogenous peptide peaks are not discernibly recognized by alloreactive bulk CTL lines. Endogenous HLA-A\*0201 binding peptides were extracted from  $2 \times 10^{10}$  JY cells and fractionated on a reverse phase HPLC column as described in *Materials and Methods*. A portion (0.5%) of each fraction, corresponding to  $1 \times 10^8$  cell equivalents, was incubated with acid-treated T2 cells for 2 h at the room temperature in the presence of 5  $\mu\text{g}/\text{ml}$  brefeldin A and 3  $\mu\text{g}/\text{ml}$   $\beta_2\text{m}$ . Bulk CTL lines MHN anti-JY (A), JS anti-JY (B), or ELH anti-JY (C) were then added at an E:T ratio of 10:1 and further incubated at 37°C for 4 h. Lysis of the positive control target cell JY is indicated by the dashed line in each panel. D: ♦, Amounts corresponding to  $2 \times 10^8$  cell equivalents from each of HPLC fractions 1 to 60 were pooled together, vacuum centrifuged to a volume of 50  $\mu\text{l}$ , and then used in a reconstitution experiment analogous to that described for A through C using CTL line MHN anti-JY. A relative peptide concentration of 1 corresponds to  $1 \times 10^8$  cell equivalents. ●, An identical reconstitution assay was conducted except that CTL clone MHN24 was used instead of CTL line MHN anti-JY, and fractions 16 and 17, which contain the sensitizing activity for this clone (see A), were omitted when the HPLC fractions were pooled and concentrated.

values occurred due to the processing and the presentation of relevant endogenous peptides (Fig. 3). Therefore, treatment of cells with acid and brefeldin A followed by addition of peptides provided us with the ability to manipulate the composition of peptides associated with cell surface class I molecules. It also allowed us to systematically evaluate the dependence of alloreactive CTL on the expression of endogenous peptides.

#### *Endogenous peptides are required to reconstitute recognition by alloreactive T cells*

The peptide-dependent refolding of acid-denatured HLA-A\*0201 molecules on the surface of T2 cells allowed us to examine the importance of specific peptides in the formation of epitopes recognized by alloreactive CTLs. We evaluated two HLA-A\*0201-specific alloreactive CTL lines at 4 wk of age: line MHN anti-JY and line MHN anti-PBL. At an E:T ratio of 10, both lines gave >60% specific lysis of C1R-A2.1 cells and >30% specific lysis of T2 (Fig. 4). However, upon acid treatment, recognition of T2 cells by these CTL was reduced to a level that was not significantly higher than the background lysis of the HLA-A\*0201-negative cell line C1R. This result strongly suggests that no significant fraction of these CTL lines is able to recognize a peptide-independent conformation of this class I MHC molecule (4).

To evaluate the role of specific peptides in Ag recognition by these lines, we incubated the acid-treated T2 cells with the same

mixture of 18 synthetic peptides (Table I) that induced refolding of HLA-A\*0201 as detected by Ab. However, incubation of target cells with this mixture did not restore recognition by either of the two HLA-A\*0201-specific alloreactive lines (Fig. 5). On the other hand, if the mixture of all endogenous peptides extracted from immunoaffinity-purified HLA-A\*0201 molecules expressed on JY cells was used in place of the synthetic peptides, they effectively reconstituted recognition in a dose-dependent manner. Similarly, the epitopes recognized by the HLA-B\*0702-specific alloreactive CTL line ST anti-PBL were lost by brief acid extraction of T2-B7 cells and were restored using a mixture of all endogenous HLA-B\*0702 peptides, but not a mixture of 9 HLA-B\*0702 binding peptides (Table I and Fig. 5C). These data indicate that the proper Ag conformation necessary for recognition by these alloreactive CTL lines is not conferred by mixtures of small numbers of structurally distinct MHC-associated peptides, but instead requires the presence of other peptides that are present in a more complex mixture. This result suggests that alloreactive T cell recognition involves specific contacts with these peptides.

#### *Complexity of peptides recognized by alloreactive T cell lines*

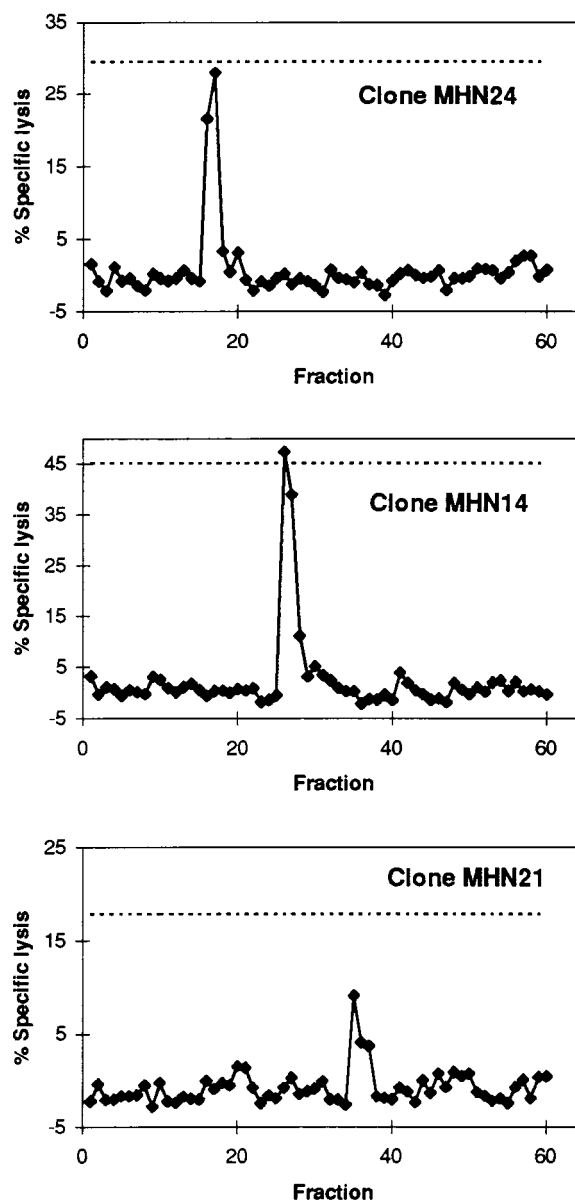
To characterize the endogenous peptides recognized by the alloreactive T cells, HLA-A\*0201-associated peptides extracted from JY cells were fractionated by reverse phase HPLC, and a portion

of each fraction was evaluated for the ability to restore CTL recognition of acid-treated target cells. Contrary to the reconstitution of recognition by unfractionated endogenous peptides shown in Figure 5, HPLC-fractionated peptides were no longer able to reconstitute recognition by any of the bulk CTL lines, even though the quantities added to the target cells were the same (Fig. 6, A–C). This was true regardless of whether the line was developed using JY cells or PBLs as stimulators (data not shown). Apparent minor “peaks” that could be discerned in some assays (Fig. 6C) were not reproducible from assay to assay. The inability of HPLC-separated peptides to reconstitute recognition by alloreactive CTL lines was not due to the loss of specific peptide species, because repooling the HPLC fractions led to the recovery of T cell recognition (Fig. 6D). On the other hand, recognition by three HLA-A\*0201-specific alloreactive clones derived from the CTL line MHN anti-JY was reconstituted by individual HPLC fractions that eluted at different positions in the gradient (Fig. 7). When the fractions that contained this reconstituting activity for CTL clone MHN24 were omitted, and the remaining fractions were pooled, this pool was unable to reconstitute recognition for the CTL clone (Fig. 6D). This demonstrates that each clone is exquisitely peptide specific, and that each recognizes a distinct peptide in the mixture extracted from HLA-A\*0201. These data suggest that bulk alloreactive CTL lines consist of a broad spectrum of T cells with distinct peptide epitope specificities, such that no specificity is sufficiently well represented to give a discernible peak of reconstituting activity when only one or a few appropriate peptides are present in an HPLC fraction (27).

## Discussion

In this study we used acid-treated target cells devoid of endogenous peptides to examine both qualitatively and quantitatively the importance of peptides in the allogeneic CTL response to HLA-A\*0201 and HLA-B\*0702. The acid treatment allowed us to manipulate the presence and the composition of peptides on the surface of target cells. Although a mixture of synthetic peptides corresponding to a subset of naturally processed HLA-A\*0201- or HLA-B\*0702-associated peptides could reconstitute recognition by specific Ab, the acid-treated target cells were no longer recognized by alloreactive bulk CTL lines unless pulsed with endogenous peptide extract. Interestingly, there was also a significant level of recognition of T2 cells before acid treatment, suggesting that at least some T cells in the bulk line could recognize peptides that continue to be expressed in association with HLA-A\*0201 in the absence of TAP (19, 26). We conclude that these human class I MHC-allospecific responses are not only peptide dependent but are collectively specific for many distinct peptide species.

These studies extended observations by several other groups who demonstrated reconstitution of epitopes for small numbers of class I-specific CTL clones (8–13). Although it has been reported that in some instances alloreactivity may not depend on the recognition of a precise self peptide but on a class I epitope influenced by the peptide (15), we believe that this is an exception rather than the rule, based on our data from three alloreactive bulk CTL lines in which the majority of alloreactive T cells are specific for endogenous peptides. Our results stand in strong contrast to a recent report by Smith et al. (4), who reported that up to 20% of primary and essentially all secondary alloreactive CTL primed by skin grafting were able to recognize H-2K<sup>b</sup> molecules expressed in T2 cells even after acid treatment and concluded that these CTL recognized a conformation of the class I molecule that was independent of peptides. One possible explanation for the discrepancy between their results and those of many other groups, including our



**FIGURE 7.** Distinct and specific endogenous peptide peaks are recognized by alloreactive clones but not by bulk CTL lines. Endogenous HLA-A\*0201 binding peptides were extracted from  $2 \times 10^{10}$  JY cells and fractionated on a reverse phase HPLC column as described in *Materials and Methods*. A portion (0.5%) of each fraction, corresponding to  $1 \times 10^8$  cell equivalents, was incubated with acid-treated T2 cells for 2 h at the room temperature in the presence of 5  $\mu\text{g/ml}$  brefeldin A and 3  $\mu\text{g/ml}$   $\beta_2\text{m}$ . The indicated CTL clones were then added at an E:T ratio of 10:1 and further incubated at 37°C for 4 h. Lysis of the positive control target cell JY is illustrated by the dashed line in each panel.

own, may lie in their observation that the generation of peptide-independent CTL was enhanced by maneuvers that are known to select for CD8-independent, and thus high avidity, CTL (28, 29). Such CTL may be able to recognize a residual level of native class I MHC molecules after acid treatment. However, it should also be noted that this group apparently did not prevent re-expression of new class I molecules on the acid-treated cells by the use of brefeldin A. As shown convincingly here (Fig. 3), re-expression of class I molecules during the time course of a CTL assay in the absence of brefeldin A is substantial and more than sufficient to allow CTL recognition.

A second issue raised by this study is the complexity of the alloreactive epitopes. It is possible to easily discern up to 11 peaks of reconstituting activity in short term bulk CTL lines reactive with human melanoma cells (30, 31). Therefore, the failure to see any significant peaks of reconstituting activity in HPLC-fractionated peptide extracts for the alloreactive CTL lines examined in the present study suggests that the number of distinct Ags that they recognize is substantially higher than this (30, 31). Our results contrast with other recent studies that have shown that the 2C peptide presented by H-2L<sup>d</sup> and the Qdm peptide presented by Qa-1<sup>b</sup> are immunodominant in alloreactive responses to these class I MHC molecules (10, 21). In addition, we observed that the 1049 peptide recognized by the clone AHIII12-2 is also recognized by a substantial fraction of HLA-A\*0201-specific murine anti-human xenoreactive CTL (26). These observations have raised the alternative possibility that a single peptide with high abundance can create a high determinant density on APC that activates a large number of T cell clones. However, the expression of H-2L<sup>d</sup> on cells is low, and the other two systems cited are not typical alloantigenic responses. It remains to be determined whether the immunodominance of a small number of peptides reflects some unique features of H-2L<sup>d</sup> or Qa-1<sup>b</sup> molecules or is a consequence of the relatively weak xenoreactive response (32, 33). In any case, our estimate of the complexity of the epitopes recognized in more conventional alloantigenic responses to HLA-A\*0201 and HLA-B\*0702 is consistent with the idea that the strength of these responses is a result of recognition of multiple unique peptide-MHC complexes.

## References

- Sherman, L. A., and S. Chattopadhyay. 1993. The molecular basis of allorecognition. *Annu. Rev. Immunol.* 11:385.
- Bevan, M. J. 1984. High determinant density may explain the phenomenon of alloreactivity. *Immunol. Today* 5:128.
- Elliot, T. J., and H. N. Eisen. 1990. Cytotoxic T lymphocytes recognize a reconstituted class I histocompatibility antigen (HLA-A2) as an allogeneic target molecule. *Proc. Natl. Acad. Sci. USA* 87:5213.
- Smith, P. A., A. Brunmark, M. R. Jackson, and T. A. Potter. 1997. Peptide-independent recognition by alloreactive cytotoxic T lymphocytes. *J. Exp. Med.* 185:1023.
- Schumacher, T. N., M. Heemels, J. J. Neeffjes, W. M. Kast, C. J. Melief, and H. L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell* 62:563.
- Ljunggren, H. G., N. J. Stam, C. Öhlén, J. J. Neeffjes, P. Hoglund, M. Heemels, J. Bastin, T. N. M. Schumacher, A. Townsend, K. Karre, and H. L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature* 346:476.
- Matzinger, P., and M. J. Bevan. 1977. Why do so many lymphocytes respond to major histocompatibility antigens? *Cell. Immunol.* 29:1.
- Rotzschke, O., K. Falk, S. Faath, and H. G. Rammensee. 1991. On the nature of peptides involved in T cell alloreactivity. *J. Exp. Med.* 174:1059.
- Udaka, K., T. J. Tsomides, and H. N. Eisen. 1992. A naturally occurring peptide recognized by alloreactive CD8<sup>+</sup> cytotoxic T lymphocytes in association with class I MHC protein. *Cell* 69:989.
- Aldrich, C. J., A. DeCloux, A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman. 1994. Identification of a TAP-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* 79:649.
- Poindexter, N. J., B. Naziruddin, D. W. McCourt, and T. Mohanakumar. 1995. Isolation of a kidney-specific peptide recognized by alloreactive HLA-A3-restricted human CTL. *J. Immunol.* 154:3880.
- Malarkannan, S., F. Gonzalez, V. Nguyen, G. Adair, and N. Shastri. 1996. Alloreactive CD8<sup>+</sup> T cells can recognize unusual, rare, and unique processed peptide/MHC complexes. *J. Immunol.* 157:4464.
- Heath, W. R., K. P. Kane, M. F. Mescher, and L. A. Sherman. 1991. Alloreactive T cells discriminate among a diverse set of endogenous peptides. *Proc. Natl. Acad. Sci. USA* 88:5101.
- Bluestone, J. A., S. Jameson, S. Miller, and R. F. Dick II. 1992. Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition. *J. Exp. Med.* 176:1757.
- Bluestone, J. A., A. Kaliyaperumal, S. Jameson, S. Miller, and R. F. Dick II. 1993. Peptide-induced changes in class I heavy chains alter allorecognition. *J. Immunol.* 151:3943.
- Guimezanes, A., T. N. Schumacher, H. L. Ploegh, and A. M. Schmitt-Verhulst. 1992. A viral peptide can mimic an endogenous peptide for allorecognition of a major histocompatibility complex class I product. *Eur. J. Immunol.* 22:1651.
- Crumpacker, D. B., J. Alexander, P. Cresswell, and V. H. Engelhard. 1992. Role of endogenous peptides in murine allogeneic cytotoxic T cell responses assessed using transfectants of the antigen processing mutant 174xCEM. T2. *J. Immunol.* 148:3004.
- Man, S., R. D. Salter, and V. H. Engelhard. 1992. Role of endogenous peptide in human alloreactive cytotoxic T cell responses. *Int. Immunol.* 4:367.
- Henderson, R. A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D. F. Hunt, and V. H. Engelhard. 1992. HLA-A2.1 associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* 255:1264.
- Wei, M. L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* 356:443.
- Connolly, J. M. 1994. The peptide p2Ca is immunodominant in allorecognition of Ld by beta chain variable region V beta 8<sup>+</sup> but not V beta 8<sup>-</sup> strains. *Proc. Natl. Acad. Sci. USA* 91:11482.
- Hunt, D. F., R. A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevilir, A. Cox, E. Appella, and V. H. Engelhard. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 255:1261.
- Chen, Y., J. Sidney, S. Southwood, A. L. Cox, K. Sakaguchi, R. Henderson, E. Appella, D. F. Hunt, A. Sette, and V. H. Engelhard. 1994. Naturally processed peptides longer than nine amino acid residues bind to the class I MHC molecule HLA-A2.1 with high affinity and in different conformations. *J. Immunol.* 152:2874.
- Hunt, D. F., W. M. Bodnar, D. Benjamin, K. Sakaguchi, N. Z. Zhu, J. Shabanowitz, R. A. Henderson, E. Appella, D. F. Hunt, and V. H. Engelhard. 1993. Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. *J. Immunol.* 151:2572.
- Storkus, W. J., H. J. Zeh III, R. D. Salter, and M. T. Lotze. 1993. Identification of T-cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution. *J. Immunother.* 14:94.
- Henderson, R. A., A. L. Cox, K. Sakaguchi, E. Appella, J. Shabanowitz, D. F. Hunt, and V. H. Engelhard. 1993. Direct identification of an endogenous peptide recognized by multiple HLA-A2.1 specific cytotoxic T cells. *Proc. Natl. Acad. Sci. USA* 90:10275.
- Wang, W., P. H. Gulden, R. Pierce, J. Shabanowitz, S. Man, D. F. Hunt, and V. H. Engelhard. 1997. A naturally processed peptide presented by HLA-A\*0201 is expressed at low abundance and recognized by an alloreactive CD8<sup>+</sup> cytotoxic T cell with apparent high affinity. *J. Immunol.* 158:5797.
- Potter, T. A., T. V. Rajan, R. F.2d. Dick, and J. A. Bluestone. 1989. Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature* 337:73.
- Connolly, J. M., T. A. Potter, E. M. Wormstall, and T. H. Hansen. 1988. The Lyt-2 molecule recognizes residues in the class I alpha3 domain in allogeneic cytotoxic T cell responses. *J. Exp. Med.* 168:325.
- Slingluff, C. L., Jr., A. L. Cox, R. A. Henderson, D. F. Hunt, and V. H. Engelhard. 1993. Recognition of human melanoma cells by HLA-A2.1-restricted CTL is mediated by at least six shared peptide epitopes. *J. Immunol.* 150:2955.
- Cox, A. L., J. Skipper, Y. Chen, R. A. Henderson, T. L. Darrow, J. Shabanowitz, V. H. Engelhard, D. F. Hunt, and C. L. Slingluff. 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 264:716.
- Holterman, M. J., and V. H. Engelhard. 1986. HLA antigens expressed on murine cells are preferentially recognized by murine cytotoxic T cells in the context of the H-2 major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* 83:9699.
- Holterman, M. J., A.-X. T. Le, and V. H. Engelhard. 1988. Weak stimulation of murine cytolytic T cells by human class I molecules maps to the  $\alpha_1$  and  $\alpha_2$  domains. In *Immunology of HLA*, Vol. 2. B. Dupont, ed. Springer-Verlag, New York, p. 140.
- Engelhard, V. H. 1994. Structure of peptides associated with MHC class I molecules. *Curr. Opin. Immunol.* 6:13.