

## Use of Combinatorial Peptide Libraries to Construct Functional Mimics of Tumor Epitopes Recognized by MHC Class I–Restricted Cytolytic T Lymphocytes

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### Summary

Identification of cytolytic T lymphocyte (CTL) epitopes presented by major histocompatibility complex (MHC) class I molecules on tumor cells is critical for the design of active immunotherapy. We describe the use of combinatorial peptide libraries with defined amino acids in two MHC anchor positions to search for epitopes that are recognized by H-2D<sup>b</sup>- and K<sup>b</sup>-restricted CTL specific for the mouse lymphoma EL4. An iterative strategy was used for screening libraries in which 16 amino acids were divided into 3 groups and 3 subgroups: α(AL, VT, FY); β(GS, P, DE); γ(KR, H, NQ). The proportions of each group and subgroup at individual peptide positions were changed in the library synthesis, and the effect of these changes on CTL activity was measured in a sensitive RMA-S cell assay. A single H-2D<sup>b</sup> epitope mimic was deduced from the original library that contained  $>2 \times 10^8$  potential peptides and was at least 9 logs more potent than the original library. Immunization of syngeneic mice with this peptide elicited CTL that lysed EL4 cells as well as RMA-S cells pulsed with peptides isolated from D<sup>b</sup> molecules of EL4 cells, indicating functional similarity between the mimicking peptide and the naturally processed CTL epitope. Furthermore, adoptive transfer of such a CTL line had a therapeutic effect in mice with EL4 established as an ascites tumor. Two H-2K<sup>b</sup>-restricted epitope mimics of the same tumor were also identified. Our method represents a novel approach for the construction of MHC class I–restricted targets that can serve as immunogens for active immunotherapy of cancer.

Most antigens recognized by CD8<sup>+</sup> cytolytic T lymphocytes (CTL) are derived from cytoplasmic proteins whose presentation involves the degradation by a proteasome proteinase complex into small peptides, transportation of peptides to the endoplasmic reticulum by peptide transporter-associated proteins, and binding to MHC class I molecules which then travel through the Golgi complex to reach the cell surface (1–3). The peptides are usually 8–10 amino acids long, with 2–3 primary anchor residues that interact with the MHC class I molecule (4, 5), and 2–3 amino acid residues that bind to the TCR (6). Further analysis of the requirement of MHC binding by a peptide indicates that several positions, besides the primary anchor and TCR binding amino acid residues, can be replaced only by a limited number of amino acids, suggesting a critical role of secondary anchor positions (7, 8). Synthetic peptides have been used for the induction of specific CTL by immunization either *in vitro* or *in vivo* (9–13), and vaccination with peptides can induce CTL-mediated immune responses against tumors (14, 15) and virus-infected cells (16) in mice. Identification of peptides that can elicit an efficient CTL response is, therefore, of practical importance for the

development of immunogens for active immunotherapy of cancer.

Several methods have been used to identify unknown CTL epitopes. If the amino acid sequence of a protein antigen is known, overlapping peptides of 8–10 amino acids in length can be synthesized and screened as CTL targets, an approach that has led to the discovery of a number of CTL epitopes derived from proteins of viral origin (17). CTL epitopes may also be identified subsequent to the search for MHC-binding motifs and measurement of the affinity of potential antigenic peptides for MHC molecules (18, 19). If the tumor antigen is not known, isolation of tumor-associated peptides from MHC class I molecules followed by purification and Edman sequencing (14, 20) or mass spectrometry (21) provides a direct way of identifying CTL epitopes. Genetic approaches, such as the screening of DNA expression libraries, provide another alternative and have led to the discovery of several human and mouse antigens recognized by specific CTL (22, 23). In many situations, however, because of the complexity and low abundance of the tumor epitopes presented, the application of these techniques is often time-consuming and sometimes problematic.

The epitope recognition by CD8<sup>+</sup> and MHC class I-restricted CTL can be degenerate. This opens the possibility of using combinatorial peptide libraries to search for peptides that mimic naturally presented epitopes. Analysis of several well-defined peptides that encode viral CTL epitopes by synthetic peptide libraries indicates that only a certain combination of amino acids is required for the interaction between MHC-peptide and TCR, whereas the amino acids in other positions can be changed without affecting function (8, 24). It is unclear from these studies, however, whether the peptide analogues can serve as functional mimics of the native peptide. We report here on combinatorial peptide libraries that were screened based on their ability to render RMA-S cells susceptible to lysis by specific CTL against unknown tumor antigens. The EL4 mouse lymphoma system, a model that we have studied in the past (25, 26), was used. Using an iterative strategy, effective peptides within the libraries were selected. Syngeneic mice immunized with these peptides generated K<sup>b</sup>- and D<sup>b</sup>-restricted CTL recognizing EL4 cells in vitro and in vivo.

## Materials and Methods

**Mice and Cell Lines.** Female C57BL/6 mice, 6–8 wks old, were purchased from Taconic Co. (Germantown, NY). The EL4 lymphoma, which is of C57BL/6 (H-2b) origin, was purchased from American Type Culture Collection (Rockville, MD). An EL4 line (B7<sup>+</sup> EL4) expressing the costimulatory molecule B7-1 was previously generated in our laboratory by infection of EL4 cells with pLXSN recombinant retrovirus containing murine B7-1 cDNA (25). RMA-S, a peptide transporter-defective lymphoma line (27), is of C57BL/6 origin. P815.K<sup>b</sup> and P815.D<sup>b</sup> are clones from the P815 mastocytoma, which had been transfected to express K<sup>b</sup> or D<sup>b</sup> genes, respectively (26). All cell lines were maintained in vitro at 37°C in IMEM containing 10% fetal bovine serum (referred to as “medium”).

**Peptide Library Synthesis.** Peptide libraries were synthesized on either a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using Boc amino acids or on a multiple peptide synthesizer (model AMS 422; Gilson Electrical Med. Inc., Middleton, WI) using Fmoc amino acids (28). Peptide mixtures corresponding to the D<sup>b</sup> motif were synthesized only by the Fmoc strategy, whereas peptide mixtures corresponding to the K<sup>b</sup> motif were synthesized by a mixed Fmoc and Boc strategy. The anchor residue positions (5) for K<sup>b</sup> peptide libraries were fixed at 5 (F, Y) and 8 (L) or for D<sup>b</sup> peptide libraries at 5 (N) and 9 (L), while all other positions were variable.

The strategy for synthesis was the same as previously described (29). Briefly, the variable X position corresponded to an approximately equimolar incorporation of 16 amino acids by a simultaneous coupling of the appropriate mixture which, when Fmoc amino acids were used, was  $\alpha(\text{A/L/V/T/F/Y})$  ratio 1:1.2:2:2:1.3:1.3;  $\beta(\text{G/S/P/D/E/})$  ratio 1:1:1.3:1:1;  $\gamma(\text{K/R/H/N/Q})$  ratio 1.3:2:1.6:1.3:1.3. Peptides were cleaved from the resin and deprotected by a 2-h reaction with TFA/water/thioanisole/ethanedithiol (100:5:5:2.5). After precipitation from cold ether, the precipitates were dissolved in formic acid and either diluted with water and lyophilized (library mixtures), or they were purified as single peptides by reverse-phase HPLC with a gradient of acetonitrile in 0.1% TFA. A previously described Th epitope, HBVc 128-140/I-A<sup>b</sup> (TPPAYRPPNAPIL) (30), was synthesized

and used for immunization. A K<sup>b</sup>-binding peptide, KHYKIRNL, from the murine p56<sup>lck</sup> sequence (31) was used as a control. Single peptides were analyzed on a Biolon AB mass spectrometer (Uppsala, Sweden).

Peptide libraries with the K<sup>b</sup> motif were synthesized by the Boc strategy as described previously (29). Free peptides were obtained from the peptide resin by reaction with 90% hydrogen fluoride/10% anisole for 1 h at 0°. After evaporation of hydrogen fluoride, the peptide-resin mixture was washed with ethyl acetate, and the peptides were dissolved in 50% acetic acid and obtained as a dry powder after lyophilization from 5% acetic acid. When the peptide libraries had been reduced to a mixture of 64 peptides (two possibilities at each of six positions; Fig. 1 B), peptide synthesis was done by the Fmoc strategy as described above. All subsequent libraries and individual peptides were synthesized by this method.

**Isolation and Purification of MHC Class I-associated Peptides.** Natural peptide mixtures and purified peptides from EL4 cells were prepared from either H-2K<sup>b</sup> or D<sup>b</sup> proteins by a combination of affinity chromatography and reverse-phase HPLC, as described (26).

**Assays for CTL Activity.** Peptide libraries, individual peptides, and peptide fractions eluted from either K<sup>b</sup> or D<sup>b</sup> molecules of EL4 cells (26) were reconstituted in PBS and further diluted in medium. RMA-S cells were incubated at 22°C for 48 h, labeled with <sup>51</sup>Cr at 22°C for 2 h, and incubated with peptides in 96-well V-shaped plates at 37°C for 1.5 h, after which they were used as targets for lysis by CTL. C2D is a CD8<sup>+</sup> CTL line specific for EL4 cells and reacts with a single peptide isolated from D<sup>b</sup> molecules (26). 4A2 is an EL4-specific CD8<sup>+</sup> CTL clone that was generated from a mouse that had been immunized with B7<sup>+</sup> EL4 cells (25, 26). It recognizes a single peptide isolated from K<sup>b</sup> molecules (see Fig. 3). The CTL activity was examined in a standard 4-h <sup>51</sup>Cr release assay with different E/T ratios as indicated.

**Generation of CTL by Immunization of Mice with Peptides.** C57BL/6 mice were injected subcutaneously with peptides at 100  $\mu\text{g}/\text{mouse}$  emulsified in IFA, with or without mixing the peptides with 100  $\mu\text{g}$  of the HBVc 128-140/I-A<sup>b</sup> Th peptide. 14 d later, spleen cells were prepared and stimulated with B7<sup>+</sup> EL4 cells for 5 d as described (25). In some experiments, the bulk-cultured spleen cells were restimulated in vitro every 7–10 d, and their cytolytic activity was examined in a standard 4-h <sup>51</sup>Cr release assay.

**Adoptive Transfer of the Mimicking Peptide-induced CTL for Treatment of Ascitic EL4 Tumor.** The method of adoptive transfer of CTL for treatment of established tumors has been described previously (32, 33). Briefly, C57BL/6 mice, in groups of five, were injected intraperitoneally with 10<sup>5</sup> EL4 cells. 3 d later, the mice were injected with 5  $\times$  10<sup>6</sup> cells of an EL4pepI-induced CTL line, followed on day 4 by an injection of 2,000 U i.p. human rIL-2 (Cetus Corp., Emeryville, CA) every day for 3 d. The control groups were injected with either IL-2 alone or IL-2 with a control CTL line that was specific for EL4pepII-A and lost CTL activity in in vitro cultures. The mice were monitored daily for survival.

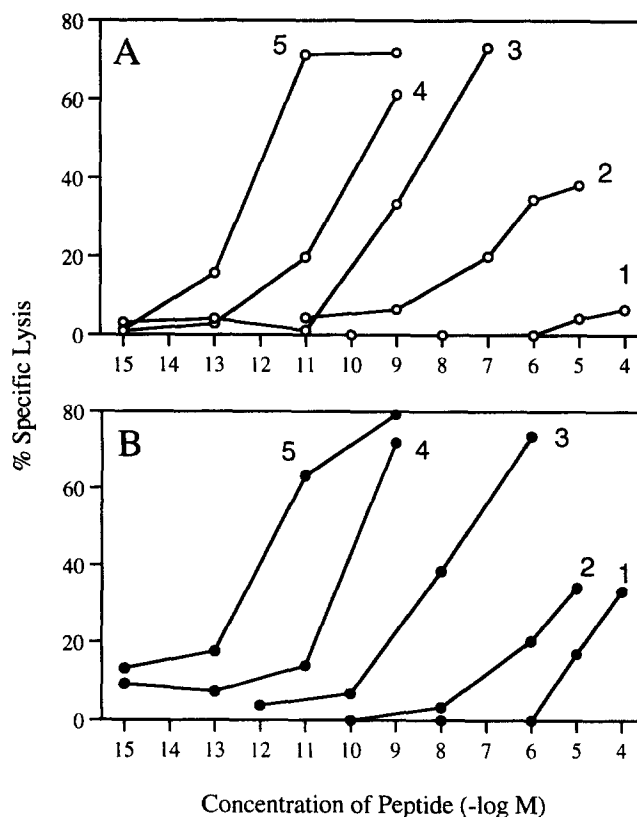
## Results

**Strategy for the Synthesis of Combinatorial Peptide Libraries.** We applied an iterative strategy (29) by dividing the 16 amino acids into three groups:  $\alpha(\text{AL, VT, FY})$ ;  $\beta(\text{GS, P, DE})$ ;  $\gamma(\text{KR, H, NQ})$ , and each group was further divided into three subgroups as can be noted by the grouping

within the parentheses. Four amino acids, C, M, I, and W, were omitted from the library for reasons previously described (29). In addition, M, I, and W were expected to decrease the solubility of the library, possibly to a concentration too low to enable us to detect biological response. The significance of our three-group strategy is that it allowed a simultaneous decrease (to 0) in the proportion of one group, an increase (two- to threefold, as indicated) in the proportion of a second group, and no change in the proportion of the third group at each of the variable residue positions in the peptide library. The effect of these changes on the biological activity of the peptide library indicated which of the groups, or amino acids within the groups, was contributing the most to the observed biological effect. Thus, a decrease in activity indicated that the first group was most important, an increase in activity indicated that the second group was most important, and an unchanged activity indicated that the third group was the most important. A repeat of the iterative strategy with the corresponding subgroups ultimately allowed the deduction of a single, highly active peptide.

**Screening of Peptide Libraries for D<sup>b</sup>- and K<sup>b</sup>-restricted Epitopes Recognized by EL4-specific CTL.** We first screened the peptide library by a D<sup>b</sup>-restricted CTL line, C2D, which lyses EL4 cells (26). The starting library was X-X-X-X-N-X-X-X-L, where X corresponded to any of 16 possible amino acids. This library consisted of a mixture of 16<sup>7</sup> = 268,435,456 peptides. The mixed peptides from this library were loaded onto RMA-S cells whose sensitivity to lysis by the C2D CTL was examined. No significant lysis was observed (Fig. 1 A, curve 1). In an attempt to increase the solubility of the library, we examined paired substitutions, DE or KR, at each of the variable X positions. As shown in Fig. 1 A (curve 2), substitution of DE at position 7 gave slightly increased activity. Substitution of DE at the other positions had no effect on activity, as was also observed for the corresponding substitution of KR (data not shown).

The library with the DE substitution at position 7 (33,554,432 peptides), X-X-X-X-N-X-DE-X-L, was subjected to an iterative strategy of variation. Each of the X positions was substituted with α<sub>2</sub>. This corresponded to a decrease (to 0) in the β group amino acids (G, S, P, D, E), an increase (twofold) in the γ group amino acids (K, R, H, N, Q), and no change in the α group of amino acids (A, L, V, T, F, Y). The results of these substitutions are shown in Table 1. Substitution of α<sub>2</sub> at positions 1, 6, and 8 had little effect, indicating that the α group was preferred at these positions; substitution of α<sub>2</sub> at position 4 decreased activity, indicating that the β group was preferred; substitution of α<sub>2</sub> at positions 2 and 3 increased activity, indicating that the γ group was preferred. This suggested that a reduced library of 54,000 peptides corresponding to α-γ-γ-β-N-α-DE-α-L would be more active. When a parallel iterative procedure was conducted by substituting β<sub>2</sub>γ at each of the X positions, the results were in agreement at positions 1, 4, 6, and 8, but differed for positions 2 and 3, where α was preferred (data not shown). Two possibilities at each of two positions indicated four combinations that



**Figure 1.** Increased potency of mimicking peptides in sensitization of RMA-S cells for lysis by EL4 antigen-specific CTL. Peptide libraries were reconstituted in PBS and further diluted in medium before use, as indicated. RMA-S cells were kept at 22°C for 48 h, labeled with <sup>51</sup>Cr at 22°C for 2 h, and incubated with peptides in 96-well V-shaped plates at 37°C for 1.5 h. They were then incubated with the EL4-specific, D<sup>b</sup>-restricted CTL line C2D (A) or with the K<sup>b</sup>-restricted CTL clone 4A2 (B) at an E/T ratio of 2:1. The cytolytic activity was examined in a standard 4-h <sup>51</sup>Cr release assay; background cpm (incubation of T cells and RMA-S cells without peptide) was subtracted. (A) The original library (labeled as 1) was X-X-X-X-N-X-X-X-L; three intermediate libraries (labeled as 2, 3, and 4) were X-X-X-X-N-X-DE-X-L; α-γ-γ-β-N-α-DE-α-L and AL-NQ-H-P-N-AL-DE-AL-L and the final peptide (labeled as 5) is EL4pepI. (B) The original library (labeled as 1) was X-X-X-X-FY-X-X-L (33,554,432 peptides); three intermediate libraries (labeled as 2, 3, and 4) were KR-X-X-X-FY-X-X-L (4,154,304 peptides), KR-γ-α-α-FY-γ-γ-L (18,000 peptides), KR-H-FY-AL-FY-KR-NQ-L (64 peptides), and the final peptide (labeled as 5) is EL4pepII.

needed to be investigated. Each of these libraries was synthesized and the results (data not shown) showed all the new libraries to be more active than the 33,554,432-peptide library. The library with γ at positions 2 and 3 was the most active and had an activity several orders of magnitude greater than that of the 33,554,432 library (Fig. 1 A, curve 3).

The 54,000-peptide library (α-γ-γ-β-N-α-DE-α-L) was subjected to further modifications (Table 2) by a logical division of each of the α, β, and γ groups into their component subgroups. Substitution of (AL)<sub>2</sub>VT at positions 1, 6, and 8 increased the activity indicating that AL was preferred at each of these positions. Substitution of GSP3 (threefold increase of proline) at position 4 also showed increased activity indicating that P was preferred at that posi-

**Table 1.** Lysis of RMA-S Cells Pulsed with Peptide Libraries by a H-2<sup>b</sup>-restricted CD8<sup>+</sup> CTL Line C2D

Peptide library sequence*									Concentration <sup>‡</sup> (-log M)	Percent lysis <sup>§</sup>	Deduced Critical Residues <sup>  </sup>
1	2	3	4	5	6	7	8	9			
No peptide										0.6	
X	X	X	X	N	X	DE	X	L	5	16.2	
									6	15.5	
									8	1.8	
$\alpha\gamma_2$	X	X	X	N	X	DE	X	L	5	17.8	$\alpha$
									6	12.9	
									8	2.5	
X	$\alpha\gamma_2$	X	X	N	X	DE	X	L	5	38.9	$\gamma$
									6	30.1	
									8	9.0	
X	X	$\alpha\gamma_2$	X	N	X	DE	X	L	5	27.0	$\gamma$
									6	29.1	
									8	9.9	
X	X	X	$\alpha\gamma_2$	N	X	DE	X	L	5	8.1	$\beta$
									6	4.2	
									8	2.7	
X	X	X	X	N	$\alpha\gamma_2$	DE	X	L	5	24.3	$\alpha$
									6	20.6	
									8	2.4	
X	X	X	X	N	X	DE	$\alpha\gamma_2$	L	5	29.9	$\alpha$
									6	19.3	
									8	3.6	

\*The intermediate libraries were synthesized with the X position incorporating an approximately equimolar mixture of 16 amino acids by a simultaneous coupling of the appropriate mixture, and with a position ( $\alpha\gamma_2$ ) replaced by the  $\alpha$  group of amino acids (AL, VT, FY) and twofold of the  $\gamma$  group amino acids (KR, H, NQ), but omitting the  $\beta$  group amino acids (GS, P, DE).

<sup>‡</sup>Peptide libraries were reconstituted in PBS and further diluted in medium. RMA-S cells were incubated at 22°C for 48 h, labeled with <sup>51</sup>Cr at 22°C for 2 h, and incubated with peptides at molar concentration as indicated in 96-well V-shaped plates at 37°C for 1.5 h.

<sup>§</sup>The lysis of the peptide-pulsed RMA-S cells by a H-2<sup>b</sup>-restricted CD8<sup>+</sup> CTL line C2D was examined in a standard 4-h <sup>51</sup>Cr release assay. E/T ratio = 4:1.

<sup>||</sup>The effect of  $\alpha\gamma_2$  amino acid substitution of peptide libraries on CTL activity was evaluated in comparison with the library X-X-X-X-N-X-DE-X-L. A decrease in CTL activity indicated that the  $\beta$  group was most important, an increase in activity indicated that the  $\gamma$  group was most important, and an unchanged activity indicated that the  $\alpha$  group was the most important. In this round screening, the deduced library was:  $\alpha$ - $\gamma$ - $\gamma$ - $\beta$ -N- $\alpha$ -DE- $\alpha$ -L.

tion. The corresponding substitutions at positions 2 and 3 gave equivocal results similar to our observation in the first iteration. As seen in Table 2, it was difficult to distinguish between KR and NQ. Other data indicated that histidine was preferred at position 3 (data not shown). Thus, there were  $2 \times 3 = 6$  possible combinations to investigate, and each of the corresponding libraries was synthesized. The results indicated that the 32 ( $2^5$ ) peptide library AL-NQ-H-P-N-AL-DE-AL-L was the most active (Fig. 1 A, curve 4). Finally, single amino acid substitutions (Table 3) indicated that the best single peptide was AQHPNAELL (referred to as EL4pepI; Fig. 1 A, curve 5). A comparison of the ability to sensitize RMA-S for lysis by the C2D CTL line between EL4pepI and the various libraries indicates

that the activity of EL4pepI was at least 9 logs higher than that of the starting library (Fig. 1 A).

A similar strategy was used to search for a K<sup>b</sup>-restricted CTL epitope. The starting library with the K<sup>b</sup> motif X-X-X-X-FY-X-X-L contained 33,554,432 ( $2 \times 16^6$ ) peptides. For screening, we used either the anti-EL4 CTL clone 4A2, which had been obtained by limiting dilution cloning of bulk-cultured spleen cells from C57BL/6 mice immunized with B7<sup>+</sup> EL4 cells (26) or bulk-cultured polyclonal T cells (25). Paired substitutions of KR, DE, or NS at position 1 showed that the KR substitution slightly increased the activity of the library. The reduced library KR-X-X-X-FY-X-X-L was subjected to the iterative process. Substitution of each of the five remaining X positions with  $\beta_2\gamma$ , fol-

**Table 2.** Lysis of RMA-S Cells Pulsed with Peptide Libraries by a H-2D<sup>b</sup>-restricted CD8<sup>+</sup> CTL Line C2D

Peptide library sequence*									Concentration (-log M)	Percent lysis	Deduced critical residues
1	2	3	4	5	6	7	8	9			
No peptide										0	
α	γ	γ	β	N	α	DE	α	L	8	73.5	
									10	10.4	
									12	0	
(AL) <sub>2</sub> VT	γ	γ	β	N	α	DE	α	L	8	72.5	AL
									10	41.9	
									12	8.9	
α	KRH <sub>3</sub>	γ	β	N	α	DE	α	L	8	68.3	KR, NQ
									10	3.9	
									12	2.0	
α	γ	KRH <sub>3</sub>	β	N	α	DE	α	L	8	68.3	KR, NQ
									10	0.8	
									12	0	
α	γ	γ	GSP <sub>3</sub>	N	α	DE	α	L	8	75.2	P
									10	82.6	
									12	11.0	
α	γ	γ	β	N	(AL) <sub>2</sub> VT	DE	α	L	8	78.0	AL
									10	74.1	
									12	32.8	
α	γ	γ	β	N	α	DE	(AL) <sub>2</sub> VT	L	8	77.6	AL
									10	79.0	
									12	29.0	

\*The intermediate libraries were synthesized and assayed as described in the legend to Table 1, except that amino acids in the subgroups from each α, β, and γ group were used for substitution. E/T ratio = 4:1.

lowed by subgroup iterations, ultimately led to a single highly active peptide, KHYLFRNL (referred to as EL4pepII). The results are summarized in Fig. 1 B, and the EL4pepII has a potency ~8 logs higher than that of the starting library.

*Induction of CTL Specific for EL4 Cells by Immunization with Epitope Mimics.* The two peptides EL4pepI and EL4pepII were used in an attempt to generate CTL against EL4 cells by immunizing C57BL/6 mice. Immunization with EL4pepI alone gave a weak CTL response against EL4 cells. However, injection of a mixture of EL4pepI and the helper peptide HBVc 128-140/I-A<sup>b</sup> elicited a strong CTL activity against EL4 cells, whereas immunization with HBVc 128-140/I-A<sup>b</sup> alone did not induce any CTL response. The CTL appeared to be specific for EL4 cells, since the P815 mastocytoma line was not lysed. In contrast, immunization of mice with either EL4pepII alone or a combination of EL4pepII and HBVc 128-140/I-A<sup>b</sup> did not induce any detectable EL4-specific CTL response (Table 4).

The failure of EL4pepII to elicit an EL4-reactive CTL suggested that it differed in the structure from the naturally

processed peptide. We observed that substitution of position 1 with A, L, G, T, S, or N had no effect on the activity in the RMA-S assay (data not shown), whereas all of the other positions were sensitive to change. Substitution of alanine at position 2 reduced activity substantially, substitution of alanine at position 3-8 reduced it much more, and conservative changes at positions 3, 4, 6, and 7 gave peptides with significantly reduced activity (data not shown). When a mixture of four peptides, EL4pepII-A, EL4pepII-L, EL4pepII-G, and EL4pepII-T (corresponding to EL4pepII with the amino terminal lysine substituted with the respective indicated amino acids), was used to immunize mice, CTL that lysed EL4 cells, but not P815.K<sup>b</sup> cells, could be generated (Fig. 2 A). Further analysis demonstrated that these CTL recognized EL4pepII-A and EL4pepII-T, but not EL4pepII-G or EL4pepII-L (Fig. 2 B). Immunization with EL4pepII-A or EL4pepII-T individually also induced EL4-specific CTL, whereas immunization with the control mlck peptide, which has a similar amino acid composition to EL4pepII and binds to K<sup>b</sup> molecules, did not (Fig. 2 C). The potency of the EL4pepII variants was further enhanced by immunization

**Table 3.** Lysis of RMA-S cells Pulsed with Peptide Libraries by a H-2<sup>b</sup>-restricted CD8<sup>+</sup> CTL Line C2D\*

Peptide library sequence									Concentration (-log M)	Percent lysis	Deduced critical residues
1	2	3	4	5	6	7	8	9			
No peptide										0.9	
AL	NQ	H	P	N	AL	DE	AL	L	8	90.3	
									10	69.6	
<b>A</b>	NQ	H	P	N	AL	DE	AL	L	8	83.3	A
									10	84.5	
AL	<b>N</b>	H	P	N	AL	DE	AL	L	8	29.7	Q
									10	3.5	
AL	NQ	H	P	N	<b>A</b>	DE	AL	L	8	97.0	A
									10	91.4	
AL	NQ	H	P	N	AL	<b>E</b>	AL	L	8	86.5	E
									10	78.1	
AL	NQ	H	P	N	AL	DE	<b>A</b>	L	8	3.2	L
									10	4.5	

\*The intermediate libraries were synthesized and assayed as described in the legend to Table 1. E/T ratio = 4:1. The deduced library was A-Q-H-P-N-A-E-L-L and designated as EL4pepI.

together with the HBVc 128-140/I-A<sup>b</sup> helper peptide (Table 5). Our results indicate that CTL induced by the mimicking peptides could recognize EL4 cells.

*Recognition of Naturally Presented EL4 Peptides by the Mimicking Peptide-induced CTL.* To demonstrate whether CTL induced by the mimicking peptides could recognize natu-

rally processed peptides on EL4 cells, we purified from EL4 cells both K<sup>b</sup>- and D<sup>b</sup>-associated peptides. Lysates were prepared from in vitro-cultured EL4 cells, and the peptide-MHC class I complex was purified by affinity chromatography using mAbs to either K<sup>b</sup> or D<sup>b</sup>, after which the eluted peptides were fractionated by reverse-phase HPLC. Individual peptide fractions were loaded onto RMA-S cells to sensitize them for lysis by CTL that had been induced by the mimicking peptides. The EL4pepI-induced CTL lysed RMA-S cells pulsed with fraction 8, 9, and 10 of peptides eluted from D<sup>b</sup> molecules, while they did not react with the other fractions. This recognition pattern was not identical, but similar to that of the C2D CTL line (Fig. 3 A). The EL4pepII-A-elicited CTL, similarly to the 4A2 CTL clone, specifically recognized fraction 9 of peptides eluted from K<sup>b</sup> molecules (Fig. 3 B). Our results demonstrate that immunization of mice with mimicking peptides can induce CTL that cross-react with natural tumor epitopes presented by EL4 cells.

*Treatment of an Established Ascites EL4 Tumor by Adoptive Transfer of the Mimicking Peptide-induced CTL.* To determine whether the CTL elicited by mimicking peptides could be used for treatment of an established tumor, we used a model in which the injection of C57BL/6 mice intraperitoneally with EL4 cells at 10<sup>5</sup>/mouse led to the rapid growth of EL4 cells as an ascites tumor. Mice treated with either IL-2 only or IL-2 combined with a control CTL line all died with ascites within 49 d of tumor cell injection. In contrast, among five mice treated with an EL4pepI-induced CTL line plus IL-2, two mice died with large ascitic tumors on days 53 and 59, respectively, and the three

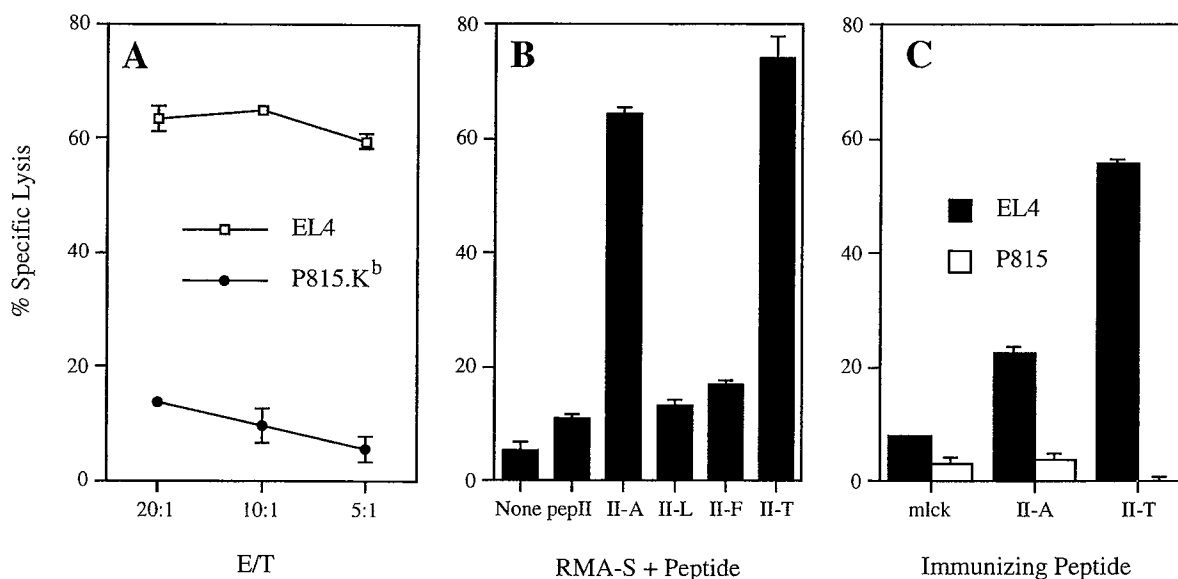
**Table 4.** Induction of EL4-specific CTL by the Mimicking Peptides EL4pepI and EL4pepII

Peptide <sup>‡</sup>	HBVc 128-140/I-A <sup>b</sup>	% Specific lysis/E:T ratio*						
		EL4			P815			
		10:1	5:1	1:1	10:1	5:1	1:1	
None	Yes	— <sup>§</sup>	4.3	0.7	—	1.4	1.3	
EL4pepI	No	16.6	—	3.5	2.3	—	0.7	
EL4pepI	Yes	65.0	—	58.3	5.1	—	1.1	
EL4pepII	No	1.6	—	0.0	3.1	—	0.8	
EL4pepII	Yes	19.0	—	5.3	16.0	—	1.3	

\*Spleen cells were prepared from the mice immunized with the peptides and were cocultured with B7<sup>+</sup> EL4 cells for two cycles of stimulation (7 d for each cycle). The cells were then tested for their CTL activity on EL4 and P815 cells in a standard 4-h <sup>51</sup>Cr release assay.

<sup>‡</sup>C57BL/6 mice were injected subcutaneously with the indicated peptides at 100 µg/mouse with or without 100 µg of HBVc 128-140/I-A<sup>b</sup> Th peptide in IFA.

<sup>§</sup>Not done.



**Figure 2.** Induction of EL4-specific CTL by immunization with variant peptides derived from EL4pepII. C57BL/6 mice were injected subcutaneously with a mixture of peptide EL4pepII-A, EL4pepII-L, EL4pepII-G, and EL4pepII-T at 100  $\mu$ g, each emulsified in IFA in a 0.05-ml vol. 2 wk later, spleen cells from immunized mice were harvested and stimulated twice in vitro with  $\gamma$ -irradiated B7<sup>+</sup> EL4 cells for 14 d. The cytolytic activity of the cultured spleen cells was examined against EL4 cells or P815.K<sup>b</sup> cells at the indicated E/T ratios (A) or against RMA-S cells pulsed with individual peptides, as indicated, at an E/T ratio of 1:1 (B). In both cases, a standard 4-h <sup>51</sup>Cr release assay was used. Mice were also immunized with an individual peptide from the control peptide mlck, EL4pepII-A, or EL4pepII-T at 100  $\mu$ g, and spleen cell cultures were prepared as described above. The cytolytic activity of the cultured spleen cells was examined against EL4 cells or P815 cells at an E/T ratio of 2:1 (C).

remaining mice were alive and had no sign of tumor on day 140, when the experiment was terminated (Fig. 4). Similar results were obtained in another experiment with five mice per group.

**Table 5.** Induction of EL4-specific CTL by the Variants of EL4pepII

Peptide <sup>‡</sup>	HBVc	Percent specific lysis/E:T ratio*					
		EL4			P815		
		10:1	5:1	1:1	10:1	5:1	1:1
None	Yes	— <sup>§</sup>	9.4	1.6	—	0.0	0.0
mlck	No	—	0.0	0.0	—	0.7	0.0
EL4pepII-A	Yes	—	8.3	4.6	—	0.0	0.0
	No	20.1	—	4.5	3.2	—	0.3
EL4pepII-T	Yes	67.6	—	54.3	0.3	—	0.0
	No	61.5	55.6	40.7	3.0	0.9	0.2

\*Spleen cells were prepared from the mice immunized with the peptides and were cocultured with B7<sup>+</sup> EL4 cells for a stimulation cycle of 7 d. After five cycles of stimulation, the cells were tested for their CTL activity on both EL4 and P815 cells in a standard 4-h <sup>51</sup>Cr release assay.

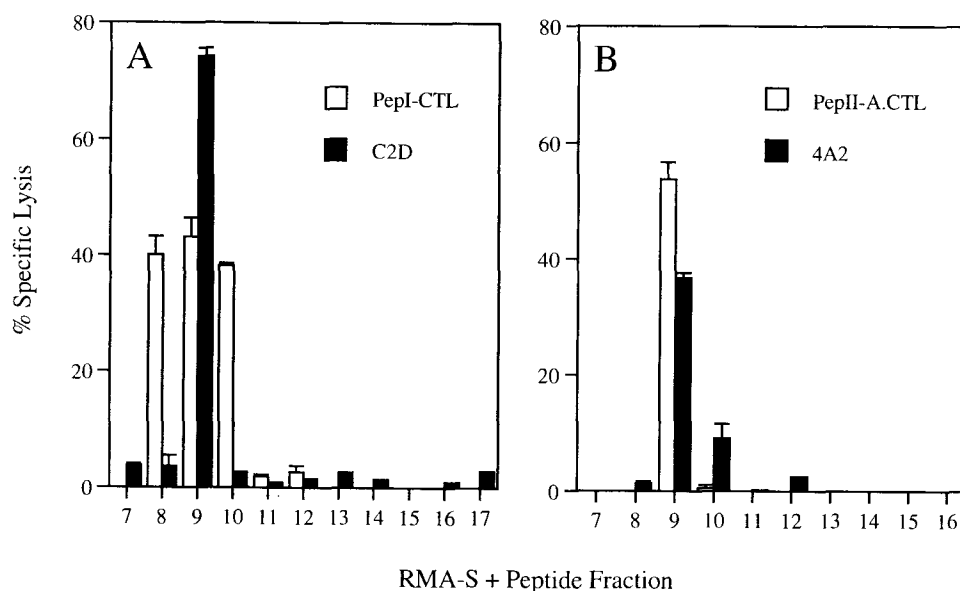
<sup>‡</sup>C57BL/6 mice were injected subcutaneously with two variants of EL4pepII, EL4pepII-A, and EL4pepII-T, or a control peptide mlck at 100  $\mu$ g/mouse with or without 100  $\mu$ g of HBVc 128-140/I-A<sup>b</sup> T helper peptide in IFA.

<sup>§</sup>Not done.

## Discussion

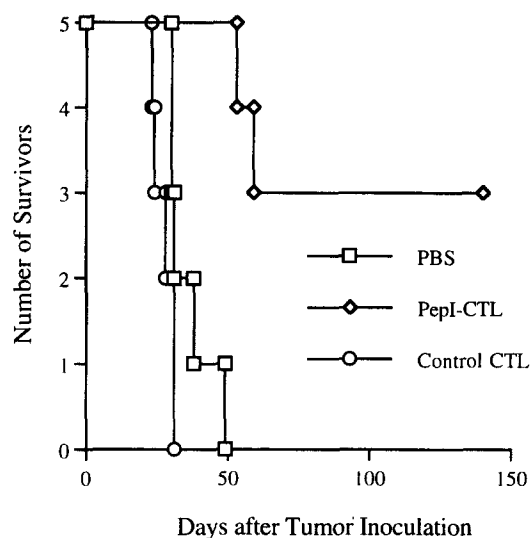
Using combinatorial peptide libraries, we have identified several functionally active peptides that can mimic the epitopes of tumor-associated antigens recognized by K<sup>b</sup>- and D<sup>b</sup>-restricted CTL and are specific for the EL4 mouse lymphoma. The mimicking peptides are recognized by CTL that were raised by immunizing mice with B7<sup>+</sup> EL4 cells, and they can induce CTL responses against EL4 cells. In principle, this method can be used to construct functional epitope mimics, provided that the presenting MHC molecules and the peptide-binding motif, are known. It thus represents a novel approach for exploring new targets for T cell-mediated immunotherapy of cancer.

Based on the motif and affinity of peptides to MHC class I molecules, a number of them have been successfully predicted from known proteins such as potential CTL epitopes (17–19). However, additional parameters appear to contribute to what constitutes a CTL epitope. For example, some peptides predicted by their motif are nonimmunogenic (34), or they are immunogenic, but the CTL induced by them do not react with naturally presented peptides (19, 35). In addition, not all high affinity peptides are immunogenic (19, 36). We showed previously that the C2D CTL line recognizes a subdominant epitope that can only induce a CTL response in the presence of costimulation by B7–CD28 interaction (26). The application of the iterative strategy using a multiple peptide synthesizer for rapid simultaneous synthesis of libraries, and the stable D<sup>b</sup>-restricted CTL clone C2D for screening, enabled us to find a single active peptide (EL4pepI) in ~10 wk. The CTL induced by



**Figure 3.** Immunization with the mimicking peptides can induce CTL that recognize naturally processed peptides on EL4 lymphoma. Reverse-phase HPLC was used for preparation and fractionation of K<sup>b</sup>- and D<sup>b</sup>-associated peptides from in vitro-cultured EL4 cells, as described in the Materials and Methods. The CTL line C2D (A) or 4A2 (B) was tested for cytolytic activity on RMA-S cells pulsed with individual HPLC fractions from either D<sup>b</sup> (A) and K<sup>b</sup> (B). Individual HPLC fractions were reconstituted in 200  $\mu$ l PBS and 1% aliquots were used to pulse RMA-S target cells. The E/T ratio was 4:1.

EL4pepI are MHC class I restricted, express CD8 (data not shown), and can lyse EL4 cells (Table 4) as well as RMA-S cells pulsed with naturally presented peptides eluted from D<sup>b</sup> molecules of EL4 cells (Fig. 3 A), but fail to lyse an allogeneic tumor, P815, or H-2D<sup>b</sup> transfectant P815.D<sup>b</sup> (data not shown). Similar results were obtained using EL4pepII-A and EL4pepII-T (Figs. 2, B and C, and 3 B). It has yet to be determined whether the mimicking peptides are more potent inducers of CTL than naturally processed peptides.



**Figure 4.** Treatment of mice with EL4 ascites tumor by adoptive transfer of EL4pepI-induced CTL. C57BL/6, in groups of five, were injected intraperitoneally with  $10^5$  EL4 cells. 3 d later, they were injected intraperitoneally with  $5 \times 10^6$  cells of an EL4pepI-induced CTL line followed the next day by an injection of 2,000 U i.p. of human rIL-2; the IL-2 injection was repeated once a day for three consecutive days. Control groups were injected with either IL-2 or IL-2 combined with a control CTL line. The mice were monitored daily for survival, and the experiment was terminated on day 140 after tumor injection.

However, the use of combinatorial peptide libraries represents a rapid and reliable approach to determine the functional mimics of unknown CTL epitopes.

Although EL4pepI itself did not produce a strong CTL response in syngeneic mice, injection of the peptide together with the Th epitope HBVc 128-140/I-A<sup>b</sup> (30), however, did induce a potent CTL response against EL4 cells (Table 4). Similarly, a P815 tumor-associated non-peptide (37) did not induce CTL unless a Th epitope was included in the immunization (38). These results underscore the importance of Th cells in the induction of EL4-specific CTL by peptides.

EL4pepII was found to be highly active in sensitizing RMA-S cells for lysis by CTL, indicating that it can trigger the cytolytic machinery through TCR ligation. However, CTL induced by immunization of mice with this peptide did not lyse EL4 cells (Table 4), although they can lyse EL4pepII-pulsed RMA-S cells (Johnston, J.V., J. Blake, and L. Chen, unpublished data). Furthermore, at least seven analogues of EL4pepII that differed only at position 1 were capable of sensitizing RMA-S cells, but only two of those (EL4pepII-A and EL4pepII-T) elicited CTL that cross-reacted with EL4 cells (Fig. 2). These results suggest that EL4pepII is immunogenic and argues that the failure of the peptide to induce anti-EL4 CTL was not caused by an inappropriate immunization. In many cases, high affinity of a peptide to the MHC class I molecule is correlated with its immunogenicity (36). Our preliminary experiments showed that EL4pepII-A binds H-2D<sup>b</sup> better than EL4pepII (Johnston, J.V., J. Blake, and L. Chen, unpublished data), suggesting this possibility.

Adoptive transfer of a CTL line from mice immunized with EL4pepI inhibited the growth of EL4 cells as an ascitic tumor and prolonged the survival of the tumor-bearing mice (Fig. 4), indicating that CTL induced by the mimicking peptide can destroy EL4 cells in vivo. Experiments are



underway to investigate the effect of the mimicking peptides in the induction of antitumor immunity in vivo. Preliminary data indicate that a weak protective immunity against challenge of EL4 cells can be achieved by simply immunizing with a mixture of EL4pepII-A with IFA. The

efficacy of immunization with mimicking peptides can probably be further increased by presenting them in conjunction with costimulation provided by B7-CD28 interaction (39, 40) or by immunizing peptide-pulsed dendritic cells (41).

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