INDUCTION OF OVALBUMIN-SPECIFIC CYTOTOXIC
T CELLS BY IN VIVO PEPTIDE IMMUNIZATION

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It is currently accepted that class I MHC-restricted CTL recognize a processed form of endogenous antigens. Initial work by Gooding and Townsend and their colleagues (1, 2) showed that expression of truncated versions of viral gene products could render target cells susceptible to CTL lysis. In conjunction with other findings, these data suggested that class I–restricted antigens are likely to be processed by mechanisms located within the cell cytoplasm (2, 3). T cell antigens can therefore be divided into two distinct groups based on separate processing pathways: endogenous, class I–restricted antigens such as viral components, which are degraded within the cytoplasm, and exogenous class II–restricted soluble antigens, which are processed by an endosomal degradation mechanism (2–4).

We have recently derived class I–restricted CTL specific for the soluble protein chicken OVA by using a transfected cell line expressing the OVA cDNA (5). Under usual circumstances of immunization with soluble OVA, this exogenous antigen is endocytosed by APC and then presented exclusively in association with class II MHC gene products. Indeed, OVA-specific class I–restricted CTL fail to recognize target cells in the presence of native OVA, whereas CTL recognition is readily achieved when OVA is introduced directly into the cell cytoplasm by using osmotic lysis of pinosomes (5). This is consistent with a strict requirement for cytosolic antigen localization for successful class I presentation.

In contast to the results obtained with whole proteins, peptide fragments can readily associate with MHC molecules on target cells surfaces in vitro and are presented for CTL recognition. This has been demonstrated for a number of viral antigens (6, 7) and transfected gene products, including OVA (5, 8). Given the potential for in vitro CTL recognition of peptide fragments, we set out to determine whether these same antigenic peptides can prime class I–restricted T cell responses in vivo. In this report we present evidence to suggest that certain peptides can elicit CTL after in vivo immunization. However, the ability of peptides to direct CTL recognition in vitro is not sufficient to predict their ability to prime CTL responses in vivo. In vivo priming appears to require peptide characteristics in addition to the presence of the CTL targeting determinants.

[1] *Abbreviations used in this paper:* CN OVA, cyanogen bromide-cleaved ovalbumin; PT OVA, partial tryptic digestion of ovalbumin; RP10, RPMI 1640 with 10% FCS and 50 μM 2-ME.

Materials and Methods

Mice.   Young adult C57BL/6 mice were obtained from the Scripps Clinic and Research Foundation vivarium (La Jolla, CA).

mAbs.   The antibodies used in this study were 13.4 (anti-Thy-1.2; reference 9), RL.172.4 (anti-CD4; reference 10), and 3.168 (anti-CD8; reference 11). These were used with guinea pig C to deplete the appropriate effector cell populations immediately before addition to cytotoxicity assays.

Antigens.   OVA (Grade VI; Sigma Chemical Co., St. Louis, MO) was used in native and denatured form or after partial cleavage with trypsin (PT OVA) or overnight cleavage with cyanogen bromide (CN OVA). CN OVA was prepared as described previously (5). Denatured OVA was the reduced and alkylated protein exposed to 8 M urea (5). Separate portions of denatured OVA were cleaved with 1% trypsin for 10, 25, and 135 min. Soybean trypsin inhibitor was added and the three digests were mixed to give the PT OVA preparation. The synthetic peptides $OVA_{229-276}$, $OVA_{242-276}$, and $OVA_{258-276}$ were synthesized on an automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). All three peptides were made in a single synthesis starting with the resin-linked carboxyl arginine. Portions of peptide resin were removed after the addition of residues 258, 242, and 229 to give the respective peptides. Peptide purity was assessed by HPLC and amino acid analysis.

Target Cells for Cytotoxicity Assays.   Target cells used were the class II negative lines, EL4 (C57BL/6, $H-2^b$ thymoma), thymidine kinase-deficient L cells (C3H, $H-2^k$ fibroblast), and L cells transfected with the $H-2K^b$ gene referred to as L/K^b (12). EL4 was transfected with a plasmid containing the OVA cDNA to generate the OVA-producing cell line E.G7-OVA, and has been described previously (5).

Priming Mice and Spleen Cell Cultures.   C57BL/6 mice were immunized intravenously with 100 μg OVA, denatured OVA, CN OVA, PT OVA dissolved in PBS, or PBS alone. Mice were also immunized intravenously with 200 or 20 nmol of synthetic peptides $OVA_{229-276}$, $OVA_{242-276}$, or $OVA_{258-276}$ dissolved in PBS. 7-15 d later, their spleens were removed and stimulated in culture. $25-35 \times 10^6$ responding spleen cells were stimulated with either 2×10^6 irradiated (20,000 rad) E.G7-OVA cells or 25×10^6 irradiated (3,000 rad) CN OVA-pulsed syngeneic spleen cells for 5 d in 10 ml of RPMI 1640 with 10% FCS and 50 μM 2-ME (RP10) in upright 25-cm² flasks (model 25100; Costar, Cambridge, MA) at 37°C in 7% CO_2/air. Syngeneic spleen cells were pulsed with CN OVA by suspending 10^8 cells in 1 ml of RP10 containing CN OVA at 500 μg/ml for 1.5 h at 37°C. CN OVA-pulsed cells were washed three times with RP10, irradiated, and then used as stimulator cells in culture.

Long-term CTL Lines.   The secondary cytotoxic effector population derived from mice immunized with 200 nmol of $OVA_{229-276}$ was harvested after 7 d of culture. 5×10^6 recovered cells were restimulated with 2×10^6 irradiated, E.G7-OVA cells and 2.5×10^7 irradiated, syngeneic spleen cells in 10 ml of RP10. Subsequent weekly restimulations were carried out with $2-4 \times 10^6$ responder cells, 2×10^6 irradiated E.G7-OVA cells, and 2.5×10^7 irradiated, syngeneic spleen cells in 10 ml of RP10 containing 5% supernatant from Con A-stimulated rat spleen cells and 50 mM α-methyl mannoside in upright 25-cm² flasks.

CTL Clone.   The E.G7-OVA-specific clone 2.4 was derived from E.G7-OVA-primed C57BL/6 mice and has been described previously (5). It recognizes $OVA_{258-276}$ in association with $H-2K^b$.

Cytotoxicity Assays.   Target cells (10^6) in 600 μl of RP10 were labeled with 300 μCi ^{51}Cr sodium chromate for 45 min. After washing, 10^4 labeled targets and serial dilutions of effector cells were incubated in 200 μl of RP10 in round-bottomed 96-well plates. Synthetic peptides were included in the appropriate assays at 10 μg/ml unless otherwise stated. After a 4-h incubation at 37°C, 100 μl supernatant was collected and specific lysis was determined as: percent specific lysis = 100 × [(release by CTL − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release in the absence of CTL was <20% of maximum release by detergent in all experiments.

Results

In Vivo Priming Using Fragmented OVA.   Class I-restricted CTL derived from C57BL/6 ($H-2^b$) mice primed with the E.G7-OVA transfectant cell line are specific

for the $OVA_{258-276}$ determinant restricted to H-2K^b (5). This appears to be the major or only OVA determinant for CTL responses on the transfected cell line. These CTL fail to recognize the syngeneic tumor target EL4 in the presence of native or denatured OVA (5). CN OVA sensitizes target cells for OVA-specific in vitro CTL lysis, as do preparations of OVA that have been trypsinized for relatively short periods. Prolonged trypsinization results in complete proteolysis at lysine 263 situated within the $OVA_{258-276}$ determinant with concomitant elimination of all CTL recognition (5).

We began by assessing the ability of native OVA, denatured OVA, and CN OVA to prime for E.G7-OVA-specific CTL in vivo. In addition, we used PT OVA that consisted of equimolar amounts of denatured OVA digested with trypsin for 10, 25, and 135 min. C57BL/6 mice were immunized intravenously with 100 μg of OVA, denatured OVA, CN OVA, or PT OVA dissolved in PBS or PBS alone. Spleen cells were taken 7–15 d later and stimulation in vitro with irradiated E.G7-OVA. Fig. 1 shows that PT OVA was the only preparation to give significant in vivo priming for a transfectant-specific CTL response. Experiments were repeated using three separate OVA preparations that had been digested with trypsin for 3.5 min, 7 min, 15 min, or overnight. All three partial digests primed for an E.G7-OVA-specific CTL response, while the overnight tryptic digest did not (data not shown). The specificity of the CTL response was identical to transfectant-primed activity; in both cases CTL were specific for $OVA_{258-276}$ restricted to H-2K^b (data not shown).

In Vivo Priming Using Synthetic OVA Peptides. CN OVA, which can sensitize EL4 cells for lysis by OVA transfectant-primed or PT OVA-primed CTL, was not capable of priming the CTL response when injected into mice in doses up to 1 mg per animal. Fractionation of CN OVA on Sephadex G50 columns in 50% formic acid and subsequently on HPLC allowed us to identify two targeting species in the digest: $OVA_{242-273}$ and $OVA_{242-285}$ (5) (methionine 273 is partly resistant to CNBr cleavage as reported by Nesbit et al. [13]). PT OVA, but not an overnight trypsin digest with cleavage at lys$_{263}$, served as an efficient in vivo immunogen. We knew that the CNBr
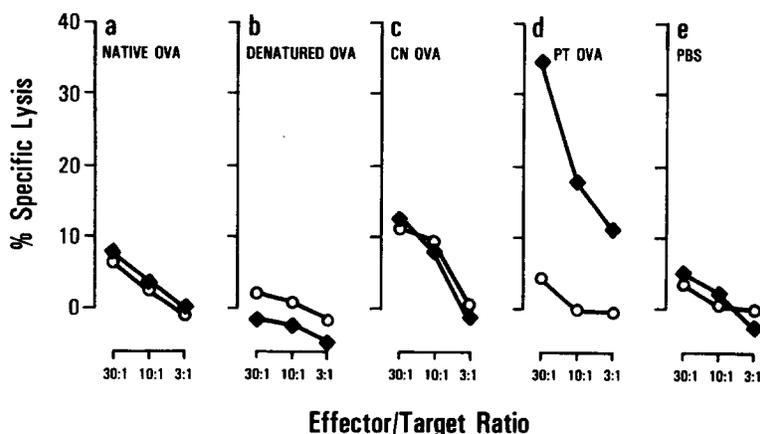

FIGURE 1. In vivo CTL priming using OVA digests. Spleen cells from C57BL/6 mice that had been injected intravenously with 100 μg of OVA (*a*), denatured OVA (*b*), CN OVA (*c*), PT OVA (*d*), or with PBS alone (*e*) were stimulated for 5 d with irradiated E.G7-OVA cells. The resulting effectors were then tested for lysis of ^{51}Cr-labeled (◆) E.G7-OVA or (O) EL4 target cells.
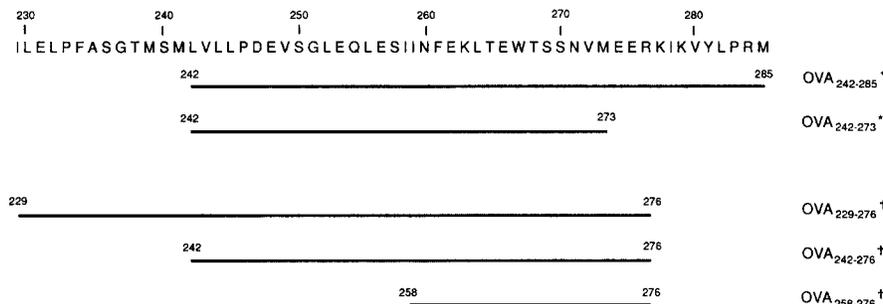
FIGURE 2.   Amino acid sequence of OVA peptides. (*) Peptides isolated from CN OVA preparations (5, 13). (+) Synthetic peptides used in this study.

fragments OVA$_{242-273}$ and OVA$_{242-285}$ were not priming peptides, yet the PT OVA peptide probably did not extend beyond 285 because of the presence of many tryptic cleavage sites just before this (arg$_{276}$, lys$_{277}$, lys$_{279}$, and arg$_{284}$) (Fig. 2). The shortest PT OVA peptide that would contain an intact targeting determinant is OVA$_{229-276}$. This peptide is an extension of the NH$_2$-terminal end of the fragments OVA$_{242-273}$ and OVA$_{242-285}$ present in the CN OVA preparations. We reasoned that the difference in in vivo priming potential observed between CN OVA and PT OVA may be controlled by the NH$_2$-terminal extension from residue 229 to 242. To directly test this hypothesis we synthesized the three OVA peptides with increasing lengths starting at residue 276 shown in Fig. 2.

The synthetic peptides offered two main advantages. First, purified synthetic fragments eliminated many of the uncertainties associated with the use of poorly defined peptide mixtures such as PT OVA. Second, all three peptides contained the same CTL determinant (OVA$_{258-276}$), since the synthesis was carried out by stepwise elongation of this core sequence. Consequently, all biological differences could then be unequivocally attributed to the NH$_2$-terminal extensions.

The data in Fig. 3 show that OVA$_{258-276}$, OVA$_{242-276}$, and OVA$_{229-276}$ were approximately equally effective in sensitizing EL4 for lysis by the E.G7-OVA-specific clone 2.4 in a 4-h ^{51}Cr-release assay. In contrast to this similarity in in vitro targeting activity, OVA$_{229-276}$ was the only one to give appreciable E.G7-OVA-specific priming in vivo after intravenous injection (Table I). In experiment 2 of Table I there was evidence of weak priming by OVA$_{258-276}$, but this peptide was still considerably less
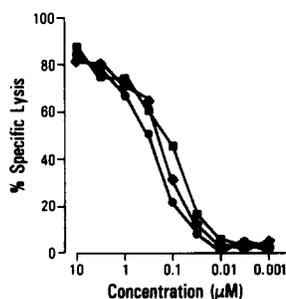

FIGURE 3.   Recognition of synthetic OVA peptides by an E.G7-OVA-specific CTL clone. Lysis of ^{51}Cr-labeled EL4 by the E.G7-OVA-specific clone 2.4 was tested in the presence of increasing concentration of (◆) OVA$_{258-276}$, (■) OVA$_{242-276}$, or (●) OVA$_{229-276}$ at an E/T ratio of 10:1.

efficient than the longer fragment OVA$_{229-276}$. We occasionally observed elevated background lysis using effectors derived from peptide immunized mice, such as the OVA$_{242-276}$-immunized animals in experiment 1. However, successful priming was evident as strong specific lysis of E.G7-OVA compared with EL4 targets. Neither E.G7-OVA- nor CN OVA-pulsed syngeneic spleen cells gave any primary in vitro response from spleen cell preparations derived from saline-injected mice. The data obtained with the synthetic peptides presented in Table I are consistent with the results obtained using the OVA digests (Fig. 1) and suggest that CN OVA fails to prime for E.G7-OVA-specific CTL because it lacks the NH$_2$-terminal extension beyond residue 242.

Characterization of CTL Derived from OVA$_{229-276}$-primed Mice. To further characterize this response, a CTL line was derived from OVA$_{229-276}$-primed mice by weekly restimulation with irradiated E.G7-OVA cells in IL-2 containing media. The effectors were CD8$^+$ T cells, since complement treatment of this line with anti-CD8 or anti-Thy-1 antibodies abrogated all CTL activity, whereas anti-CD4 had no effect (Fig. 4). The T cells recognized the OVA$_{258-276}$ determinant, and no activity could be revealed with the synthetic peptide OVA$_{229-263}$ (Fig. 5 *a*). Finally, CTL reactivity to OVA$_{258-276}$ was restricted to H-2K^b, demonstrated by the peptide-specific lysis of L cell targets transfected with the K^b gene (Fig. 5 *b*).

TABLE I

CTL Priming after Intravenous Injection of Synthetic OVA Peptides

| Exp.* | Peptide used for in vivo priming | Target cell | Percent specific lysis of targets following secondary in vitro culture with:‡ | | | |
| | | | E.G7-OVA | | CN OVA-pulsed spleen cells | |
			60:1§	20:1	60:1	20:1
1	OVA$_{242-276}$	E.G7-OVA	26	14	16	9
		EL4	25	16	12	8
	OVA$_{229-276}$	E.G7-OVA	59	39	63	58
		EL4	15	8	8	7
	None	E.G7-OVA	2	0	6	2
		EL4	3	1	5	0
2	OVA$_{258-276}$	E.G7-OVA	21	10	15	9
		EL4	11	7	5	6
	OVA$_{242-276}$	E.G7-OVA	8	3	0	0
		EL4	3	2	3	3
	OVA$_{229-276}$	E.G7-OVA	53	29	64	52
		EL4	10	6	4	5
	None	E.G7-OVA	- 1	- 2	- 4	- 3
		EL4	2	4	2	1

* C57BL/6 mice were immunized with 200 nmol of each peptide in Exp. 1 and 20 nmol of each peptide in Exp. 2. Controls received PBS alone. Animals were killed 7 d after immunization.

‡ Responding spleen cells were stimulated in vitro for 5 d in the presence of irradiated E.G7-OVA cells or irradiated, syngeneic spleen cells that had been pulsed with 500 μg/ml CN OVA.
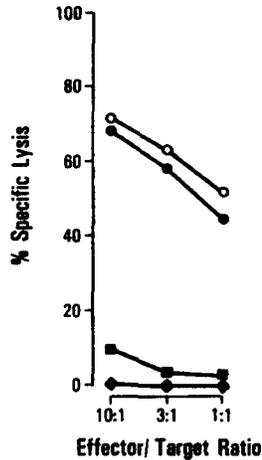
§ E/T ratio.

FIGURE 4.   Peptide-primed, E.G7-OVA-specific effectors are CD8$^+$ T cells. A CTL line derived from OVA$_{229-276}$-primed mice was treated with complement in the presence of (◆) anti-Thy-1, (■) anti-CD8, (●) anti-CD4 antibody, or (O) no antibody before addition to ^{51}Cr-labeled E.G7-OVA.

Discussion

We have shown that intravenous injection of C57BL/6 mice with the OVA$_{229-276}$ peptide in saline elicits CTL that are identical to those derived from E.G7-OVA-primed mice in terms of antigen specificity and MHC restriction. These effectors clearly differ from those induced by primary, in vitro stimulation of C57BL/6 spleen cells with OVA peptides that we have described previously (14). In vitro culture of naive splenocytes in the continuous presence of CN OVA elicits CTL that are largely specific for a determinant distinct from OVA$_{258-276}$ and that show a broad MHC crossreactivity pattern (14). A minor component within this bulk population is specific for OVA$_{258-276}$ in association with H-2K^b (data not shown). All CTL induced against OVA digests or synthetic OVA peptides by primary in vitro stimulation fail to lyse E.G7-OVA targets (14), in contrast to those derived by in vivo priming with OVA$_{229-276}$ (Table I) or PT OVA (Fig. 1). Neither CN OVA–pulsed cells (Table I) nor E.G7-OVA (Fig. 1 and Table I) stimulate OVA-specific primary CTL responses in vitro. We suspect that this failure reflects low densities of cell surface MHC-peptide complexes as discussed earlier (14).

E.G7-OVA-specific effectors from OVA$_{229-276}$-primed mice express CD8, not CD4, and are specific for the OVA$_{258-276}$ determinant in association with H-2K^b. Our data
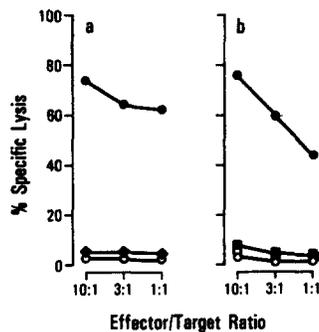

FIGURE 5.   MHC-restricted peptide reactivity of peptide-primed, E.G7-OVA-specific CTL. (a) A long-term CTL line derived from OVA$_{229-276}$-primed mice was tested for lysis of EL4 in the presence of 10 μg/ml (●) OVA$_{258-276}$, (◆) OVA$_{229-263}$, or (O) media alone. (b) The same CTL line was tested for lysis of L/K^b cells (closed symbols) or L cells (open symbols) in the presence (●, O) or absence (■, □) of 10 μg/ml OVA$_{258-276}$.

do not rule out the possibility that peptides longer than $OVA_{229-276}$ in PT OVA can also prime a similar CTL response, only that whole OVA is unable to do so. We have failed to induce any transfectant-specific CTL using native OVA at doses from 100 μg to 1 mg per animal. It should be emphasized that we set out to compare exogenous peptide immunization with priming by cells that endogenously express the protein. Consequently, we have concentrated exclusively on E.G7-OVA-specific T cells, whereas OVA priming may elicit CTL that have other specificities (15). It has also been reported previously that complete OVA digests fail to prime CTL in vivo (15). This is consistent with our current studies. We extend these earlier findings by suggesting that the failure in $H-2^b$ mice results from deletion of residues in the N terminal to position 242. Thus, $OVA_{229-276}$ can prime a CTL response, whereas $OVA_{242-276}$ or the CN OVA peptides $OVA_{242-285}$ and $OVA_{242-273}$ fail to do so.

It is possible that the OVA segment between residues 229 and 242 represents a Th determinant that is required for optimal CTL induction. To our knowledge no such helper determinant has been mapped to this region. Furthermore, this explanation would appear unlikely since native OVA that clearly contains this region is not an immunogen in this assay system. A second possibility to explain the immunogenicity of $OVA_{229-276}$ could involve an enhanced peptide-MHC association for the longer peptides. The dose titrations presented in Fig. 3 would argue against this since all three peptides have the same potency for CTL targeting in vitro.

Given their potential for CTL activation in vitro, it might at first appear surprising that $OVA_{242-276}$ and $OVA_{258-276}$ fail to elicit CTL in vivo. We have recently demonstrated that peptides can sensitize glutaraldehyde-fixed target cells for lysis by the E.G7-OVA-specific clone 2.4 (16). Therefore, in vitro peptide-MHC association can obviously occur at the cell surface and does not require access to the intracellular pathway of class I antigen presentation. Recent work by Hosaka, Bennink, Yewdell, and coworkers (17, 18) on the traditional class I-restricted antigen, influenza virus, demonstrated that access to this intracellular pathway is essential for in vivo CTL priming. While it is generally accepted that noninfectious virus preparations fail to immunize CTL, they demonstrated that inactivated influenza virus can successfully vaccinate CTL so long as it introduces sufficient viral components directly into the cell cytoplasm (17, 18). We have previously shown that native OVA fails to provide peptides that associate with class I MHC molecules unless it is directly introduced into the target cell cytoplasm for intracellular degradation (5). Our results presented here show that $OVA_{229-276}$ is distinct from whole OVA and the shorter targeting peptides, such as CN OVA or $OVA_{258-276}$, in its ability to prime CTL in vivo. We suggest that this priming reflects a capacity for $OVA_{229-276}$ to enter the class I presentation pathway in vivo. Antigenic peptides that can only associate with MHC at the cell surface may behave like other exogenous molecules and fail to elicit a class I-restricted response.

To date there have been no other reports of successful in vivo priming of CTL using synthetic targeting peptides. We have injected C57BL/6 mice with the 16-residue, D^b-restricted influenza nucleoprotein peptide (residues 365–380) originally identified by Townsend et al. (6) and found no evidence for influenza-specific CTL priming with doses comparable with the ones used in this study (data not shown). Most class I-restricted targeting peptides could therefore resemble $OVA_{258-276}$ rather than $OVA_{229-276}$ and fail to prime CTL in vivo.

The mechanism by which $OVA_{229-276}$ may enter the class I presentation pathway is completely unknown. It is intriguing that this same peptide, but not the native protein, was shown to inhibit protein translocation across the endoplasmic reticulum in an in vitro translation system (19). The authors concluded that residues 234–254 contained the signal sequence for OVA secretion. While the significance of this observation remains controversial in terms of OVA secretion (20, 21), the fact remains that this peptide exhibits a second unusual biological characteristic that could be associated with its immunological function that we have uncovered. Perhaps $OVA_{229-276}$ is unusual in that it can pass through the cell membrane from the outside, possibly after endocytosis. Thus, the enhanced immunogenicity observed as the peptide length is increased from $OVA_{258-276}$ to $OVA_{229-276}$ may reflect the conformational constraints thought to maintain correct peptide-bilayer interaction during membrane translocation (22, 23).

There is good evidence that the class I and class II MHC-encoded molecules function as restriction elements for peptides that derive from separate antigen processing and presentation systems (3, 24, 25). Most exogenous soluble proteins, such as OVA and inactivated virus particles that cannot fuse with cells, fail to activate class I–restricted T cells in vivo or in vitro. The peptide $OVA_{229-276}$ is an obvious exception to this rule since it can prime $H-2K^b$-restricted CTL after injection into mice. There are other examples in the literature of successful in vivo induction of class I–restricted T cells by peptide (26) or purified viral protein preparations (27–29). Whether these successes depend on special properties of the polypeptides themselves or on effects of the adjuvants used is not known. It has been suggested that specialized APC can phagocytose other cells and cycle the degradation products into their class I presentation system (4). Thus, soluble proteins that become associated with cells that are then phagocytosed could enter the class I pathway by this route. It has also been reported that virus-specific, class I–restricted T cells can be restimulated in vitro with purified proteins. For example, influenza hemagglutinin (30, 31) and hepatitis B surface antigen (32) reactivate memory CTL during in vitro culture. One explanation for this may be that the proteins are processed (i.e., degraded) extracellularly to expose the class I–restricted epitope that binds the MHC molecule on the surface of the stimulator cell. These would then be examples of cell-free antigen processing and would not reflect the ability of soluble antigen to enter the class I pathway of antigen processing and presentation. Indeed, we have reported that serum components can process denatured OVA (5).

We have defined the minimum sequential requirement for direct priming within the $OVA-H-2K^b$ system. Analysis of the conformational characteristics associated with this priming peptide will provide us with valuable structural data that could then be applied to other class I–restricted systems such as infectious virus preparations.

Summary

CTL recognize peptide forms of processed, foreign antigens in association with class I molecules encoded by the MHC and are usually directed against endogenously synthesized "cellular antigens," such as those expressed by virus-infected cells. In vitro studies have shown that small exogenous peptides can directly associate with class I molecules on the cell surface and mimic the target complex derived by intracellular processing and presentation. We have recently generated OVA-specific, $H-2K^b$-restricted CTL by immunizing C57BL/6 mice with a syngeneic tumor line transfected

with the OVA cDNA. The CTL recognize the OVA transfectant E.G7-OVA and the synthetic peptide $OVA_{258-276}$, but fail to recognize the native protein. We reasoned that given the potential for direct peptide/class I association observed in vitro, $OVA_{258-276}$ may induce CTL after in vivo priming. However, we found that this is not the case. $OVA_{258-276}$ and peptides of increasing lengths up to $OVA_{242-276}$ and $OVA_{242-285}$, which are all able to form the target complex in vitro, are inefficient at priming E.G7-OVA-specific CTL responses after intravenous injection. This is also true for both native and denatured OVA. In contrast to these results, the synthetic peptide $OVA_{229-276}$ corresponding to a peptide in a partial tryptic digestion of OVA can efficiently prime C57BL/6 mice in vivo after intravenous injection. This peptide elicits CTL that appear identical to those derived from animals immunized with syngeneic cells producing OVA endogenously. These results are discussed in terms of separate class I and class II antigen presentation pathways and the ability of only certain, exogenous antigens to enter the cytoplasmic, class I pathway.

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