

CLASS I-RESTRICTED PROCESSING AND PRESENTATION OF EXOGENOUS CELL-ASSOCIATED ANTIGEN IN VIVO

BY FRANCIS R. CARBONE AND MICHAEL J. BEVAN

*From the Department of Immunology, Research Institute of Scripps Clinic,
La Jolla, California 92037*

It is now well accepted that both class I and class II MHC-restricted T cells recognize denatured or fragmented forms of protein antigens derived as a consequence of intracellular processing events (1-3). While there exists some evidence to suggest that class I and class II presentation rely on distinct processing mechanisms (2, 4, 5), the extent that this difference limits the accessibility of certain types of antigens to one or another presentation pathway remains unclear. In the case of class I presentation it has been shown that, at least in vitro, exogenous nonreplicating antigens fail to sensitize targets for CTL recognition (6). In contrast, direct introduction of the same proteins into the cell cytoplasm results in effective CTL lysis (7, 8). Despite these in vitro studies, there is some evidence to suggest that in vivo, exogenous antigens may be processed for class I-restricted T lymphocyte recognition. For example, it has been shown that in MHC heterozygous F₁ mice, CTL restricted to one parental MHC-encoded product can be primed against foreign minor histocompatibility antigens or viral antigens by introducing the antigens on cells expressing the MHC of the other parent (9, 10). Consequently, these cell-associated antigens must be taken up, processed, and then presented to CTL precursors by host cells.

In this article we address whether this observation can be extended to include soluble proteins. We found that exogenous protein antigens can be processed in vivo for presentation to class I-restricted T cells provided that the antigen is introduced in a cell-associated form.

Materials and Methods

Mice. C57BL/6 (H-2^b), BALB.B (H-2^b), BALB/c (H-2^d), and (BALB.B × BALB/c)F₁ mice were from the Scripps Clinic and Research Foundation vivarium.

Antigens. OVA (Grade VI; Sigma Chemical Co., St. Louis, MO) was used in the native form. β-GAL (Grade VIII; Sigma Chemical Co.) was used in the native form and after boiling in 1 M NaOH for 2 min to give an alkali digest.

Tumor Cells and Transfectants. The tumor cells used in this study were the Ia⁻ lines EL4 (C57BL/6, H-2^b thymoma) and P815 (DBA/2, H-2^d mastocytoma). Derivation of the OVA-producing EL4 transfectant, E.G7-OVA, has been described previously (7). The β-GAL producing transfectant, P13.1, was derived by electroporation of 10⁷ P815 cells in 1 ml of PBS with 10 μg of Pst I linearized pCH110 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ)

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Address correspondence to Dr. F. R. Carbone, Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

and 1 μg of Pvu I linearized pSV2 neo (11) followed by selection in 400 $\mu\text{g}/\text{ml}$ of G418. The C3-4 transfectant, kindly provided by Drs. H.-G. Rammensee and U. Theopold, was derived from the BALB/c hybridoma igm 662 by transfecting with a plasmid encoding the β -GAL gene fused to the third and fourth exon of IgM heavy chain (12).

Immunization. Mice were immunized intravenously with a 200 μl suspension of 25×10^6 splenocytes after a protein pulse or cytoplasmic loading. For isotonic pulsing, 120×10^6 splenocytes were treated with $\text{NH}_4\text{Cl}/\text{Tris}$ to remove red blood cells and then suspended in a 1 ml HBSS solution of protein at 10 mg/ml. After a 10 min incubation at 37°C , the suspension was diluted with HBSS, washed twice, and the cells were irradiated (1,000 rad) before their use for immunization. For cytoplasmic loading, 120×10^6 red blood cell depleted splenocytes were suspended in 1 ml of a 10 mg/ml hypertonic solution of protein for 10 min at 37°C . The hypertonic medium was made up as follows: 0.5 M sucrose, 10% w/v polyethylene glycol 1000, 10 mM Hepes, pH 7.2, in RPMI 1640 medium. The cells were rapidly diluted in a hypotonic solution of 60% HBSS and 40% water, incubated for a further 2 min at 37°C , pelleted, and washed twice in HBSS. The cells were irradiated (1,000 rad) before their use for immunization. Soluble proteins were injected intravenously in a volume of 200 μl solution in HBSS.

In Vitro Stimulation of Effector Populations. Spleen cells (35×10^6 cells) from normal or immunized mice which had been primed 7–11 d earlier were incubated for 5 d with 3×10^6 E.G7-OVA cells (irradiated with 20,000 rad) for OVA responses or 3×10^6 C3-4 cells (irradiated with 20,000 rad) for β -GAL responses. Cultures were maintained in 10 ml of RP10 (RPMI 1640 with 10% FCS and 50 μM 2-ME) in upright 25-cm² flasks at 37°C in 7% CO_2/air . For the experiments using in vitro depletion, spleen cells from naive C57BL/6 mice and C57BL/6 mice primed with OVA pulsed cells were depleted of CD4 and CD8 positive cells using guinea pig complement and the mAbs 2B6 (anti-CD4, reference 13) and 3.168 (anti-CD8, reference 14), respectively. Surviving cells were stimulated either alone or in various combinations for 5 d with irradiated E.G7-OVA cells as described above.

Cytotoxicity Assay. Target cells were used without prior treatment, or after pulsing 10^7 cells for 10 min with a 1 ml solution of protein in RPMI (10 mg/ml), or after cytoplasmic loading with a 10 mg/ml hypertonic solution as described previously (7). The cells were then labeled with 300 μCi sodium chromate for 60 min. After washing, 10^4 labeled targets and serial dilutions of effector cells were incubated in 200 μl of RP10 for 4 h at 37°C . After a 4 h incubation at 37°C , 100 μl of supernatant was collected and the specific lysis was determined as: Percent specific lysis = $100 \times [(\text{release by CTL} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release})]$. Spontaneous release in the absence of CTL was <25% of maximal release by detergent in all experiments.

Results

Class I-restricted CTL Priming by Protein-pulsed and Protein-loaded Splenocytes. We have derived an OVA-expressing EL4 transfectant, E.G7-OVA, that primes C57BL/6 mice in vivo for an OVA-specific, class I-restricted CTL response. The CD8⁺ effectors, specific for a determinant mapped by the peptide OVA₂₅₈₋₂₇₆ in association with H-2K^b, lyse EL4 in the presence of soluble OVA only if the protein is introduced into the cell cytoplasm by osmotic lysis of pinosomes (7). To assess whether this technique could be used for in vivo immunization, we introduced soluble OVA into the cytoplasm of splenocytes from C57BL/6 mice by cytoplasmic loading with a 10 mg/ml OVA solution. These cells were injected intravenously into syngeneic mice. In addition, we also injected mice with syngeneic splenocytes that had been simply "pulsed" with OVA, i.e., incubated for a short time in isotonic saline containing OVA. Other mice were injected intravenously with 10–500 μg soluble OVA. Spleen cells were taken from these mice 7–11 d after immunization and restimulated in vitro with the E.G7-OVA transfectant. After 5 d in culture, priming was assessed by the presence

of OVA-specific effectors capable of lysing E.G7-OVA. Uninjected or saline injected responder mice gave no OVA transfectant-specific CTL response under these conditions (Fig. 1 *d*). Similarly, mice injected with 10–500 μg soluble OVA showed no evidence of CTL priming confirming our previous results (7, 15) and shown here for the 100 μg dose (Fig. 1 *c*). In contrast to this, spleen cells from responder mice that had been injected with syngeneic OVA-loaded cells showed an excellent transfectant-specific CTL response (Fig. 1 *a*). Optimal protein concentrations were 3–10 mg/ml of OVA in hypertonic media and reproducible in vivo priming could be achieved at concentrations as low as 1 mg/ml. To our initial surprise, an injection of spleen cells that had been simply incubated with OVA under isotonic conditions also primed the CTL response (Fig. 1 *b*). This latter result was unexpected, since previous in vitro experiments, using transfectant-specific effectors, demonstrated that whereas cytoplasmic loading introduced soluble protein into the class I pathway of presentation, exogenous protein addition, or pulsing, did not. Variation of pulsing times between 5 and 30 min did not appreciably alter the effectiveness of in vivo priming. However, it was generally observed that OVA-pulsed splenocytes were somewhat less effective in vivo immunogens than were splenocytes that had been put through the cytoplasmic loading procedure (Fig. 1, *a* and *b* and data not shown).

Experiments using a second soluble protein, β -GAL from *Escherichia coli* combined with a different mouse strain, BALB/c, paralleled those shown for OVA and C57BL/6 mice. For assaying β -GAL-specific cytolysis, the target used was the P13.1 cell line (P815 [DBA/2, H-2^d] transfected with the pCH110 β -GAL expression vector). Un-

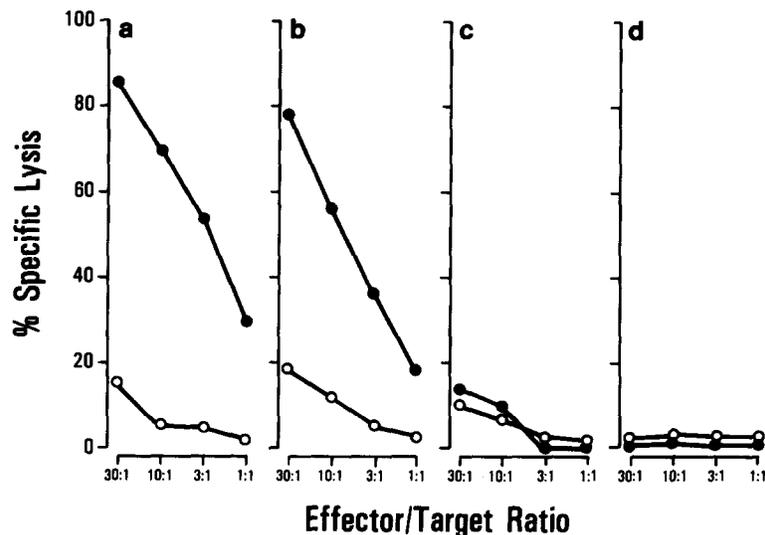


FIGURE 1. In vivo CTL priming by OVA. Spleen cells from C57BL/6 mice that had been injected intravenously with (a) 25×10^6 syngeneic spleen cells loaded with a 10 mg/ml hypertonic solution of OVA, (b) 25×10^6 syngeneic spleen cells pulsed with a 10 mg/ml OVA solution in HBSS, (c) 100 μg of OVA, or (d) PBS were stimulated in vitro for 5 d with E.G7-OVA cells. The resulting effectors were then tested for lysis of ^{51}Cr -labeled (●) E.G7-OVA or (○) EL4.

fortunately, this cell line induced an unacceptable background CTL response in vitro that crossreacted on the control P815 targets and was not β -GAL specific. To circumvent this problem, we made use of a completely unrelated β -GAL-producing transfectant of BALB/c origin, C3-4, for secondary in vitro stimulations. Fig. 2 shows that cytoplasmic loading or pulsing with β -GAL generated BALB/c spleen cell preparations that were equally immunogenic for a P13.1-specific CTL response. These effectors also recognized the β -GAL-expressing C3-4 line (data not shown) and an alkali digest of β -GAL (see below) showing that they are β -GAL specific. In contrast to the successful priming with cell-associated β -GAL, an injection of 100 μ g soluble β -GAL did not prime the response (Fig. 2 c). Since neither EL4 nor P815 cells express class II MHC gene products, and the lysis shows a syngeneic restriction, these OVA- and β -GAL-specific effectors are class I MHC restricted.

Protein-specific CTL Fail to Detect the Presentation of Exogenous Soluble Antigen. OVA-specific CTL lines and clones derived from C57BL/6 mice primed with the E.G7-OVA transfectant failed to lyse targets pulsed with the native protein (7). This is also true for β -GAL-specific, H-2^d-restricted CTL derived by C3-4 priming (12, 16). It was possible that using protein-pulsed splenocytes as immunogen may have primed for a completely different CTL effector population that could recognize exogenous antigens presented in association with class I. Figs. 3 and 4 show that this was not the case. The E.G7-specific effectors derived from cytoplasmic loading or protein pulsing could lyse EL4 targets only after the protein was introduced into the cell cytoplasm by osmotic lysis of pinosomes (Fig. 3) or after the H-2K^b-binding peptide was released from OVA by CNBr treatment (data not shown).

Similar results were found for the H-2^d-restricted β -GAL-specific effectors (Fig.

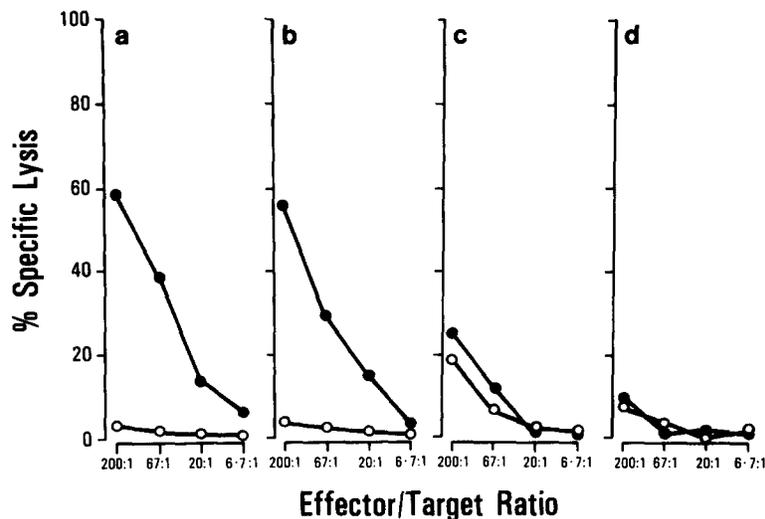


FIGURE 2. In vivo CTL priming by β -GAL. Spleen cells from BALB/c mice that had been injected intravenously with (a) 25×10^6 syngeneic spleen cells loaded with a 10 mg/ml hypertonic solution of β -GAL, (b) 25×10^6 syngeneic spleen cells pulsed with a 10 mg/ml β -GAL solution in HBSS, (c) 100 μ g of β -GAL, or (d) PBS were stimulated in vitro for 5 d with C3-4 cells. The resulting effectors were then tested for lysis of ^{51}Cr -labeled (●) P13.1 or (○) P815.

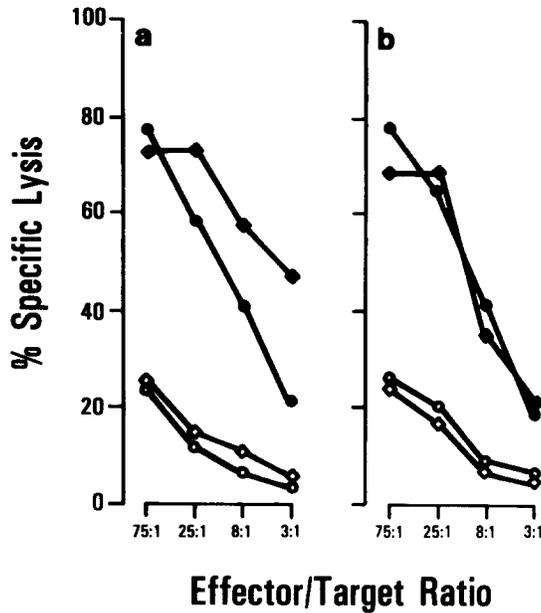


FIGURE 3. In vitro recognition by OVA-specific CTL requires cytosolic localization. Effectors derived from C57BL/6 mice primed with (a) syngeneic cells after cytoplasmic loading with a 10 mg/ml solution of OVA or (b) syngeneic cells pulsed with a 10 mg/ml solution of OVA were assayed in a ⁵¹Cr-release assay with (●) E.G7-OVA, (○) EL4 cells alone, (◆) EL4 loaded with a 10 mg/ml solution of OVA by osmotic lysis of pinosomes, or (◇) EL4 pulsed with a 10 mg/ml solution of OVA in HBSS.

4). The only significant difference is the relatively weak lysis of target cells presenting the cytoplasmic β -GAL. This effect appears to be a function of the protein rather than the effectors since CTL derived from transfectant primed mice also show a comparable low level of lysis of these same cells (16). Recognition of the β -GAL alkali

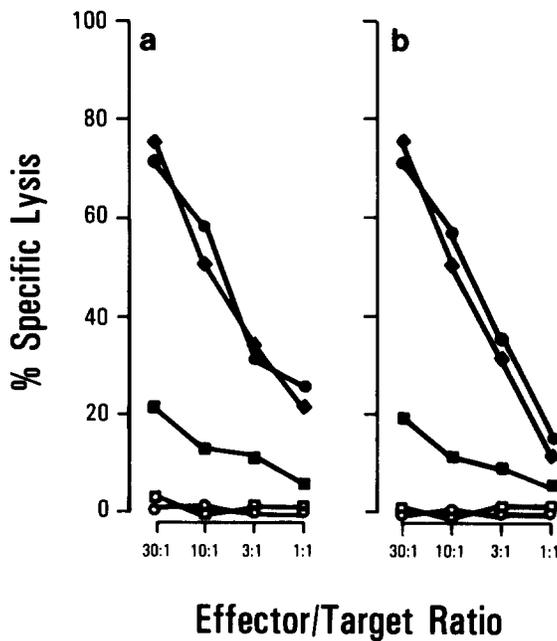


FIGURE 4. In vitro recognition by β -GAL-specific CTL requires cytosolic localization. Effectors derived from BALB/c mice primed (a) with syngeneic cells after cytoplasmic loading with a 10 mg/ml solution of β -GAL or (b) with syngeneic cells pulsed with a 10 mg/ml solution of β -GAL were assayed in a ⁵¹Cr-release assay with (●) P13.1, (○) P815 cells alone, (◆) P815 plus 100 μ g/ml of an alkali β -GAL digest, (■) P815 cells loaded with a 10 mg/ml solution of β -GAL by osmotic lysis of pinosomes, or (□) P815 cells pulsed with a 10 mg/ml solution of β -GAL in HBSS.

digest with P815 targets confirms that the effectors are specific for a degraded form of β -GAL (Fig. 4).

Cross-priming by Protein-pulsed Splenocytes. Previous work on in vivo priming of class I-restricted CTL against minor histocompatibility antigens (9) showed that these antigens, introduced on H-2 different stimulating cells, could "cross-prime" a CTL response in vivo. For example, B10 (H-2^b) cells injected into a (BALB/c \times BALB/B)F₁ (H-2^d \times H-2^b) responder effectively primed H-2^d-restricted CTL precursors specific for B10 minor antigens. This cross-priming was explained as the presentation of foreign, minor histocompatibility antigens by host APCs. We devised similar experiments to ascertain whether in vivo presentation of the soluble proteins OVA and β -GAL involves analogous APCs in the responder animal.

OVA pulsed BALB/c (H-2^d), BALB.B (H-2^b), and (BALB/c \times BALB.B)F₁ splenocytes were used to immunize F₁ (BALB/c \times BALB.B) mice. Spleen cells from these and control mice were restimulated in vitro with the H-2^b transfectant E.G7-OVA for 5 d. The same H-2^b target cell was used to assay the CTL effectors. As shown in Fig. 5, the three protein-pulsed spleen cell preparations primed OVA-specific, H-2^b-restricted CTL. Spleen cells from mice injected with control BALB/c cells (Fig. 5 *d*) or BALB.B cells (data not shown) showed no evidence of priming.

In vivo priming using β -GAL-pulsed splenocytes revealed the same cross-priming effect (Fig. 6). In this case the significant result is that β -GAL-pulsed H-2^b cells can be used to prime the H-2^d-restricted response assayed on the P815 transfectant. These results clearly showed that MHC class I-restricted presentation of the soluble proteins introduced in association with splenocytes can involve host APCs.

Protein-pulsed Splenocytes Prime CD8⁺ T Cells In Vivo. It was conceivable that in

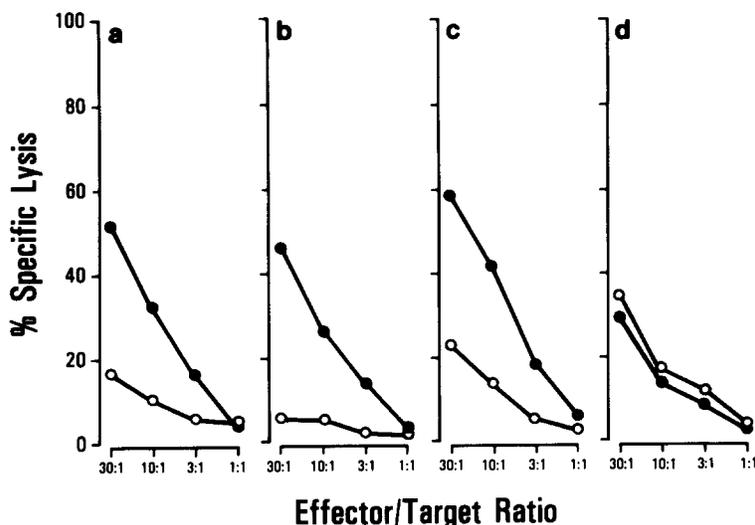


FIGURE 5. Cross-priming by OVA-pulsed splenocytes. (BALB.B \times BALB/c)F₁ mice were immunized with OVA pulsed (a) BALB.B, (b) BALB/c, or (c) BALB.B \times BALB/c splenocytes or (d) control BALB/c cells. Spleen cells from these mice were stimulated in vitro for 5 d with E.G7-OVA and the resultant effectors were used in a ⁵¹Cr-release assay with (●) E.G7-OVA and (○) EL4.

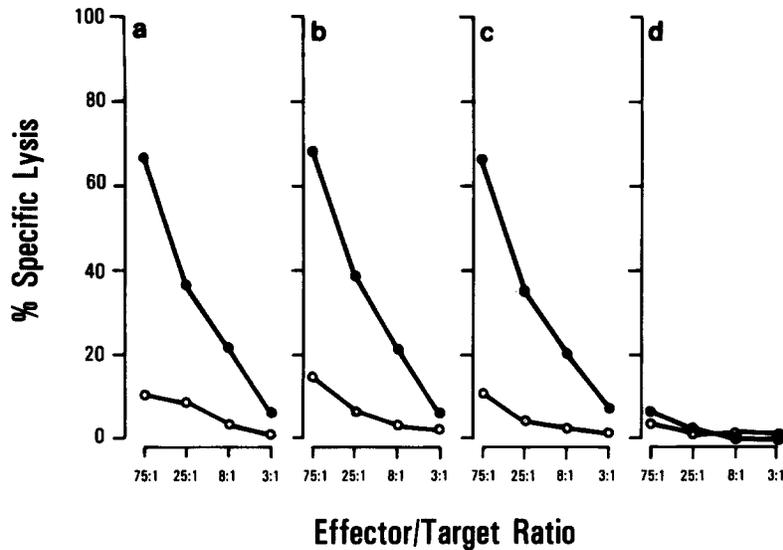


FIGURE 6. Cross-priming by β -GAL-pulsed splenocytes. (BALB.B \times BALB/c) F_1 mice were immunized with β -GAL pulsed (a) BALB.B, (b) BALB/c, or (c) BALB.B \times BALB/c spleen cells, or (d) control BALB.B cells. Spleen cells from these mice were stimulated in vitro for 5 d with C3-4 and the resultant effectors were used in a ^{51}Cr -release assay with (●) P13.1 and (○) P815.

vivo injections of cell-associated protein did not lead to priming class I-restricted CTL precursors at all, but had its effect entirely on class II-restricted helper cells. To ascertain which of the in vivo primed cell populations was critical for the subsequent secondary in vitro response, we separately depleted the CD4^+ and CD8^+ cells from OVA-immune splenocytes. These treated populations were then stimulated in vitro with E.G7-OVA alone, together, or in association with naive CD4^+ and CD8^+ populations. Table I shows that primed CD8^+ cells were essential for the in vitro

TABLE I
The Secondary In Vitro CTL Response to OVA Requires
In Vivo-primed CD8^+ T Cells

Source and treatment of responder cells*	Percent specific lysis of:†	
	E.G7-OVA	EL4
Naive, anti-CD8 treated (A)	4/1	4/4
Naive, anti-CD4 treated (B)	1/1	1/3
Primed, anti-CD8 treated (C)	5/2	5/3
Primed, anti-CD4 treated (D)	12/6	3/2
C plus D	39/24	6/4
C plus B	7/3	9/6
A plus D	33/21	5/3
Primed control	45/26	11/8
Naive control	14/9	16/9

* Splenocytes from naive and OVA-pulsed splenocyte primed C57BL/6 mice before or after antibody plus complement treatment were cultured for 5 d with irradiated E.G7-OVA.

† ^{51}Cr -labeled targets used in a 4-h assay at E/T ratios of 30:1/10:1.

OVA-specific response. CD8⁺ cells from unprimed mice did not respond to the OVA transfectant when cultured together with primed CD4⁺ cells. It was evident that CD4⁺ cells were necessary for optimal CTL stimulation *in vitro*, but these CD4⁺ cells did not require prior *in vivo* priming and most likely provided a source of nonspecific lymphokine stimulation.

Discussion

In the work presented here we have shown how a class I-restricted CTL response can be primed against a soluble protein antigen. In all respects the CTL induced by immunization with protein mimic the activity of effectors generated by immunization with cells expressing the proteins endogenously as a result of gene transfection. The first method of *in vivo* priming was arrived at rationally. We had previously shown that cytoplasmic loading by the lysis of hypertonic pinocytotic vesicles was a way to get soluble protein into the MHC class I pathway of antigen processing (7). As expected, syngeneic spleen cells loaded with OVA or β -GAL could be used to prime a class I-restricted CTL response (Figs. 1 *a* and 2 *a*). The second method of priming the same response was discovered by chance. In control experiments we found that simply incubating spleen cells with OVA and β -GAL under isotonic conditions ("pulsing") also gave an immunogenic preparation (Figs. 1 *b* and 2 *b*). This was surprising, since the same treatment does not produce appropriate target cells for class I-restricted CTL (7), nor are the pulsed cells that prime *in vivo* capable of stimulating a secondary CTL response *in vitro* (data not shown).

It was conceivable that the *in vivo* priming effect was actually due to primed, protein-specific CD4⁺ helper T cells that were necessary for the *in vitro* activation of primary CTL. However, the results presented in Table I ruled this out. CD4⁺ cells from primed mice had no effect on the *in vitro* activation of CTL from naive, CD8⁺ populations. Furthermore, only cultures containing CD8⁺ cells from immunized animals showed any OVA-specific CTL activity, consistent with the requirement for *in vivo* priming of CD8⁺ T cells.

Formal proof that protein presentation for class I CTL induction could actually occur *in vivo* was provided by the demonstration that antigen-pulsed splenocytes were capable of cross-priming CTL responses in F₁ mice (Figs. 5 and 6). Cross-priming has been demonstrated previously for endogenous cell-associated antigens such as minor histocompatibility antigens (9) and SV40 T antigen (10). In these earlier studies it was also concluded that cross-priming reflected host antigen presentation. However, while cross-sensitization of these cellular products could readily occur *in vivo*, no such cross-presentation was detected *in vitro* (9, 10). An analogous situation is found with OVA and β -GAL responses, i.e., splenocytes pulsed with the antigen prime the CTL response *in vivo* but do not reactivate CTL during 5 d *in vitro* culture nor do pulsed cells serve as effective targets.

Other investigators have demonstrated that certain exogenous antigens can effectively prime class I-restricted CTL responses (17-20). However, whether the previously observed priming required *in vivo* processing was never directly assessed. At least in the case of OVA, this has been shown to be of particular importance (15). OVA and β -GAL were used here as examples of intact and native antigens that clearly required processing for effective class I presentation (Figs. 3 and 4, and reference

7). In this form both proteins repeatedly failed to generate CTL responses *in vivo*. Consequently the two approaches that we have presented, cell pulsing and osmotic loading, could be generally applied to a variety of solubilized antigens for effective CTL priming.

Our explanation for the host presentation of antigens introduced on syngeneic or allogeneic cells is that specialized cells phagocytose the injected cell or cellular debris and the degradation products are fed into the class I pathway of presentation. Preliminary experiments demonstrating successful CTL priming using OVA-pulsed and glutaraldehyde-fixed or disrupted cells agree with this suggestion (data not shown). Pinocytosis of soluble antigen does not usually allow access to this pathway. The postulated phagocytic pathway of class I-restricted presentation would allow CTL epitopes expressed in peripheral cells (e.g., fibroblasts or hepatic cells) to be presented more efficiently in the central lymphoid organs. The nature of the phagocytic cells is not known. It could be a macrophage or a dendritic cell. The latter, are not phagocytic *in vitro* after isolation (21). However, studies using *in vivo* transfer of radiolabeled allogeneic lymphocytes have documented the movement of radiolabel into dendritic cell lysosomes (22). Unfortunately, this approach did not unequivocally establish that the dendritic cells became labeled due to phagocytic activity nor did it extend the observation to syngeneic cell systems such as those described here. Nonetheless, the suggestion of potential differences between *in vivo* and *in vitro* phagocytic capabilities for dendritic cells would fit the phenomenology of class I-restricted cross-priming. Further analysis using other antigens and disrupted cell preparations should provide some insight into the mechanism of this form of *in vivo* antigen presentation.

Summary

MHC class I-restricted T lymphocyte responses are usually directed to cellular antigenic components resulting from endogenous gene expression. Exogenous, non-replicating antigens, such as soluble proteins, usually fail to enter the class I pathway of antigen processing and presentation. Consistent with this notion, we have recently shown that soluble, exogenous proteins can be efficiently processed for class I presentation *in vitro* only if they are introduced directly into the target cell cytoplasm. In this report we extend this work to the *in vivo* situation by introducing soluble protein into the cytoplasm of mouse splenocytes via the osmotic lysis of pinosomes and then using these cells for *in vivo* immunization. Our results show that cytoplasmic loading of OVA and β -GAL into H-2^b and H-2^d splenocytes respectively, resulted in effective *in vivo* immunogens for class I-restricted CTL. To our surprise, control spleen cell preparations simply incubated with the exogenous, native protein for 10 min at 37°C in isotonic medium and then washed could also induce a comparable class I-restricted CTL response following intravenous injection. Experiments using (H-2^b × H-2^d)F₁ mice showed that protein pulsed splenocytes from one parental strain could effectively "cross prime" T cells restricted to the MHC of the other parental strain. In all cases, target cell recognition by the effector CTL generated by immunization with spleen cell-associated antigen required the antigen to be present in the cell cytoplasm. Thus the CTL do not recognize target cells exposed to soluble, exogenous antigen. These results, reminiscent of analogous experiments with cross priming by minor histocompatibility antigens, argue that class I-restricted processing

and presentation of exogenous antigen can occur in vivo following immunization with cell-associated antigen.

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Author/s:

Carbone, FR; Bevan, MJ

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