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Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages

(vaccine/cytotoxic T lymphocyte/viral immunity/antigen presentation)

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ABSTRACT **Antigens in extracellular fluids can be processed and presented with major histocompatibility complex (MHC) class I molecules by a subset of antigen presenting cells (APCs). Chicken egg ovalbumin (Ova) linked to beads was presented with MHC class I molecules by these cells up to 10^4-fold more efficiently than soluble Ova. This enhanced presentation was observed with covalently or noncovalently linked Ova and with beads of different compositions. A key parameter in the activity of these conjugates was the size of the beads. The APC that is responsible for this form of presentation is a macrophage. These cells internalize the antigen constructs through phagocytosis, since cytochalasin B inhibited presentation. Processing of the antigen and association with MHC class I molecules appears to occur intracellularly as presentation was observed under conditions where there was no detectable release of peptides into the extracellular fluids. When injected *in vivo* in C57BL/6 mice, Ova-beads, but not soluble Ova, primed $CD4^-$ $CD8^+$ cytotoxic T lymphocytes (CTLs). Similar results were obtained in BALB/c mice immunized with β-galactosidase-beads. The implications of these findings for development of nonliving vaccines that stimulate CTL immunity are discussed.**

Major histocompatibility complex (MHC) class I molecules bind fragments of endogenously synthesized proteins and transport these peptides to the cell surface. In this manner, cells display to the immune system a sampling of their endogenous proteins (1–3). When a viral peptide, to which the host is not tolerant, is presented, cytotoxic T lymphocytes (CTLs) are stimulated and kill the antigen-bearing cell. The immune system thereby purges the pathologically affected cell from the host. This CTL response plays a major role in host defense against pathogenic viruses and tumors (4, 5).

The peptides presented on MHC class I molecules are generated in a nonlysosomal compartment in cells, probably the cytosol (3). Antigens in the extracellular fluid do not gain access into this processing compartment in most cells (6–8). Accordingly, CTL immunity is not generally elicited when proteins or nonliving viruses are injected into animals (8). This anatomical segregation of the class I pathway, while potentially important for preserving the immunological identity of healthy cells, has been a major barrier to the development of nonliving vaccines that elicit CTL immunity. It has also been unclear how $CD8^+$ immunity is elicited against certain pathogens that reside in the phagolysosomes of macrophages (9) and why sometimes exogenous antigens prime CTLs (10–14).

Although exogenous proteins are excluded from the class I presenting pathway in most cells, there is an antigen presenting cell (APC) that can process and present these antigens on class I molecules (15). This activity was first defined *in vitro*; however, when these cells are recovered from mice injected with soluble antigen, they present this antigen to class I-restricted T cells (16). The APC that mediates this unique antigen presenting activity is a macrophage (16, 17) (unpublished data). Previous studies, reporting that the depletion of macrophages with silica or carrageenan reduces the capacity of mice to generate a CTL response even to live viruses, indicate that macrophages in general may be key components in the initiation of all CTL responses (18, 19).

The present studies were initiated to investigate how antigens enter into the class I presentation pathway in these macrophages and whether this pathway could be exploited to elicit CTL immunity with nonliving antigens.

MATERIALS AND METHODS

Mice. Female C57BL/6 and BALB/c 5- to 8-week-old mice were purchased from The Jackson Laboratory.

Cell Lines. RF33.70 is an anti-Ova+K^b hybridoma (20). EL4 is a C57BL/6 T lymphoma, and EG7 is a chicken egg ovalbumin (Ova)-transfected clone of EL4 (7). P815 is a DBA/2 mastocytoma and P13.4 is a β-galactosidase (β-Gal)-transfected clone of P815. EG7 and P13.4 were kindly provided by M. Bevan (University of Washington, Seattle). BMA3.1A and BMC2 are C57BL/6 bone marrow macrophage cell lines (unpublished data).

Reagents. Ova, β-Gal, azide, 2-deoxyglucose, and cytochalasin B were purchased from Sigma. Monoclonal antibodies (mAbs) were kindly provided by the laboratories of origin: anti-Thy-1 (M5/49) (21), anti-CD4 (GK1.5) (22), and anti-CD8 (HO2.2ADH4) (23).

Antigenic Constructs. Iron oxide beads (0.5–1.5 μm) were purchased from Advanced Magnetics (Cambridge, MA). Ova or β-Gal was covalently linked to these beads via an amino group bound according to the manufacturer's instructions. These conjugates are referred to as Ova-Fe beads or β-Gal-Fe beads. Ova was linked to latex beads (Polybeads) (diameter, 0.5–10 μm) (Polysciences) either by a covalent amino bond, Ova-latex(C), or by passive adsorption, Ova-latex(A), according to the manufacturer's directions. The amount of Ova bound to the beads was determine based on the difference in optical density between the solution of Ova before and after the linkage, and/or by using ^{125}I-labeled Ova, and typically varied from 0.1 to 9 mg/ml.

Cell Culture. APCs were exposed to antigen and T–T hybridoma cultures were prepared essentially as described (20), except that indomethacin (0.25 μM) was added to the culture medium. Serum-free cultures were performed in Opti-MEM (GIBCO) supplemented with Nutridoma (1%)

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Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; APC, antigen presenting cell; Ova, chicken egg ovalbumin; β-Gal, β-galactosidase; mAb, monoclonal antibody.

(Boehringer Mannheim). In some experiments, cultures were prepared in Transwell plates (Corning) with T–T hybrids and live or fixed APCs in the upper chamber separated by 0.4-μm filters from APCs and antigen in the lower chamber in serum-free medium (500 μl). After 24 hr, an aliquot of supernatant (100 μl) was collected and subjected to freeze–thawing, and interleukin 2 was assayed (24).

Inhibition Studies. In some experiments, macrophage cell lines were preincubated with or without azide (30 mM) and 2-deoxyglucose (5 mM) or cytochalasin B (10 μM) for 1 hr at 37°C, and then antigens were added for 5 hr in the continuous presence of inhibitors. Subsequently, cells were fixed with 1% paraformaldehyde, washed with medium, and put into T–T hybridoma cultures.

***In Vivo* Immunization.** C57BL/6 or BALB/c mice were injected subcutaneously with antigen in 0.1 ml of phosphate-buffered saline. The amount of antigen is indicated in the individual experimental protocols. One to two weeks later, splenocytes were restimulated with EG7 (15×10^6 cells per flask) as described (7) and splenic CTL activity was measured 5 days later (20). For β-Gal, the same procedure was used except that P13.4 cells (6×10^6 cells per flask) were used instead of EG7 cells. ^{51}Cr release assays were performed as described (20).

RESULTS

Analysis of the Presentation of Exogenous Soluble Versus Particulate Ova with MHC Class I Molecules. Antigens in the extracellular fluids can be internalized into macrophages by two distinct mechanisms. Soluble antigens are internalized by endocytosis while particulate antigens can be taken up through phagocytosis. To analyze how antigens are internalized and presented with MHC class I, we incubated APCs with soluble Ova or Ova conjugated to a phagocytic substrate. The latter conjugates were generated by linking Ova to beads that are avidly phagocytosed. Antigen presentation was assayed using the RF33.70 hybridoma, which secretes interleukin 2 upon stimulation with Ova–K^b complexes on the surface of APCs.

As shown in Fig. 1*A*, thioglycolate-elicited peritoneal exudate cells present soluble Ova added to the extracellular fluids with MHC class I molecules. In comparison, Ova linked to iron oxide (Fe) beads is presented ≈1000-fold more efficiently. The macrophages in these cultures avidly internalize the antigen-Fe beads (unpublished observations).

We have previously shown that the APC that presents exogenous soluble antigen with MHC class I is a macrophage. This is the predominant cell in the thioglycolate-induced exudates analyzed in Fig. 1*A*. The activity of the particulate Ova preparation with these APC populations implies that macrophages are responsible for presenting the exogenous Ova-Fe beads. To verify that point, we assayed the presentation of exogenous Ova beads by cloned macrophage cell lines. We have previously shown that these cell lines present exogenous soluble Ova with MHC class I molecules (unpublished data). The cloned macrophage presents the Ova-Fe beads with K^b ≈10,000-fold more efficiently than soluble Ova (Fig. 1*B*). Similar results were obtained with other macrophage cell lines (see below; unpublished observations). These results unambiguously identify the macrophage as an APC that can present antigens of particulate origin with MHC class I. These results do not indicate whether this is a property of all or only a subset of macrophages.

We next analyzed whether APCs of other cell lineages could present Ova-Fe beads with MHC class I. As shown in Fig. 1*B*, the T-cell line EL4 does not present the exogenous Ova-Fe bead to RF33.70 cells. This particulate antigen preparation was also not presented by the LB27.4 B-lymphoblastoid cell line (unpublished data). However, both of these cell lines are capable of processing and presenting endogenous Ova with MHC class I molecules (15, 25). These results suggest that macrophages may be uniquely capable of presenting the antigen-bead preparations to class I MHC-restricted T cells.

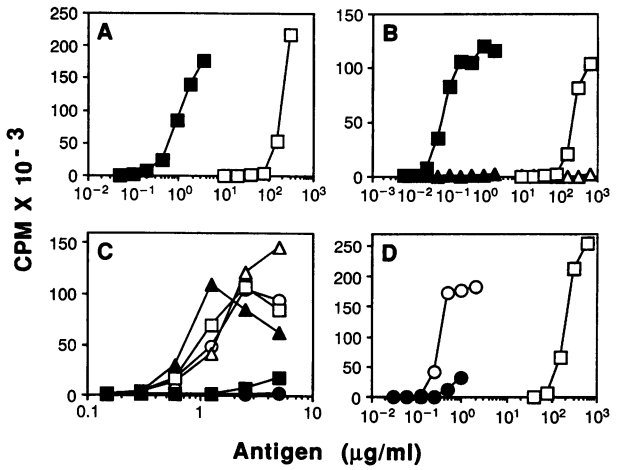

FIG. 1. Presentation of exogenous antigens with MHC class I molecules. (*A*) Cultures were prepared with RF33.70 T-cell hybrid (anti-Ova + K^b) (5×10^4 cells per well), thioglycolate-elicited peritoneal exudate cells (10^4 cells per well), and soluble Ova (open squares) or Ova-Fe beads (solid squares) in 96-well plates (200 μl) in duplicate. Cultures were then handled as described. (*B*) Cultures contained RF33.70 cells (5×10^4 cells per well), soluble Ova (open symbols), or Ova-Fe beads (solid symbols) and, as APCs (5×10^4 cells per well), either EL4 (triangles) or BMA3.1A (squares). Cultures were otherwise prepared and handled as described in *A*. (*C*) Cultures contained RF33.70 cells (5×10^4 cells per well), thioglycolate-elicited macrophages (5×10^4 cells per well), and Ova-latex(A). The size of the beads was 0.5 μm (open circles), 0.75 μm (open triangles), 2 μm (open squares), 3 μm (solid triangles), 6 μm (solid squares), and 10 μm (solid circles). Cultures were prepared and handled as described in *A*. (*D*) Cultures were prepared with BMA3.1A (5×10^4 cells per well), RF33.70 T-cell hybrid (4×10^4 cells per well), and soluble Ova (open squares), 3-μm Ova-latex(A) (open circles), or 3-μm Ova-latex(C) (solid circles). Otherwise, cultures were prepared and handled as described in *A*.

We next examined how several of the physical parameters of the antigen-bead constructs influenced their presentation by macrophages. In one set of experiments, Ova was conjugated to beads of different compositions. Ova bound to latex beads (Fig. 1*C*) and silica beads (unpublished data) are also presented efficiently with MHC class I. Stimulation of the T–T hybrid is not observed if macrophages are incubated with beads that do not contain the Ova antigen. Taken together, these results demonstrate that beads of very different compositions are active in this system, although there are some differences in the efficiency with which these different antigen-beads are presented.

We next investigated the effect of particle size on the presentation of exogenous antigen with MHC class I. As shown in Fig. 1*C*, Ova is optimally presented when bound to beads of diameter 2–3 μm. Beads of diameter 10 μm are poorly presented (Fig. 1*C*), as are particles of much smaller size (unpublished data). The Ova-Fe beads used in Fig. 1 *A* and *B* are 0.5–1.5 μm.

In another set of experiments, we analyzed whether the nature of the linkage of the antigen to the beads was critical to its presentation. As illustrated in Fig. 1*D*, Ova was efficiently presented whether it was covalently or noncovalently bound to the latex beads. The adsorbed Ova was somewhat more active than the covalently bound Ova. The

linkage to the Fe beads described above was also through amino groups on the antigen.

The Presentation of Ova-Beads by Macrophages Is Energy Dependent and Involves Phagocytosis. Native antigens require energy-dependent processes to be expressed at the cell surface with MHC molecules. To determine whether internalization and processing of Ova-beads are required for the display of MHC class I–peptide complexes at the cell surface, we preincubated the BMA3.1A macrophage cell line with or without azide and 2-deoxyglucose for 90 min before addition of antigen. After 5 hr of incubation with Ova-Fe beads in the continued presence of the inhibitors, the APCs were fixed with paraformaldehyde and the presence of Ova-K^b complexes on the cell surface was assayed. Treatment with these two drugs inhibited totally the capacity of these cells to process Ova-Fe particles with MHC class I molecules (Fig. 2*A*), while it inhibited only partially their capacity to present Ova added as a peptide (Fig. 2*B*).

Next, we investigated whether the Ova-Fe particles were internalized by phagocytosis. To examine this issue, we preincubated BMA3.1A cells with or without 10 μM cytochalasin B, a phagocytosis inhibitor, for 1 hr before addition of either Ova-Fe beads or soluble Ova. After 5 hr of incubation in the continued presence of the inhibitors, the APCs were fixed with paraformaldehyde and the presence of Ova–K^b complexes on the cell surface was assayed. (Fig. 2 *C* and *D*). Only the presentation of Ova-Fe beads was inhibited by the drug treatment. This clearly establishes that phagocytosis is needed for presentation of exogenous antigen-beads with MHC class I molecules. BMA3.1A cells were still capable of presenting soluble Ova to the RF33.70 cells. This indicates that cytochalasin B has not generally inhibited the class I pathway and that endocytosis can also direct antigens to the class I pathway, albeit much less efficiently.

MHC I–Peptide Complexes Generated from Ova-Beads Are Formed Within the APC. We next investigated whether macrophages when incubated with Ova-beads were generating or releasing peptides into the extracellular fluids that could be bound and presented by MHC class I molecules on the cell surface. For this purpose, we used double-chamber culture plates that were separated by a 0.4-μm filter, which allows a free exchange of soluble proteins but excludes the transfer of cells or of the antigen-beads. Live BMC2 macrophage cell line incubated with Ova-Fe beads in the lower chamber did not generate Ova or Ova peptides that could be transferred into the upper chamber, where either live or fixed APCs were cocultured with RF33.70 cells (Fig. 3*A*). When Ova-Fe beads were added to the upper chamber, BMC2 cells stimulated RF33.70 cells, which serves as a positive control for the activity of these cells. As another positive control, the Ova-(257–264) peptide, added to either chamber, bound to MHC class I molecules and stimulated the T–T hybridoma (Fig. 3*B*). These data indicate that under our experimental conditions the generation of peptide–MHC complexes from Ova-beads is occurring within the cell and not through peptide binding to MHC molecules on the cell surface.

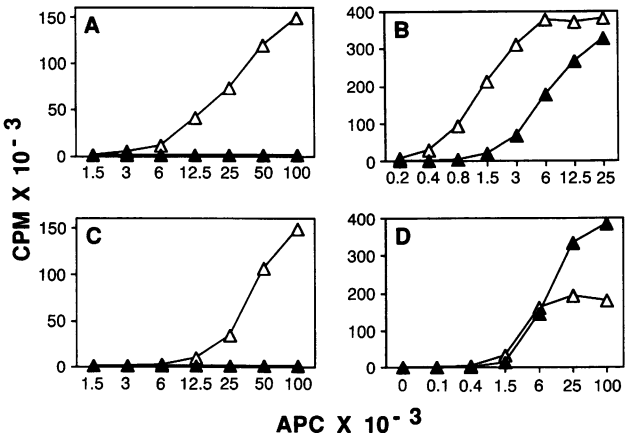

FIG. 2. Azide and 2-deoxyglucose or cytochalasin B affect the presentation of particulate antigens with MHC class I. (*A* and *B*) BMA3.1A cells were preincubated for 90 min with (solid triangles) or without (open triangles) 2-deoxyglucose (5 mM) and azide (30 mM), and then Ova-Fe beads (45 μg/ml) (*A*) or Ova-(257–264) peptide (15 ng/ml) (*B*) was added for 5–6 hr in the continuous presence of the two drugs. Cells were fixed with 1% paraformaldehyde and APC activity was assayed in culture with RF33.70 cells (5×10^4 cells per well) as described in Fig. 1. (*C* and *D*) BMA3.1A cells were preincubated for 1 hr with (solid triangles) or without (open triangles) cytochalasin B (10 μM). Ova-Fe beads (45 μg/ml) (*C*) or soluble Ova (2.4 mg/ml) (*D*) was then added for 5 hr in the continuous presence of the drug. Cells were then fixed with 1% paraformaldehyde and APC activity was assayed as described in *A* and *B*.

Antigen-Beads Prime an Efficient CTL Response *in Vivo*. The remarkable efficiency through which the Ova-beads are processed and presented with MHC class I molecules *in vitro* led us to examine whether these particles might target Ova into the class I presenting pathway *in vivo* and prime CTL responses. We immunized mice subcutaneously with antigen-Fe beads or soluble antigen and the generation of CTLs was assayed in a ^{51}Cr release assay as described. Generation of CTLs under these conditions is dependent on priming *in vivo* (7, 32). CTLs were primed in C57BL/6 mice injected with antigen-Fe beads containing 90 or 18 μg of Ova (Fig. 4*A*). In contrast, mice injected with an equivalent amount of soluble Ova were unable to generate a CTL response (Fig. 4*B*).

To determine whether a CTL response could be primed with other antigen-beads or could be observed with other strains of mice, we immunized BALB/c ($H2^d$) mice with β-Gal-Fe beads (Fig. 4 *C* and *D*). The priming of CTLs was assayed on P13.4, as described above for Ova. In both of these cases, immunization with the antigen-beads primed antigen-specific CTLs. In contrast, CTLs were not primed in mice immunized with soluble β-Gal (Fig. 4*D*). Similar results have been obtained with beads conjugated with the hen egg lysozyme antigen (unpublished data).

The CTLs generated upon priming with Ova-Fe beads and β-Gal-Fe beads were assayed on target cells in which the antigen was expressed from a transfected cDNA. The antigen–MHC complexes displayed on these target cells are generated from the processing of the endogenously synthesized antigen. The observation that these targets are killed

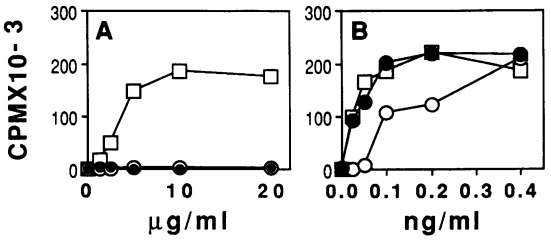

FIG. 3. MHC I–peptide complexes are formed within the APC. Hybridoma cultures were prepared in double-chamber plates (500 μl), in serum-free medium as described. To investigate release of immunogenic peptides, we cultured in the lower chamber live BMC2 cells (1.25×10^5 cells per well) and Ova-Fe beads (*A*) or Ova-(257–264) peptide (*B*) and in the upper chamber RF33.70 cells (1.25×10^5 cells per well) with live (open circles) or fixed (1% paraformaldehyde) (solid circles) BMC2 cells (1.25×10^5 cells per well). As a positive control, live BMC2 cells (1.25×10^5 cells per well) (open squares) were cultured with RF33.70 cells (1.25×10^5 cells per well) and Ova-Fe beads (*A*) or Ova-(257–264) peptide (*B*) together in the upper chamber.

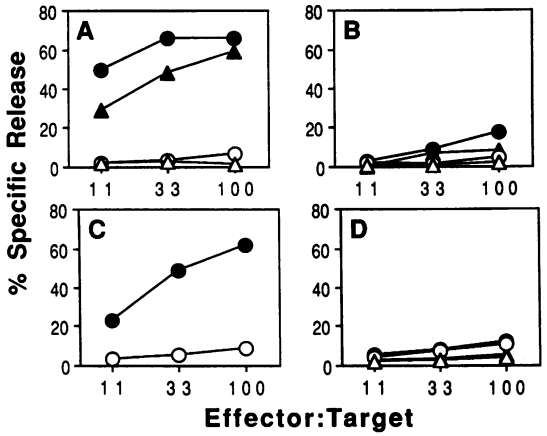

FIG. 4. *In vivo* priming of CTL response. (*A*) Splenocytes from C57BL/6 mice injected with 90 μg (circles) or 18 μg (triangles) of Ova-Fe beads were restimulated and assayed for their cytolytic activity on ^{51}Cr-labeled target cells—EL4 (open symbols) or EG7 (solid symbols). (*B*) Similar to *A* except that soluble Ova (90 or 18 μg) was used instead of Ova-Fe beads. Restimulation of CTLs and the ^{51}Cr release assay was carried out as described. (*C*) Splenocytes from BALB/c mice injected with 13 μg (circles) of β-Gal-Fe beads were assayed for their cytolytic activity on P815 (open symbols) or P13.4 (solid symbols). Targets were prepared as described in *A*. (*D*) Similar to *C* except that 13 μg (circles) or 130 μg (triangles) of soluble β-Gal was used instead of β-Gal-Fe beads.

indicates that the antigen-beads primed CTLs with specificity for endogenously processed antigenic sequences.

In a final set of experiments, we investigated the phenotype of the effector cells generated from priming with Ova conjugated to beads. For this purpose, CTL effector cells were treated with various anti-T-cell mAbs and complement just before the ^{51}Cr release assay. As shown in Fig. 5, the CTL effector cells are eliminated by treatment with anti-Thy-1 and anti-CD8 but not with anti-CD4 mAb and complement. These results indicate that the effector cells primed by the antigen-bead conjugates have the phenotype of classic CTLs.

DISCUSSION

In this report, we demonstrate that antigens conjugated to beads are efficiently taken up from the extracellular fluids and presented with MHC class I molecules. This phenomenon is observed with both normal heterogeneous and cloned macrophage cell lines and is operative *in vitro* and *in vivo*.

Several lines of evidence indicate that the presentation of antigen conjugates by macrophages requires phagocytosis or

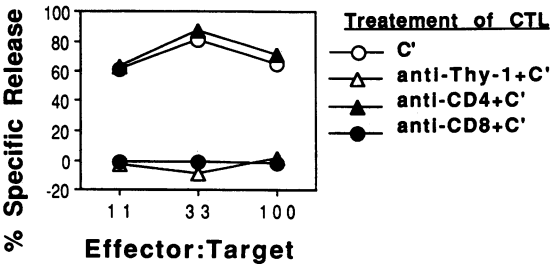

FIG. 5. CTL effector cells that are primed with Ova-Fe beads are CD4$^-$ CD8$^+$ T cells. Splenocytes from mice injected subcutaneously with Ova-Fe beads (110 μg) were restimulated with EG7 cells as described in Fig. 4. Cells from these cultures were then treated with medium—anti-Thy-1 mAb (M5/49), anti-CD8 mAb (ADH4), or anti-CD4 mAb (GK1.5)—and complement as described (26) and were assayed for cytolytic activity against EG7 cells. Background percentage specific release using EL4 cells as targets was 0.6, 1.3, and 5.2 at ratios of 11:1, 33:1, and 100:1, respectively.

related processes. First, these conjugates are presented by phagocytic but not nonphagocytic APCs. Second, the size of the beads that are active in this process corresponds to those that are internalized through phagocytosis, although it is possible that macropinocytosis is involved in internalization of the 0.5- to 1-μm beads. Third, the presentation of the antigen-conjugated beads is selectively inhibited by cytochalasin B. These observations are consistent with the recent findings of Pfeifer *et al.* (27) on the phagocytosis and presentation of bacterial antigens with MHC class I.

When compared to soluble antigen, the Ova-beads are presented 10^3- to 10^4-fold more efficiently. This increased capacity to be presented with MHC class I molecules might be due to enhanced antigen uptake. This would be analogous to our previous findings on the enhanced uptake of antigens by the surface immunoglobulin receptor on B cells (28). Alternatively, the delivery of antigens from the phagolysosomes to the class I pathway might be more active than from other endocytic compartments. The mechanisms underlying the enhanced presentation of antigen conjugates will be of interest for future studies.

Our findings that soluble antigens are presented with MHC class I suggest that antigens entering macrophages through endocytic mechanisms can also enter the class I presentation pathway. It is possible, however, that the presentation of the soluble Ova is actually accounted for by some insoluble material in these antigen preparations; however, the presentation of such "soluble" antigen is not inhibited by cytochalasin B.

After internalization into macrophages, Ova-beads are processed and peptides derived from them associate with MHC class I molecules. Pfeifer *et al.* (27) have reported that macrophages can regurgitate peptides derived from phagocytosed antigens, which bind and are presented by class I molecules on the cell surface. From our experiments, MHC class I presentation of phagocytosed antigen cannot be fully accounted for by this mechanism. The implication of our findings is that the processed peptide is associating with MHC class I molecules in an intracellular compartment. The cell biology of this antigen presenting pathway will be of interest for future study.

The present findings have two important implications. First, the display of phagocytosed antigen with MHC class I may explain how CD8$^+$ T-cell responses are generated *in vivo* against intracellular pathogens that reside in the phagolysosomes of macrophages (9). The presented antigens should stimulate CD8 T cells to produce γ-interferon leading to macrophage activation and/or to eliminate reservoirs of infected cells through killing. This could also be an important mechanism for displaying antigens from infected or transformed somatic cells with class I molecules in the central lymphoid organs.

Second, antigen conjugated to phagocytic substrates may be useful in the development of antiviral vaccines. CTLs play a key role in host defense against virus and tumors; however, this form of immunity is not generally elicited with traditional nonliving vaccines (8), but it can be generated by using live pathogens or viral vectors, which can themselves cause significant morbidity. Based on our findings with three unrelated antigens and two distinct mouse strains we predict that this approach may be generally applicable to virtually any immunogenic viral or tumor antigen.

It has been found recently that antigens incorporated into immunostimulating complexes or encapsulated into certain liposomes can stimulate CTL immunity (29–31). Although the mechanisms by which these preparations are presented are not clearly understood, it is thought that they fuse or disrupt cell membranes, since lipid and/or saponin are critical constituents. The mechanism underlying the present

approach is likely to be different and may explain priming of CTLs obtained with cell-associated antigens (10, 11).

The use of the kind of antigen construct described in this report has several potentially attractive features for vaccine development. (*i*) This approach does not require adjuvants or potentially toxic agents. (*ii*) The antigens are effective upon subcutaneous injections in aqueous buffers, which is a clinically acceptable procedure. (*iii*) Finally, given the cell type that is involved in antigen presentation, it is not unlikely that this form of immunization will also stimulate concomitant $CD4^+$ T-cell immunity.

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