

Major Histocompatibility Complex Class I Presentation of Peptides Derived from Soluble Exogenous Antigen by a Subset of Cells Engaged in Phagocytosis

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Summary

Major histocompatibility complex (MHC) class I molecules generally present peptides derived from cytoplasmic proteins, but recent reports have suggested that macrophages (M ϕ) may be uniquely able to present exogenous antigens via these molecules, and that particle-associated antigens show a marked increase in the efficiency of such presentation. We confirm here that particle uptake by M ϕ permits exogenous ovalbumin (OVA) to gain access to the endogenous class I processing pathway, an event that occurs rarely, if at all, in the absence of phagocytic stimuli. Presentation of soluble protein antigens by MHC class I molecules, however, is not limited to M ϕ , nor is direct coupling of antigen to the particle required. A variety of unconjugated particles promoted presentation of simultaneously offered soluble OVA to K^b-restricted T cells by both M ϕ and non-M ϕ antigen-presenting cells (APC), provided the latter could phagocytose the particles. Enhancement of presentation by phagocytic stimuli could not be explained by greater delivery of soluble antigen to endosomal compartments because such stimuli did not increase soluble tracer accumulation, nor did they improve presentation of OVA to an MHC class II-restricted T cell hybridoma. OVA presentation induced by cophagocytosis of particles and free antigen was nevertheless very inefficient in comparison to presentation of OVA peptide, and even modest responses required high concentrations of protein and particles. Furthermore, only a fraction of APC exposed to OVA and particles were lysed by anti-OVA cytotoxic T lymphocytes, despite virtually all cells showing OVA accumulation, particle uptake, and K^b expression. Titration experiments were most consistent with a model in which, by disrupting membrane integrity, phagocytic overload ("indigestion") allows escape of OVA into the cytosol of some APC, rather than with a model in which phagocytosis activates a novel antigen processing pathway that has evolved to permit class I loading of exogenous antigen. These data suggest caution in the development of vaccine strategies based on use of particle conjugates for elicitation of CD8⁺ T cell immunity, but, at the same time, may be relevant to understanding class I-restricted responses to some intracellular pathogens normally resident in membrane-bound vesicles.

Class I proteins of the MHC display at the cell surface a sample of peptides derived from cellular proteins, allowing T lymphocytes to respond to intracellular pathogens or mutated cellular antigens that would otherwise remain undetectable. Because MHC class I molecules are generally involved in presenting peptides to CTL, whose effector function is best restricted to actively infected cells, it makes biological sense that these MHC molecules should be limited in their ability to acquire peptides derived from proteins in the extracellular milieu (exogenous antigens). This would help prevent healthy cells from becoming sensitized for CTL lysis by foreign, noninfectious proteins released from neighboring infected or transformed cells. This notion is supported by numerous experimental observations that soluble intact ex-

ogenous proteins can be presented by class I only when administered in fusogenic form or deliberately introduced into the cytosol by *in vitro* manipulations (1). If exogenous antigens were completely excluded from the class I pathway, however, it is unclear how immune responses could be mounted against intracellular parasites residing in the endosomal compartment of MHC class II-negative cells or against viruses that do not infect professional class II-positive APC. Moreover, several instances of MHC class I presentation of peptides from putatively nonfusogenic exogenous antigens have been reported (2-8) and class I-restricted CTL responses against endosomal parasites are essential in controlling many parasitic infections (9, 10). Together, these observations suggest the existence of mechanisms for delivering

peptides from exogenous antigens to class I molecules, and they have led to the hypothesis that there exist APC that are specialized to do so (11).

One such APC has been tentatively identified as a macrophage (M ϕ)¹ (8, 12–14). Recent studies have suggested that exogenous antigens in particulate form markedly increase the efficiency of class I presentation by M ϕ , but it is not clear whether phagocytosis merely augments antigen uptake or has a qualitative effect on antigen handling (15–18). Furthermore, two different pathways have been described as contributing to exogenous antigen presentation by MHC class I molecules, one involving peptide regurgitation from phagosomes and binding to cell surface class I (15, 17), and another involving antigen transfer into the cytosol and processing via the conventional MHC class I pathway (16, 18). The latter studies suggested that a novel mechanism might exist for transport of proteins or their partial degradation products from endosomes/phagosomes to the cytosol. In this report, we provide evidence that phagocytosis can induce a qualitative change in antigen handling, not only by M ϕ , but also by a variety of phagocytically competent, non-M ϕ APC that is independent of a quantitative effect on antigen delivery and that permits class I presentation of soluble exogenous antigens by a fraction of APC. The change in antigen handling induced by phagocytosis appears to result from a loss of phagosomal membrane integrity and escape of antigen into the cytosol, however, rather than upregulation of a novel specific transport system designed for delivery of exogenous antigens into the endogenous antigen processing pathway. These observations are discussed in the context of immune responses to replicating endosomal parasites and of vaccine development.

Materials and Methods

Mice. Female C57BL/6 (B6) and B10.A mice were obtained from the National Cancer Institute (National Institutes of Health [NIH], Bethesda, MD), and they were used between 6 and 18 wk of age.

Media. RPMI 1640 or DMEM medium (BioFluids, Rockville, MD) was supplemented with glutamine, nonessential amino acids, Hepes, sodium pyruvate, antibiotics, 10% FCS (all from Biofluids), and 2-ME. RPMI 1640 complete medium was used for all functional assays and for culturing all cell types, except BO-97.11 and E3 K^b, which were cultured in complete DMEM medium.

Reagents. OVA grades V, VI, or VII was purchased from Sigma Chemical Co. (St. Louis, MO). It was dissolved in complete medium at a stock concentration of 40 mg/ml and prepared fresh periodically. Different batches of OVA gave varying degrees of extracellular generation of class I peptides, independently of grade (see Results). BSA grade VIII was purchased from Sigma. OVA peptides 257–264 (SIINFEKL) and 323–339 (ISQAVHAAHAEINEAGR) were synthesized by Dr. J. Coligan (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). Brefeldin A was purchased from Epicentre Technologies (Madison, WI). FITC-OVA

was purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents were purchased from Sigma.

OVA and BSA beads or mock-conjugated beads were BiomagTM amino-terminated magnetic particles (PerSeptive Diagnostics, Cambridge, MA) of 1- μ m nominal diameter (range = 1–10 μ m) coupled covalently or mock-coupled to protein per the manufacturer's recommendations. Briefly, the beads were washed three times by magnetic separation in 10 mM pyridine, pH 6, activated with 5% glutaraldehyde, and washed again. BSA, OVA, or no protein were added to ~20% of the bead weight in pyridine buffer and the suspension was rotated overnight at room temperature. Any remaining activated sites were quenched with 1 M glycine for 1 h, and the conjugated beads were washed three times in PBS/1% FCS followed by a wash in complete medium. The amount of protein bound to the particles was estimated from the difference in protein content of the supernatant before and after coupling, measured using a colorimetric protein assay (Bio Rad Laboratories, Richmond, CA). Coupling efficiency ranged from 2.3 to 7.2 mg protein/ml (0.1–0.3 μ g protein per bead). FluoresbriteTM carboxylated latex beads of 1-, 1.5-, and 2- μ m diameter and PolybeadsTM latex beads of 1.5- μ m diameter were purchased from Polysciences (Warrington, PA). Zymosan A was purchased from Sigma and prepared as described (19). Fixed *Staphylococcus aureus* (PansorbinTM) was from Calbiochem (La Jolla, CA). All particles were washed in complete medium, and stocks were prepared in the same medium and kept at 4°C.

APC. Thioglycollate-induced inflammatory M ϕ (thio-M ϕ) were obtained by adherence to tissue culture plastic of peritoneal exudate cells (PEC) obtained through lavage of the peritoneal cavity of mice injected intraperitoneally 4–5 d previously with 1 ml thioglycollate broth (NIH Media Unit). In some experiments, unfractionated PEC were used; there was no difference in antigen presentation between adherent and unfractionated PEC (Reis e Sousa, C., and R. N. Germain, unpublished observations).

Epidermal cells (EC) were prepared as described (19) and were depleted of Langerhans cells by treatment with mAbs M5/114 (anti-I-A^b [20]) and NLDC-145 (21) followed by immunomagnetic separation with sheep anti-rat DynabeadsTM (Dyna, Oslo, Norway) per the manufacturer's instructions. Depletion was assessed by loss of presentation of OVA or OVA 323–339 peptide to the BO-97.11 T cell hybridoma (not shown).

1-3 (K^b) is an L cell line transfected with K^b (22). EE2H3 is a cell line derived from H-2^b mouse embryos that was transfected with K^b to obtain E3 K^b (23). E3 K^b cells were pretreated with 2,000 U/ml IFN- α , β to upregulate class I expression before use in antigen presentation assays (24).

T Cell Hybridomas and Lines. B3Z, a CD8⁺ T cell hybridoma specific for the OVA 257–264 peptide (SIINFEKL) in the context of K^b (25), was a generous gift from Dr. N. Shastri (University of California, Berkeley, CA). BO-97.11, a CD4⁺ T cell hybridoma that responds to the peptide OVA 323–339 presented by I-A^b (26), was a kind gift from Dr. P. Marrack (National Jewish Center for Respiratory Medicine, Denver, CO).

CTL lines specific for SIINFEKL + K^b were generated from B6 mice immunized with a vaccinia construct expressing the SIINFEKL epitope (27). Briefly, spleen cells from immunized mice (kind gift from Dr. J. Bennink, NIAID, NIH, Bethesda, MD) were restimulated in vitro with SIINFEKL peptide and polyclonal CTL effectors were generated. Lines were established from these effectors by culturing with irradiated (2,000 rad) B6 spleen cells pulsed with SIINFEKL in 24-well plates (Costar Corp., Cambridge, MA) in medium containing 7.5% T-StimTM (Collaborative Biomedical Products, Bedford, MA). Cultures were restimulated every 2–3 wk or were expanded for CTL assays by transferring cells to 25-cm²

¹ Abbreviations used in this paper: BFA, brefeldin A; CCD, cytochalasin D; CHX, cycloheximide; EC, epidermal cells; ER, endoplasmic reticulum; M ϕ , macrophages; PEC, peritoneal exudate cells; TAP, transporter associated with antigen processing; Thio-, thioglycollate-elicited.

flasks (Corning Inc., Corning, NY) with peptide-pulsed irradiated stimulators and culturing for 7 d, increasing the concentration of T-Stim™ to 15% on day 3.

Antigen Presentation Assays. APC (generally $5\text{--}10 \times 10^4$) were cultured in 96-well flat-bottom plates (Costar) in 200 μl complete RPMI 1640 medium with the concentrations of antigen and particles indicated in each experimental protocol. In some experiments, $5\text{--}10 \times 10^4$ T hybridoma cells were cocultured with antigen and APC, whereas in others, T cells were added to antigen-pulsed, fixed, or live APCs as indicated for each figure. Indomethacin (0.25–2 μM) was sometimes included in assays with live APCs. Adherent M ϕ were fixed in 1% paraformaldehyde in PBS for 10 min at room temperature followed by quenching with 0.1 M glycine in PBS for a similar length of time and washing thoroughly in medium before culture with T hybridoma cells.

IL-2 production by T cell hybrids was measured in most experiments as the ability of serial dilutions of assay supernatant to stimulate incorporation of [^3H]thymidine by the indicator cell line CTLL-2. In most figures, data are expressed as mean cpm of CTLL-2 cultured in a 1:4 dilution of supernatants from triplicate cultures; all error bars represent one SD from the mean. Where possible (see Fig. 5 B), IL-2 units were calculated from supernatant dilutions, as described (28). In a few experiments (see Fig. 7 B), IL-2 concentrations were determined by ELISA using antibodies purchased from PharMingen (San Diego, CA) per the manufacturer's instructions with minor modifications. Data from those experiments are expressed as IL-2 U/ml, calculated by comparison with an IL-2 standard curve. The sensitivity of the IL-2 ELISA was comparable to that of the CTLL-2 assay (Reis e Sousa, C., and R. N. Germain, unpublished results).

Since B3Z contains the *lacZ* reporter gene under the control of the human NF-AT element (25), activation of this hybridoma was assayed in some experiments through measurement of β -galactosidase activity induced in activated cells using *o*-nitrophenyl- β -D-galactopyranoside, exactly as described (29).

CTL Assays. Target cells that had been prepulsed with antigen were labeled with $\sim 100 \mu\text{Ci}$ ^{51}Cr sodium chromate (Amersham Corp., Arlington Heights, IL) in 50% FCS for 1 h at 37°C, washed in PBS, and cultured in medium for 1 h before washing again. 2×10^4 targets were added to a titration of CTL effectors in 96-well round-bottom plates in a final volume of 200 μl complete medium, centrifuged to promote cell contact, and incubated at 37°C for 4 h. Supernatants were harvested manually (100 μl) or mechanically using a supernatant collection system (Skatron Instruments, Sterling, VA). Radioactivity released into the supernatant was measured in a gamma counter (Beckman Instruments, Irvine, CA), and percent specific release was calculated from the mean of triplicate cultures according to the following formula:

$$\text{percent specific release} = \frac{100 \times (\text{experimental release} - \text{spontaneous release})}{(\text{maximal release (1\% NP-40)} - \text{spontaneous release})}$$

Flow Cytometry. Cells were analyzed using a FACScan™ cytometer and Lysys™ software (Becton Dickinson & Co., Mountain View, CA). Staining for K^b was performed using mAb AF6-88.5 directly conjugated to FITC or FITC–mouse IgG $_a$ as an isotype-matched negative control (both from PharMingen). Flow cytometric phagocytosis and endocytosis assays were performed essentially as described (19); the exact experimental protocol is given in the figure legends. Note that although the phagocytosis assay does not discriminate internalized particles from bound particles, most particles associated with cells have been internalized (19).

Results

Phagocytic Stimuli Allow K^b Presentation of OVA by Thio-M ϕ . Weak but reproducible presentation of soluble OVA by class I K^b molecules of M ϕ has been reported (13). Phagocytosis of particles containing covalently coupled or adsorbed OVA (OVA beads) greatly augments the efficiency of such presentation (16–18), an effect ascribed to enhanced delivery of antigen to the endosomal–phagosomal pathway. To examine whether augmented antigen delivery to the APC fully accounted for the superiority of OVA beads compared to soluble OVA or whether phagocytosis might affect the qualitative nature of exogenous antigen handling, we compared presentation by the thio-M ϕ of OVA directly conjugated to beads (OVA beads) and of free soluble OVA given concomitantly with control beads (OVA + BSA beads).

Surprisingly, we detected little stimulation of a K^b -restricted T cell hybridoma, B3Z, after OVA bead uptake by H-2 b thio-M ϕ (Fig. 1 A). This was true even though OVA-beads were extremely efficient at delivering antigen for presentation by the same thio-M ϕ to I-A b -restricted T hybridoma cells (Fig. 1 B). The lack of presentation using OVA beads and thio-M ϕ was not caused by chemical modification of the OVA epitope during covalent coupling because the same OVA beads led to stimulation of B3Z after uptake by non-M ϕ APC (see below). Soluble OVA coadministered with BSA beads, however, was presented on K^b about 100-fold more efficiently than OVA alone (Fig. 1 A). BSA beads either did not affect I-A b presentation of OVA (Fig. 1 B) or had a detrimental effect at high particle concentrations (Fig. 1 D and data not shown). This suggested that the simultaneous exposure of thio-M ϕ to soluble OVA and BSA beads did not increase uptake into at least those portions of the endocytic pathway associated with class II presentation, and raised the possibility that the BSA beads affected soluble OVA presentation by class I via a qualitative change in the handling of this protein by the M ϕ . In agreement with this hypothesis, phagocytosis of beads did not increase the pinocytic accumulation of FITC-OVA or lucifer yellow by thio-PEC (Fig. 1 E). The effect of feeding BSA beads together with soluble OVA on class I presentation depended on both OVA concentration (Fig. 1 A) and on the number of particles administered (Fig. 1 C). Similar results were obtained using OVA + mock-conjugated beads lacking associated protein instead of BSA beads (data not shown and see below). BSA-beads did not stimulate either hybridoma in the absence of OVA (Fig. 1, A and B). Enhanced presentation of OVA in the presence of beads did not result from upregulation of K^b or to an increased proportion of “empty” K^b molecules receptive to exogenous peptide because exposure to BSA beads did not affect the dose–response curve for presentation of the OVA peptide SIINFEKL (Fig. 2 A) or the response to a fixed concentration of the same peptide (Fig. 2 B). Effective presentation required relatively simultaneous exposure to beads and OVA; even a 30-min delay in antigen addition after bead exposure (together with cytochalasin to block further phagocytosis) resulted in little or no stimulation of B3Z (data not shown).

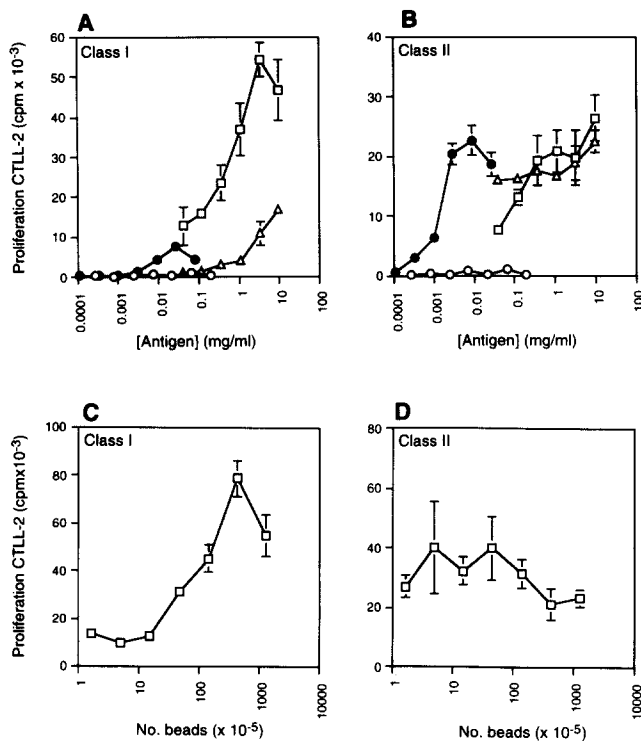


Figure 1. Presentation of ovalbumin to T cell hybridomas by thio-MΦ. (A) Presentation of particulate and soluble OVA to B3Z (K^b restricted). (B) Response of BO-97.11 ($I-A^b$ -restricted) to the same antigens. (C) Presentation of soluble OVA to B3Z is dependent on the dose of BSA beads. (D) BSA beads do not enhance presentation of soluble OVA to BO-97.11. Thioglycollate-elicited PEC were plated at 5×10^4 per well in 96-well plates. After 4 h incubation at 37°C , nonadherent cells were washed off, and the indicated concentrations of soluble or particulate antigens were added together with B3Z or BO-97.11 T hybridoma cells (5×10^4 per well) in $200 \mu\text{l}$ complete medium containing $0.25 \mu\text{M}$ indomethacin. OVA + BSA beads wells received the indicated concentration of OVA + 1.25×10^7 beads per well in A and B, and the indicated number of beads per well + 10 mg/ml OVA in C and D. The concentrations of protein in OVA beads and BSA beads added in A and B were calculated from the amount bound to beads (see Materials and Methods), and the top concentration of each antigen corresponded to 1.25×10^8 beads per well. Supernatants from wells were assayed for IL-2 content after 26 h by [^3H]thymidine incorporation into CTL-2 indicator cells. All the data come from the same experiment. $-\circ-$, BSA beads; $-\square-$, soluble OVA + BSA beads; $-\bullet-$, OVA beads; $-\triangle-$, soluble OVA. (E) Uptake of soluble OVA is not affected by phagocytosis of BSA beads. 3.5×10^5 thio-PEC were incubated for 2 h with 0.2 mg/ml of FITC-OVA in the presence or absence of BSA beads. Cells were washed and analyzed for FITC fluorescence using propidium iodide and live gating to exclude dead cells. Several doses of BSA beads were tested (only three representative ones are shown): curves for 0 (- beads), 9.3×10^6 (+ beads), or intermediate doses of beads (not shown) were superimposable, indicating that phagocytosis had no effect on pinocytic accumulation of FITC-OVA. At higher doses, the signal from the soluble tracer was decreased (shown for 8.3×10^7 [hi beads]). Equivalent results were obtained with lucifer yellow (not shown).

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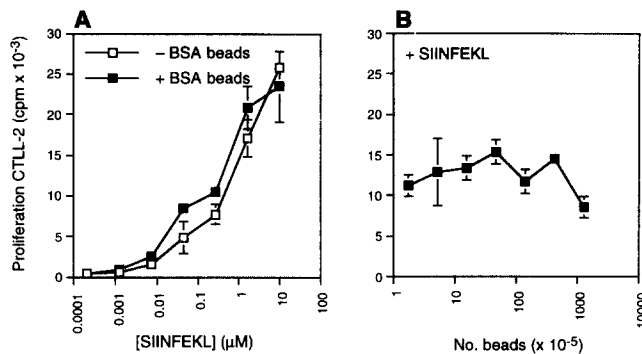


Figure 2. Beads do not affect the presentation of peptide by thio-MΦ. (A) Dose response to SIINFEKL presented by MΦ with or without 1.25×10^7 BSA beads per well. (B) Effect of bead concentration on presentation of $2 \mu\text{M}$ of SIINFEKL peptide. Thio-PEC (5×10^4 per well) were cultured with the indicated concentrations of SIINFEKL and BSA beads. After 24 h, adherent cells were washed thoroughly with RPMI 1640 and B3Z (5×10^4 per well) were added in $200 \mu\text{l}$ complete medium and incubated for a further 24 h. IL-2 was measured as indicated in Fig. 1. BSA beads promoted presentation of soluble OVA in the same experiment (not shown).

Mechanism of Antigen-Processing of Soluble OVA + Beads. Light paraformaldehyde fixation of APC results in a marked enhancement of the ability of surface class I molecules to bind antigenic peptides (30) and allows a 100–1,000-fold increase in the stimulatory activity of offered SIINFEKL (see below). This provided a sensitive test for the extracellular generation of class I-binding peptides from soluble OVA, and was used as a control to be sure that the effects being observed involved an intracellular processing step. All OVA preparations used in these experiments were first screened by this assay to ensure that they did not give rise to such extracellular class I presentation. Only occasional batches of OVA, with no correlation to nominal grade, passed this stringent test (Fig. 3). Interestingly, when OVA that did not show activity with fixed MΦ was tested with live MΦ in the absence of beads, this material was unable to reproducibly stimulate B3Z to produce IL-2 (data not shown). We were thus unable to confirm the existence of a pathway for class I presentation of soluble exogenous antigen in MΦ that had not been exposed to phagocytic stimuli (8, 13), under conditions in which the possibility of extracellular processing was rigorously excluded.

Fixed MΦ also did not present soluble OVA to B3Z in the presence of BSA beads, making it very unlikely that the beads carried proteases capable of generating functionally significant levels of OVA peptides extracellularly (Fig. 4 A and data not shown). To determine whether presentation in the presence of beads involved regurgitation of OVA peptides generated endocytically (15, 17), live MHC-incompatible MΦ were mixed with fixed K^b -expressing MΦ and were exposed to BSA beads and OVA. This combination was ineffective in stimulating B3Z cells; only when K^b -expressing MΦ were pulsed live were they capable of stimulating IL-2 production by B3Z (Fig. 4 A), despite the bias towards detection

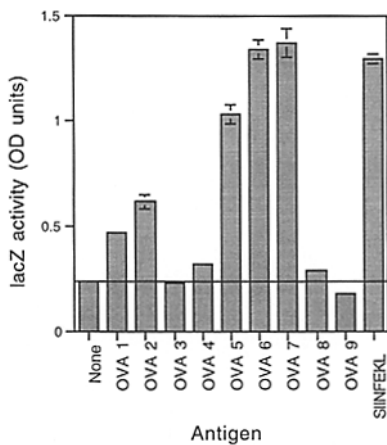


Figure 3. Several batches of ovalbumin allow presentation by K^b that is independent of cellular processing. Adherent thio- $M\phi$ were fixed in 1% paraformaldehyde, quenched with glycine, and washed with complete medium. Fixed $M\phi$ were incubated for 24 h with B3Z (10^5 per well) in the presence or absence of 20 μM SIINFEKL or different batches of OVA at 10 mg/ml. Activation of B3Z was measured by induction of β -galactosidase activity (see Materials and Methods), and the results are expressed as units of absorbance at 415 nm. Only two batches of OVA (OVA 3 and OVA 9) were not presented by fixed $M\phi$; OVA 3 was used in most subsequent experiments. All preparations of OVA were purchased from Sigma and dissolved in complete medium at a stock concentration of 40 mg/ml before use. The nominal grade was as follows: OVA 1, 2, 3, 6, 7, grade V; OVA 4, 9, grade VI; OVA 5, 8, grade VII.

of peptide transfer when fixed $M\phi$ expressing K^b were used (Fig. 4 B). These results suggest that presentation resulting from exposure to OVA + beads does not involve extracellular processing or peptide regurgitation by $M\phi$.

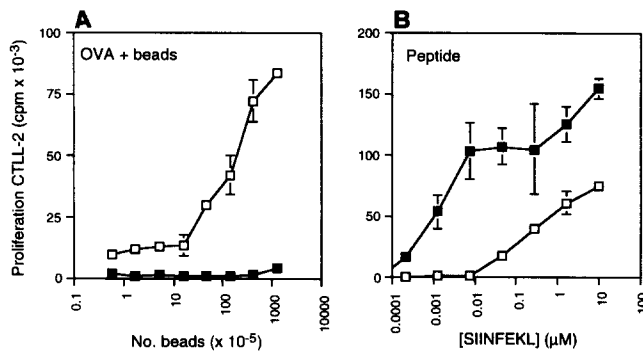


Figure 4. Absence of detectable peptide regurgitation during presentation in response to OVA + beads. (A) Live MHC-mismatched $M\phi$ (B10.A; H-2^s) pulsed with OVA + beads do not transfer OVA peptide to fixed MHC-matched $M\phi$ (B6; H-2^b) despite the fact that (B) fixed B6 $M\phi$ present SIINFEKL 100–1,000 \times better than live cells. Thio-PEC from B6 or B10.A mice were plated at 5×10^4 per well for 6 h at 37°C. Nonadherent cells were discarded and adherent cells were fixed as in Fig. 3. Live $M\phi$ (5×10^4 per well) of the other haplotype were added, together with the indicated concentrations of SIINFEKL or BSA beads + 10 mg/ml OVA. Cultures were incubated for 20 h and were subsequently washed and fixed again. B3Z (10^5 per well) were added in 200 μl medium and IL-2 was measured 24 h later as in Fig. 1. \square , live B6/fixed B10.A; \blacksquare , live B10.A/fixed B6.

The presentation pathway for OVA offered to $M\phi$ in the presence of beads was dissected further by analyzing its susceptibility to inhibition by different drugs (Fig. 5). Attachment of particles to $M\phi$ cell surface receptors was not sufficient to permit access to the MHC class I processing pathway because cytochalasin D (CCD), an inhibitor of phagocytosis, prevented presentation (Fig. 5 A). Presentation was also dependent on newly synthesized proteins because it was blocked by cycloheximide, suggesting that OVA peptides were not being generated in phagosomal compartments, binding to K^b internalized from the plasma membrane, and recycling back to the cell surface (Fig. 5 A). Consistent with this observation, presentation was also inhibited by brefeldin A (BFA;

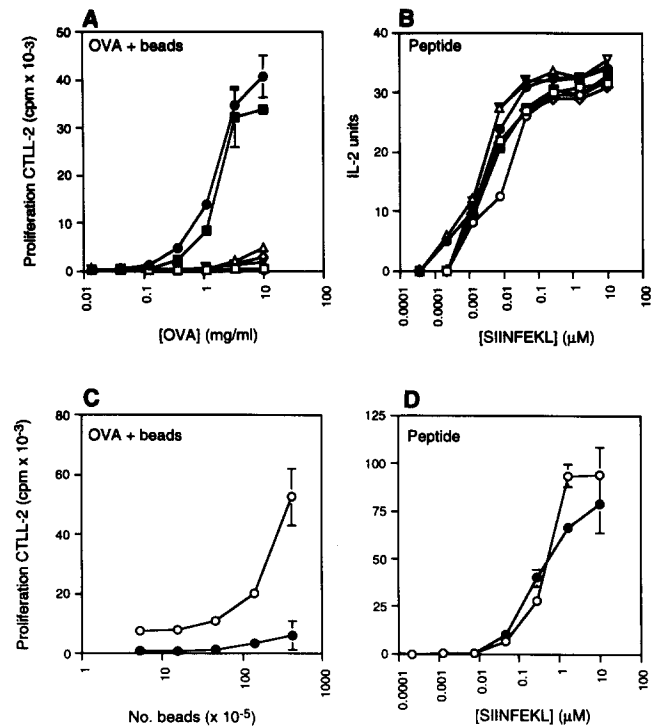


Figure 5. Effect of drugs on presentation of OVA + BSA beads. (A and B) Thio-PEC (10^5 per well) were plated, incubated for 24 h, and nonadherent cells were discarded. Adherent $M\phi$ were preincubated for 60 min at 37°C with medium alone (No Tx \blacksquare), or medium containing 0.5 $\mu g/ml$ BFA \square , 10 $\mu g/ml$ CCD \triangle , 10 $\mu g/ml$ cycloheximide (CHX \circ), 20 mM ammonium chloride (NH_4Cl \diamond), 200 $\mu g/ml$ leupeptin ∇ , or 100 μM chloroquine (CHQ \blacksquare). The indicated concentration of OVA + 1.25×10^7 mock-conjugated beads (A) or SIINFEKL (B) was then added in medium containing the respective drug, as necessary to maintain a constant drug concentration. After 4 h incubation, adherent cells were washed and fixed as described in Fig. 3. B3Z (6×10^4 per well) were added and IL-2 content of supernatants was measured 22 h later. (C and D) Thio-PEC were plated at 10^5 per well with the indicated number of $1.5 \mu m$ Polybeads + 10 mg/ml OVA (C) or the indicated concentration of SIINFEKL (D) in the presence or absence of 1 μM gelonin. After 22 h incubation, adherent $M\phi$ were washed and fixed as in A and B, and B3Z (10^5 per well) were added for 22 h. IL-2 content of supernatants was measured as in Fig. 1. The data in A and B and C and D come from the same experiments. \circ , without gelonin; \bullet , with gelonin. The data in B are expressed as IL-2 units as calculated from supernatant dilutions (28) and do not have error bars.

Fig. 5 A), a drug that prevents egress of proteins from the endoplasmic reticulum (ER) and Golgi while allowing plasma membrane recycling (31). Neither drug affected presentation of the SIINFEKL peptide, which is dependent on preexisting plasma membrane K^b rather than newly synthesized molecules (Fig. 5 B). It is noteworthy that the ability of BFA to inhibit presentation of OVA + beads is in disagreement with the results reported by Harding and Song for OVA directly conjugated to beads (17), but that it is in agreement with a recent report by Kovacovics-Bankowski and Rock (18) using the same antigen. Also in agreement with the latter report, chloroquine did not affect presentation by M ϕ exposed to OVA + beads (Fig. 5 A). However, the interpretation of this result is complicated by the fact that chloroquine also promoted class I presentation of soluble OVA in the absence of phagocytic stimuli (data not shown). Other endosomal degradation inhibitors such as ammonium chloride or leupeptin did inhibit K^b presentation of OVA by M ϕ after exposure to OVA + beads (Fig. 5 A), but not peptide (Fig. 5 B), although it is not clear if this was caused by inhibition of proteolysis or alterations in endosomal traffic.

These results were consistent with a model in which phagocytosis of beads allowed delivery of intact or partially proteolyzed OVA into the cytosol for further processing as a conventional endogenous antigen and import into the ER in the form of peptides via transporter associated with antigen processing (TAP). An alternative explanation was that peptides were generated in the endo/phagosomal cisternae and bound to newly synthesized K^b in a TAP-independent manner as the class I trafficked through the post-ER portion of the secretory pathway. To differentiate between these models, we examined the susceptibility of presentation to gelonin, a membrane-impermeant protein synthesis inhibitor that is usually nontoxic to living cells because it has no access to the cytosol (13, 18, 32). Gelonin inhibited presentation of OVA offered to M ϕ in the presence of beads (Fig. 5 C) while leaving intact the ability of the M ϕ to present peptide (Fig. 5 D). This result suggests that phagocytosis of beads allows access of relatively intact exogenous proteins to the cytosol, where they can be acted upon by the conventional MHC class I processing pathway.

Phagocytosis Allows K^b Presentation of OVA by Nonprofessional Phagocytes. Because presentation by K^b of peptides derived from soluble OVA appeared to result from a qualitative change in antigen handling induced by phagocytosis rather than simply from increased delivery of antigen, we examined whether phagocytic stimuli could induce other APC to present OVA to B3Z. Langerhans cell-depleted EC containing phagocytic keratinocytes (19) were pulsed with OVA beads or OVA + beads and assayed for activation of B3Z. Like thio-M ϕ , EC stimulated B3Z after exposure to OVA + beads, but unlike the former APC, EC also stimulated the hybridoma after exposure to OVA beads (Fig. 6). Similar results were obtained with the fibroblast line 1-3 (K^b) and, after IFN- α,β treatment to induce TAP expression, with the embryonic cell line E3 K^b , both of which were capable of phagocytosing particles (data not shown). Neither phagocytic stimulus, how-

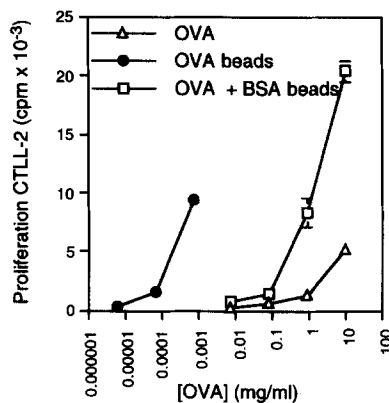


Figure 6. Nonprofessional phagocytes can also present OVA upon phagocytic stimulation. Epidermal cells depleted of Langerhans cells were cultured at 3.5×10^5 per well with the indicated concentrations of antigen. After 24 h, B3Z (5×10^4 per well) were added, incubated for 24 h, and IL-2 was measured as in Fig. 1. Concentration of protein added as OVA beads was calculated as in Fig. 1; the top concentration shown corresponded to 1.1×10^6 OVA beads per well. Higher concentrations of beads inhibited the response (not shown). Cultures incubated with OVA + BSA beads received 1.25×10^7 BSA beads per well in addition to the indicated concentration of OVA.

ever, promoted presentation of OVA to B3Z by LB 27.4, a nonphagocytic B cell hybridoma (33), supporting the notion that presentation is related to the ability to internalize particles (data not shown).

Different Phagocytic Stimuli Promote K^b Presentation of OVA. We next tested the ability of different phagocytic stimuli to induce presentation of soluble OVA to B3Z by thio-M ϕ . Phagocytosis of 1–2- μ m latex particles promoted presentation, although 1- μ m particles appeared to be somewhat less efficient than larger beads (Fig. 7 A). DynabeadsTM (4.5- μ m) were as efficient as BSA beads at promoting presentation (Fig. 7 A). All of these particles inhibited presentation when used in amounts greater than $1\text{--}50 \times 10^7$ particles per well, possibly caused by steric interference with the interaction between APC and B3Z (data not shown). In contrast to latex or magnetic particles, degradable particles such as zymosan (yeast cell walls, 2–4- μ m diameter) or formaldehyde-fixed bacteria (*Staphylococcus aureus*, *Escherichia coli*) did not have any effect at concentrations at which latex and magnetic beads gave high dose inhibition (Fig. 7 A and data not shown). Zymosan and *S. aureus*, however, were able to stimulate K^b presentation of OVA by M ϕ when 100-fold more particles were used than with nondegradable particles (Fig. 7 B; other particles not tested at these doses). The relative inefficiency of the biological particles in promoting presentation of coadministered OVA was not caused by competition for K^b binding by peptides derived from these particles because cofeeding zymosan did not alter the dose response to OVA + latex beads (data not shown).

Phagocytic Stimuli Induce Class I Presentation by Only a Subset of APC Taking up Both Particles and Antigen. Although phagocytosis of particles was clearly able to induce class I presentation of OVA to B3Z, the total amount of IL-2 produced at

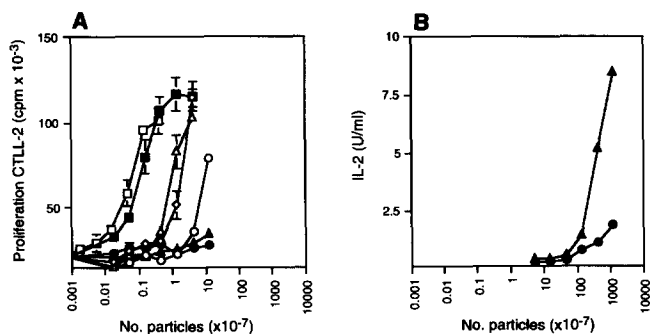


Figure 7. Different phagocytic particles stimulate class I presentation by thio-M ϕ with varying efficiencies. (A) Thio-PEC were plated with the indicated number of particles per well + 10 mg/ml OVA, cultured for 22 h, and washed and fixed as in Fig. 3. B3Z were added at 5×10^4 per well and IL-2 in supernatants was measured 24 h later by proliferation of CTLL-2 indicator cells as in Fig. 1. (B) Thio-PEC were cultured for 2 d, nonadherent cells were discarded, and the indicated number of zymosan or *S. aureus* particles + 5 mg/ml OVA were added to each well before overnight incubation. Adherent M ϕ were washed and fixed and B3Z were added for 24 h. IL-2 in supernatants was measured by ELISA and the means of triplicate cultures were converted to IL-2 U/ml by comparison to a standard curve. Note that the scale of the x axis is the same in both A and B to facilitate comparison. The data in A and B come from separate experiments. \circ —, 1.0 μ m latex; \diamond —, 1.5 μ m latex; \triangle —, 2.0 μ m latex; \blacksquare —, BSA beads; \square —, Dynabeads™; \blacktriangle —, *S. aureus*; \blacksquare —, zymosan.

optimal beads numbers and maximal available concentrations of soluble OVA was much lower than that which could be obtained using SIINFEKL-pulsed APC in the same experiment. For example, supernatants from stimulation assays using APC exposed to OVA + beads could seldom induce half-maximal proliferation of CTLL-2 indicator cells at a 1:4 dilution, whereas supernatants from peptide-stimulated cells did so at 1:30 dilution or greater (see Fig. 5, A and B).

Peptide reconstruction experiments demonstrated that the response obtained with OVA + beads could be replicated using APC uniformly pulsed with low concentrations of SIINFEKL or by diluting APC pulsed with high concentrations of peptide into unpulsed APC (data not shown). To differentiate between these models (a low average density of peptide-K^b on all cells versus a few cells with substantial levels and many cells with essentially no complexes), we used lysis by CTL specific for OVA + K^b to determine the absolute number of thio-M ϕ that presented immunologically relevant numbers of OVA peptides in association with class I after exposure to OVA + BSA beads. These CTL responded to offered SIINFEKL at peptide concentrations 100 times lower than necessary to obtain a measurable response from B3Z (data not shown). In three consecutive experiments, at all tested E/T ratios, the maximal amount of specific killing of PEC pulsed with OVA + BSA beads was 10–15% above the background obtained with OVA alone, although 80–100% of PEC pulsed with SIINFEKL peptide were capable of being lysed by the same CTL population (Fig. 8 A). These results suggested that after pulsing with OVA + BSA-beads, only a fraction of PEC were able to present OVA peptides in associ-

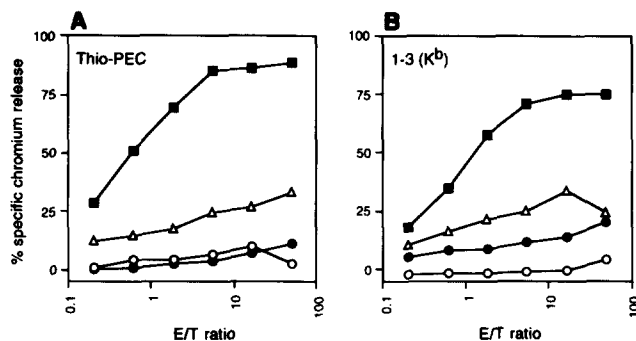


Figure 8. Anti-OVA CTL kill only a fraction of APC pulsed with OVA + beads. 3×10^6 thio-PEC (A) or 5×10^6 1-3 (K^b) (B) were incubated in sterile polypropylene tubes in medium alone (No Tx) or in medium containing OVA \pm BSA beads or 1 μ M SIINFEKL. OVA concentrations were 1 mg/ml in A and 5 mg/ml in B, and BSA beads were added at 1.25×10^8 per tube in A or 2.5×10^8 per tube in B. After overnight culture, cells were washed, labeled with ⁵¹Cr, and used as targets for a CTL line against SIINFEKL + K^b in a standard 4-h chromium release assay at different E/T ratios. The data in A and B come from separate experiments. \circ —, no Tx; \bullet —, OVA; \triangle —, OVA + BSA beads; \blacksquare —, SIINFEKL.

ation with K^b at levels adequate for stimulation of highly sensitive CTL.

Class I Presentation Induced by Phagocytic Stimuli may be a Stochastic Event. Thio-PEC contain activated and resting M ϕ in addition to inflammatory cells. Conceivably, one of these cell types might account for the subpopulation of PEC that presented OVA-derived peptides in association with class I after exposure to soluble antigen plus beads, perhaps by expressing higher levels of K^b, or because of quantitative differences in the amount of OVA or particles internalized. Flow cytometric analysis, however, revealed that thio-PEC had a unimodal distribution of surface K^b expression (Fig. 9 A), FITC-OVA and lucifer yellow uptake (Fig. 1 E), and uptake of fluorescent latex beads (Fig. 9 B).

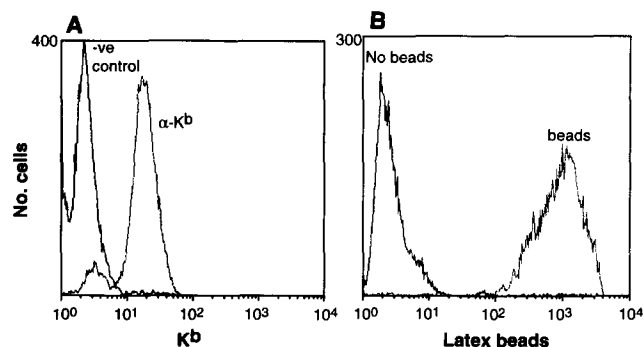


Figure 9. FACS[®] analysis of thio-PEC. (A) Uniform expression of K^b by thio-PEC. Fixed cells were stained with FITC-conjugated α -K^b or FITC-conjugated mouse IgG2a as a negative control. (B) Uniform uptake of fluorescent latex beads by thio-PEC. 5×10^6 thio-PEC were incubated overnight with 2.5×10^8 fluorescent latex beads (1.5 μ m), and propidium iodide-negative cells were analyzed for association with particles.

This analysis thus offered no obvious phenotypic explanation for the existence of only a subset of thio-PEC with the ability to present exogenous antigen-derived peptides via class I, but it could not address the possibility that the PEC contained a subset of cells with a distinct intracellular pathway for delivery of phagosomal antigens into the class I processing pathway. To explore this issue, we turned to our observation that non-MØ were also capable of presenting OVA peptides via K^b after exposure to OVA + beads. Such cloned cell lines should not show marked heterogeneity in the activity of any such putative transport pathway. When 1-3 (K^b) cells pulsed with OVA + particles were tested for killing by CTL, however, a low level of specific lysis was again observed (Fig. 8 B) despite the fact that more than 90% of 1-3 (K^b) cells were associated with particles (data not shown). Thus, even a clonal cell population showing extensive bead and antigen uptake generates only a few cells with substantial OVA peptide-K^b display on the cell surface.

Discussion

The hypothesis (34) that MHC class I molecules present peptides derived from cytosolic antigens ("endogenous antigens"), whereas MHC class II molecules present peptides derived from antigens in the endocytic pathway ("exogenous antigens"), has become the generally accepted paradigm for antigen processing (1). The segregation between the two antigen presentation pathways, however, is not absolute and there are examples of MHC class II presentation of endogenously synthesized, cytosolic antigens (35), as well as several instances of class I responses to exogenous antigens (36). Although some examples of this type have been claimed to represent binding to class I or II in sites other than the ER or endosomes, respectively, most appear to represent unexpected routing of an exogenous antigen into the cytosol or an endogenous antigen to the endocytic pathway, followed by MHC class I or class II molecule loading in their preferred compartments (1). It is possible that these exceptions represent the activity of specialized APC possessing different mechanisms for translocating protein antigens or their fragments. In this respect, MØ have been suggested to process a unique pathway for presentation of peptides derived from exogenous soluble antigens in association with MHC class I molecules (13, 14). Because the discovery of novel protein or peptide transport mechanisms involved in antigen presentation is of substantial importance to both immunology and cell biology, we undertook a reexamination of soluble antigen presentation by MHC class I molecules of MØ.

Unlike previous studies using the same protein antigen (OVA) and MHC class I presentation element (K^b) (8, 13), presentation by MØ of peptides derived from soluble OVA was not observed consistently. Because a different assay T cell (B3Z) was used in our study compared to that used by Rock and colleagues, we cannot rule out the possibility that their assay system was more sensitive, and detected presentation at a level that we could not. We have noticed, however,

that many batches of OVA purchased commercially (seven of nine tested, Fig. 3) allow presentation by fixed MØ after pulsing in serum-containing medium, possibly as a result of extracellular processing by serum proteases (37, 38). Paraformaldehyde fixed MØ are a very sensitive indicator of extracellular proteolysis, as they present peptide 100–1,000× better than live cells (Fig. 4 B and reference 30) and better than other fixed cell types with higher levels of K^b such as 1-3 (K^b) (Reis e Sousa, C., and R. N. Germain, unpublished observations). When we used ultra-"clean" preparations of OVA that could not be presented by fixed MØ, we no longer were able to reproducibly obtain presentation of soluble antigen to B3Z by live MØ without addition of beads, although weak presentation of unscreened antigen was occasionally seen (e.g., Figs. 1 A and 6). Thus, it does not appear that MØ are constitutively capable of efficient processing and presentation of free soluble protein antigens via the class I pathway, although it can readily be shown by use of labeled antigen or assays of MHC class II presentation that these cells take up substantial amounts of protein into the endocytic pathway.

Class I responses to exogenous antigens *in vivo*, however, appears most often to involve particulate rather than soluble antigens (whole cells or cell debris in cross-priming [2–4, 6–12], bacteria and protozoa during infections [9, 10]). Therefore, much attention has been focused on recent reports suggesting that phagocytosis markedly increases the efficiency of processing of exogenous antigens for MHC class I presentation by MØ (15–18). It is possible that inefficient delivery of exogenous antigens to the class I pathway can be compensated for by phagocytic uptake which increases the effective delivery of antigen to the cell, compared to fluid phase uptake (16, 17). Surface Ig uptake of antigen is known to increase class II antigen presentation efficiency by three to four orders of magnitude (39), and this is consistent with the data of Barnaba et al., who found that antigen-specific, but not unspecific B cells, could present hepatitis surface antigen via class I (7). In accord with this, class I presentation was not observed by either Rock or ourselves using antigen-unspecific B cells and soluble antigen. Alternatively, the process of active phagocytosis could stimulate a cell to turn on a specific protein/peptide transport pathway that would deliver antigen into the class I pathway.

To distinguish between these possibilities, it was necessary to dissociate antigen uptake from particle delivery, to examine several cell types in the presence and absence of phagocytic stimuli, and to vary these stimuli. When these experiments were performed, we found that several types of inert unconjugated particles could promote class I presentation of soluble OVA via class I. This gain in presentation activity could not be accounted for by increased antigen uptake because the accumulation of soluble tracers by cells pulsed with particles was not increased (Fig. 1 E) and OVA directly conjugated to particles was less efficient at promoting presentation to B3Z by thio-MØ despite being more efficient at delivering antigen for class II presentation (Fig. 1). These results argue strongly in favor of a qualitative rather than quantitative effect of phagocytosis on class I presentation of

exogenous antigen. This effect required simultaneous administration of particles and antigen (Reis e Sousa, C., and R. N. Germain, unpublished results), implying either a physical basis to the activity rather than the induction of a novel transport pathway, or the exclusive localization of any putative transport system to the phagosomes formed at the time of bead exposure.

The inability of thio-M ϕ to show effective class I presentation of OVA directly conjugated to beads is in contrast to previous results (16–18) also using inert particles conjugated to comparable amounts of antigen. This did not result from a failure to enhance delivery of OVA to the thio-M ϕ because we observed greatly augmented class II presentation of an OVA-derived peptide by the same cells. Nor did it result from a chemical inactivation of the SIINFEKL determinant within the conjugated OVA, since the same OVA beads were able to sensitize non-M ϕ for class I presentation to the same assay hybridoma. We are thus unable to explain the difference between our studies and those of others, other than to suggest, based on the detectable activity of the conjugates in non-M ϕ environments, that there may have been more extensive degradation of the OVA by the M ϕ populations, leading to less presentation than with soluble OVA + beads. This reduced level of presentation may nevertheless have been sufficient to trigger the particular hybridomas used in the other studies (16–18).

Despite this clear difference in the behavior of OVA beads between the two studies, the mechanism of presentation of OVA + beads had the characteristics of the endogenous pathway for class I processing (Figs. 4 and 5), as was reported by Rock and colleagues for presentation of OVA directly conjugated to beads (16, 18), and in contrast to the mechanism of peptide regurgitation invoked by Harding et al. (15, 17). This capacity was not unique to M ϕ , however, as previously suggested (16–18). Rather, several different cell types capable of phagocytosis (keratinocytes, embryonic cell lines, fibroblasts), but not the one nonphagocytic cell we tested (a B hybridoma), were able to present OVA peptides in association with K^b when coexposed to phagocytic stimuli, but not soluble OVA alone. Several particle types were effective in promoting class I presentation of OVA. These findings, together with the absence of a quantitative explanation for the effect of bead exposure on class I presentation of soluble OVA, encouraged us to further examine whether phagocytosis induced the activity of a novel pathway for importing endosomal antigens into the cytosol, analogous in reverse to the import of cytosolic antigens for degradation in lysosomes (40). Such a pathway from endosomes to cytosol has not been described, although isolated lysosomes have been reported to release peptides *in vitro*, apparently in the absence of damage to lysosomal membranes (41). A powerful biological argument for the existence of such a pathway is that tying MHC class I processing of exogenous antigens to phagocytosis would allow presentation of antigens derived from endosomal parasites while excluding presentation of soluble macromolecules taken up as part of a cell's normal pinocytic traffic; this would make it possible to discriminate between cells infected with

endosomal parasites and those that have taken up parasite proteins but are not infected.

It was thus surprising to note that the magnitude of the response to OVA + particles was at most only a few percent of that achieved using peptide antigen, despite a unimodal expression of K^b, the very efficient uptake of beads by virtually all M ϕ , and the ready detection of cointernalized protein by the same cells. If phagocytosis could activate a phagosome-to-cytosol protein/peptide delivery system, presentation under these conditions should be much more effective. The inefficiency of presentation raises questions about the suitability of this approach for the development of vaccines intended to elicit strong CTL responses (16–18), particularly in view of alternative (possibly more effective) strategies, including viral vectors or liposomes. Nonetheless, because thio-PEC are heterogeneous, other factors that are the property of a particular stage of activation or subpopulation of cells (for example, levels of TAP-1 and TAP-2 expression) might limit the function of this pathway. Experiments using CTL did suggest that the low presentation resulted from having only a small subpopulation of cells presenting OVA via class I, rather than the inefficient presentation of antigen by all the cells in the M ϕ population. We have so far been unable, though, to find substantial differences between thio-PEC before or after treatment with IFN- γ , or between thio-PEC and bone marrow-derived M ϕ , in the presentation of OVA offered in the presence of beads (not shown). Moreover, analysis of CTL killing of a clonal, nonprofessional phagocytic APC [1-3 (K^b)], also revealed a similar low level of sensitization by OVA + beads that again resulted in CTL lysis of only a small fraction of all phagocytically active cells in the population (Fig. 8 B).

These results are difficult to reconcile with the hypothesis that phagocytosis per se activates a novel antigen-processing pathway that has evolved to respond to infections by endosomal parasites. It clearly remains possible that the immune system has developed a specific mechanism for sensing (live) intracellular pathogens that induces an efficient pathway for delivery of endosomal/phagosomal antigens into the class I pathway, a mechanism for which we have not provided the proper inductive stimulus. A simpler explanation, however, is that among APC that have taken up large numbers of particles, escape of antigen from some vesicles into the cytosol occurs in a few cells, with consequent access to the endogenous pathway for MHC class I presentation, in a manner similar to that achieved by osmotic lysis of pinosomes (37). We term this the "indigestion" model because it involves particle overloading of the phagosomal compartment. This "indigestion" model is consistent with the observation that larger, non-degradable particles are much more efficient than smaller, degradable ones at promoting class I presentation in M ϕ (Fig. 7).

Antigen escape might be affected by whether a particle aggregate enters a single phagosome or by membrane composition and, consequently, by the stage of the cell cycle or the stage of differentiation of the cell. It could involve incomplete fusion events between two phagosomal vesicles, al-

lowing soluble contents to leak into the cytoplasm or gross disruption of the phagosomal membrane. The latter mechanism is used by *Listeria* and *Trypanosoma* organisms to establish intracellular infection through secretion of specific hemolysins (9, 10). Cytosolic entry, however, can be observed with microorganisms not known to possess hemolysins, such as some strains of *Mycobacteria*, which are normally confined to the endosomal compartment (42, 43). Cytosolic escape of *Mycobacteria tuberculosis* occurs in alveolar M ϕ from naive rabbits but not in M ϕ from rabbits immunized with *M. bovis* bacillus Calmette-Guérin, demonstrating that escape is dependent, at least in part, on properties of the infected cell (43). Thus, "indigestion" after uptake of inert particles might actually mimic what happens during active replication of an organism in the phagosomal compartment. Disruption of

membrane integrity (an "ulcer") would develop if the phagocyte could not control parasite replication, or if the parasite arrested lysosomal degradation by preventing fusion of the phagosomal vacuole with lysosomes or by neutralizing phagosomal pH (9, 10). Such a mechanism would specifically mark for CTL lysis only those cells in which parasite replication went unchecked. Normal scavenging activity in the absence of infection should not give "indigestion" and, hence, should not sensitize M ϕ for CTL killing. A critical event in determining escape could therefore be the inability to condense phagosomes and recycle membrane to the cell surface (44). Further studies are clearly warranted to determine if "indigestion" during infection by intracellular parasites contributes to the recruitment of CD8 effectors to the site of infection.

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References

1. Germain, R.N. 1994. MHC-dependent antigen processing and peptide presentation: providing signals for T lymphocyte activation. *Cell* 76:287-299.
2. Bevan, M.J. 1976. Cross-priming for a secondary response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143:1283-1288.
3. Gooding, L.R., and C.B. Edwards. 1980. H-2 antigen requirements in the in vitro induction of SV40-specific cytotoxic T lymphocytes. *J. Immunol.* 124:1258-1262.
4. Staerz, U.D., H. Karasuyama, and A.M. Garner. 1987. Cytotoxic T lymphocytes against a soluble protein. *Nature (Lond.)* 329:449-451.
5. Jin, Y., W.K. Shih, and I. Berkower. 1988. Human T cell response to the surface antigen of hepatitis B virus (HBsAg). Endosomal and nonendosomal processing pathways are accessible to both endogenous and exogenous antigen. *J. Exp. Med.* 168:293-306.
6. Carbone, F.R., and M.J. Bevan. 1990. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171:377-387.
7. Barnaba, V., A. Franco, A. Alberti, R. Benvenuto, and F. Balsano. 1990. Selective killing of hepatitis B envelope antigen-specific B cells by class I-restricted, exogenous antigen-specific T lymphocytes. *Nature (Lond.)* 345:258-260.
8. Rock, K.L., S. Gamble, and L. Rothstein. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science (Wash. DC)* 249:918-921.
9. Kaufmann, S.H. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* 11:129-163.
10. Scott, P., and A. Sher. 1993. Immunoparasitology. In *Fundamental Immunology*. W. Paul, editor. Raven Press Ltd., New York. pp. 1179-1210.
11. Bevan, M.J. 1987. Class discrimination in the world of immunology. *Nature (Lond.)* 325:192-194.
12. Debrick, J.E., P.A. Campbell, and U.D. Staerz. 1991. Macrophages as accessory cells for class I MHC-restricted immune responses. *J. Immunol.* 147:2846-2451.
13. Rock, K.L., L. Rothstein, S. Gamble, and C. Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J. Immunol.* 150:438-446.
14. Denkers, E.Y., R.T. Gazzinelli, S. Hieny, P. Caspar, and A. Sher. 1993. Bone marrow macrophages process exogenous *Toxoplasma gondii* polypeptides for recognition by parasite-specific cytolytic T lymphocytes. *J. Immunol.* 150:517-526.
15. Pfeifer, J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark, and C.V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature (Lond.)* 361:359-362.
16. Kovacsics-Bankowski, M., K. Clark, B. Benacerraf, and K.L. Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. USA* 90:4942-4946.
17. Harding, C.V., and R. Song. 1994. Phagocytic processing of

- exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J. Immunol.* 153:4925–4933.
18. Kovacovics-Bankowski, M., and K.L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science (Wash. DC)*. 267:243–246.
 19. Reis e Sousa, C., P.D. Stahl, and J.M. Austyn. 1993. Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med.* 178:509–519.
 20. Bhattacharya, A., M.E. Dorf, and T.A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488–2495.
 21. Kraal, G., M. Breel, M. Janse, and G. Bruin. 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* 163:981–997.
 22. Allen, H., D. Wraith, P. Pala, B. Askonas, and R.A. Flavell. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature (Lond.)*. 309:279–281.
 23. Catipovic, B., J. Dal Porto, M. Mage, T.E. Johansen, and J.P. Schneck. 1992. Major histocompatibility complex conformational epitopes are peptide specific. *J. Exp. Med.* 176:1611–1618.
 24. Bikoff, E.K., L. Jaffe, R.K. Ribaud, G.R. Otten, R.N. Germain, and E.J. Robertson. 1991. MHC class I surface expression in embryo-derived cell lines inducible with peptide or interferon. *Nature (Lond.)*. 354:235–238.
 25. Karttunen, J., S. Sanderson, and N. Shastri. 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. USA*. 89:6020–6024.
 26. Hugo, P., J.W. Kappler, D.I. Godfrey, and P.C. Marrack. 1992. A cell line that can induce thymocyte positive selection. *Nature (Lond.)*. 360:679–682.
 27. Bennink, J.R., J.W. Yewdell, G.L. Smith, C. Moller, and B. Moss. 1984. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. *Nature (Lond.)*. 311:578–579.
 28. Racioppi, L., F. Ronchese, L.A. Matis, and R.N. Germain. 1993. Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. *J. Exp. Med.* 177:1047–1060.
 29. Shastri, N., and F. Gonzalez. 1993. Endogenous generation and presentation of the ovalbumin peptide/K^b complex to T cells. *J. Immunol.* 150:2724–2736.
 30. Rock, K.L., L. Rothstein, S. Gamble, C. Gramm, and B. Benacerraf. 1992. Chemical cross-linking of class I molecules on cells creates receptive peptide binding sites. *J. Immunol.* 148:1451–1457.
 31. Miller, S.G., L. Carnell, and H.-P.H. Moore. 1992. Post-Golgi membrane traffic: brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. *J. Cell Biol.* 118:267–283.
 32. Stirpe, F., S. Olsnes, and A. Pihl. 1980. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. *J. Biol. Chem.* 255:6947–6953.
 33. Kappler, J., J. White, D. Wegmann, E. Mustain, and P. Marrack. 1982. Antigen presentation by Ia⁺ B cell hybridomas to H-2-restricted T cell hybridomas. *Proc. Natl. Acad. Sci. USA*. 79:3604–3607.
 34. Germain, R.N. 1986. Immunology. The ins and outs of antigen processing and presentation. *Nature (Lond.)*. 322:687–689.
 35. Long, E.O. 1992. Antigen processing for presentation to CD4⁺ T cells. *New Biol.* 4:274–282.
 36. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* 52:1–123.
 37. Moore, M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell*. 54:777–785.
 38. Kozlowski, S., M. Corr, T. Takeshita, L.F. Boyd, C.D. Pendleton, R.N. Germain, J.A. Berzofsky, and D.H. Margulies. 1992. Serum angiotensin-1 converting enzyme activity processes a human immunodeficiency virus 1 gp160 peptide for presentation by major histocompatibility complex class I molecules. *J. Exp. Med.* 175:1417–1422.
 39. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 8:773–793.
 40. Dice, J.F. 1992. Selective degradation of cytosolic proteins by lysosomes. *Ann. NY Acad. Sci.* 674:58–64.
 41. Isenman, L.D., and J.F. Dice. 1993. Selective release of peptides from lysosomes. *J. Biol. Chem.* 268:23856–23859.
 42. Mor, N. 1983. Intracellular location of *Mycobacterium leprae* in macrophages of normal and immune-deficient mice and effect of rifampin. *Infect. Immun.* 42:802–811.
 43. Leake, E.S., Q.N. Myrvik, and M.J. Wright. 1984. Phagosomal membranes of *Mycobacterium bovis* BCG-immune alveolar macrophages are resistant to disruption by *Mycobacterium tuberculosis* H37Rv. *Infect. Immun.* 43:443–446.
 44. Steinman, R.M., I.S. Mellman, W.A. Muller, and Z.A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1–27.