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# MHC Class I-Restricted Presentation of Maleylated Protein Binding to Scavenger Receptors[1]

**Pratima Bansal,[2,3] Paushali Mukherjee,[2] Sandip K. Basu, Anna George, Vineeta Bal, and Satyajit Rath[4]**

**Pathways for loading exogenous protein-derived peptides on MHC class I are thought to be present mainly in monocyte-lineage cells and to involve phagocytosis- or macropinocytosis-mediated antigenic leakage into either cytosol or extracellular milieu to give peptide access to MHC class I. We show that maleylation of OVA enhanced its presentation to an OVA-specific MHC class I-restricted T cell line by both macrophages and B cells. This enhanced presentation involved uptake through receptors of scavenger receptor (SR)-like ligand specificity, was TAP-1-independent, and was inhibited by low levels (2 mM) of ammonium chloride. No peptide loading of bystander APCs by maleylated (maleyl) OVA-pulsed macrophages was detected. Demaleylated maleyl-OVA showed enhanced MHC class I-restricted presentation through receptor-mediated uptake and remained highly sensitive to 2 mM ammonium chloride. However, if receptor binding of maleyl-OVA was inhibited by maleylated BSA, the residual presentation was relatively resistant to 2 mM ammonium chloride. Maleyl-OVA directly introduced into the cytosol via osmotic lysis of pinosomes was poorly presented, confirming that receptor-mediated presentation of exogenous maleyl-OVA was unlikely to involve a cytosolic pathway. Demaleylated maleyl-OVA was well presented as a cytosolic Ag, consistent with the dependence of cytosolic processing on protein ubiquitination. Thus, receptor-specific delivery of exogenous protein Ags to APCs can result in enhanced MHC class I-restricted presentation, suggesting that the exogenous pathway of peptide loading for MHC class I may be a constitutive property dependent mainly on the quantity of Ag taken up by APCs.** *The Journal of Immunology,* 1999, 162: 4430–4437.

P eptides from proteins exogenous to APCs are usually presented on MHC class II, while those from APC-endogenous proteins are presented on MHC class I. Exogenous proteins are taken up by various endocytic processes from the extracellular milieu and degraded in the endolysosomal compartments, and the resultant peptides are bound to MHC class II in these compartments (1). Endogenous cytosolic proteins are degraded predominantly by a ubiquitin-ATP-proteasome-dependent pathway (2) in the cytosol, and the peptides are then transported by TAP (3, 4) into the endoplasmic reticulum (ER)[5] (5–8), where they bind to nascent MHC class I and the stable assembly is transported to the cell surface (9).

However, several exceptions to these rules segregating MHC class I and class II presentation pathways have been reported.

Some endogenous protein-derived peptides are known to be presented on MHC class II (10–14), and there are reports of presentation of peptides derived from soluble, exogenous proteins on MHC class I. Cytotoxic CD8 responses could be generated by pathogens resident in the phagolysosomal compartments of APCs (15, 16), as well as by soluble OVA (17), suggesting the existence of a mechanism for presentation of exogenous Ags on MHC class I. Macrophages were initially thought to be the only APC capable of such presentation, and B cells were shown to be incapable of it (18–20). However, similar properties have now been described for dendritic cells (21, 22), fibroblasts (23), and mast cells (24).

The ability of most of these APC types to present exogenous Ag on MHC class I still appears dependent on their phagocytic abilities (18–24). It is not clear whether this correlation with phagocytosis is simply due to the advantage of achieving high concentrations of Ag in the APC, or whether specific cell biological properties of phagosomes are crucial, since the processing mechanisms involved are not yet unambiguously understood. Various pathways have been reported for processing of exogenous Ag for presentation on MHC class I. According to the endosomal processing model, exogenous proteins are processed in the endosomal compartments, and peptides are then regurgitated, as a peculiar property of macrophages and dendritic cells, into the extracellular milieu to associate with surface MHC class I (25). The cytosolic processing model depends on formation of leaky phagosomes (23, 26) and/or leaky macropinosomes (27), allowing escape of proteins from endosomal fluid into the cytosol, with subsequent cytosolic processing of Ags via the conventional proteasome- and TAP-dependent pathway (26, 27). In accordance with the endosomal model, one report finds Ags encapsulated in acid-sensitive liposomes to be presented on MHC class I more efficiently than acid-resistant ones (28), while another report finds Ags in both

acid-sensitive and acid-resistant liposomes presented equally well on MHC class I, as would be expected by the cytosolic model (29).

We have been analyzing the consequences of Ag delivery to various APCs via SR-mediated endocytosis (30–32) for presentation on MHC class II to various APCs and have now extended these studies to the effects of such SR-mediated uptake on MHC class I-restricted presentation and to the pathways used in such presentation.

## Materials and Methods

### Mice

C3H/HeJ (H-$2^k$) and C57BL/6 (H-$2^b$) mice (The Jackson Laboratory, Bar Harbor, ME), as well as TAP-1-deficient (TAP-1-KO) mice (H-$2^b$, gift of Dr. L. van Kaer, Vanderbilt University, Nashville, TN) bred in the small animal facility of the National Institute of Immunology (New Delhi, India), were used for experiments at 6–10 wk of age.

### Antigens

OVA (Sigma, St. Louis, MO) was dialyzed extensively against PBS to remove small degradation products. OVA or BSA were maleylated with maleic anhydride (Sigma) at alkaline pH as previously described (33), followed by extensive dialysis against PBS. The degree of maleylation was assessed by estimating the loss of free $\epsilon$-amino groups as measured by trinitrobenzenesulfonic acid (34). Maleyl-OVA or maleyl-BSA were used only when maleylation was >95%. Demaleylation was performed at pH 3.5 as described (33). The demaleylated maleylated (de-maleyl)-OVA used showed regeneration of >90% of the active $\epsilon$-amino groups of OVA as measured by the trinitrobenzenesulfonic acid assay (34). In addition to maleyl-BSA, the SR ligands fucoidin and polyguanylic acid (polyG) (Sigma) were also used where appropriate.

### Cell lines

The MHC class I (H-$2K^b$)-restricted, OVA-specific T cell transfectant (B3.4.5) was a gift from Drs. C. Hogquist and M. Bevan (University of Washington, Seattle, WA). It recognizes the OVA peptide 257–264, SIINFEKL. The IL-2-dependent T cell line, CTLL-2, was obtained from the American Type Culture Collection (ATCC; Manassas, VA).

### Cytosolic delivery of Ags by osmotic lysis of pinosomes

Proteins were delivered into the cytosol using osmotic lysis of pinosomes as described (35). Briefly, APCs were incubated in hypertonic solution (0.5 M sucrose, 10% polyethylene glycol 800, and 10 mM HEPES) containing 10 mg/ml of Ag in serum-free medium (DMEM; Life Technologies, New Delhi, India) for 10 min at 37°C, followed by incubation in isotonic serum-free medium for 5 min. Cells were then thoroughly washed before being fixed with 1% paraformaldehyde (Sigma) for 1 min, washed, and used as APCs in T cell stimulation assays.

### Preparation of cellular subpopulations

Peritoneal exudate cells were elicited from C57BL/6 and TAP-1-KO mice by i.p. injection of 4% Brewer's thioglycollate broth (HiMedia, Mumbai, India) and by performing peritoneal lavage 72 h later with 10 ml chilled PBS. Macrophages were isolated by plastic adherence for 1 h. Splenic B cells were isolated from plastic adherence-depleted single-cell suspensions of splenic cells by labeling them with either anti-B220-biotin (PharMingen, San Diego, CA) followed by streptavidin-coupled magnetic beads or anti-CD19-coupled magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for separation on magnetic columns (Miltenyi Biotec). Macrophage and B cell preparations were monitored flow cytometrically and used at >95% purity.

### Ag presentation assays

B3.4.5 cells (1 × $10^5$/well) were stimulated either with titrated doses of OVA, maleyl-OVA, or de-maleyl-OVA presented by various APC types, or with titrated numbers of APCs exogenously or cytosolically loaded with various Ags in triplicate cultures. Culture supernatants were collected 24–36 h later. The amount of IL-2 induced was measured by the proliferative responses of CTLL-2 cells and is shown as proliferation (mean ± SE) of triplicate CTLL-2 cultures.

All T cell cultures were done in 200 $\mu$l of tissue culture medium (DMEM) containing 10% FCS (Life Technologies), antibiotics, and 0.05 mM 2-ME in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ). Proliferative responses of CTLL-2 cells were measured by stimulating 1 × $10^4$
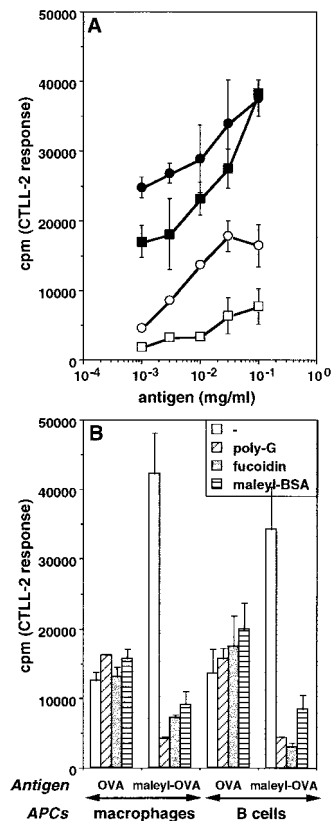


**FIGURE 1.** MHC class I-restricted presentation of maleyl-OVA is better than that of native OVA and is receptor mediated. *A*, Shows the responses of the MHC class I-restricted T cell line B3.4.5 to peritoneal macrophages (circles) or splenic B cells (squares) presenting exogenous maleyl-OVA (filled symbols) or native OVA (open symbols). Background [$^3$H]thymidine incorporation in the absence of any Ag was 2500 cpm. *B*, Shows inhibition of MHC class I-restricted presentation of native and maleyl-OVA by SR ligands. Peritoneal macrophages or splenic B cells were pulsed with 100 $\mu$g/ml native OVA or maleyl-OVA in the presence or absence (−) of 100 $\mu$g/ml of the various inhibitors shown. Background [$^3$H]thymidine incorporation in the absence of Ag was 1500 cpm. The data are representative of three independent experiments.

CTLL-2 cells per well with B3.4.5 culture supernatants, incubating for 24 h, and pulsing the plates with 0.5 $\mu$Ci/well of [$^3$H]thymidine (NEN Life Science Products, Boston, MA) for 12–16 h. Plates were harvested onto glass fiber filters for scintillation counting (Betaplate; Wallac, Turku, Finland).

## Results

### SR-mediated Ag uptake enhances MHC class I-restricted presentation by both macrophages and B cells

Peritoneal exudate cell macrophages or splenic B cells were used to present either native OVA or maleyl-OVA in medium to the OVA-specific H-$2K^b$-restricted T cell transfectant line B3.4.5. Fig. 1*A* shows that macrophages presented native OVA to B3.4.5, while B cells exhibited almost no presentation. However, when maleyl-OVA was used as the Ag, both macrophages and B cells showed excellent presentation to B3.4.5 (Fig. 1*A*).

We have previously shown that both macrophages and B cells can take up maleylated Ag via receptor-mediated uptake (32). To confirm that the enhanced presentation of maleyl-OVA in the present experiments was indeed receptor-mediated, various structurally unrelated SR-binding competitor ligands were used to inhibit both OVA and maleyl-OVA presentation by macrophages or
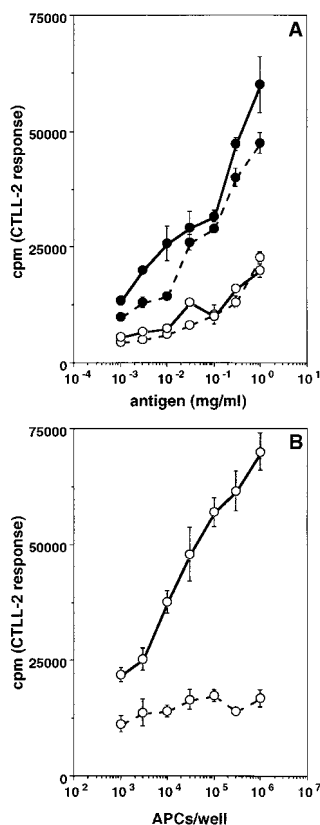
**FIGURE 2.** MHC class I-restricted presentation of exogenous native or maleyl-OVA does not require TAP-1. *A*, Shows the response of the T cell line B3.4.5 to peritoneal macrophages from TAP-1-KO (broken lines) or wild-type (continuous lines) mice presenting native OVA (hollow circles) or maleyl-OVA (filled circles) exogenously. *B*, Shows the responses of B3.4.5 to titrated numbers of wild-type (continuous lines) or TAP-1-KO (broken lines) peritoneal macrophages loaded cytosolically with native OVA (10 mg/ml). Background [$^3$H]thymidine incorporation in the absence of Ag was 3000 cpm. The data are representative of three independent experiments.



**FIGURE 3.** Processing of exogenous maleyl-OVA is more sensitive to inhibition of endosomal acidification by ammonium chloride than processing of exogenous native OVA. Peritoneal macrophages were pulsed with 1 mg/ml of native OVA (hollow circles) or maleyl-OVA (filled circles) in the presence or absence of graded concentrations of ammonium chloride as shown and used after washing and fixation to stimulate B3.4.5 T cells. The inhibition of the B3.4.5 response by various doses of ammonium chloride added to the macrophages during processing is shown. The response to native OVA was 10,500 cpm, and that to maleyl-OVA was 30,700 cpm, while the background counts were 2,000 cpm. The data are representative of five independent experiments.

B cells. The known SR ligands fucoidin, polyG, and maleyl-BSA (36, 37) all significantly inhibited the presentation of maleyl-OVA by both macrophages and B cells, although they did not decrease the efficiency of OVA presentation to B3.4.5 by either APC type (Fig. 1*B*).

### Presentation of exogenous OVA and maleyl-OVA is TAP independent

If exogenously administered Ags were to leak into the cytosol from endosomes and be processed there, TAP would be required for transport of peptides into the ER lumen for binding to MHC class I, as reported earlier (26). However, there are also reports of the presentation of exogenous Ags being TAP independent (25, 38). To determine the role of TAP in the presentation of maleyl-OVA, we used peritoneal macrophages from either TAP-1-KO or wild-type mice to present either OVA or maleyl-OVA in the culture medium to B3.4.5. The results (Fig. 2*A*) show that TAP-1-KO macrophages presented both native OVA and maleyl-OVA as efficiently as wild-type macrophages. They did not, however, present cytosolically loaded OVA, establishing their mutant phenotype (Fig. 2*B*).

### Processing of maleyl-OVA is sensitive to endosomal pH

An alternative to the TAP-dependent cytosolic processing model for MHC class I-restricted presentation of exogenously adminis-
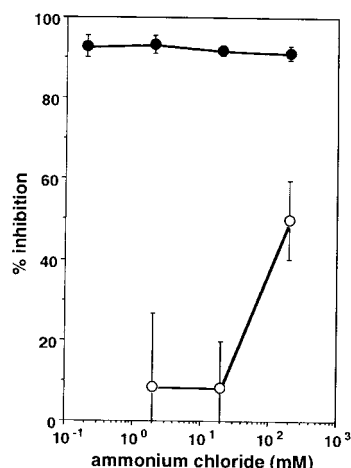
tered Ags is that this processing takes place in phagosomes and the resultant peptides are regurgitated into extracellular medium where they associate with surface MHC class I (25). Protein processing in the endolysosomal compartment is likely to utilize the acid proteases available there (39). Some reports have shown that processing of exogenous Ag is not sensitive to inhibition of endosomal acidification (24, 26), lending weight to the argument that the exogenous Ag may leak from the phagosome/endosome to the cytosol and be processed there. Since the presentation of both pinocytosed and receptor-binding protein in the present system was TAP-1 independent, we examined its dependence on endosomal acidification. Processing of exogenous native or maleyl-OVA in the presence of ammonium chloride was assessed by pulsing H-2$^b$ macrophages with 1 mg/ml OVA or maleyl-OVA in the presence or absence of various concentrations of ammonium chloride for 3 h, washing and fixing the cells, and using them to stimulate B3.4.5. The presentation of native OVA was reproducibly inhibited only at 200 mM ammonium chloride, and then only partially, but the presentation of maleyl-OVA was greatly inhibited even by 0.2 mM ammonium chloride (Fig. 3).

We next examined whether macrophages could take up and process maleyl-OVA and regurgitate peptides for binding to surface MHC class I (25). H-2$^b$ or H-2$^k$ macrophages were pulsed with 1 mg/ml of native or maleyl-OVA in the culture medium for 1 h, washed, and titrated (the H-2$^b$ APCs by themselves and the H-2$^k$ APCs in the presence of $1 \times 10^5$ H-2$^b$ macrophages per well as bystander APCs that could potentially bind to OVA peptides regurgitated by the Ag-pulsed H-2$^k$ macrophages). B3.4.5 cells were used to detect Ag presentation as usual. Fig. 4 shows that, while H-2$^b$ APCs could elicit a response when Ag-pulsed, they could not do so when present as bystander APCs along with Ag-pulsed APCs of the wrong MHC haplotype. Not only was this true of OVA-pulsed APCs but also of maleyl-OVA-pulsed APCs, which
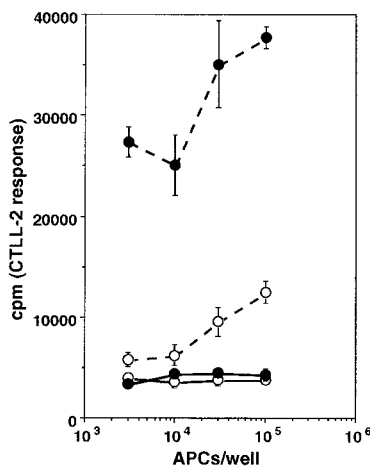
**FIGURE 4.** MHC class I-restricted presentation of native or maleyl-OVA does not involve peptide regurgitation. H-2$^b$ (broken lines) or H-2$^k$ (continuous lines) peritoneal macrophages pulsed with 1 mg/ml of native (hollow circles) or maleyl-OVA (filled circles) in the culture medium for 1 h were washed and titrated with B3.4.5 cells (the H-2$^b$ APCs by themselves and the H-2$^k$ APCs in the presence of $1 \times 10^5$ H-2$^b$ macrophages per well). Background [$^3$H]thymidine incorporation in the absence of Ag was 2000 cpm. The data are representative of two independent experiments.

triggered much higher responses when of the right haplotype (Fig. 4).

*Ammonium chloride sensitivity of maleyl-OVA presentation is due to requirement for receptor-ligand uncoupling*

We then further explored the extreme sensitivity of maleyl-OVA presentation to the inhibition of endosomal acidification (Fig. 3). We initially used de-maleyl-OVA in these experiments. De-maleyl-OVA was presented by macrophages with greater efficiency than native OVA, although not quite as well as maleyl-OVA (Fig. 5A). Maleyl-BSA (10 mg/ml) was used as a competitor SR ligand with de-maleyl-OVA (100 μg/ml) to pulse H-2$^b$ macrophages, which were washed and fixed 3 h later and used to stimulate B3.4.5. The results (Fig. 5B) established that the enhanced efficiency of presentation of de-maleyl-OVA was due to receptor-mediated uptake. When presentation of native OVA, maleyl-OVA, and de-maleyl-OVA was inhibited with ammonium chloride, de-maleyl-OVA continued to be highly sensitive to 2 mM ammonium chloride, as was maleyl-OVA, while native OVA presentation was inhibited only by 200 mM ammonium chloride (Fig. 5C).

Next, to deliver maleyl-OVA to macrophages through pinocytosis rather than through receptor-mediated endocytosis, APCs were pulsed with maleyl-OVA (100 μg/ml) in the presence or absence of maleyl-BSA (10 mg/ml) with or without 2 mM ammonium chloride. Fig. 5D shows the results of one such experiment expressed as percentage inhibition of the presentation of maleyl-OVA by 2 mM ammonium chloride, and it can be seen that the sensitivity of maleyl-OVA presentation to 2 mM ammonium chloride is decreased from 85% to 45% in the presence of competing maleyl-BSA.

*Maleyl-OVA is processed poorly as a cytosolic protein*

We also used another approach to address the possibility that some maleyl-OVA was indeed leaking into the cytosolic presentation pathway. Native OVA or maleyl-OVA were loaded into macrophage cytosol via osmotic lysis of pinosomes, and these macrophages were then used to stimulate B3.4.5. In contrast to the find-

ings obtained when the two proteins were given exogenously, cytosolic native OVA was well presented, but cytosolic maleyl-OVA generated a very poor response (Fig. 6). If there had indeed been any cytosolic leakage of exogenously administered maleyl-OVA from endosomes, these data confirmed that such leaked maleyl-OVA could not be processed cytosolically.

It was of interest to address the question of why maleyl-OVA was so resistant to cytosolic processing. It has been shown that processing of a cytosolic protein is inhibited by blocking potential ubiquitination sites on it by methylation of amino groups (40). Since maleyl residues attach primarily to amino groups, it is possible that this blocks ubiquitin-binding sites, leading to poor cytosolic degradation of maleyl-OVA. However, it was also possible that maleyl-OVA is processed by a ubiquitination-independent pathway (41) but that the peptide repertoire generated from it and loaded on MHC class I in the absence of demaleylation is quite different from that generated from native OVA, and does not include the SIINFEKL sequence recognized by B3. This possibility is reinforced by the fact that the subterminal lysine residue in the nominal target peptide for B3.4.5 is important for TCR contact (42, 43).

The poor processing of cytosolic maleyl-OVA could be either due to a direct effect of the maleyl groups per se, or indirectly due to the conformational change that maleylation imposes. It has been shown that demaleylating maleyl-proteins still permits them to retain the SR-binding conformation provided by the maleylation process (33). This allows us to distinguish between the direct and the indirect effects of maleyl groups on cytosolic processing, since if the direct effect of maleyl residues was the main factor involved, a prediction would be that de-maleyl-OVA would be better processed cytosolically than maleyl-OVA. We therefore demaleylated maleyl-OVA and loaded it cytosolically for presentation to B3.4.5. Fig. 6 also shows that de-maleyl-OVA is presented much better than maleyl-OVA, although not quite as well as native OVA itself, through the cytosolic pathway, lending support to the probability that the poor cytosolic processing of maleyl-OVA may be due to blockade of ubiquitin-binding sites by the maleyl groups.

## Discussion

The data here demonstrate that receptor-mediated endocytosis of a fluid-phase protein by macrophages as well as by B cells enhances its MHC class I-restricted presentation and that this processing and presentation is likely to be an acidification-dependent endosomal event.

The basis for the processing of exogenous Ags by macrophages for presentation on MHC class I has been much debated. While fluid-phase proteins are processed and presented by APCs of the monocytic lineage for MHC class I-restricted presentation (27), the efficiency of this presentation has been shown to improve markedly if the Ag is taken up by phagocytosis in either *Salmonellae* (44), *Escherichia coli* (45), acid-resistant liposomes (46), or latex beads (25). In fact, even in unconventional APC types that have been shown to process exogenous Ag for MHC class I, the phagocytic ability of the tumor cell lines involved has been speculated to be a relevant factor (20, 23). Phagocytosis would clearly increase the efficiency of Ag loading into APCs, and, if endosomal Ag processing pathways for MHC class I are relatively inefficient, increase in Ag uptake would enhance their efficacy significantly.

However, it is not clear whether the improvement in MHC class I-restricted presentation by phagocytosis of the Ag is principally a quantitative effect achieved by augmented Ag uptake, or whether phagocytic uptake has qualitative effects on Ag handling. The latter possibility revolves in part around the fact that Ag delivery

**FIGURE 5.** The sensitivity of exogenous maleyl-OVA presentation to ammonium chloride is dependent on receptor-ligand decoupling rather than maleylation per se. *A*, Shows the response of B3.4.5 cells to titrated doses of native OVA (hollow circles), maleyl-OVA (filled circles), and de-maleyl-OVA (hollow squares) presented by peritoneal macrophages. Background [³H]thymidine incorporation in the absence of Ag was 5000 cpm. *B*, Shows the response of B3.4.5 cells to titrated numbers of peritoneal macrophages pulsed with 100 $\mu$g/ml de-maleyl-OVA in the presence (filled squares) or absence (hollow squares) of 10 mg//ml of maleyl-BSA. Background [³H]thymidine incorporation in the absence of Ag was 4000 cpm. *C*, Shows the responses of B3.4.5 to peritoneal macrophages pulsed with 1 mg/ml of native, maleyl-, or de-maleyl-OVA as shown in the presence of various doses of ammonium chloride (0 mM, hollow bars; 2 mM, hatched bars; 200 mM, stippled bars). Background counts in the absence of Ag were 4000 cpm. *D*, Shows the percentage inhibition in the response of B3.4.5 cells due to the presence of 2 mM ammonium chloride during macrophage APC pulsing with 100 $\mu$g/ml maleyl-OVA in the presence or absence of 10 mg/ml maleyl-BSA as shown. The data are representative of three independent experiments.
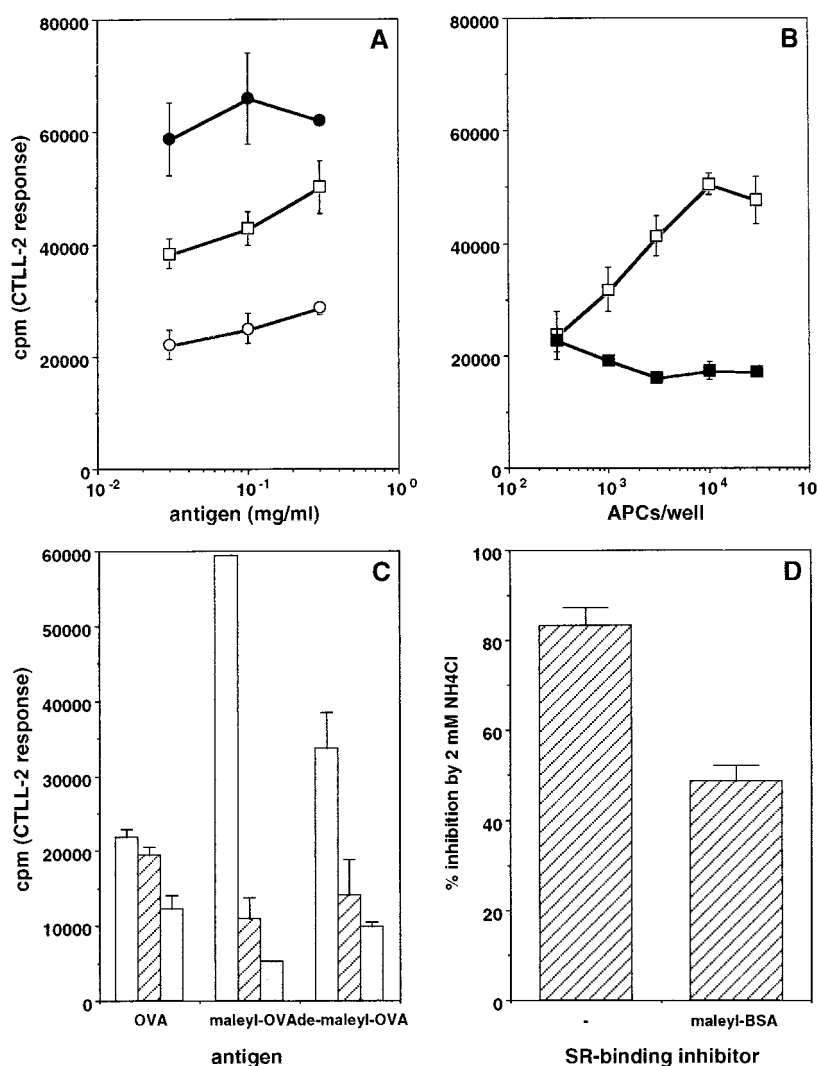
vehicles for phagocytosis used in some of these experiments, such as latex beads or iron beads, induce rupturing of the phagosomal membrane, leading to discharge of the endosomal contents into the cytosol, followed by conventional proteasomal processing for peptide loading on MHC class I (23). An allied possibility suggested has been that a unique cell biological property of endocytosis in macrophages, namely macropinocytosis, may be crucial in permitting them to present exogenous Ags on MHC class I, since macropinosomes, again, are leaky and can deliver their contents to the cytosol (38, 47).

If the quantitative effects of phagocytosis in enhancing Ag uptake are more important than the qualitative effects of phagosome or macropinosome leakiness, receptor-mediated endocytosis, which would provide the quantitative improvements but not the qualitative alterations, should be adequate to enhance MHC class I-restricted presentation of exogenously administered Ags by macrophages. SRs on macrophages are known to internalize bound ligand rapidly and to deliver it efficiently to the lysosomes (48) and such receptor-mediated endosomes are not known to be leaky (49). We have already reported that SR-mediated presentation of protein Ags improves their presentation on MHC class II (30). We have therefore used SR-specific delivery of an exogenous protein to test the dominance of quantitative vs qualitative effects in MHC class I-restricted exogenous Ag presentation. Our data provide evidence

that delivering OVA to receptors with SR-like ligand specificities by maleylating it enhances its presentation on MHC class I and that this is dependent on receptor binding, since the enhancement is inhibited by multiple structurally unrelated competing SR ligands (Fig. 1). Clearly, the quantitative enhancement of Ag uptake by receptor-mediated endocytosis is adequate to permit better endosomal MHC class I-restricted presentation, and phagosomes or macropinosomes are unlikely to be unique compartments permitting access to the peptide-loading pathway for MHC class I.

Further, presentation of exogenous Ags on MHC class I is clearly not a unique property of macrophages, since B cells present receptor-bound exogenous Ag efficiently. In fact, we find that B cell tumor lines also show similar properties (data not shown), thus eliminating any chances that contaminating macrophages were responsible for the enhanced presentation of maleyl-OVA by purified B cell populations (Fig. 1). While the classical SRs, SR-AI/II, are found almost exclusively on monocytic cells, other members of the SR superfamily such as CD36 or CD5 are found at least on subsets of B cells (50, 51). There are other, less well-characterized molecules expressing SR-related cysteine-rich domains expressed by B and other lymphoid cells (52, 53). Further, dG-oligomers, related to the SR ligand polyG, are also reported to stimulate mouse B cells by direct binding (54–56), although the receptor has not been characterized. It therefore appears probable that a variety
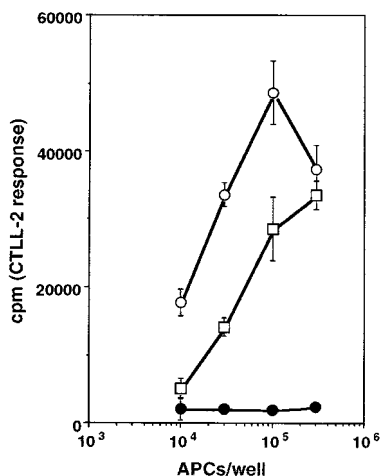
**FIGURE 6.** Cytosolic maleyl-OVA is presented very poorly to B3.4.5 due to the presence of maleyl residues. Responses of B3.4.5 cells to peritoneal macrophages cytosolically loaded with 10 mg/ml of native OVA (hollow circles), maleyl-OVA (filled circles), or de-maleyl-OVA (hollow squares) by osmotic lysis of pinosomes are shown. Background counts in the presence of mock-loaded APCs were 2000 cpm. The data are representative of five independent experiments.

of pattern-recognition receptors of the SR superfamily with distinct but overlapping ligand specificities may be present on B cells. Our data showing enhanced presentation of maleylated proteins on MHC class II (32) or class I (Fig. 1) are consistent with this possibility. Thus, receptor-specific delivery of exogenous protein Ags to any APC may lead to enhanced MHC class I-restricted presentation, suggesting that the exogenous pathway of peptide loading for MHC class I may be a constitutive property dependent mainly on the quantity of Ag taken up by the APC.

Our finding that endosomal bodies with specialized leaky properties such as phagosomes or macropinosomes are not absolutely required for MHC class I-restricted presentation of exogenous proteins also bears on the question of the pathway of processing involved, since it becomes more likely that this is purely endosomal rather than cytosolic. Both endosomal and cytosolic pathways have been invoked in various reports for the phenomenon, phagosomal processing followed by peptide regurgitation for binding to cell surface MHC class I (25), or leaky phagosomes or macropinosomes delivering protein to the cytosolic proteasome-TAP-mediated classical pathway (23, 26). There are a number of points in our data suggesting that the processing of Ag taken up by macrophages via receptor-mediated endocytosis is in fact endosomal. Processing and presentation of exogenous maleyl-OVA is very sensitive to ammonium chloride (Fig. 3), which serves to neutralize the low pH in maturing endosomes and inhibits endosomal processing (1). This shows that exogenous maleyl-OVA needs to be retained in the endosomes until exposure to the low pH of late endosomal compartments to gain access to the peptide-loading pathway for MHC class I. While it has been reported that ammonium chloride can also inhibit cytosolic processing of OVA (57), the probability of its effects being mainly endosomal in the current data is supported by the finding that maleyl-OVA, when directly introduced into the cytosol, virtually cannot be processed for MHC class I-restricted presentation (Fig. 6). Clearly, if unprocessed maleyl-OVA in the cytosol cannot be proteasomally processed to generate SIINFEKL, leakage of maleyl-OVA from the endosomes to the cytosol is unlikely to be responsible for its MHC class I-restricted presentation.

We have attempted to examine the basis of the exquisite sensitivity of maleyl-OVA presentation to ammonium chloride in comparison with native OVA. One explanation for this sensitivity is that successful processing requires demaleylation of the maleyl-protein, which is known to take place at acidic pH (33). This is unlikely to be an explanation, since de-maleyl-OVA is also sensitive to ammonium chloride (Fig. 5). Demaleylated proteins have been reported to maintain the ability to bind to SRs despite the loss of maleyl groups (33). In keeping with those reports, there is enhanced presentation of de-maleyl-OVA as well via SRs (Fig. 5). Since the presence of maleyl groups was thus ruled out as a cause for the sensitivity of maleyl-OVA presentation to 2 mM ammonium chloride, we next examined whether this sensitivity was due to the maleyl-OVA being receptor bound. Converting the mode of delivery of maleyl-OVA from a receptor-mediated one to a pinocytic one by using an excess of an SR-binding competitor does reduce its sensitivity to 2 mM ammonium chloride (Fig. 5), although the decrease, while consistently seen in multiple experiments, is not dramatic. This suggests that at least part of this sensitivity may be due to the dependence of receptor-ligand uncoupling on acidification.

It can be argued that there is some amount of leakage of maleyl-OVA after demaleylation and degradation even from acidic endosomes to the cytosol for proteasomal degradation. If such maleyl-OVA-derived peptides were generated endosomally and then delivered to the cytosol for MHC class I loading, their transport from the cytosol to the ER would be TAP dependent (4–6). To test this possibility, we have used macrophages from TAP-1-KO mice as APCs to present native or maleyl-OVA to the MHC class I-restricted T cell line B3.4.5. We find that TAP-1-KO macrophages present both Ags as efficiently as TAP-1-sufficient macrophages do (Fig. 2). This suggests that neither exogenous proteins nor peptides generated from exogenous fluid-phase or receptor-binding proteins are transferred to the cytosol for MHC class I loading.

Previous demonstrations of endosomal processing for MHC class I-restricted presentation have argued for a peptide regurgitation model in which peptides are thrown out into the medium and bound to cell surface MHC class I (25). We have examined this possibility in our experimental system using bystander H-2$^b$ macrophages to present peptides processed and regurgitated by non-H-2$^b$ macrophages pulsed with Ag, but have been unable to detect any such regurgitation (Fig. 4). While the endosomal pathway for MHC class I-restricted presentation seen here may not be the only such pathway, given the many reports of cytosolic generation of peptides from exogenous proteins, our data make it probable that processing as well as MHC class I loading of peptides from receptor-binding proteins may take place predominantly in the endosomal compartment.

Another point of interest in our data relates to the basis for the nonprocessing of maleyl-OVA when introduced directly into the cytosol (Fig. 6). There are two possible explanations, not necessarily mutually exclusive. One is that maleyl-OVA is simply not processed well by the proteasomal route at all. The other possibility is that it is indeed processed but does not generate the SIINFEKL peptide, either because a completely different peptide repertoire is generated, or at least because maleyl-SIINFEKL (peptide maleylated at the subterminal lysine residue) is generated. The subterminal lysine residue in SIINFEKL has been shown to be essential for recognition by the TCR of B3.4.5, although it is not needed for H-2K$^b$ binding (42, 43, 58). Interestingly, maleylation of this lysine residue abrogates binding of the peptide to H-2K$^b$ (data not shown), suggesting that influences on peptide conformation by non-MHC-contacting amino acid residues may modulate peptide repertoires of MHC molecules. However, it also appears

that maleyl-OVA is not processed well by the proteasomal route, since maleyl-OVA-loaded spleen cells used for priming in vivo cannot generate a CTL response even against maleyl-OVA (data not shown). Of the two possible explanations for this, 1) that the altered conformation that permits the maleyl-protein to bind SRs (33) also inhibits proteasomal processing, and 2) that the presence of the maleyl groups directly hinders processing, our data suggest that the latter is correct, since maleyl-protein demaleylation, which is known to spare the SR-binding capability, nonetheless restores cytosolic processing (Fig. 6). Maleylation is mainly directed at the $\epsilon$-amino groups of lysine residues in the protein being maleylated (33), and it is the methylation of precisely these groups that is needed for the binding of ubiquitin (2). These data therefore support the dominant role played by ubiquitination in delivering cytosolic proteins to the proteasomal degradatory pathway shown earlier (40), although there have also been reports of ubiquitination-independent pathways of cytosolic protein degradation (41).

## Acknowledgments

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