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Class I MHC Presentation of Exogenous Soluble Antigen via Macropinocytosis in Bone Marrow Macrophages

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Summary

Extracellular proteins are not generally presented on class I MHC molecules in vitro, yet many studies show that a pathway exists in vivo for the presentation of extracellular material on class I molecules to prime $CD8^+$ T cell responses. Here, we provide morphological evidence that proteins taken up by macropinocytosis can gain access to the cytosol and therefore into the conventional class I MHC pathway. Class I presentation of soluble ovalbumin by mouse bone marrow macrophages was dramatically enhanced by MCSF or phorbol ester and blocked by amiloride, which stimulate and inhibit membrane ruffling and macropinocytosis, respectively. Brefeldin A, gelonin, and a peptide aldehyde inhibitor of proteasomal processing each blocked presentation of macropinocytosed antigen, demonstrating that unusual access to the conventional class I MHC pathway was occurring. This novel cell type-specific endocytic pathway may facilitate presentation of exogenous material on class I MHC molecules, allowing induction of $CD8^+$ T cell responses to soluble proteins, tumor cell fragments, and some pathogens.

Introduction

The conventional view of antigen presentation to T cells is that endogenous proteins are presented on class I major histocompatibility complex (MHC) molecules and exogenous proteins on class II MHC molecules (Morrison et al., 1986; Bevan, 1987; Townsend and Bodmer, 1989; Germain and Marguiles, 1993). However, an increasing number of reports demonstrate that this division is not absolute and that a significant level of cross-over can occur, such that peptides from endogenous cytosolic proteins may appear on class II MHC molecules (Jaraquemada et al., 1990; Dodi et al., 1994) and peptides from exogenous proteins on class I MHC molecules. $CD8^+$ T cell responses can be elicited in vivo in the absence of de novo antigen synthesis within host antigen-presenting cells (APCs). These responses have been observed following immunization with purified proteins (Wraith et al., 1987; Staerz et al.,1987; Bachmann et al., 1994; Schirmbeck et al., 1995; Blum-Tirouvanziam et al., 1994; Ke et al., 1995) and in cross-priming experiments where CD8 T cell responses to antigens carried uniquely by immunizing donor cells are restricted to MHC molecules expressed only on recipient cells (Bevan, 1976; Gooding and Edwards, 1980; Fink et al., 1983; Huang et al., 1994.).

Based on these findings, it has been suggested that a pathway must exist for the processing and presentation of exogenous material on class I MHC molecules (Bevan, 1987; Townsend and Bodmer, 1989). However, it has been difficult to analyze this unusual mode of class I peptide loading in vitro where exogenous proteins are usually presented on class II MHC and not on class I MHC molecules (Morrison et al., 1986). Rock and colleagues (Rock et al., 1990) have demonstrated class I MHC-restricted presentation in vitro of exogenous soluble antigen and much more efficient presentation of antigen conjugated to latex beads (Kovacsovics-Bankowski et al., 1993). However, the mechanistic basis for class I MHC presentation of phagocytosed particulate antigens is somewhat unclear, since different studies, both using latex bead–associated antigen, report a brefeldin A-insensitive pathway in one case (Harding and Song, 1994) but brefeldin A-sensitive presentation in another (Kovacsovics-Bankowski and Rock, 1995). Brefeldin-insensitive presentation of bacterial antigen (Pfeifer et al., 1993) and heat-inactivated virus preparations in some cell types (Liu et al., 1995) have also been described. Thus, the question of how exogenous physiological antigens, both particulate and nonparticulate, might access the class I MHC pathway following endocytosis is not clearly resolved.

Endocytic pathways distinct from the clathrin-mediated route have now been described in animal cells (reviewed by van Deurs et al., 1990; Watts and Marsh, 1992). Macropinocytosis is a cell type–specific endocytic pathway associated with plasma membrane ruffling activity (Swanson and Watts, 1995). It can be induced by growth factors, phorbol esters (West et al., 1989; Swanson, 1989; Racoosin and Swanson, 1993; Hewlett et al., 1994), or activated forms of the small GTP binding protein rac (Ridley et al., 1992) and appears to operate constitutively in cultured human dendritic cells (Sallusto et al., 1995). Macropinocytosis, but not other endocytic pathways, is blocked by the Na^+/H^+ channel inhibitor amiloride (West et al., 1989) or its analogs (Hewlett, 1995). Macropinosomes are heterogeneous in size (Swanson, 1989; Hewlett et al., 1994) and interact with lysosomes in macrophages (Racoosin and Swanson, 1993), but in human A431 epithelial cells they comprise a distinct endosome population refractory to fusion with other endosomes (Hewlett et al., 1994). We made the surprising observation that extracellular tracers taken up during ruffling and macropinocytosis in human A431 cells were later found in the cytosol, which led us to test the hypothesis that this endocytic pathway may provide a mechanism for presentation of exogenous antigen on class I MHC molecules.

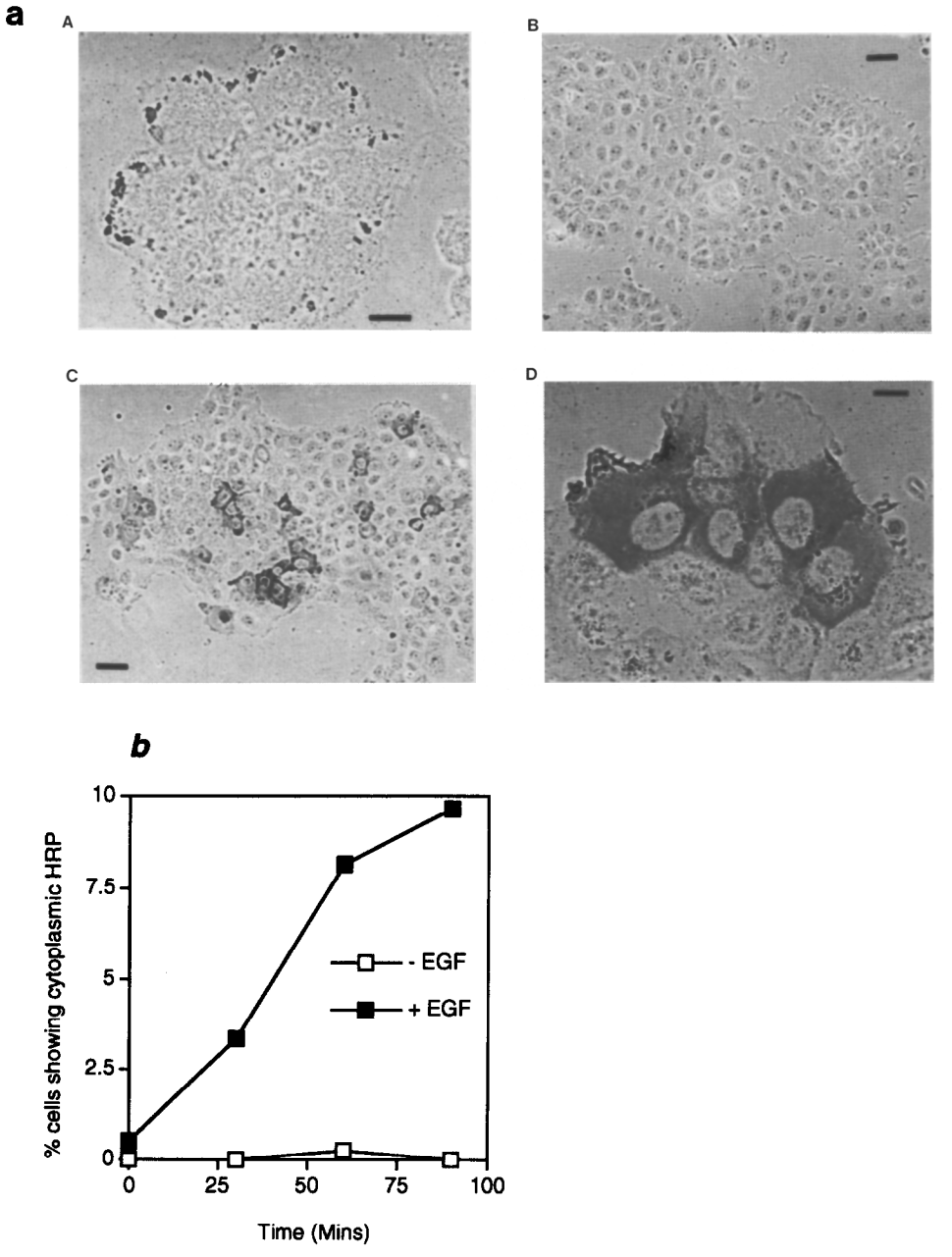

Figure 1. Release of Solute Markers into the Cytosol following Induction of Macropinocytosis

(a) A431 cells were incubated with HRP in the presence (A, C, D) or absence (B) of EGF for 10 min, then fixed and peroxidase cytochemistry performed immediately (A) or following a 90 min chase (B, C, D). Macropinosomes, heterogeneous in size, can be seen close to the ruffling margins of a group of cells (A).

(b) Quantitation, after different chase times of cells showing cytosolic HRP reaction product. A cell was scored if it excluded trypan blue and the brown HRP precipitate was excluded from the nuclear region. In all cases, a minimum of 500 cells were counted. Scale bars, (a), 20 μm (A, D); 50 μm (B, C). A431 cells were grown on sterile 13 mm coverslips, then pulsed with 10 mg/ml HRP in the presence or absence of 300 ng/ml EGF as described previously (West et al., 1989; Hewlett et al., 1994) and, where indicated, chased at 37°C. After fixation, HRP cytochemistry was performed with diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01%).

Results

Detection of Macropinocytosed Markers in the Cytosol

In human A431 cells stimulated with epidermal growth factor (EGF) in the presence of horseradish peroxidase (HRP), macropinosomes of various sizes can be observed in the cytosol, frequently in close proximity to the cell margins where ruffling activity is maximal (Figure 1a, A) (West et al., 1989; Hewlett et al., 1994). Although most macropinocytosed tracer is recycled out of these cells during a chase period at 37°C, we detected HRP activity in the

cytosol in a proportion of cells (Figure 1a, C and D). These cells were viable since HRP was excluded from the nucleus (Figure 1, C and D) and the cells did not take up trypan blue (data not shown). Cytosolic HRP was seen only in EGF-stimulated cells (Figure 1a, compare B and C), which also contained intact macropinosomes and were therefore alive during the HRP pulse. Most importantly, HRP only appeared in the cytosol following a chase period, i.e., after removal of exogenous HRP, demonstrating a time-dependent release into the cytosol. Approximately 9% of cells were visibly loaded with cytosolic HRP following a 90 min chase period (Figure 1a, C and D; Figure 1b).

This surprising observation raised the possibility that macropinocytosis might provide a pathway for the presentation of exogenous antigens on class I MHC molecules via the cytosol. We tested this hypothesis using mouse bone marrow macrophages (BMM) in which macropinocytosis has been well characterized (Swanson, 1989; Racoosin and Swanson, 1993) and which constitute a physiologically relevant cell type in the context of antigen presentation and CTL priming. Mouse BMM were stimulated with either MCSF or phorbol ester in the presence of fluorescein isothiocyanate (FITC)–dextran. HRP is less suitable as a fluid phase endocytic marker in BMM since it stimulates its own uptake (Swanson et al., 1985). As in A431 cells, macropinosomes in BMM were heterogeneous in size (Figures 2A and 2B) and their formation could be blocked by amiloride or its analogs (data not shown; Figure 3c; see below). In addition, we observed FITC–dextran release into the cytosol of some living cells (see Figures 2C and 2D). However, since cytosolic release was again seen only in a minority of cells it seemed that although BMM would not be efficiently lysed in a CTL assay following induction of this pathway they might nonetheless be able to trigger T cell activation. We therefore utilized the Lac Z-inducible T cell hybridoma B3Z (Karttunen et al., 1992), specific for the ovalbumin epitope 257–264 presented on the murine class I MHC molecule H-2K^b (Moore et al., 1988). B3Z activation is detectable when the frequency of presenting cells is as low as 0.1%–0.01% (Karttunen et al., 1992; Sanderson and Shastri, 1994).

Macropinocytosed Antigen Is Loaded onto Class I MHC Molecules

BMM were pulsed with soluble ovalbumin for 10 min under conditions that stimulate or inhibit macropinocytosis. The cells were then incubated without ovalbumin for different times, fixed, and overlaid with B3Z T cells. Presentation of the ovalbumin$_{257-264}$ epitope was assessed by scoring the number of activated (blue) B3Z T cells. Exposure of BMM to ovalbumin in the presence of phorbol myristate acetate (PMA) gave a time-dependent increase in triggering of the B3Z T cells (Figure 3a). Amiloride added during the antigen pulse markedly reduced subsequent T cell triggering (Figure 3b). Thus, presentation of exogenous ovalbumin was enhanced or blocked, respectively, by stimulators and inhibitors of macropinocytosis.

To eliminate the possibility that PMA and amiloride were modulating presentation of ovalbumin via generalized effects, for example, on the level of class I MHC or adhesion molecules, we compared the effect of PMA and the more potent amiloride analog, dimethyl amiloride (DMA) on ovalbumin presentation versus presentation of a synthetic ovalbumin$_{257-264}$ peptide (SIINFEKL) recognized by B3Z cells. The DMA concentrations that blocked PMA-stimulated macropinocytosis of FITC–dextran (Figure 3d) also inhibited PMA-stimulated and constitutive presentation of intact ovalbumin (Figure 3c). In contrast, even high DMA concentrations had no detectable effect on peptide presentation. Moreover, in contrast with ovalbumin presentation, peptide presentation was, if anything, slightly inhibited by PMA (Figure 3e). The inhibitory effect of DMA on class I presentation correlates strictly with its parallel effects on macropinocytosis, since it inhibited ovalbumin presentation only when it was included during the antigen pulse and not when it was present for the same length of time either before or after pulsing (Table 1). BMM exhibit a significant level of constitutive ruffling and macropinocytosis, which presumably accounts for the DMA-inhibitable presentation of ovalbumin seen in the absence of PMA (e.g., Figure 3c). Thus, PMA and DMA modulate antigen presentation via their effects on ruffling and macropinocytosis and not by modulating cell surface expression of class I MHC or accessory molecules.

Unconventional Access to the Conventional Class I MHC Pathway

Release of exogenous markers into the cytosol following macropinocytosis seemed likely to account for the increased expression of exogenous ovalbumin on class I MHC molecules. However, other studies have suggested that exogenous antigens may be loaded onto mature class I MHC molecules (Pfeifer et al., 1993; Harding and Song, 1994; Liu et al., 1995). To assess whether newly synthesized class I molecules and a functional secretory pathway are required for presentation of macropinocytosed ovalbumin, we tested the effects of gelonin, a protein synthesis inhibitor (Stirpe et al.,1980), and brefeldin A, which blocks secretory protein traffic and class I MHC antigen presentation (Nuchtern et al., 1989; Yewdell and Bennink, 1989). Gelonin added during the antigen pulse inhibited ovalbumin presentation (Figure 4a) but not presentation of the synthetic peptide ovalbumin$_{257-264}$ (data not shown), indicating that newly synthesized class I MHC molecules are required for presentation of macropinocytosed ovalbumin. Since gelonin is impermeant to intact cells (Stirpe et al., 1980; Rock et al., 1993), these data provide independent evidence that macropinocytosis provides access of soluble proteins to the cytosol. When ovalbumin-pulsed macrophages were subsequently incubated with brefeldin A, triggering of B3Z cells was strongly inhibited (Figure 4b). However, following a period of recovery in the absence of brefeldin A and without further exposure to ovalbumin, the macrophages became competent to activate the B3Z cells (Figure 4b), demonstrating that the effect of brefeldin A is reversible and that presentation of macropinocytosed ovalbumin depends on a functional secretory pathway.

We next assessed whether, prior to class I MHC loading,

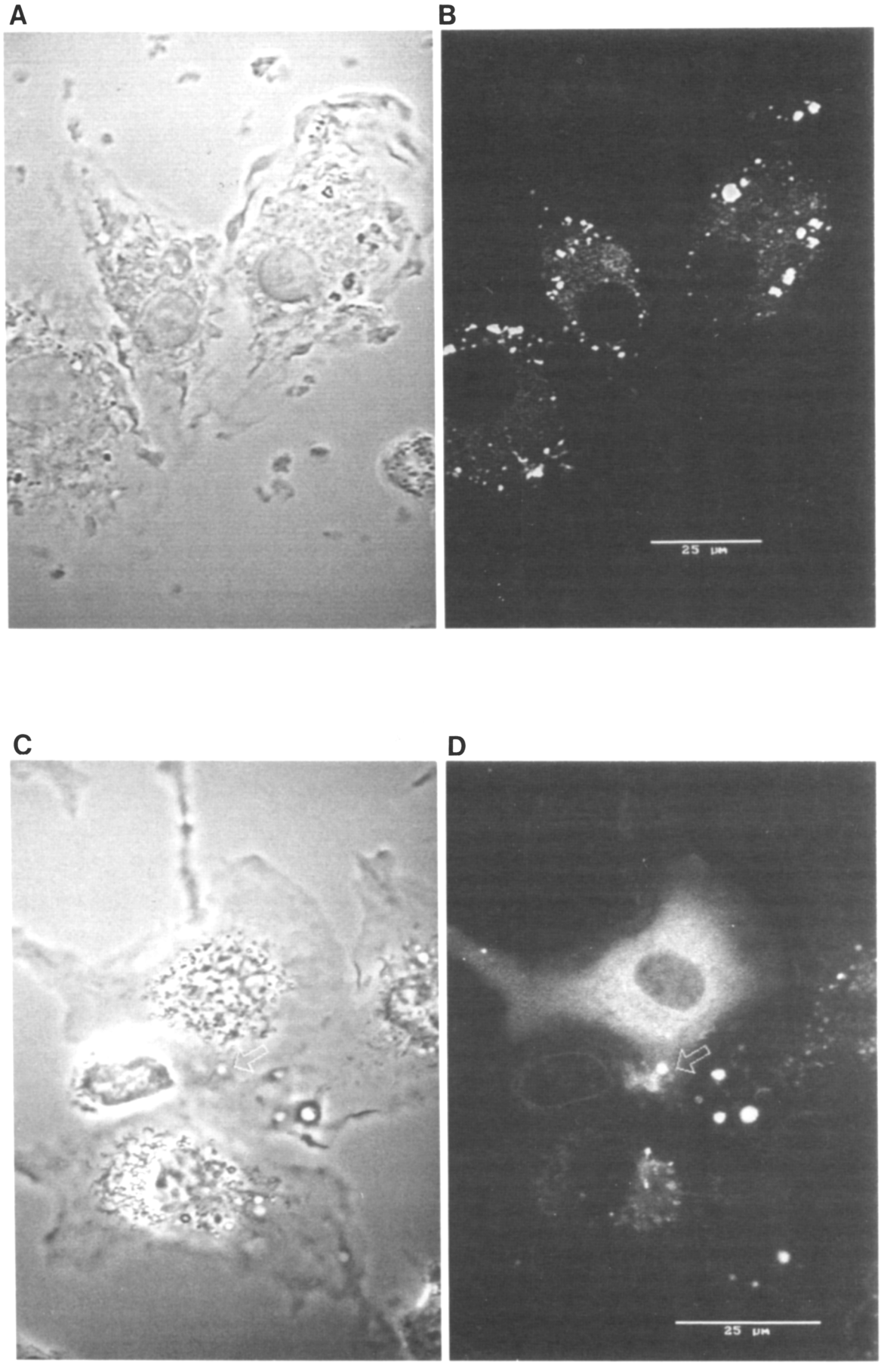

Figure 2. BMM Pulsed with FITC–Dextran in the Presence of MCSF

Phase contrast (A, C) and confocal fluorescence (FITC) fields of the same cells showing macropinosomes (B, D) and, less frequently, cells showing cytosolic staining (C, D). Intact macropinosomes are also observed within cells showing cytosolic fluorescence (arrow in C and D).

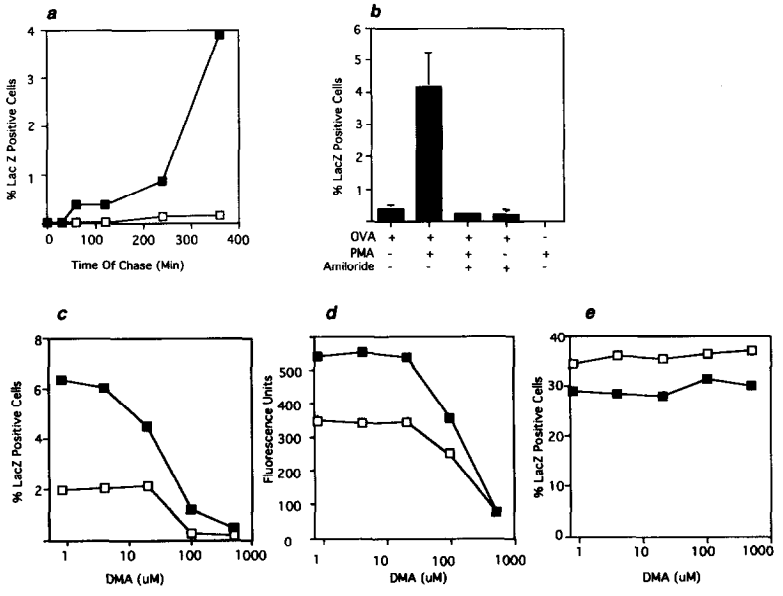

Figure 3. Effects of PMA and Amiloride on Macropinocytosis and Class I Presentation of Exogenous Antigens

BMM untreated (open square) or treated (closed square) with 30 ng/ml PMA to induce macropinocytosis 45 min prior to pulsing with either ovalbumin (a, b, c), ovalbumin 257–264 (e), or FITC–dextran (d). Time-dependent presentation of native ovalbumin by ovalbumin-pulsed cells (a). Effect on ovalbumin (b, c) or ovalbumin 257–264 (e) presentation by amiloride (b) or dimethyl amiloride (c, e). Effect of dimethyl amiloride on FITC–dextran uptake into BMM (d).

BMM were replated at 3 × 10^5 cells/well in 24-well plates 6–18 hr and PMA added where indicated (closed square) 45 min prior to pulsing with batch-tested (see Experimental Procedures) ovalbumin (10 mg/ml) or ovalbumin$_{257-264}$ (0.1 μM) or FITC–dextran (10 mg/ml). For presentation studies, BMM were washed extensively with PBS/BSA following ovalbumin pulsing, then incubated for 2 hr at 37°C. B3Z cells were overlaid at a density of 4 × 10^5 cells/well and incubated overnight at 37°C, then harvested by vigorous pipetting. β-galactosidase activity was assayed using X-Gal as described (Sanderson and Shastri, 1994; Karttunnen et al., 1992). In (a), cells were fixed with 1% paraformaldehyde for 30 min and quenched with 0.2 M glycine in PBS, washed, and then incubated with B3Z indicator cells. The percentage of activated blue B3Z cells was determined by cell counting. At least 1500–3000 total cells were counted. Experiments were performed in triplicate and the results presented as means ± SD. In (d), PMA-treated cells were washed extensively following exposure to FITC–dextran (5 mg/ml), then lysed in 0.2% Triton-X 100 and microfuged to remove aggregates. FITC content was measured in a Perkin-Elmer fluorimeter (excitation, 490 nm; emission, 520 nm; 2.5nm, bandpass). Controls without PMA or amiloride received equivalent amounts of DMSO carrier.

processing of ovalbumin occurred within macropinosomal compartments or in the cytosol via the action of the proteasome complex. Presentation of exogenous ovalbumin to B3Z cells was blocked by the peptide aldehyde inhibitor LLnL (Figure 4c). Since LLnL and other peptide aldehydes also inhibit some classes of lysosomal protease (Sasaki et al, 1990), we also tested the analog LLM, which has a selectively reduced activity against the proteasome and, in contrast with LLnL, had no effect on presentation of ovalbumin introduced into the cytosol by electroporation (Rock et al., 1994). As shown in Figure 4c, LLM also had no effect on presentation of macropinocytosed ovalbumin. Similarly, chloroquine, a pleiotropic inhibitor of acid pH-dependent proteases did not inhibit ovalbumin presentation and in fact showed a reproducible stimulation, indicating that macropinosomal antigen processing is unnecessary and might actually reduce the efficiency of class I MHC presentation of exogenous antigens (see Figure 3d). Thus, cytosolic rather than macropinosomal processing of ovalbumin was necessary for class I presentation.

Discussion

Here, we demonstrate that macropinocytosed soluble antigen can gain access to the cytosol for presentation on class I MHC molecules. Presentation was regulated by the level of macropinocytic activity, utilized the conventional class I pathway and required no special antigen formulation. A pathway for the presentation of exogenous material on class I MHC molecules was predicted (Bevan, 1987; Townsend and Bodmer, 1989) based on reports of CD8 T cell priming by soluble proteins (Wraith et al., 1987; Staerz et al., 1987) and cross-priming of host CD8 T cells to donor cell determinants restricted by MHC molecules found only on recipient bone marrow–derived cells (Fink et al., 1983; Gooding and Edwards, 1980). Such a pathway may also be necessary to explain how tumor-derived soluble heat shock proteins elicit tumor-specific macrophage-dependent immunity (Udono et al., 1994), and how tumor cells induce CD8 responses restricted by the MHC of the host bone marrow rather than that of the inducing tumor cells (Huang et al., 1994).

These in vivo observations suggest that there are exceptions to the general embargo on class I presentation of exogenous antigens. Usually, this blockade is considered necessary because cells that passively acquire CTL determinants, but are not themselves infected, would be killed by activated CTLs recognizing the exogenous material in the context of class I MHC molecules. However, it may be desirable to override this embargo to prime CTL under certain conditions. For example, professional APCs are

BMM were grown as described (Racoosin and Swanson, 1989), detached from the plastic, and replated at 1.5 × 10^5 cells/ml onto sterile 24 mm coverslips. Following pulsing with FITC–dextran (5 mg/ml) for 10 min in the presence of 3000 U/ml MCSF the cells were washed in PBS/BSA, then mounted in 0.4% trypan blue in HBSS/BSA in a Dvorak–Stotler perfusion chamber for viewing in Biorad MRC 500 laser confocal microscope at 30°C–37°C.

Table 1. Dimethyl Amiloride Inhibits Presentation During but Not Before or After an OVA Pulse

		Window of DMA exposure		
PMA	None	Preëxposure	Coexposure	Postexposure
		Percentage Lac-Z +ve cells		
+ PMA	2.73	1.78	0.39	2.42
− PMA	0.39	NT	NT	NT

BMMs were stimulated with PMA 45 min prior to OVA pulsing. Dimethyl amiloride (DMA; 100 μM) was included in separate incubations during different 20 min periods and then removed. Exposure to DMA was either at the start of PMA stimulation (Preëxposure), 10 min prior to and during the 10 min OVA pulse (Coexposure) or after removal of OVA (Postexposure). Following a 2 hr chase, cells were overlaid with B3Z cells and presentation was quantitated as described in Figure 3, legend and in Experimental Procedures. NT, not tested.

required to initiate most CTL responses (Lafferty et al., 1983; Schwartz, 1989; Ohashi et al., 1991; Chen et al., 1992; Townsend and Allison., 1993), suggesting that a pathogen may evade an immune response by not infecting or replicating within professional APCs or within cell types that traffic to lymphoid areas (Kundig et al., 1995). In this case, the professional APC would not be able to use the conventional class I MHC pathway, which requires infection of the APC and subsequent de novo synthesis of pathogen-encoded proteins. To initiate a CTL response against such a pathogen, the professional APC would be required to present exogenous material on its class I MHC molecules.

What features should such a pathway for presentation of exogenous antigen on class I MHC molecules have? First, it should be mostly confined to professional APCs so that uninfected cells do not become targets for activated effector CD8 cells. Second, in contrast with the constitutive clathrin-mediated endocytic pathway, it should be upregulatable to boost presentation of exogenous antigens in defined situations. Third, the loading of class I MHC should be cell autonomous, in that only class I MHC molecules on the cell taking up antigen should be loaded to avoid passive transfer of material to bystander cells. Fourth, the conventional proteasome- and TAP-dependent class I MHC pathway should be involved to ensure that CTLs are induced to epitopes that will be recognized when endogenously synthesized material is processed and presented. In other words, T cells elicited by exogenous material must be useful as effector cells.

All of these features are provided by the macropinocytic pathway described here. It is most active in professional APCs and can be up- and down-regulated (Swanson, 1989; Sallusto et al., 1995). Importantly, we have shown that presentation of soluble antigen on class I MHC molecules was similarly modulated upwards or downwards depending on ruffling and macropinocytic activity. Although we have no direct evidence that presentation on class I MHC molecules occurs via this pathway in vivo, we suggest that it constitutes a likely route for CTL induction by exogenous antigen.

In a series of studies, Rock and colleagues have characterized the ability of macrophages and macrophage cell

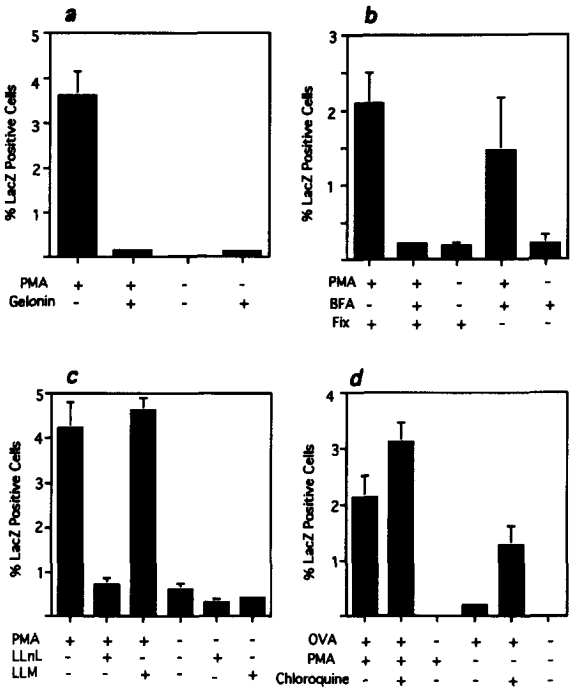

Figure 4. Effects of Gelonin, Brefeldin A, LLnL, LLM, and Chloroquine on Presentation of Macropinocytosed Ovalbumin on Class I MHC Molecules Assessed Using B3Z Indicator Cells

(a) BMM pulsed with ovalbumin in the presence or absence of gelonin. (b) BMM pulsed with ovalbumin and chased in the presence or absence of brefeldin A for 6 hr. B3Z cells were overlaid on either fixed cells or live cells washed free of brefeldin A. Ovalbumin pulsing of BMM in the presence or absence of LLnL or LLM (c) or chloroquine (d).

BMM were pretreated with PMA 45 min prior to ovalbumin pulsing. Times of addition of inhibitors prior to ovalbumin pulsing were the following: gelonin (1 μM), 20 min; LLnL or LLM (N-acetyl-L-leucinyl-L-leucinal-L-norleucinal and N-acetyl-L-leucinyl-L-leucinal-methional, respectively; both 50 μM) or chloroquine (100 μM), all at 60 min. Brefeldin A (5 μg/ml) was added after ovalbumin pulsing and was replenished in the culture medium at 120 min intervals. Cells were chased for 6 hr following ovalbumin pulsing and then overlaid with B3Z cells either with (c, d, and where indicated in b) or without (a) prior fixation. Following an overnight incubation with B3Z cells, T cell triggering was measured as described and Figure 3 legend. Samples without drugs contained equivalent amounts of DMSO or ethanol carrier.

lines to present exogenous antigen coupled to latex beads (Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1995). As in the studies reported here, this particulate antigen was also presented via the classical class I pathway, although it was not clear how antigen coupled to latex beads gained access to the cytosol. In a recent study, it was reported that coadministration of soluble antigen together with phagocytosable particles gave enhanced presentation compared with equivalent amounts of antigen conjugated to beads (Reis e Sousa and Germain, 1995). Again, presentation was via the conventional class I pathway, indicating phagosome to cytosol transfer. In contrast, using a preparation of latex bead–coupled antigen, a distinct brefeldin A-insensitive mechanism was described and was attributed to loading of mature post-Golgi class I MHC molecules either within phagocytic vacuoles or following regurgitation and recap-

ture of processed material (Harding and Song, 1994). The reasons for these discrepant results using synthetic particulate antigens are not clear, but unconventional access to the conventional class I pathway as described here and in other recent studies (Kovacsovics-Bankowski and Rock, 1995; Reis e Sousa and Germain, 1995) would seem a more appropriate strategy for the immune system to adopt.

As discussed above, class I MHC loading following extracellular or endosomal processing, although clearly demonstrated in several in vitro studies, might not provide the best match between class I–restricted determinants at the induction and effector phases of the response. Moreover, the efficiency of post-Golgi loading of mature class I MHC might be dictated by the availability of empty class I molecules, reusable class I MHC molecules, or class I molecules targeted to the endocytic/phagocytic pathway by association with the invariant chain (Sugita and Brenner, 1995). In contrast, class I MHC availability would be ensured by regulating access of exogenous antigen to the conventional pathway. Processing in the inductive and effector phases of the response would be uniform and potentially damaging sensitization of bystander cells would be avoided. Interestingly, some bacteria stimulate macropinocytosis (Francis et al., 1993), resulting in nonphagocytic sequestration within macropinosomes (Alpuche-Aranda et al., 1994). This observation raises the possibility that organisms, which themselves have no obvious means of entering the cytosol, but to which CD8 T cell responses can nonetheless be made, might transfer material through macropinosome membranes into the cytosol. Yersinia (Starnbach and Bevan, 1994) and Listeria monocytogenes mutants that lack listeriolysin (Szalay et al., 1994) constitute two possible examples. However, it should be stressed that several studies have demonstrated distinct nonclassical loading of class I MHC molecules, which is usually (Schirmbeck et al., 1995; Harding and Song, 1994), though not always (Liu et al., 1995; de Bruijn et al., 1995), brefeldin A insensitive. Strikingly, the antigen preparations used are mostly particulate, suggesting that the nature of the antigen may affect the pathway used. Thus, intracellular pathogens confined to vacuoles may load class I molecules at these sites following processing by endosomal rather than proteasomal proteases (Pfeifer et al., 1993; de Bruijn et al., 1995). The key point is that a good match between epitopes inducing CTLs and those presented by other cell types needs to be made. Perhaps two distinct pathways for loading class I MHC molecules with extracellular antigens exist: one for pathogens confined to vacuoles and a second, described here, which may be most important for pathogen-derived class I determinants that are loaded via the conventional class I pathway.

In summary, we have begun to define a pathway that allows unconventional access to the conventional class I MHC pathway in a regulatable and cell type–specific manner. It will be important to define the stimuli of macropinocytosis, presumably acting through rac and related GTPases, that might operate in vivo. The mechanism described here may constitute an important arm of the normal CD8 T cell response and may be useful for vaccination purposes.

Experimental Procedures

Reagents

EGF was purchased from AMS Biotechnology (Witney, Oxon, England). Cell culture media and G418 were from GIBCO BRL (Paisley, Scotland). Lab-Tek tissue culture dishes (100 mm) were from Nunclon (Roskilde, Denmark) and all other tissue culture plastics were from Costar (Bucks, England). Brefeldin A and hygromycin B were purchased from Boehringer Mannheim (East Sussex, England). Fetal calf serum (FCS) was from Advanced Protein Products (West Bromwich, England). 5-Bromo-4- chloro-3-indolyl-β-D-galactoside (X-Gal) was from Calbiochem-Novabiochem (Nottingham, England). Glass cover slips and slides were from BDH Chemicals Ltd. (Poole, England). All other reagents were from Sigma Immunochemicals (Poole, England).

Cell Culture

A431 cells (a gift from Dr. P. Smith, University College, London) were maintained in DMEM containing heat-denatured (FCS) (10%), kanamycin (100 U/ml) and glutamine (2 μM). B3Z cells were maintained in RPMI 1640, 10% FCS supplemented with sodium pyruvate (1 mM), nonessential amino acids (1 mM), glutamine (2 μM), kanamycin (100 U/ml), hygromycin B (400 U/ml), and geneticin (G418; 1 mg/ml). BMM were obtained from C57BL/6J female mice, and expanded in 30% L cell supernatant for 5–7 days as described by Racoosin and Swanson (1989). The macrophages were detached from dishes by washing briefly with ice-cold phosphate-buffered saline (PBS), incubation with 10 ml PBS containing 10 mM EDTA, 20% FCS at 37°C for 10 min, and agitating vigorously with an automatic pipettor. For assays, BMM were cultured at $10^{5-2} \times 10^5$ per well in 24-well plates coated with poly-L-lysine or onto sterile 24 mm circular glass cover slips for microscopy. Greater than 95% of the cells were macrophages as determined by immunofluoresence staining with a monoclonal antibody (M1/70.15.1, Serotec, Oxford, England) to the macrophage cell surface marker Mac-1.

Uptake of Fluid Phase Markers

Uptake of the fluid phase markers HRP and FITC-conjugated dextran was detected by microscopy. Exposure to these solute markers was performed as previously described (West et al., 1989; Hewlett et al., 1994, respectively). Prior to use the HRP and FITC–dextran solutions used were passed through a 0.22 μm filter. A431 cells were grown on 13 mm diameter glass cover slips, thickness number 2. Cells were incubated with HRP for 10 min, washed, then chased in culture medium in the absence of HRP for the times indicated before rinsing in PBS, exposure to 0.4% trypan blue in PBS for 2 min, and then fixation in 0.5% glutaraldehyde. HRP activity was developed with 0.5 mg/ml diaminobenzidine (DAB) and 0.01% hydrogen peroxide in PBS.

BMM were grown on 24 mm cover slips and exposed to the solute marker FITC–dextran (5 mg/ml) in a similar manner, in the presence or absence of either 30 ng/ml PMA or 3000 U/ml M-CSF. Cover slips were mounted in a Dvorak–Stotler perfusion chamber in PBS, 0.4% trypan blue and viewed at 30°C–37°C on a Nikon Microphot-SA attached to a confocal laser scanning microscope (MRC-600 series, Bio-Rad Laboratories, Cambridge, England).

Fluid phase pinocytosis was quantitated by measuring the uptake of the solute marker FITC–dextran by fluorimetry, essentially as described by Racoosin and Swanson (1989). Quantitation of FITC–dextran uptake, after a 2 hr exposure, was performed by excitation at 488 nm (2.5 nM bandpass) and measurement of emmision at 520 nm (2.5 nM bandpass).

Antigen Uptake and Presentation

Presentation of ovalbumin 257–264 on K^b (Moore et al, 1988) was detected using the T cell hybridoma B3Z, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter (Sanderson and Shastri, 1994; Karttunnen et al., 1992). Ovalbumin (Lorne Labs, batches 30C571N or 34M877) gave a background of T

cell triggering upon incubation with fixed APCs of <0.1% activated B3Z cells. BMM, obtained as described above, were pulsed with 10 mg/ml ovalbumin dissolved in serum-free DMEM for 10–30 min and fixed with 1% paraformaldehyde for 30 min. Excess fixative was quenched with 0.2 M glycine in PBS and the BMM were overlaid with B3Z cells overnight. The B3Z cells were assayed for β-galactosidase production with X-Gal as described by Sanderson et al. (1994). Activation of the B3Z cells was quantitated by counting the number of blue (LacZ-positive cells) on a Neubauer counting chamber and expressing the result as a percentage of the total number of cells present. In all cases a minimum of 10^3 cells were counted.

For antigen presentation assays, BMMs in a 24-well plate were pulsed with 250 μl prewarmed ovalbumin (10 mg/ml) in DMEM with or without 30 ng/ml PMA for a period of 10 min. The cells were washed three times with PBS/BSA (1 mg/ml). Following loading, the cells were chased for 2 hr in complete DMEM before overlaying with 3.5×10^5 B3Z cells in complete RPMI and incubating overnight. To remove the B3Z cells, the plates were spun at 400 rpm for 3 min and the majority of the medium then removed. Vigorous pippetting of the remainder of the medium removed the majority of B3Z cells and incubation at room temperature in trypsin/EDTA dislodged the remainder. After washing twice, LacZ activity within the cells was developed as described above.

Where indicated, the following inhibitors were included in the antigen presentation assays. Amiloride and DMA were stored as stock solutions of 0.6 M and 10 mM, respectively, in DMSO, at 0°C, and diluted in culture media for use. Gelonin was used at a final concentration of 1 μM after dilution, in culture medium, from a 20 μml stock solution, in DMSO. BMMs were incubated with amiloride and DMA and gelonin for 10 min and 20 min, respectively, prior to OVA pulsing and during the ovalbumin pulse. Brefeldin A was prepared at 5 mg/ml in DMSO and was used at 5 μg/ml in complete DMEM for 6 hr. As Brefeldin A has a short lifetime in culture, the medium containing it was replaced every 2 hr. Following a 6 hr chase, cells were either fixed with paraformaldehyde, as outlined above, or overlaid with B3Z and incubated overnight as indicated. The peptide aldehydes LLnL and LLM (50 μM) and the lysosomotropic amine chloroquine (100 μM) were preincubated for the same period with BMM as PMA, and the cells were chased in the presence of these inhibitors for a further 6 hr before fixation.

In all cases, control cultures were incubated in DMSO at a concentration equivalent to that in the inhibitor preparation.

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