

Functional and ultrastructural evidence for intracellular formation of major histocompatibility complex class II–peptide complexes during antigen processing

(endosomes/immunocytochemistry/endocytosis)

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ABSTRACT Antigen presentation requires intracellular processing of native antigens to produce immunogenic peptides that bind to major histocompatibility complex class II (MHC-II) molecules. In functional studies of antigen processing by elicited peritoneal macrophages, MHC-II–peptide complexes were formed intracellularly. Immunogenic peptides were not released to bind surface MHC-II molecules. Ultrastructural studies employing immunogold staining in ultrathin cryosections of these macrophages showed large amounts of MHC-II molecules in intracellular sac-like vacuoles in the peripheral cytoplasm; most of these were negative for the lamp 1 lysosomal/endosomal membrane protein and cathepsin D. MHC-II molecules were also present in endosomes containing cathepsin D and lamp 1 as well as previously internalized gold-transferrin. The intracellular pool of MHC-II molecules was only slightly decreased by treatment with cycloheximide for 3 hr, indicating that it consisted mainly of endocytosed, recycling molecules, as opposed to nascent ones. These ultrastructural studies support the notion that there is endocytosis of MHC-II molecules into endocytic compartments, consistent with our earlier biochemical data. Furthermore, we have defined the distinct endocytic compartments that must mediate important functions in antigen processing, including the formation of MHC-II–peptide complexes.

The recognition of foreign antigens by CD4⁺ T lymphocytes requires that these proteins be processed intracellularly by antigen-presenting cells (e.g., macrophages, dendritic cells, and B cells) into constituent peptides that can bind to major histocompatibility complex class II (MHC-II) molecules, including murine I-A and I-E. Antigen processing involves endocytosis and endosomal processing of antigen and MHC-II molecules with transport of MHC-II–peptide complexes to the plasma membrane. In macrophages and B cells MHC-II molecules are endocytosed and are subsequently confined to an intracellular compartment of light density (1), although some B-cell lines may manifest little or no endocytosis of them (2). Macrophages and B cells differ in their ability to “functionally” recycle MHC-II molecules as defined by turnover of MHC-II–peptide complexes (peptide exchange), a mechanism utilized by some B cells but to a lesser extent by macrophages (3). Thus, macrophages exhibit a dichotomy between continued endocytosis of MHC-II and lack of “functional” recycling of MHC-II molecules.

The site of formation of the MHC-II–peptide complex remains unresolved; we now present functional evidence that this occurs within an endosomal compartment. We have also applied immunogold staining of ultrathin cryosections of unperturbed, fixed cells to ultrastructurally define the endo-

somal compartments involved in MHC-II endocytosis and processing. Intracellular MHC-II resides within two distinct intracellular compartments that must play important roles in antigen processing and the formation of MHC-II–peptide complexes.

MATERIALS AND METHODS

Cells and Functional Assays. Peritoneal macrophages were elicited by intraperitoneal injection of *Listeria monocytogenes* and peptone from CBA/J (H-2^k) and BALB/c (H-2^d) mice (1). These cells were plated (5×10^4 per well of each type utilized) and incubated with the antigen hen egg lysozyme (HEL), followed by fixation in 1% paraformaldehyde (1). The amount of HEL peptides bound to I-A^k was detected functionally by the response of a T-cell hybridoma. 3A9 T-hybridoma cells (10^5 per well) respond to I-A^k–HEL-(46–61) complexes by secretion of interleukin 2, measured by a CTLL cell proliferation bioassay (1).

Incubation with Tracers and Fixation. Macrophages were incubated with 17-nm gold-transferrin particles (20 μ g of transferrin per ml) for 3–30 min at 37°C or for 1 hr, on ice, prior to fixation for an additional 1 hr on ice in 2% formaldehyde/0.5% glutaraldehyde or 2% formaldehyde/1% acrolein, both in 0.1 M sodium phosphate (pH 7.4). The uptake of the gold-transferrin is transferrin receptor-specific (4), but its intracellular sorting may not reflect the true path of transferrin sorting. Other samples of viable macrophages were first incubated with 10% bovine serum albumin for 1 hr at 37°C and then fixed, whereas others were fixed, as above, without prior exposure to an exogenous tracer. To label surface MHC-II, formaldehyde/acrolein-fixed cells were extensively washed in phosphate-buffered saline (PBS), then treated with anti-MHC-II and 7-nm protein A-gold before cryosectioning, and finally postfixed in the same fixative prior to immunoelectron microscopy. Some macrophages were incubated with cycloheximide (10 μ g/ml) for 3 hr prior to fixation; this treatment abrogates antigen processing in these cells (1).

Immunoelectron Microscopy. Fixed macrophages were washed in PBS/0.15 M glycine and embedded in 10% gelatin at 4°C (5). Small blocks of gelatin were infiltrated with 2.5 M sucrose and mounted for cryotomy. The preparation of immunogold-labeled ultrathin cryosections was performed as described (6). The triple immunolabeling procedure included important blocking steps wherein unconjugated protein A and glutaraldehyde were introduced after protein A-gold binding to saturate previously applied antibody and prevent cross-labeling in subsequent staining steps. For immunolabeling MHC-II molecules we used protein A affinity-purified

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Abbreviations: HEL, hen egg lysozyme; MHC, major histocompatibility complex.

10.3.6.2 (anti-I-A monoclonal antibody) (7). We also used antigen affinity-purified rabbit antibodies against human cathepsin D (8), chicken 215-kDa mannose-6-phosphate receptor (9), and bovine serum albumin. Rat anti-mouse lamp 1 monoclonal culture supernatant was a kind gift of L. Arterburn and T. August (The Johns Hopkins University School of Medicine, Baltimore). The protein A-gold particles had sizes of 5 nm, 10 nm, 12 nm, and 15 nm \pm 10% (6).

For a quantitative evaluation of MHC-II distribution, cryosections were sequentially labeled with anti-I-A^k, 10-nm protein A-gold, anti-lamp 1, and 5-nm protein A-gold. In two experiments MHC-II-gold particles were counted on the microscope screen at \times 15,000. In both experiments 20 randomly selected profiles each of control and cycloheximide-treated cells were evaluated. Gold particles were attributed to the nearest of the compartments listed in Table 1. Totals of 1840 and 1544 particles were obtained from control and cycloheximide-treated cells, respectively. Background labeling was <3% and was subtracted from the values given in Table 1.

RESULTS

MHC-II–Peptide Complexes Are Formed Within an Intracellular Compartment. Potentially, antigen-derived peptides could bind to MHC-II molecules either within an endosomal compartment or on the plasma membrane following their release via a “secretion-recapture” mechanism. To distinguish between these possibilities we used two sets of macrophages. CBA/J macrophages (H-2^k) process HEL and present the 52–61 peptide bound to I-A^k. Such macrophages, if paraformaldehyde-fixed, will present the 52–61 peptide but not native HEL. BALB/c macrophages (H-2^d), on the other hand, process HEL but will not present 52–61. [The peptide 52–61 will not bind to I-A^d (10).] Therefore, we mixed viable BALB/c and fixed CBA/J macrophages with HEL to determine whether HEL processing by the viable BALB/c macrophages would result in the secretion of antigenic peptides that could bind to the surface I-A^k on fixed CBA/J macrophages, for presentation to 3A9 T cells. Fig. 1 shows that this did not occur. As expected, the addition of exogenous HEL-(52–61) peptide resulted in its presentation to 3A9. This assay is sensitive to 10–100 nM peptide or 1–10 μ g of HEL per ml; yet processing of up to 1000 μ g of HEL per ml by BALB/c macrophages failed to release sufficient peptide for presentation by surface I-A^k on fixed CBA/J macrophages. These findings indicate that immunogenic peptides of HEL are not released to bind to surface MHC-II molecules. Instead, the peptides must bind to them within an intracellular compartment, followed by transport of the complex to the plasma membrane.

Table 1. Distribution of MHC-II in macrophages: Percent contained in various subcellular compartments

Compartment	Morphology		Biochemistry*	
	Control	Cycloheximide [†]	Control	Cycloheximide [†]
Plasma membrane [‡]	60	73	78	63
Intracellular	40	27	22	37
Sacs [‡]	28	22		
Endosomes [§]	9	4		
Lysosomes [¶]	2	1		
Golgi complex [‡]	1	—		
Coated pits	—	—		

*Ref. 1.

[†]Incubation with cycloheximide (10 μ g/ml) for 3 hr.

[‡]Lamp 1-negative.

[§]Lamp 1-positive.

[¶]Lamp 1-positive, homogeneous electron-dense.

Analysis of MHC-II Distribution by Immunoelectron Microscopy. Macrophages were harvested and fixed immediately or were incubated first, at 37°C, with colloidal gold-transferrin or bovine serum albumin and subsequently fixed. To delineate surface I-A^k molecules, aliquots were stained, before sectioning, with anti-I-A^k and protein A-gold. To delineate intracellular molecules, aliquots were stained after cryosectioning with antibodies to I-A^k, cathepsin D, the mannose-6-phosphate receptor, or the lamp 1 lysosomal/endosomal membrane protein (11).

MHC-II molecules were distributed widely over the plasma membrane and were often concentrated at one pole of the cell characterized by ruffled plasma membrane overlying an accumulation of sac-like endocytic structures (hereafter referred to as sacs to distinguish them from typical endosomes). The sacs extended deeply into the cytoplasm. The deepest sacs were closely associated with endosomes and lysosomes (Fig. 2). Intracellular MHC-II molecules were distributed largely in the membranes of the sacs, with lesser amounts in smaller endosomes (below) (Table 1). Interestingly, MHC-II was absent from coated pits forming from the plasma membrane and the membrane of the sacs (see Figs. 2 and 5).

To investigate whether the sacs represent deep invaginations of the plasma membrane in contact with the extracellular milieu or are closed intracellular spaces, intact fixed cells were incubated with anti-I-A^k and small protein A-gold particles to label surface MHC-II. The cells were then cryosectioned and immunolabeled for intracellular MHC-II with larger gold particles. Fig. 3 shows that the exogenous tracer added to intact, fixed cells (or to viable cells at 4°C, not shown) did not penetrate to the sacs, establishing their true intracellular nature.

The presence of MHC-II in the endocytic/degradative pathway was investigated in sections that were simultaneously immunolabeled for cathepsin D, a marker of endosomes and lysosomes, and lamp 1, a major membrane constituent of late endosomes and lysosomes (11). Lamp 1 and cathepsin D were undetectable in Golgi compartments, at the plasma membrane, and in the sacs. MHC-II colocalized with cathepsin D and lamp 1 in multivesicular endosomes at the periphery (Fig. 4A) and center (Fig. 4B) of the cells. These endosomes were also positive for the large mannose-6-phosphate receptor (not shown).

Quantitation of MHC-II Distribution. The relative distribution of MHC-II was quantitated in sections that were double immunolabeled for MHC-II and lamp 1 to discriminate between sacs, endosomes, and Golgi compartments. Of the total MHC-II, 60% occurred at the plasma membrane and 40% occurred in intracellular compartments, in concordance with previously obtained biochemical data (Table 1). Sacs contained 28% and endosomes contained 9% of the cellular MHC-II. Coated pits did not show significant MHC-II labeling (of 62 coated pits in 20 random cell sections, none showed MHC-II labeling).

Cells incubated with cycloheximide for 3 hr had little change in the amount of MHC-II in the intracellular sacs, although the sacs were structurally disrupted into smaller, more vesicular structures (that remained deficient in cathepsin D and lamp 1). MHC-II in endosomes did decline from 9% to 4%. Thus, the latter compartments may contain, in part, newly synthesized MHC-II, whereas the sacs apparently contain MHC-II derived by endocytosis from the plasma membrane (1).

Viable macrophages were incubated for various periods at 37°C with colloidal gold-transferrin or bovine serum albumin to label the endocytic pathway. At all time points gold-transferrin was found on the plasma membrane, within the MHC-II-positive sacs, and in coated pits (Fig. 5). MHC-II appeared to be excluded from gold-transferrin-labeled coated

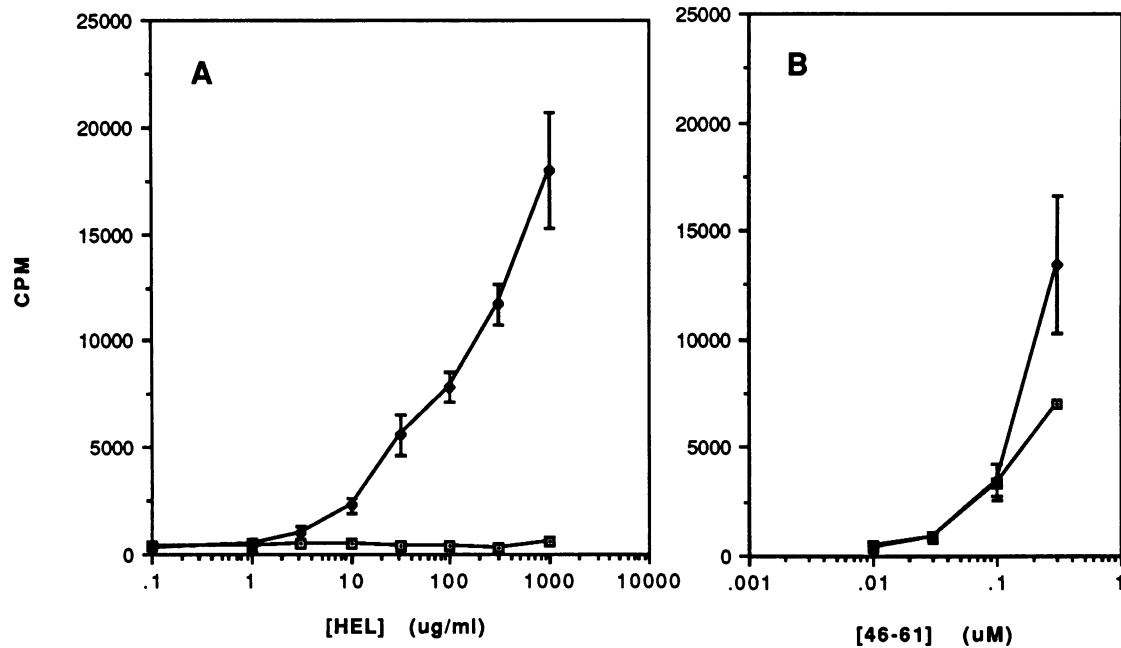


FIG. 1. (A) HEL was incubated at various concentrations for 2 hr with viable macrophages from CBA/J mice (H-2^k) (squares) or with viable BALB/c (H-2^d) macrophages plated with fixed CBA/J macrophages (closed diamonds). The cells were then fixed and washed, and 3A9 T-hybridoma cells were added. (B) Similar cell populations were incubated without antigen and then fixed. HEL-(46-61) was then added at various concentrations, and the response of 3A9 cells was determined. Squares, fixed BALB/c plus fixed CBA/J macrophages. Closed diamonds, fixed CBA/J macrophages.

pits (Fig. 5, Table 1). Within 5–10 min gold-transferrin also appeared in multivesicular endosomes and small vesicular and tubular endosomes. Immunostaining for bovine serum albumin after its uptake demonstrated a distribution similar to gold transferrin (not shown).

DISCUSSION

This paper indicates that peptides of HEL are not released and captured by surface I-A^k molecules. It implies that the

site of coupling of peptide and MHC-II is intracellular. This study also shows two distinct intracellular compartments that bear MHC-II molecules and are the potential sites of coupling. The intracellular distribution of MHC-II shown by immunoelectron microscopy is entirely consistent with our biochemical studies of MHC-II localization (1). Both approaches demonstrate that 30–40% of MHC-II is intracellular, and this distribution is only slightly altered after incubation of cells with cycloheximide. The biochemical and morphological approaches indicate that MHC-II is largely

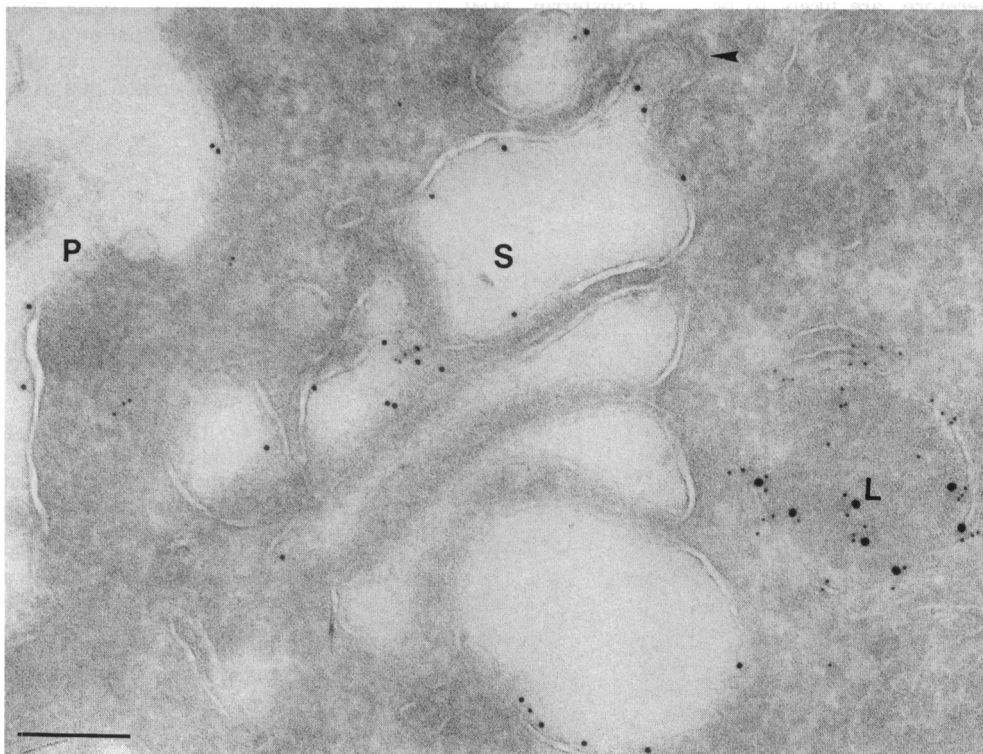


FIG. 2. Triple immunolabeled cryosection of a macrophage to show the presence of MHC-II (10-nm gold) at the plasma membrane (P) and in endocytic sacs (S). MHC-II is absent from a coated pit at the sac membrane (arrowhead) and from the homogeneously dense lysosome (L). The latter shows cathepsin D (15-nm gold) and lamp 1 (5-nm gold), which are both absent from the sacs. (Bar = 0.2 μ m; \times 72,800.)

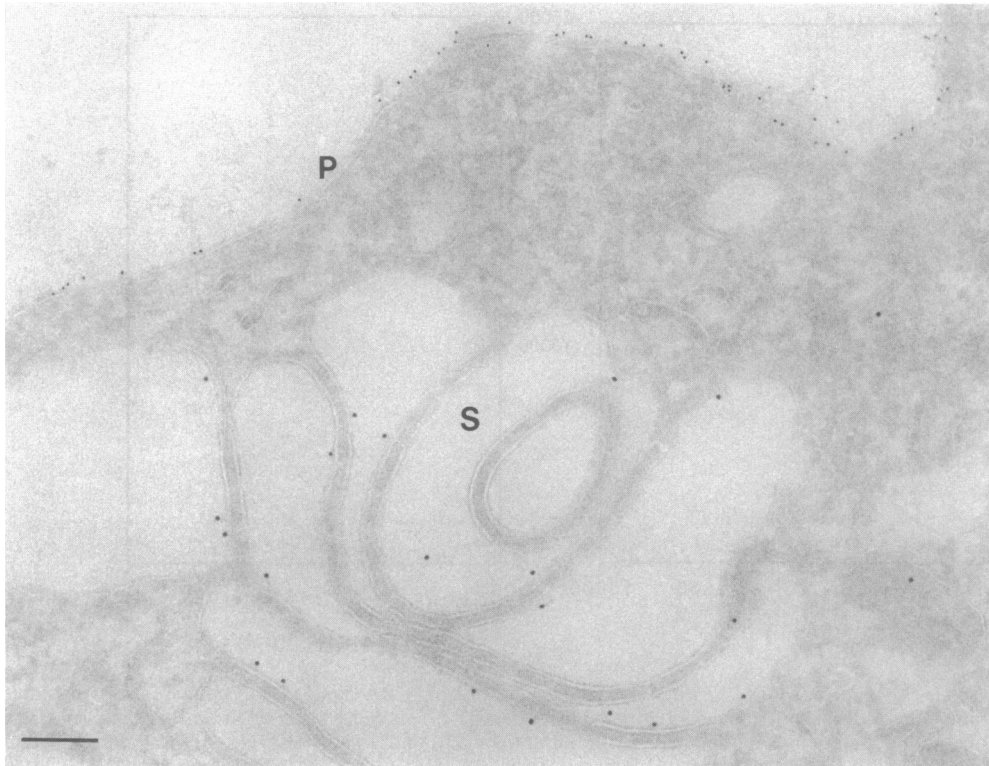


FIG. 3. Intact, fixed macrophages were incubated with anti-MHC-II and 7-nm protein A-gold, followed by cryosectioning and immunolabeling of intracellular MHC-II with 12-nm gold. MHC-II can be seen at the plasma membrane (P) and membranes of the sacs (S). The small gold particles are restricted to the cell surface, indicating that the sac lumina were not in continuity with the extracellular milieu. (Bar = 0.2 μm ; $\times 50,600$.)

confined to prelysosomal compartments with little, if any, in lysosomes.

Approximately 70% of the intracellular MHC-II was contained within large sac-like endocytic structures that showed no staining for lamp 1 or cathepsin D. These sacs were sealed internal compartments without connection to the extracellular milieu, as they were not penetrated by exogenous tracers applied to the cells at 4°C or following fixation. They are probably engaged in the initial steps of endocytosis as shown by their content of exogenous tracers. The sacs also contain many coated pits that accumulate gold-transferrin but appear to exclude MHC-II. The sacs, therefore, are likely to be internalized ruffles of the plasma membrane with abundant MHC-II. Exogenous tracers may leave the sacs via coated pits for transport to endosomes or lysosomes, whereas MHC-

II could be recycled by fusion of the sacs with the plasma membrane. The sac membranes may serve as a reservoir of membrane rich in MHC-II that can be utilized during events such as cell-cell interactions, formation of phagosomes, or cell adherence and spreading. Indeed, our previous biochemical data showed that adherence of macrophages decreased the intracellular pool of MHC-II by an amount similar to that measured in the sacs (1). Cycloheximide disrupts the sac structure and also inhibits macrophage adherence and antigen processing, further suggesting a role of the sacs in these processes. It is interesting to note that unlike gold-transferrin, MHC-II was not concentrated in coated pits, suggesting that its internalization and the formation of the sacs were largely independent of these structures. In contrast, gold-transferrin was concentrated in coated pits asso-

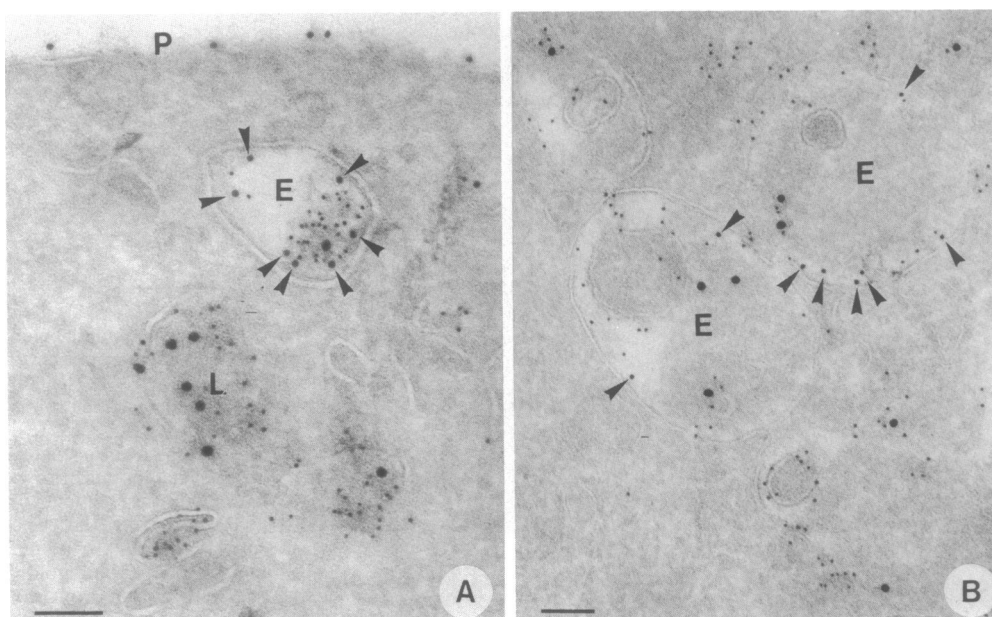


FIG. 4. Triple immunolabeled cryosections of macrophages showing the colocalization of MHC-II (10-nm gold, arrowheads), cathepsin D (15-nm gold), and lamp 1 (5-nm gold) in a peripheral endosome (E in A) and a central multivesicular endosome (E in B). The lysosome (L) in A and the other endosomes in B are only reactive for cathepsin D and lamp 1. (A: Bar = 0.1 μm ; $\times 90,000$. B: Bar = 0.1 μm ; $\times 69,000$.)

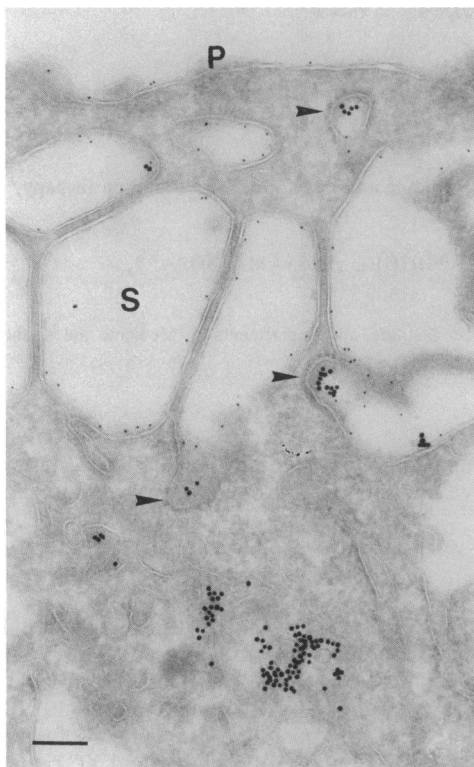


FIG. 5. Viable macrophages were incubated for 10 min at 37°C with 17-nm gold-transferrin, followed by fixation and cryosectioning. The cryosections were immunolabeled for MHC-II with 5-nm protein A-gold. Although gold-transferrin is clearly accumulated in coated pits (arrowheads) of the sacs (S), MHC-II appears to be excluded from the pits. P, plasma membrane. (Bar = 0.2 μ m; \times 35,300.)

ciated with the plasma membrane and the intracellular sacs, from which it was transferred to multivesicular endosomes and then lysosomes.

About 10% of the cell's MHC-II was observed in smaller, multivesicular endosomes that also contained cathepsin D and lamp 1, but MHC-II was rarely present in lysosomes. These endosomes also appear to contain newly synthesized MHC-II (cycloheximide decreased MHC-II by 50%). The lack of MHC-II in lysosomes implies limited delivery of MHC-II from endosomes to lysosomes or possibly a rapid recycling of MHC-II from lysosomes. Rapid degradation of a large proportion of MHC-II delivered to lysosomes is not a sufficient explanation, since MHC-II molecules are long-lived ($t_{1/2}$ = 10–12 hr) (3).

During antigen processing, the proteolytic generation of immunogenic peptides may occur within the same prelysosomal compartment in which the complexes are formed (in the presence of cathepsin D and other proteases), or peptides may be "rescued" and recycled from other endocytic compartments or from lysosomes. We have no morphological evidence that the rescue of peptides from lysosomes could be mediated by MHC-II. Peptides generated in lysosomes could utilize other novel mechanisms for retrograde transport to the MHC-II-containing endosomes for complexing. The mechanisms and possible roles of MHC-II in recycling peptides from phagolysosomes are also unexplored, and the pathways may differ from those in phagocytically inactive cells.

Differences in compartmentalization and function of MHC-II may be found among antigen-presenting cells. In macrophages, MHC-II-peptide complexes are clearly formed intracellularly, within the sacs or within the lamp 1 positive, cathepsin D-positive endosomes. The latter compartment is similar to endosomes in human B-lymphoblastoid cells recently studied by Guagliardi *et al.* (12) (containing cathepsin D, invariant chain, and MHC-II); they propose that this compartment could be the site of MHC-II-peptide complexing. Neeffjes *et al.* (5) propose that in human B-lymphoblastoid cells the complexes are formed in cathepsin D-negative, MHC-II-enriched vesicles associated with the trans-Golgi complex. In macrophages MHC-II-peptide complexes may form during postsynthetic transport rather than following the internalization of MHC-II from the plasma membrane, since functional studies imply little or no MHC-II-peptide turnover in these cells (3). Indeed, macrophages exhibit less recycling of MHC-II molecules than some B cells, which appear to recycle these molecules by turnover of MHC-II-associated peptides (3, 13). An explanation may lie in differences in intracellular targeting of MHC-II molecules. Most internalized MHC-II within macrophages is contained in the lamp 1-negative, cathepsin D-negative sacs, which may be deficient in proteins or factors necessary for turnover of MHC-II-peptide complexes. Our preliminary evidence suggests that murine B cells do not have sacs and that intracellular MHC-II resides in typical endosomes. These endosomes could mediate MHC-II-peptide turnover, but further analysis of the endocytic and secretory pathways in these cells is essential.

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