

Immunocytochemical Characterization of the Endocytic and Phagolysosomal Compartments in Peritoneal Macrophages

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Abstract. We have used endocytic and phagocytic tracers in an EM immunocytochemical study to define the compartments of the phagocytic and endocytic pathways in mouse peritoneal macrophages. Endocytosed BSA-gold appeared successively in early endosomes, spherical endosomal vesicles, a late endosomal tubulo-reticular compartment (TC), and terminal lysosomes. The TC appeared as an elaborate structure enriched for the lysosomal membrane glycoproteins Lamp 1 and Lamp 2, and expressing significant levels of rab7, a late endosome-specific GTP-binding protein. The cation-independent mannose-6-phosphate receptor was restricted to specialized regions of the TC that were predominantly adjacent to the Golgi complex. Both the early endosome and the TC had coated bud structures whose composition and function are presently unknown.

Phagolysosomes containing latex beads expressed the same membrane antigens and received endocytic tracers simultaneously with the TC. Since the membrane surrounding both organelles was also in direct continuity,

we assume that both structures form one functional compartment. Macrosialin, an antigen confined to macrophages and dendritic cells, was heavily expressed in TC and phagolysosomal membranes with low levels being detected in other endosomal compartments and on the cell surface. Treatment of cells with wheat germ agglutinin drastically altered the morphology of the TC, giving rise to sheets of tightly adherent membrane and greatly expanded vesicles, in which cell-associated wheat germ agglutinin was concentrated. The spherical endosomal carrier vesicles loaded with internalized gold tracers clustered nearby, often making contact without fusing. Since the delivery of endocytic tracer to the TC was significantly delayed these experiments suggest that the lectin is somehow preventing the endosome vesicles from fusing with the TC. Collectively, our data argue first that the PLC is equivalent to the "tubular lysosomes" commonly described in macrophages, and second that the meeting of the phagocytic and endocytic pathway occurs in this compartment.

MACROPHAGES (M ϕ) and other phagocytes take up extracellular components by two distinct mechanisms. Extracellular fluid, solutes, and receptor-bound ligands are internalized by endocytosis, a process common to all eukaryotic cells, while large particles are ingested by phagocytosis, predominantly a function of neutrophils and mononuclear phagocytes. These processes are mechanistically distinct. Endocytosis occurs continually via coated pits on the cell surface (Goldstein et al., 1985) and varies linearly with temperatures down to $\sim 4^{\circ}\text{C}$ (Steinman et al., 1974), whereas phagocytosis is a local response to a segment of membrane (Griffin and Silverstein, 1974) to en-

gagement of appropriate cell surface receptors by particles such as bacteria (Griffin et al., 1975) and requires a temperature $>18\text{--}21^{\circ}\text{C}$ (Rabinovitch, 1967).

These processes also differ in their intracellular consequences. Endocytosis is followed by complex sorting events which rapidly return some internalized components to the cell surface while delivering others to a series of distinct endosomal compartments and ultimately, to lysosomes (for reviews see Storrie, 1988; Kornfeld and Mellman, 1989; Gruenberg and Howell, 1989). Phagosomes usually fuse extensively with lysosomes (Cohn and Wiener, 1963), but some intracellular pathogens, e.g., *Mycobacterium tuberculosis* (Armstrong and Hart, 1971), *Toxoplasma gondii* (Jones and Hirsch, 1972; Joiner et al., 1990), and *Chlamydia trachomatis* (Friis, 1972), survive intracellularly by inhibiting phagosome-lysosome fusion. This fusion block can be reproduced in vitro by the addition to the culture medium of sulfatides from Mycobacterial extracts (Goren et al., 1976), or of polyanions like polyglutamic acid or dextran sulphate (Hart and Young, 1979). Such studies reveal differences be-

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1. *Abbreviations used in this paper:* BSA-Au₅, bovine serum albumin-5-nm gold conjugates; BSA-Au₁₆, BSA-16-nm gold conjugates; ECV, endosome carrier vesicle; MPR, mannose-6-phosphate receptor; M ϕ , macrophage(s); PBS-C, PBS with Ca²⁺ and Mg²⁺; PLC, prelysosomal compartment; TC, tubulo-reticular compartment; WGA, wheat germ agglutinin.

Table I. Antigenic Markers Used

Antigen	Antibody	Reference
Lamp 1	ID4B (Rat IgG2a)	Chen et al., 1985
Lamp 1	IG11 (Rat IgG1)	H. Rosen and S. Gordon, unpublished*
Lamp 2	ABL-93 (Rat IgG2a)	Chen et al., 1985, 1988
Macrosialin	FA/11 (Rat IgG2a)	Smith and Koch., 1987
CI-MPR	Rabbit antiserum	Griffiths et al., 1988
rab7	Rabbit antiserum (affinity purified)	Chavrier et al., 1990
WGA	Rabbit antiserum	Louvard, D., unpublished

* The 20 NH₂-terminal amino acids of the affinity-purified antigen were identical to the sequence reported for murine Lamp 1 (Chen et al., 1988).

tween mechanisms of phagosome-lysosome and endosome-lysosome fusion; dextran sulphate inhibits phagosome-lysosome fusion without delaying the degradation of endocytic tracers like HRP, or lysosomal delivery of thorotrast (Kielian et al., 1982). Conversely, the lectin con A spares phagosome-lysosome fusion, but causes the formation of large vacuoles by inhibiting lysosome-endosome fusion (Kielian and Cohn, 1981). Similar morphological changes are produced by several other lectins, including wheat germ agglutinin (WGA), wax bean agglutinin, and cross-linked peanut agglutinin (Goldman et al., 1976).

Recent EM studies in non-phagocytic cells have concentrated on the morphology of endosomal compartments defined by kinetic and antigenic markers (Griffiths et al., 1988, 1989, 1990; Geuze et al., 1988; Gruenberg et al., 1989; Parton et al., 1989; Bomsel et al., 1990; see Kornfeld and Mellman, 1989 for review). Collectively these data argue for four distinct structures beyond the coated vesicle through which internalized material on route to lysosomes appear to pass sequentially. These are the early endosome, the spherical endosome "carrier" vesicle (ECV) (commonly referred to as multivesicular bodies), the cation-independent mannose 6-phosphate- (MPR) enriched late endosome/prelysosomal compartment (PLC), and the MPR-negative lysosome. Here, these studies are extended to phagocytes and show that the phagocytic and endocytic pathways converge at the level of an extensive tubulo-reticular compartment (TC). We show that this TC contains markers expected for a late endosomal, prelysosomal compartment. However, it also has features expected of a functional lysosome, as well as being kinetically and structurally similar to the tubular lysosomes described by Swanson et al. (1987). Macrosialin, an antigenic marker for mononuclear phagocytes and related cells (Smith and Koch, 1987; Rabinowitz and Gordon, 1991), is shown to be predominantly localized in the TC. Finally, vacuoles induced by WGA are identified as elements of the TC and the associated functional block is defined as a delay in the fusion of the spherical endosome vesicles with the TC.

Materials and Methods

Media, Sera, and Reagents

PBS (137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.3) was reconstituted from tablets (Oxoid Ltd, Basingstoke, UK) and autoclaved; complete PBS (PBS-C) was made by adding 0.9 mM CaCl₂ and 0.49 mM MgCl₂. FCS (from Sera Lab Ltd, Crawley Down, UK) was heat inacti-

vated at 56°C for 30 min. Dulbecco's MEM (Gibco Laboratories, Paisley, Scotland) was supplemented with 2 mM L-glutamine, 50 µg/ml penicillin G, 50 µg/ml streptomycin, and either 5% FCS or 0.1% BSA. A list of antibodies used in this study is given in Table I. BSA was conjugated to colloidal gold of different sizes prepared according to Slot and Geuze (1985). For this conjugation, 0.2 g/100 ml colloidal gold was used. The 16-nm gold conjugate (for late endocytic structures) was used at a dilution corresponding to an OD₅₂₀ of 0.5. For the 5-nm gold traversing the endocytic pathway, an OD₅₂₀ of 1.4 was used.

Isolation and Culture of Macrophages

Male C57Bl/6 mice were bred at the Sir William Dunn School of Pathology (Oxford). Peritoneal exudates were harvested by lavage 5 d after i.p. injection of 1 ml 50% (vol/vol) sterile Biogel P-100 beads, 100–200 mesh (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), prepared as described (Fauve et al., 1983). Exudate cells were washed in PBS, resuspended in DME with FCS, and cultured in 9-cm-diam bacteriologic Petri dishes (Flow Laboratories, Rickmansworth, UK). After 4 h, the monolayers were washed three times in PBS-C and re-fed with culture medium. The use of bacteriologic rather than tissue culture-treated plastic facilitated subsequent recovery of macrophages.

Endocytic Markers, Phagocytic Stimuli, Fixation, and Cryosectioning

MØ were simultaneously loaded with endocytic and phagocytic markers (Fig. 1). To label late endocytic compartments, BSA-16-nm gold conjugate (BSA-Au₁₆) was added to the culture medium for 9 h. The monolayers were then washed three times in PBS-C, re-fed with medium, and cultured for 10 h. A phagocytic meal of sterile latex beads (1:300 [vol/vol]) was offered; after 30 min, the monolayers were washed three times in PBS-C and re-fed DME with BSA. FCS was omitted from all subsequent incubations to avoid interactions between WGA and fetuin. After 2.5 h, some plates received 1–40 µg/ml WGA (Vector Laboratories, Inc., Peterborough, UK); 1 h later, all plates were washed three times with PBS-C and re-fed with medium. After 6.0–6.5 h, early endocytic compartments were labeled with BSA-5-nm gold conjugate (BSA-Au₅). In each experiment, every plate was individually timed to ensure constant pulse and chase periods. Tracer was added to the medium for 5 or 8 min, some plates were washed with cold buffer and fixed immediately as described below, while others were rapidly washed in PBS-C, re-fed with warm (37°C) medium, and cultured for periods up to 3 h. In some experiments a 5-min internalization was used to label only the early endosomes. Each plate was then placed on ice, washed once with ice-cold PBS-C, an removed on ice to a 4°C-cold room. All further manipulations occurred strictly at 4°C until the cells were fixed. The buffer was replaced with 5 mM EDTA/PBS and the monolayers incubated 45–60 min to allow cell detachment. The MØ were removed by vigorous pipetting, pelleted in a bench-top centrifuge, and resuspended in PBS. 1-ml aliquots were transferred to microfuge tubes; 100 µl 20% formaldehyde in 200 mM Pipes, pH 7, was added, and the cells gently mixed and briefly pelleted in a microfuge. The tubes were removed to room temperature, and the buffer was replaced with 8% formaldehyde/200 mM Hepes, pH 7. After a further 10 min, the pellets were compacted by microfugation and stored at 4°C for 24–48 h before preparation for cryosectioning and labeling with antibodies followed by protein A-gold, as described previously (Griffiths et al., 1984). Double labeling was carried out according to Geuze et al. (1981) with the additional modification that glutaraldehyde (1% in PBS for 5 min) was used to treat the sections (instead of protein A) between the two labeling steps (Slot et al., 1991).

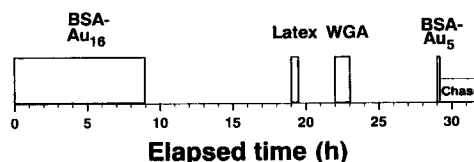


Figure 1. Flow chart showing the administration of the endocytic and phagocytic markers used. MØ monolayers were exposed to BSA-gold conjugates and latex beads as detailed in Materials and Methods. Some cultures were also treated with WGA. A short pulse of BSA-Au₅ was followed by culture in fresh medium for 0–180 min before fixation.

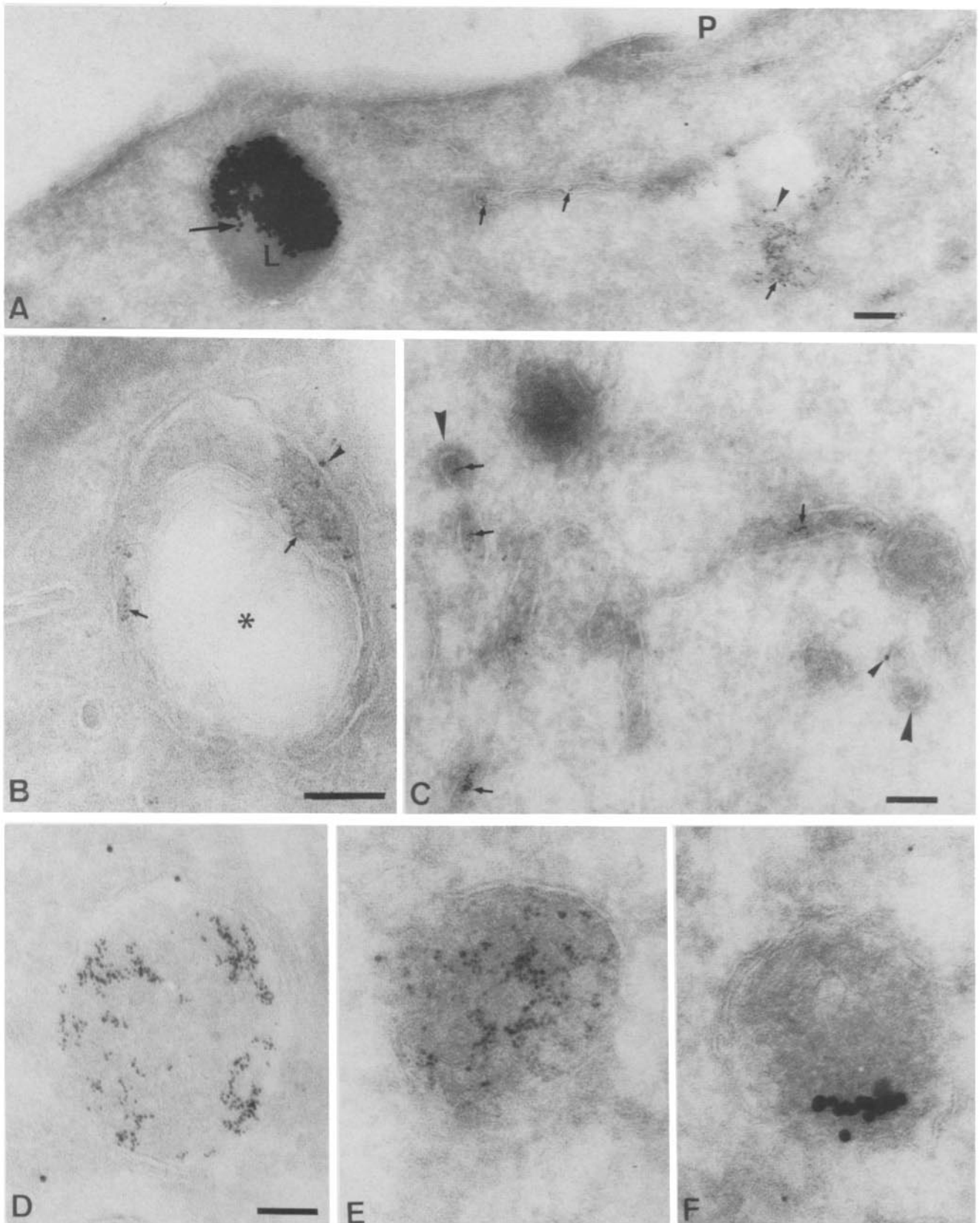


Figure 2. Appearance of early endosomes, spherical endosome vesicles and lysosomes in macrophages. In *A–C*, 5-nm gold-BSA (*small arrows*) had been internalized for 5 min and can be observed in early endosomes that are predominantly cisternal structures. These may be flat sheets, as in *A*, or can form crescent shaped structures, as in *B*, that enclose an electron-transparent zone (*asterisk*). In *C* two coated “buds” are seen, one of which contains 5-nm gold particles (*large arrowheads*). In *A* a structure we classify as a lysosome (*L*) is seen as a spherical, electron-dense vesicle that has accumulated 16-nm gold-BSA (*large arrow*) that was internalized and chased overnight. The small arrowheads in *A* and *B* show the low labeling (9-nm gold) typically seen in early endosomes for the MΦ-specific antigen macroscia-lin. *D–F* show examples of the spherical endosome vesicles labeled with 5-nm gold (*D* and *E*) or 16-nm gold (*F*) for 5 min followed by a 10-min chase. *P*, plasma membrane. Bars, 100 nm.

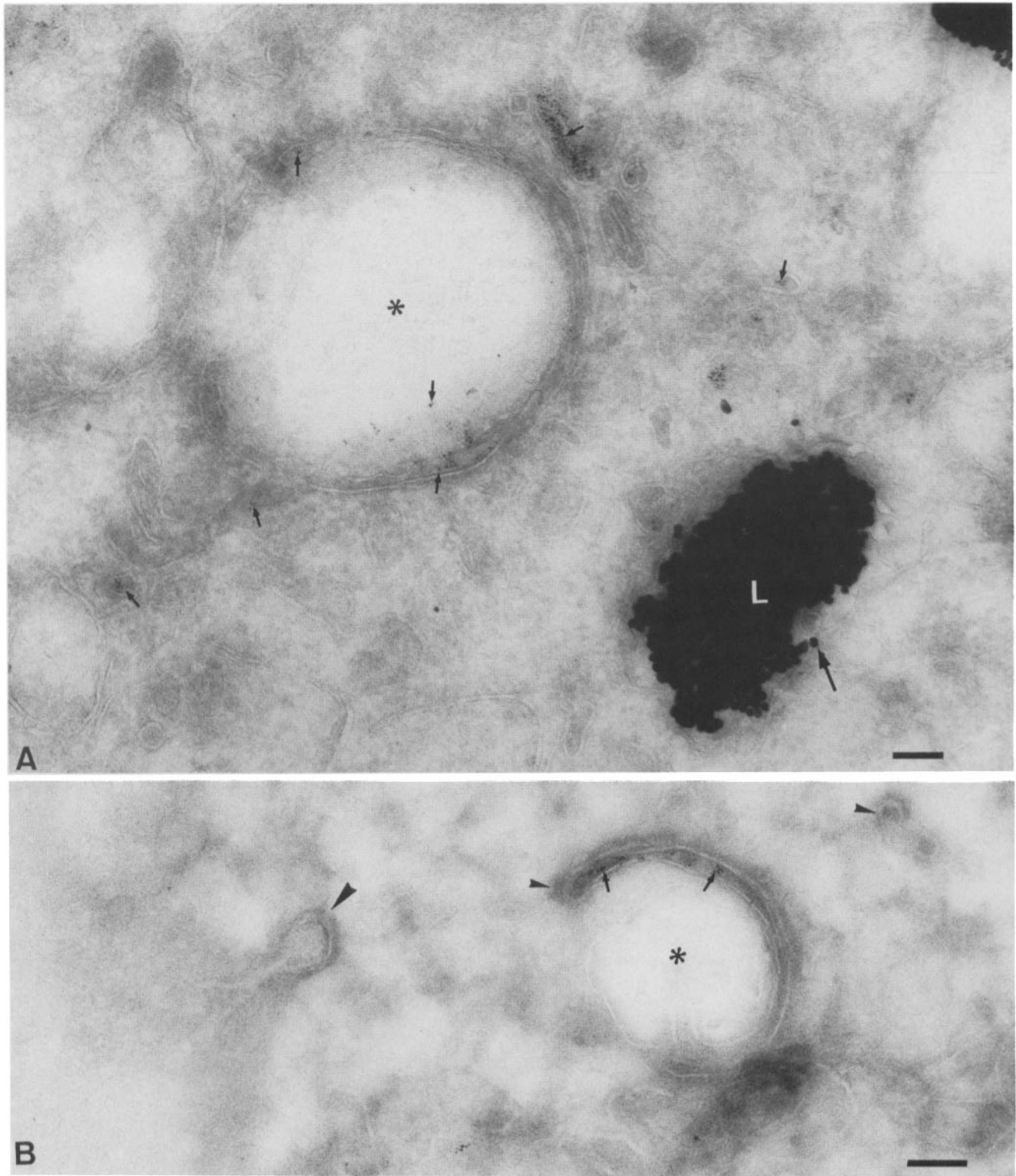


Figure 3. Appearance of early endosomes and lysosomes. The early endosomes are identified by the presence of 5-nm gold-BSA (*small arrows*) and show the characteristic curved cisternal appearance, enclosing a clear space (*asterisk*). Note the small coated "buds" associated with the early endosome (*small arrowheads*) are about half the size (~ 50 nm) of a plasma membrane coated pit (~ 100 nm; *large arrowhead*). *L* indicates a lysosome that had an aggregate of the 16-nm gold-BSA (*large arrow*) after an overnight chase. Bars, 100 nm.

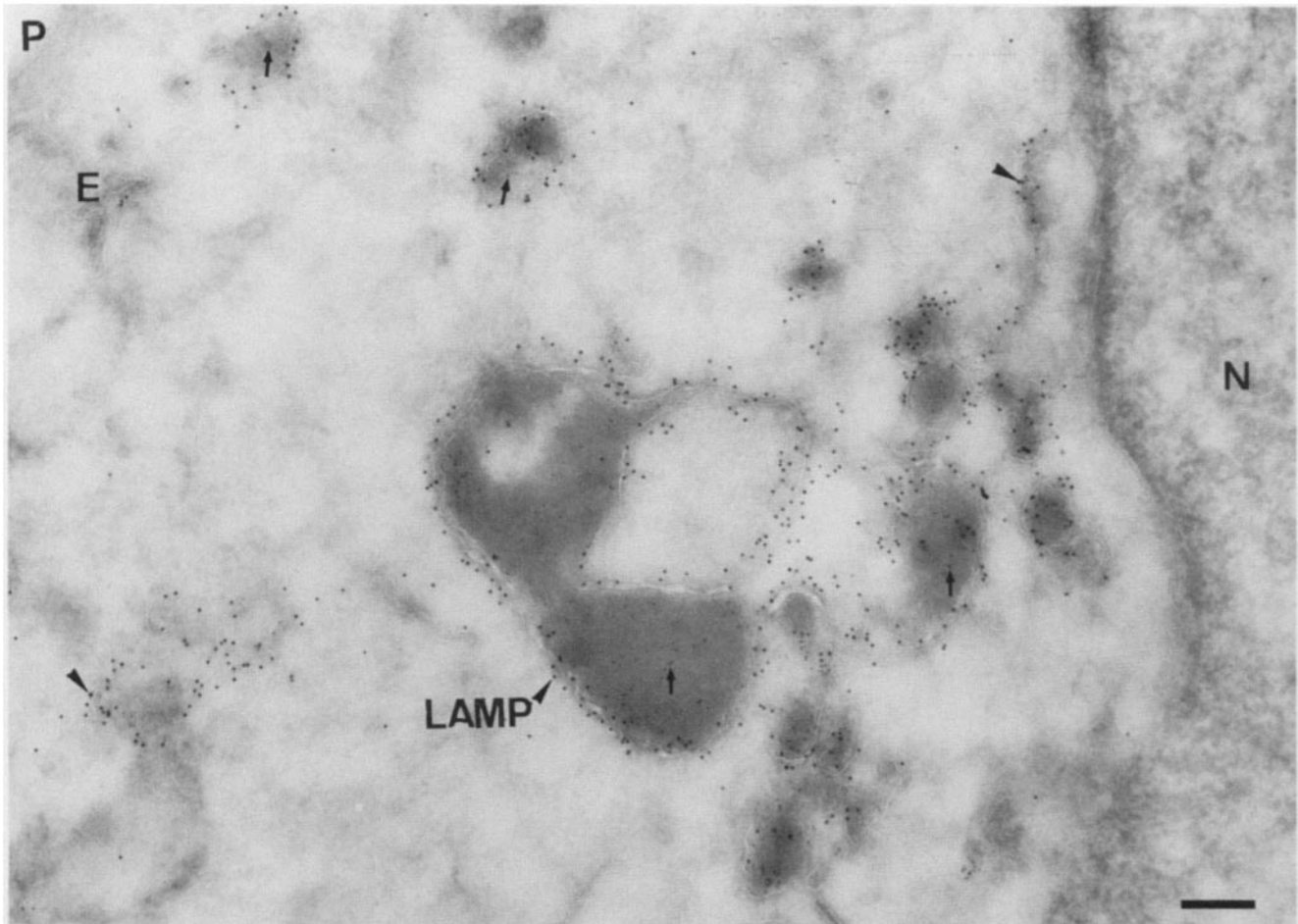


Figure 4. LAMP 1-labeling of the TC with 9 nm-gold (arrowheads). This cell took up 5-nm gold-BSA for 8 min followed by a chase of 30 min. This gold marker (arrows) fills the extensive tubulo-vesicular cisternal structures adjacent to the nucleus (N). A structure near the plasma membrane (P) that is probably an early endosome (E) is seen that has low labeling for LAMP but no 5-nm gold. In the close vicinity is a heavily labeled TC profile that contains 5-nm gold. Bar, 200 nm.

Functional Effects of WGA

Biogel-elicited M ϕ were cultured on sterile glass coverslips in DME with BSA. WGA was added to a concentration of 1–40 μ g/ml, and the cells cultured for 1–7 h, rinsed in PBS, and fixed in 0.25% glutaraldehyde/PBS. Some coverslips received 0.125–1.0 mM chitotriose 30 min before addition of WGA. Fixed preparations were mounted in distilled water and examined under phase-contrast optics with a microscope (Axiophot, Carl Zeiss, Inc., Thornwood, NY).

Uptake of FITC-WGA

WGA was conjugated with FITC isomer I (Sigma Chemical Co, Poole, UK) as described (Johnson and Holborow, 1986), to an OD_{280:495} ratio of 0.62. Coverslip preparations of Biogel-elicited M ϕ were dipped in ice-cold PBS and inverted onto drops of FITC-WGA (20 g/ml in 0.1% BSA/PBS) on parafilm at 4°C for 100 min, then rinsed in cold PBS. Some coverslips were immediately fixed in ice-cold 2% paraformaldehyde/PBS; others were transferred to warmed culture medium (with BSA) and incubated at 37°C for 3–30 min before rinsing and fixation. For prolonged chase periods, cells were exposed to FITC-WGA for 1 h at 37°C, then washed and cultured in fresh medium up to 50 h. Coverslips were mounted in Moviol M4-88 (Hoechst Chem. Co., Frankfurt, Germany) for fluorescence microscopy with an epifluorescence microscope (Axiophot; Carl Zeiss, Inc.). In control experiments, addition of 0.5 mM chitotriose inhibited labeling.

Quantitation

(A) To quantify the passage of 5-nm gold from the early endosomes to the TC, cells were allowed to internalize 16-nm gold-BSA for 5 min only or

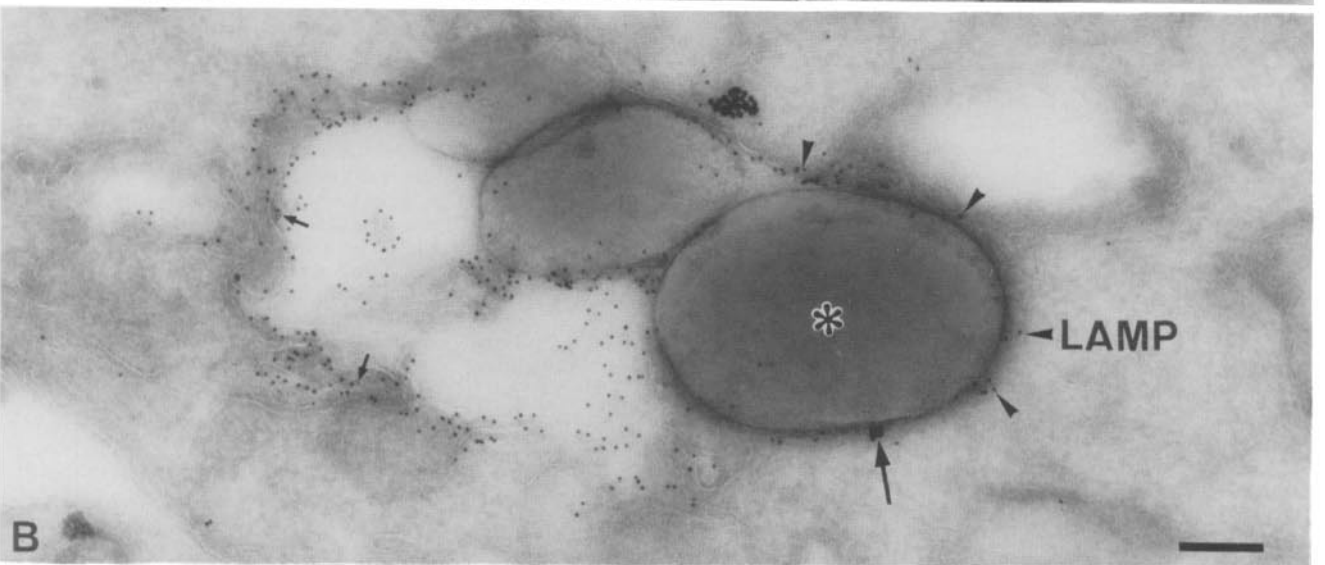
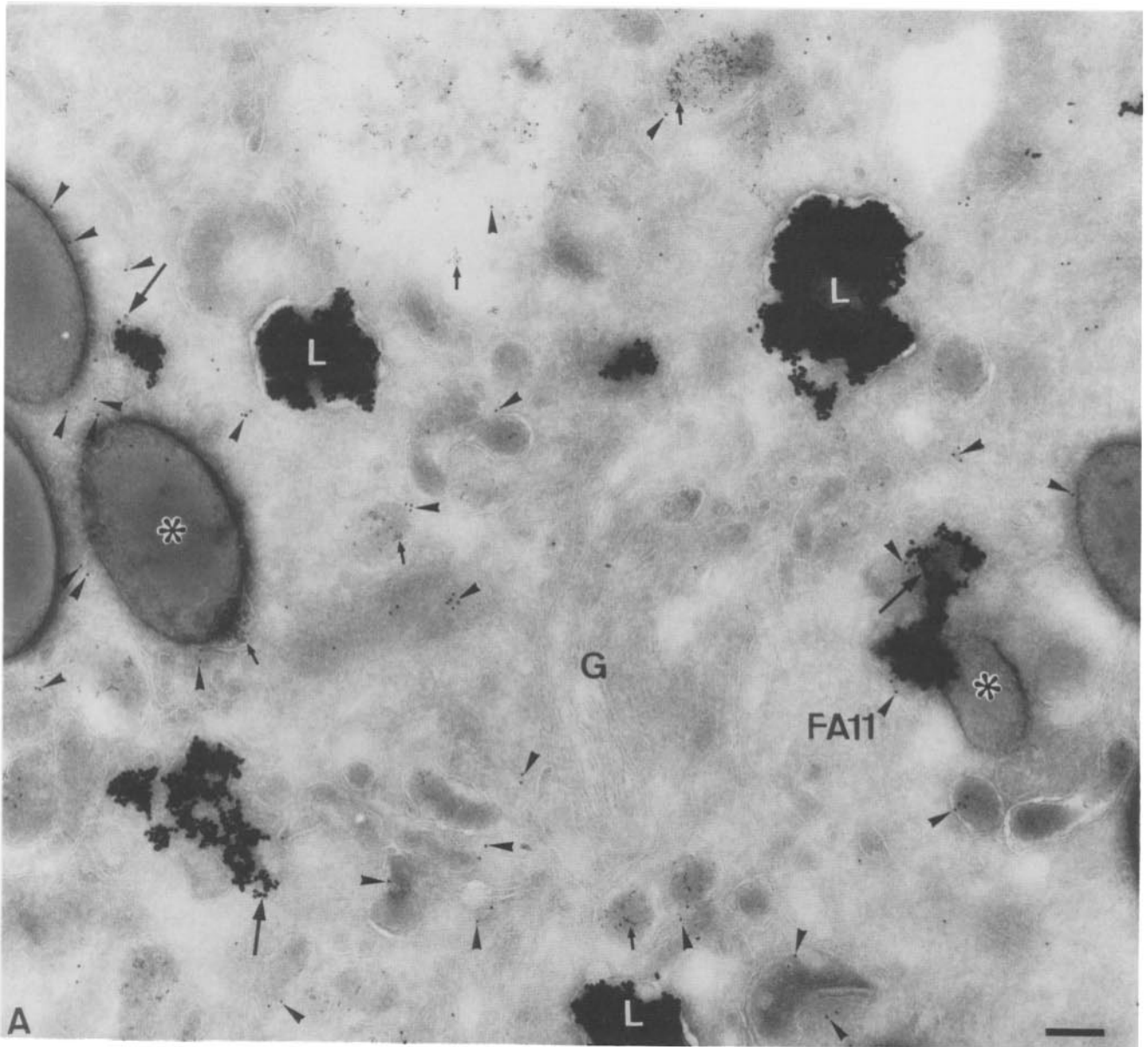
for a 5-min pulse followed by either a 10- or 20-min chase. After fixation and cryosectioning the sections were labeled with anti-Lamp 2 and protein A-gold (9 nm). Thin sections having a uniform thickness (as determined from their overall electron density) were selected. 20 micrographs of the cytoplasm were taken in a systematic fashion at a primary magnification of 17,000. These were enlarged 4.09 \times and three different structures were identified and photographed: first, early endosome profiles (low labeling with Lamp 2) that are easy to identify in these cells; second, the enlarged vesicular parts were scored separately; third, the Lamp 2-enriched TC. When they were labeled with internalized gold, the spherical endosome vesicles were relatively easy to identify. However, when they were not labeled the profiles of these vesicles were not easily recognized. For this reason, these structures were not included in this analysis. (B) To quantify the amount of internalized gold that entered the TC in the presence and absence of WGA, 22 micrographs of systematically sampled areas of the TC were taken at a primary magnification of 28,000. These were enlarged 4.09 \times . In both A and B the number of gold particles was related to the area of the organelle by point counting using a square lattice grid, as described by Griffiths and Hoppeler (1986).

Results

The overall plan of the experiments described here is summarized in Fig. 1, and a list of the antigenic markers used is given in Table I.

The Early Endocytic Pathway

BSA-Au₁₆ was administered and chased overnight to its



final endocytic destination. A short pulse of BSA-Au₅ was used to mark kinetically defined early endosomal compartments. Within 5 min, endocytosed BSA-Au₅ was found in a reticular structure consisting of tubules attached to cisternae that were either sheet-like (Fig. 2 *A*) or curved around a central electron-lucent area (Fig. 2 *B* and Fig. 3, *A* and *B*); these structures resemble early endosomes described in other cell types (Griffiths et al., 1989; McDowall et al., 1989; Parton et al., 1989). Early endosomal tubules bore small coated buds with roughly half the diameter of coated pits associated with the plasma membrane (Figs. 2 *C* and 3 *B*). As in other cells, the tubulo-reticular structures were continuous with larger, irregularly shaped vesicular structures (see Fig. 11 *B*).

With longer chase intervals, endocytic marker was followed through later endocytic compartments. From 8 to 30 min, BSA-Au₅ was found in spherical vesicles (Figs. 2, *D-F*, and 11, *A* and *C*), resembling the ECVs described in other cell types (Gruenberg et al., 1989; Griffiths et al., 1988, 1990). These structures have often been referred to as multivesicular bodies because of their densely packed membrane profiles. Although these internal membranes were evident in some images (e.g., Fig. 11 *C*), they were less prominent in the lumen of the vesicles in MΦ than in other cells we have investigated (Fig. 2, *D-F*; note that Fig. 2 *F* was from an experiment where 16-nm gold was used instead of 5-nm gold as a marker for the early structures). These structures became much more frequent after the WGA treatment (see below).

Late Components of the Endocytic Pathway

After a 5–8 min pulse and chase of 10 min or longer, in addition to the filling of the spherical ECVs, a significant amount of the gold marker entered an extensive tubulo-cisternal network that is, in part, closely adjacent to the nucleus (Fig. 4), centrioles (not shown), and the Golgi complex (Figs. 5 *A*, 6 *C*, 7 *B*, and 9 *B*). For the sake of simplicity, we will subsequently refer to this structure as the TC. In the present study it took ~60 min in order to chase all of the gold from the early endosomes and ECVs into the TC. At this time label was also detected in spherical dense structures that we classify as lysosomes (see below); characteristically these accumulated the 16-nm-gold conjugate after an overnight chase (Figs. 2 *A*, 3 *A*, and 5 *A*). It should be noted that under this condition a significant amount of 16-nm gold remained associated with the TC. A similar phenomenon is seen with the late endosome/prelysosomal compartment in other cells (Griffiths et al., 1988, 1990).

A striking feature of the TC was the presence of many coated buds (Fig. 6, *A*, *D*, and *E*) as well as internal membrane profiles in its lumen, which could represent invagina-

tions or internal vesicles. This compartment was heavily enriched for the lysosomal membrane antigens Lamp 1 (Fig. 4) and Lamp 2 (Fig. 5 *B*). Specific labeling on the membranes of the TC was also seen with antibodies to rab7, a GTP-binding protein that is restricted to the cytosolic side of membranes of late endosomes (Chavrier et al., 1990; Gorvel et al., 1991) (Fig. 6). This labeling was fairly uniform throughout the whole structure. We could see no evidence of significant reactivity on the coated bud structure although small amounts of labeling were occasionally seen at or near these structures (not shown). Small amounts of labeling for rab 7 were also consistently associated with the Golgi complex (Fig. 6 *C*), but early endosomes, the spherical endosome vesicles, and putative lysosomes were devoid of labeling (not shown).

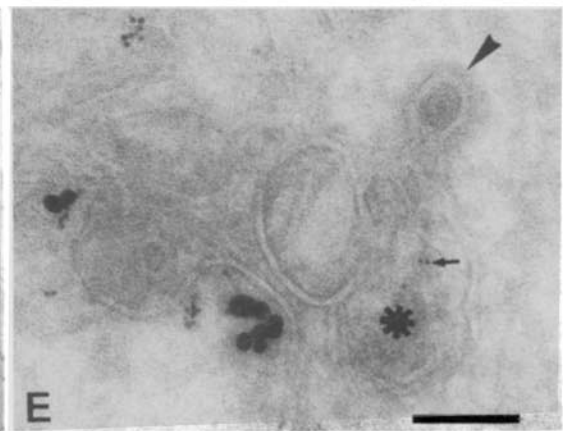
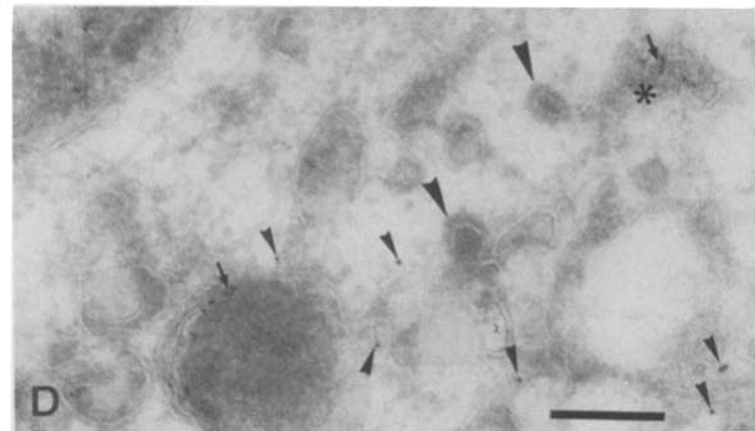
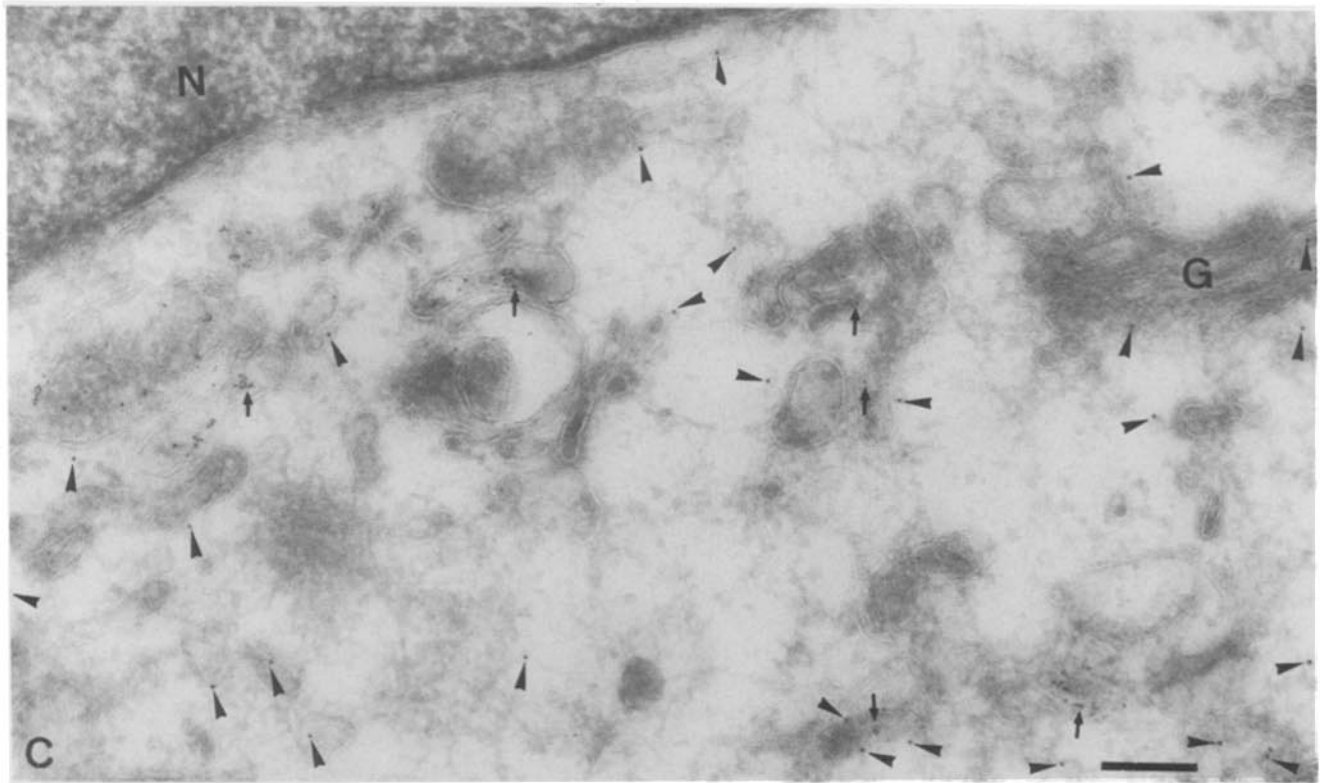
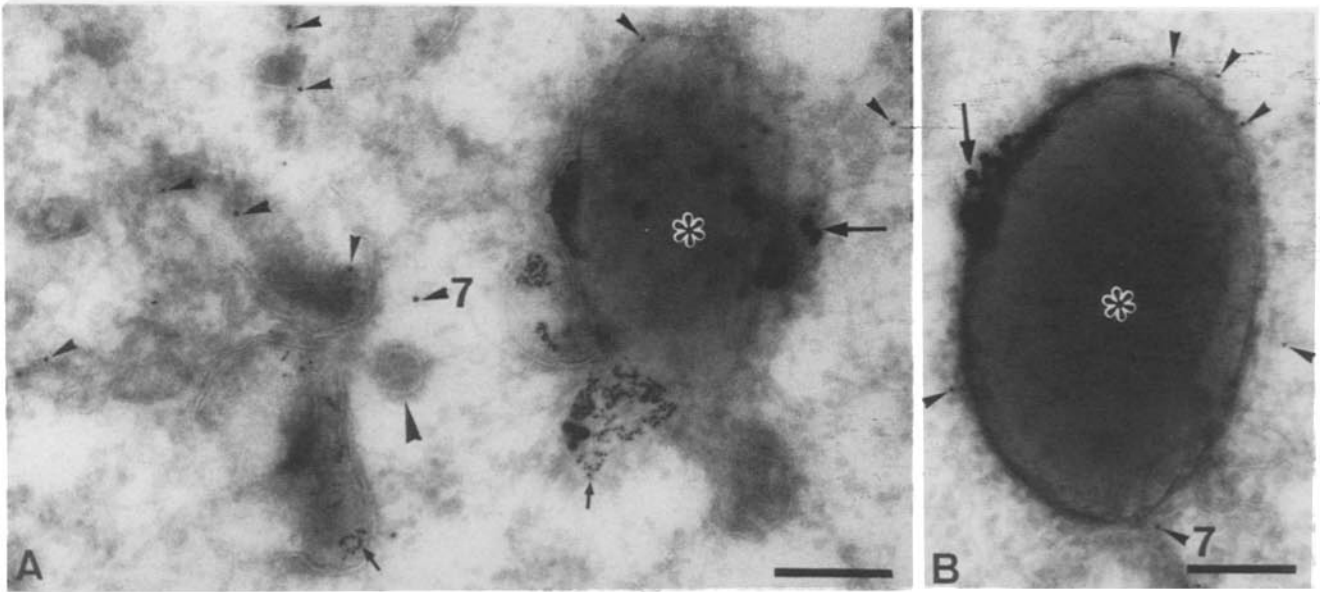
The CI-MPR was detected only in regions close to the Golgi complex (Fig. 7); antibodies to this receptor did not label the bulk of the TC, even though extensively labeled and unlabeled regions were continuous and filled simultaneously with BSA-Au₅. Both of these regions of the TC contained significant levels of residual BSA-Au₁₆, even after an overnight chase (Fig. 7, *A* and *C*), and both labeled extensively with the Lamp antibodies (Fig. 7 *C*) as well as with rab 7 (not shown). The vesicles we tentatively define as lysosomes were positive for the Lamp antibodies, negative for CI-MPR, and morphologically similar to those structures better defined in other cell types. Another striking feature of these lysosome vesicles is that internalized gold particles within the lumen are usually clustered into large aggregates (Figs. 2 *A*, 3 *A*, 5 *A*, and 9 *B*).

The MΦ specific marker Macrosialin (Smith and Koch, 1987; Rabinowitz and Gordon, 1991) was found at low levels on the plasma membrane (not shown) and in early endosomes (Fig. 2 *A* and *B*). The bulk of this marker was found on the membranes of the TC (Fig. 5 *A*). The putative terminal lysosome structures were devoid of labeling (Fig. 2 *A* and 5 *A*).

Quantitation of Internalization through the Endocytic Pathway

To follow the pathway of internalization up to the TC in a quantitative fashion, cells were allowed to internalize 16-nm gold-BSA for either 5 min only or for a 5-min pulse followed by a 10-min or a 20-min chase in medium without gold. To facilitate the identification of the TC, the section was additionally labeled with anti-Lamp 2- and 9-nm gold-protein A. In addition to the TC, two different kinds of structures were classified, both showing relatively low labeling for Lamp 2. First, the tubulo-cisternal early endosomes (e.g., Fig. 2 *A* and *B*), and second, early endosome vesicular profiles that were more irregular in shape (e.g., the enlarged

Figure 5. (A) Labeling of the TC with FA-11, the antibody against the MΦ specific marker macrosialin and 9-nm gold (*arrowheads*). This cell internalized took up 5-nm gold BSA for 8 min followed by a chase of 22 min, while the 16-nm gold was taken up after an overnight chase into both lysosomes (*L*), which are devoid of macrosialin labeling, and the TC (*large arrows* indicate this gold in the TC) which labels significantly for macrosialin (*small arrowheads*). The 5-nm gold is in TC elements after an 8-min pulse and a 22-min chase. The asterisks indicate latex particles that are in the TC as seen by macrosialin labeling and by the presence of both 5-nm and 16-nm gold. The whole of the Golgi complex (*G*) is completely devoid of labeling for the internalized gold and has only one or two gold particles labeled for macrosialin. In *B* three latex particles (*asterisk*) are seen in the phagolysosome/TC that is heavily labeled for Lamp 2 (9-nm gold, *arrowheads*) and contains both 16-nm gold (*large arrow*) as well as small amounts of the 5-nm gold (*small arrows*) after labeling conditions identical to *A*. Bars, 200 nm.



endosome profile in Fig. 11 B). Many of the profiles are continuous with the early endosome tubulo-cisternal parts, as in other cells (Griffiths et al., 1989; Gruenberg et al., 1989). We assume they represent stages in the formation of the spherical endosomal vesicles. Since we could not unequivocally identify unlabeled spherical endosome vesicles, we did not include these structures in the analysis.

As shown in Fig. 8, after a 5-min pulse, label was only detected in the early endosomal tubulo-cisternal as well as the irregular vesicular profiles. After a 10-min chase, almost all the label had left these structures (note however that small amounts of label often remained in these structures for long periods). After a 10-min chase, essentially all the gold was distributed between the spherical vesicles (not shown) and the Lamp 2-enriched TC. Although not easy to quantify, we had the clear impression that the number of labeled spherical vesicles decreased with the longer chase period. While it is difficult from these data to determine the position of the spherical vesicles in the pathway, the analysis shows clearly that the gold marker passes kinetically from early endosome structures to the TC.

Phagolysosomes Are Continuous with the Tubular Compartment

Latex beads served as a nondegradable phagocytic stimulus readily detected by EM, facilitating a comparison of phagocytic compartments with endocytic structures defined kinetically and antigenically. After 10 h, phagolysosomes contained both latex particles and BSA-Au₁₆ previously chased into terminal endocytic elements (Figs. 5, A and B; 6, A and B; and 7 A). These phagolysosomes received newly ingested BAS-Au₅ at the same time as the TC (Figs. 5, A and B, and 7 A); they were enriched for Lamp 2 (Fig. 5 B), Lamp 1 (not shown), macrosialin (Fig. 5 A), and rab 7 (Fig. 6, A and B). In many images, the membrane surrounding a latex particle was continuous with that of a typical TC structure (Fig. 5 B). The membrane around the latex beads was usually devoid of MPR although sometimes in close apposition to, and often continuous with, the MPR enriched membranes (Fig. 7 A). In a few cases (e.g., Fig. 9 A), the TC contained a partly digested bacterium. Collectively, these data indicate that the late phagolysosomes and the TC are the same compartment.

WGA Produces Persistent Vacuoles

WGA, used at high concentrations (up to 30 $\mu\text{g}/\text{ml}$) has been reported to induce formation of vacuoles in thioglycollate-elicited M ϕ (Goldman et al., 1976). The Biogel-elicited M ϕ used in this study were very sensitive to the effects of WGA and showed dramatic effects at lectin concentrations as low as 1 $\mu\text{g}/\text{ml}$ (Fig. 10). Within 15–30 min, enhanced spreading and formation of short, broad processes were evident. In densely plated cultures, initial small contacts between neighboring cells enlarged over the next few hours to involve ex-

tensive areas of the cell surface, occasionally leading to cell-cell fusion and formation of multinucleate giant cells. In sparse cultures, cells showed circumferential spreading. Small phase-lucent vesicles appeared at the cell periphery in the first 2 h. These became progressively larger and fewer, while moving towards the nucleus (Fig. 10 b), where they remained for several days in culture. All these lectin-induced changes were completely inhibited by the haptenic sugar, chitotriose (GlcNAc)₃ (Fig. 10 a).

To follow the movement and fusion of these WGA-induced vacuoles, M ϕ were incubated at 4°C with FITC-WGA and washed in the cold and transferred to warm medium for various intervals before fixation. In the cold medium (Fig. 10 c), cells were rounded and showed a ring of surface fluorescence. Upon warming, FITC-WGA was rapidly cleared from the cell surface, initially into small, poorly resolved, peripheral structures (Fig. 10 d), and then into progressively larger perinuclear vesicles outlined by the lectin (Fig. 10 e).

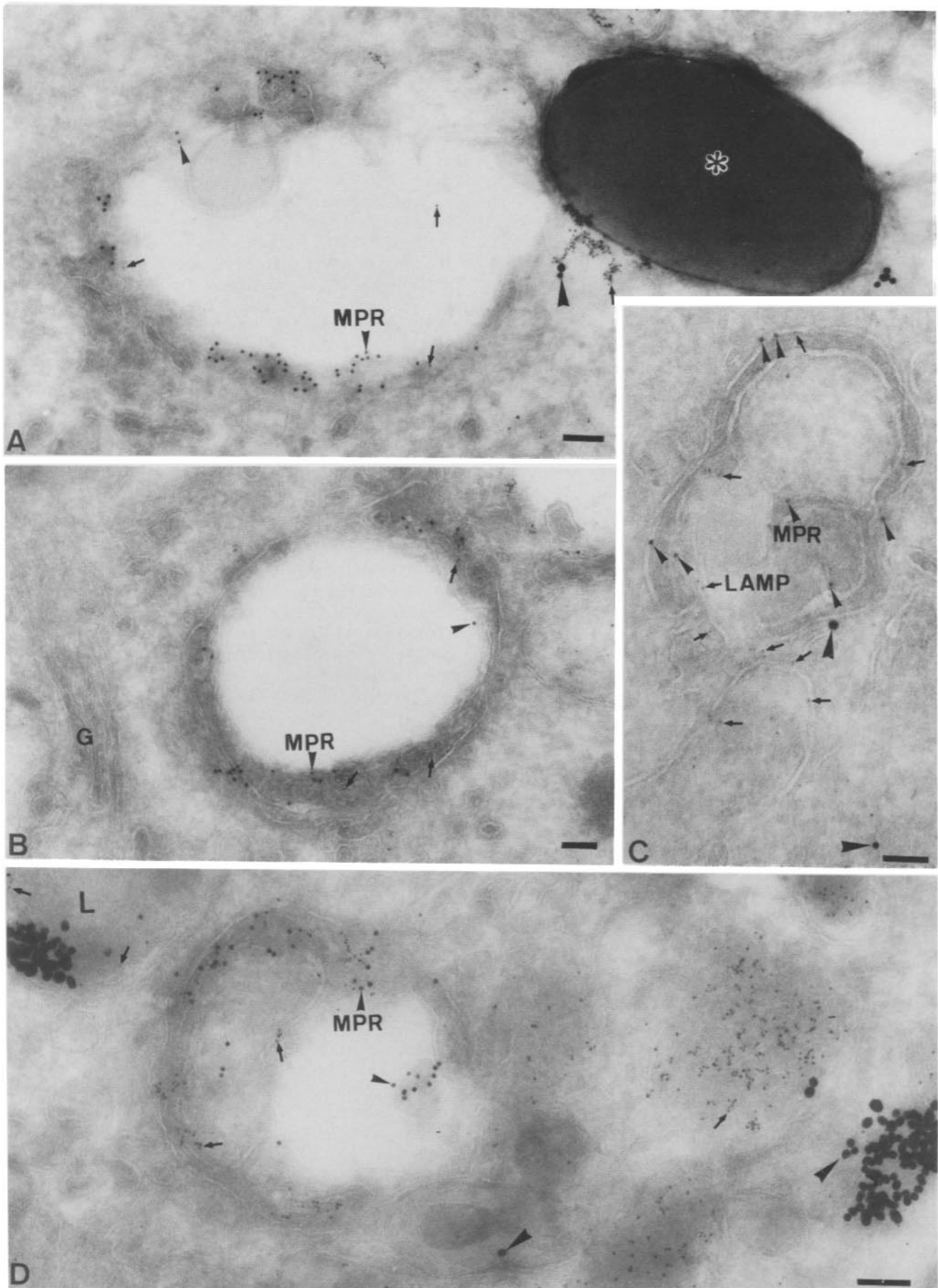
These vacuoles persisted without apparently impairing cell viability, and continued to enlarge until by 50 h they filled much of the cytoplasm (Fig. 10 f).

EM Localization of WGA Binding Sites and Internalized WGA

In M ϕ not treated with WGA we could detect WGA binding sites by treating thawed cryosections with WGA followed by anti-WGA and protein A-gold. Using this procedure binding sites for this lectin were present at low levels in two or three cisternae of the Golgi complex (Fig. 9 B), in early endosomes, and in ECVs (not shown). Denser labeling was seen over the plasma membrane (not shown) and the TC was the most heavily labeled compartment (Fig. 9 B). Low but variable labeling was seen in those structures we tentatively define as lysosomes (see above; Fig. 9 B).

After treatment with WGA in culture and treatment of thawed cryosections with anti-WGA and protein A-gold, vacuolated cells had low levels of WGA on the cell surface (not shown), in the early endosomes (Fig. 11 B), and in spherical endosome vesicles (Fig. 11, A and C). Most cell-associated WGA was located in the strikingly deformed TC (Figs. 11, A and B, and 12), defined by its morphology and by its strong reactivity with anti-Lamp 1 and 2 and macrosialin (not shown). In some areas the cisternae appeared collapsed, comprising sheets of membrane tightly apposed, perhaps due to the lectin cross-linking the membranes (Fig. 12); in others, there was massive dilation of vesicles (Figs. 11, A and B, and 12), and often these were continuous with phagolysosomal membrane (Fig. 11 A). These morphological changes were accompanied by a delay in endocytic trafficking: by 20–40 min, a time in which significant amounts of marker would normally have entered the TC, little or no BSA-Au₅ had entered this compartment. A quantitation of this effect is shown in Fig. 13. In many images,

Figure 6. Rab 7 labeling of the TC (7, arrowheads, 9-nm gold). The large gold (large arrows, unmarked in E) is again marker taken in after an overnight chase and the 5-nm gold (small arrows) was internalized for 8 min followed by a 22-min chase. Note that most of the rab 7 labeling (small arrowheads) is associated with membranes of TC elements, including the membrane enclosing the latex particles (white asterisks in A and B). In A, D, and E coated "bud" structures are evident (large arrowheads) that are in continuity with 5-nm gold-containing structures (see black asterisk in D and E). In C rab7 labeling is also associated with the Golgi complex (G). Bars: (A–D) 200 nm; (E) 100 nm.



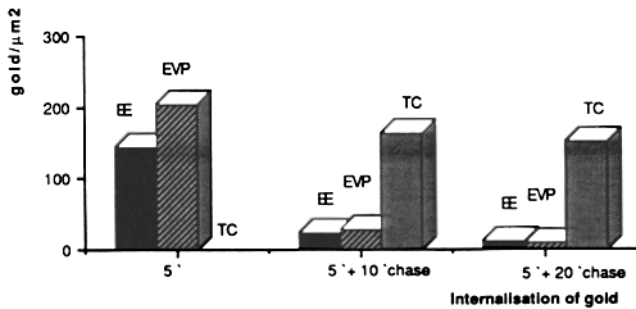


Figure 8. Quantitation of the passage of internalized 16-nm gold-BSA conjugate from early endosomes to the TC, following a 5-min pulse only or a 5-min pulse followed by either a 10- or 20-min chase. The sections were labeled with anti-lamp 2 antibodies and 9-nm protein A-gold to identify the TC. The early endosomes tubulo cisternal structures (EE) and the enlarged, vesicular parts of the early endosome were identified on a morphological basis (as well as having only sparse labeling for Lamp 2). These endosome vesicular profile (EVP) were classified as profiles that were irregular in outline that we believe are precursor structures to the ECV (see Fig. 11 B); they are mostly seen in continuity with the early endosome tubular-cisternal structure. The ECV were not quantified for reasons explained in the text.

spherical ECVs, packed with BSA-Au₅, were closely associated with PLC, but had apparently not fused (Fig. 11, A and C). Some of these structures retained BSA-Au₅ for as long as 180 min (not shown). We were not successful in labeling these preparations with antibodies to MPR and rab 7 since we obtained a spurious co-localization of these antibodies with WGA, presumably because the free binding sites on the lectin bound to oligosaccharides on these IgGs. We were unable to block this reaction with sugars.

Discussion

In this EM study of a typical phagocytic cell, we have used a variety of antibodies in combination with endocytic tracers to define the endosomal compartments and to show their relationship to the late stages of the phagocytic pathway. The organization of the endosomal system in MΦ is essentially identical in outline to that described in other cultured cells (Fig. 14).

We should caution that our discussion of the endocytic and phagocytic pathways here does not take into account the phenomenon of macropinocytosis (Swanson, 1989; Racoosin and Swanson, 1989). As described by these authors, this is a phenomenon similar to the phagocytosis whereby a relatively small number of extremely large vesicles (0.5–5 μm in diameter) are internalized, especially at sites where mem-

brane ruffling is apparent. As shown by Racoosin and Swanson (1989) growth factors such as MΦ Colony Stimulating Factor are able to increase significantly the intake of fluid by this process in a receptor-mediated fashion. Unlike clathrin-coated vesicle uptake, macropinocytosis is inhibited by nocadazole and cytochalasin D. According to Swanson (1989) the large macropinosomes eventually shrink and possibly condense into a dense, lysosome-like vesicle. More studies are needed to determine how this process relates to the endocytic and phagocytic structures described in our study.

Endosomal Compartments

Endocytic tracers filled the early endosomes after a 5-min pulse. These organelles consist of tubules and cisternae that were often curved around electron-lucent structures. Tracer was excluded from the central electron-lucent structures, whose nature and function are obscure. Though previously described in other cell types (Griffiths et al., 1989) they were especially prominent in MΦ. In classic studies with MΦ (Steinman and Cohn, 1972; Steinman et al., 1976) and other cells (Steinman et al., 1974), the reaction product of HRP, internalized for brief periods, was thought to be bound to the luminal side of a pinosome (early endosome) membrane; our observations actually show that the electron-lucent structures were inaccessible to endocytic tracers, and that the apparent membrane association reflects the flattened crescentic shape of early endosomes *cisternae* (see also Griffiths et al., 1989). Many coated buds decorated this compartment; these had approximately half the diameter of coated pits and vesicles associated with the plasma membrane. We are presently investigating whether these coats contain clathrin.

Tracer passed from early endosomes to spherical, endosome vesicles. These structures showed trace amounts of the lysosomal antigens Lamp 1 and Lamp 2, and the CI-MPR, as in other cells we have studied. In those cells the evidence suggests that these vesicles are transported via microtubules from early endosomes to late endosomes (PLC) (Gruenberg et al., 1989; Bomsel et al., 1990). Two observations argue that these vesicles are kinetically between the early endosomes and the TC in these, as in other cells. First our qualitative observations suggest that the number of labeled vesicles decreased with time while the marker remained in elements of the TC. The second was the effects of WGA in blocking delivery into the TC while label persisted in the endosomal vesicles. The internal membranous structures, prominent in these vesicles in other cell types, were not so distinct in MΦ (see Fig. 2, D–F). This made it difficult to identify these vesicles when they were not selectively labeled with internal-

Figure 7. Labeling of specific areas of the TC with anti-MPR (9-nm gold, arrowheads) adjacent/continuous with unlabeled areas. In all figures 16-nm gold (large arrowheads) was internalized after an overnight chase, and in all figures except C 5-nm gold was taken in for 8 min followed by a 22- (A), 45- (B), and 60- (D) min chase. In A a latex particle (asterisk) is evident that is surrounded by a membrane that also encloses both 5-nm and 16-nm gold but is devoid of MPR labeling. In B the MPR-enriched part of the TC is directly adjacent to the unlabeled Golgi complex (G). In C, double labeled on the section with anti-MPR (small arrowhead) and Lamp 2 (arrows), a cisternal part of the TC is evident that is labeled Lamp 2 and MPR. While the MPR labeling is restricted to one cisternal part of this profile the Lamp labeling extended beyond the area shown in this figure. One internalized 16-nm gold is evident (large arrowhead). In D an MPR-labeled TC structure is evident next to unlabeled regions. A part of a possible lysosome (L) is seen that has the 16-nm gold but only small amounts of the 5-nm gold. Bars, 100 nm.

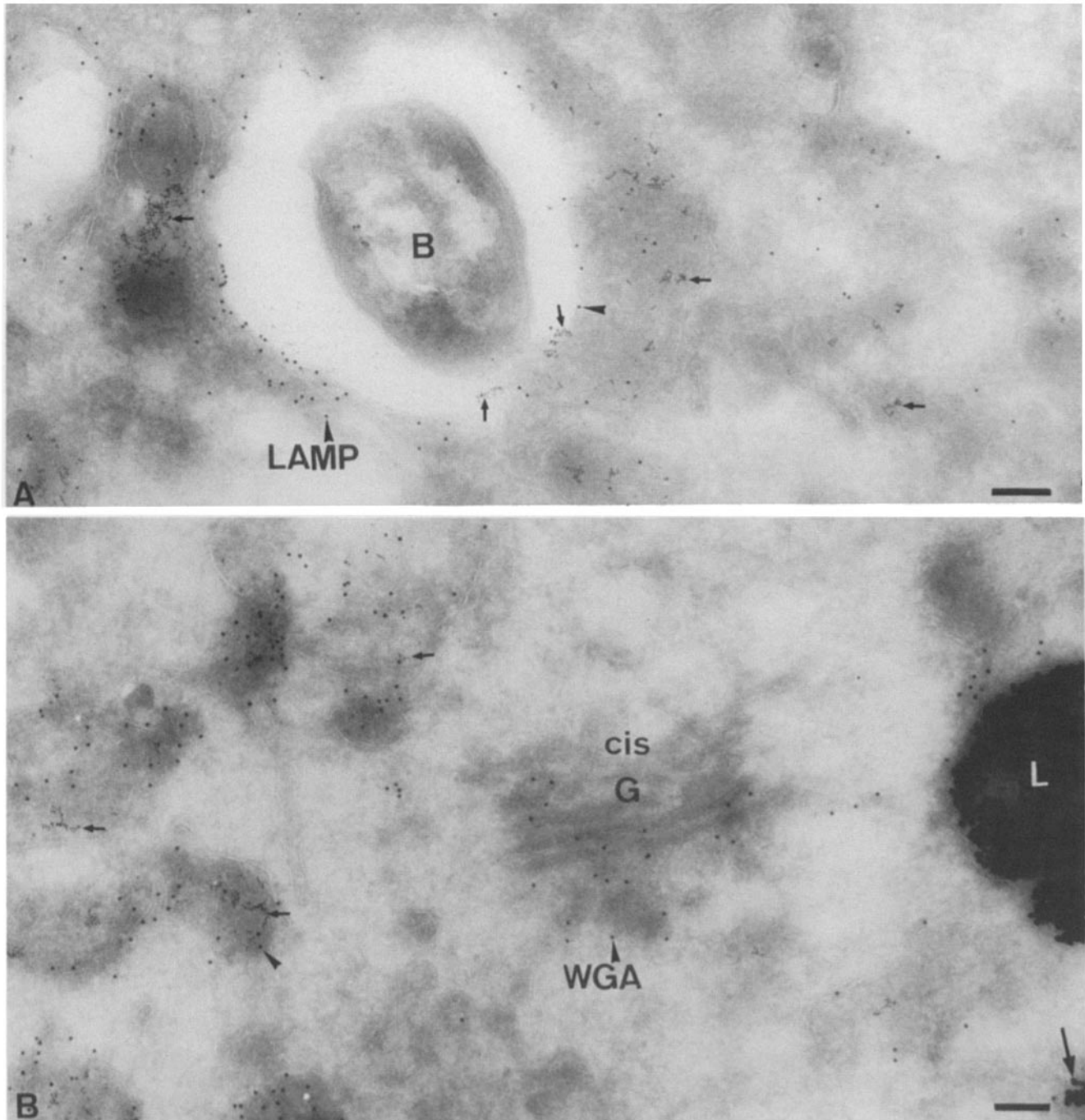
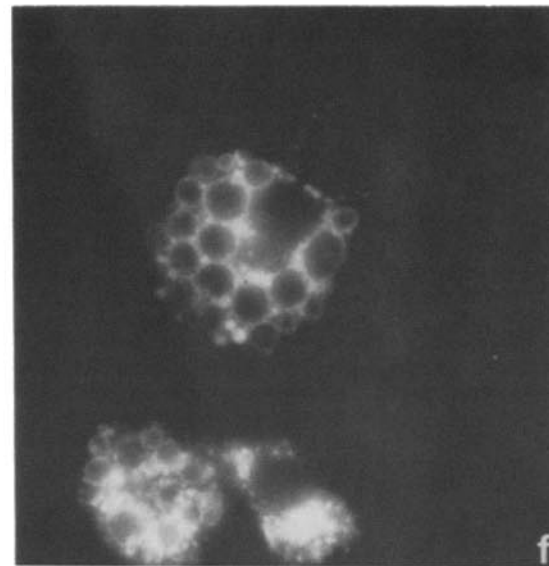
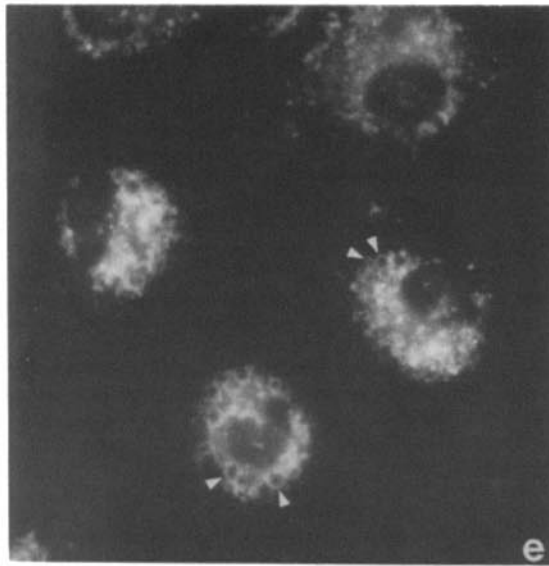
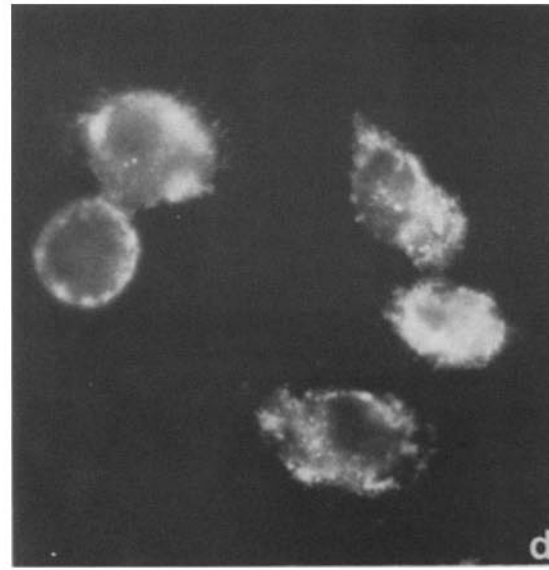
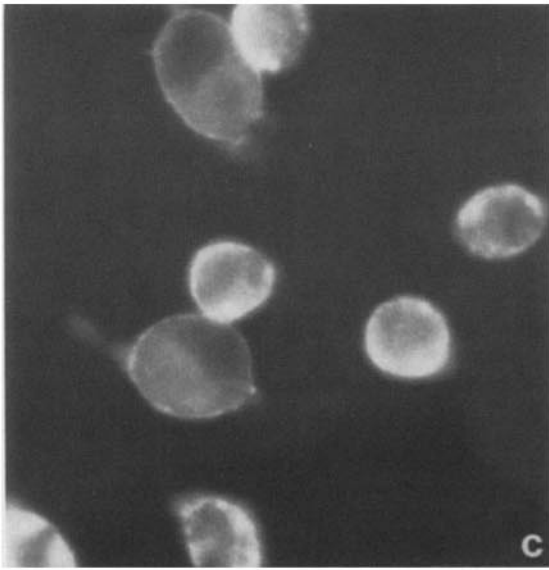
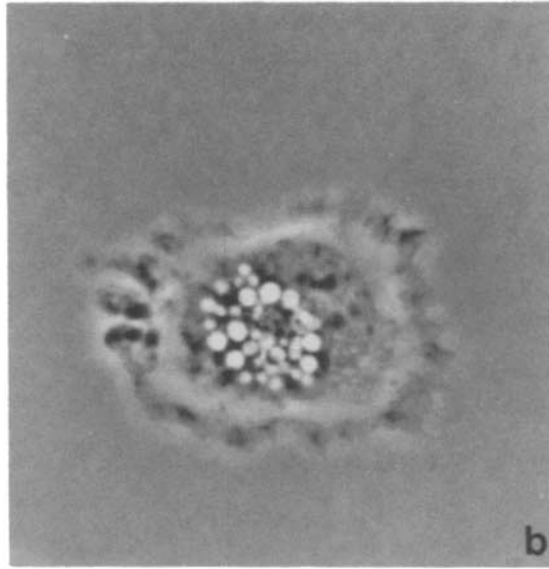
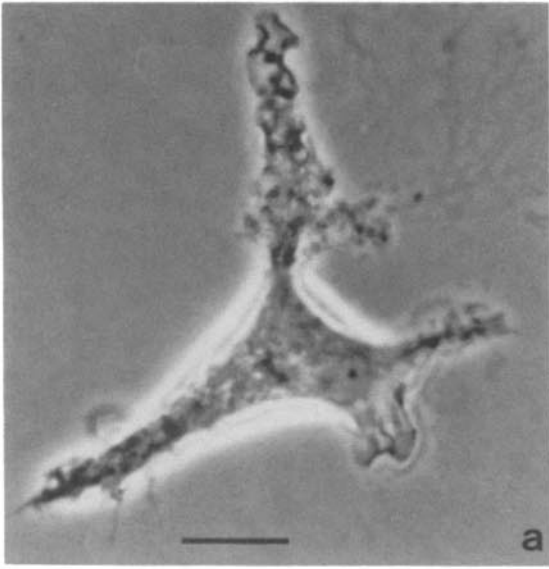
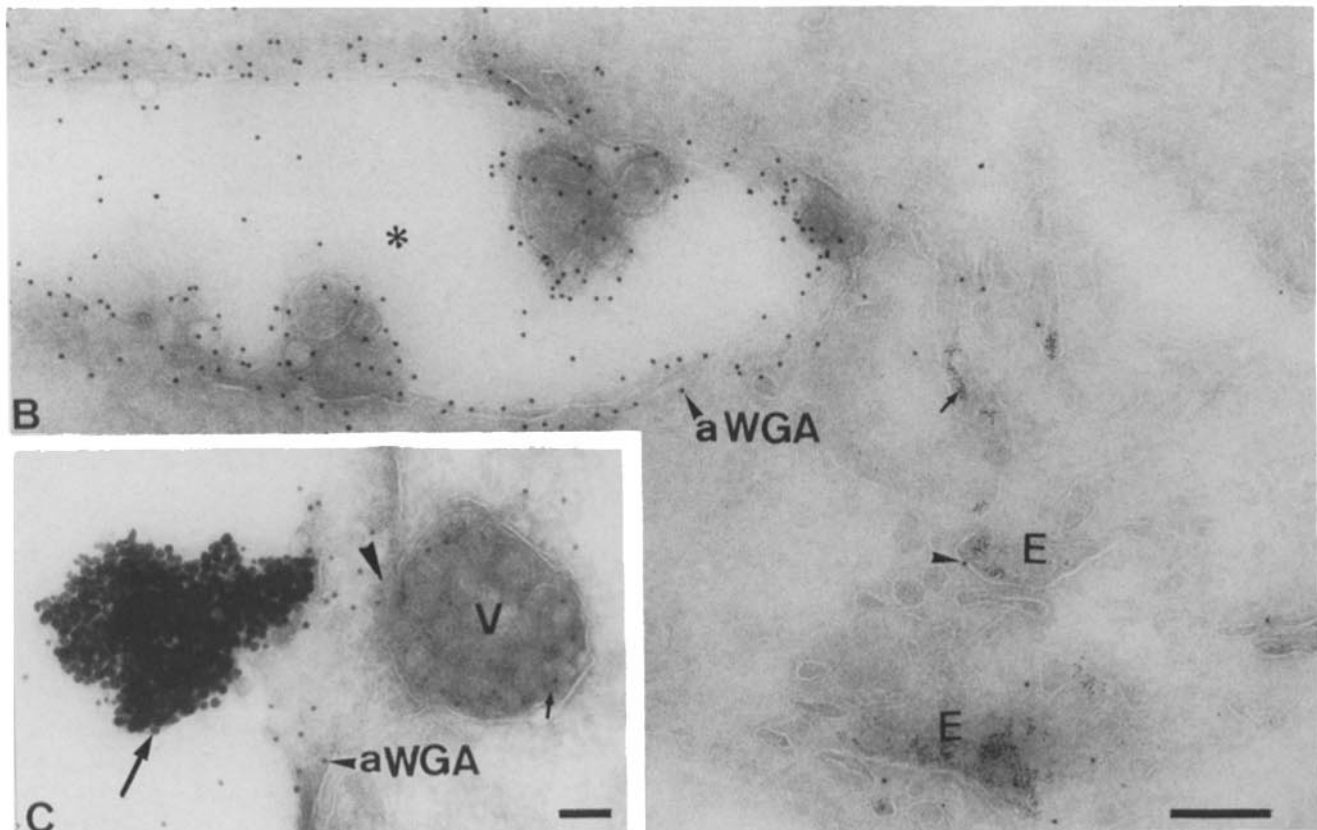


Figure 9. Bacteria in the TC; WGA binding sites. In *A* a bacterium is evident in an enlarged area of the TC that contains 5-nm gold (8-min pulse, 30-min chase; *small arrows*) and is heavily labeled for Lamp 1 (*arrowheads*, 9-nm gold). The few bacteria that were observed in this study were always in the TC; we presume they were present in the M Φ before isolation. In *B* the section was labeled with WGA followed by anti-WGA and 9-nm gold (*arrowheads*). Extensive labeling is evident over TC elements that contain large amounts of the 5-nm gold (8-min pulse, 22-min chase). The lysosome (*L*) with a large aggregate of the 15-nm gold (*large arrow*, overnight chase) is typically only weakly labeled. Note the strong labeling for WGA over approximately one-half of the Golgi stack that is devoid of any internalized gold markers. The unlabeled *cis* side is indicated. Bars, 200 nm.

Figure 10. Functional effects of wheat germ agglutinin on Biogel-elicited macrophages. (*a* and *b*) Phase-contrast micrographs of macrophage coverslip preparations after 6 h treatment with (*a*) 1- μ g/ml WGA and 0.25-mM chitotriose, or (*b*) 1- μ g/ml WGA alone. Lectin treatment induces circumferential spreading and vacuolation; haptenic sugar inhibits the lectin effect. (*c-e*) Fluorescence microscopy of macrophages treated at 4°C with 20 μ g/ml FITC-WGA and fixed (*c*) without warming, or after warming to 37°C for 3 (*d*) or 30 (*e*) min. Surface-bound lectin is rapidly internalized initially (*d*) into poorly resolved peripheral vesicles and subsequently (*e*) into larger vesicles completely outlined by fluorescent lectin (*arrowheads*). (*f*) Macrophages exposed to FITC-WGA at 37°C for 10 h and cultured 50 h further. Greatly expanded vacuoles around the nucleus are outlined by lectin. Bar, 10 μ m.





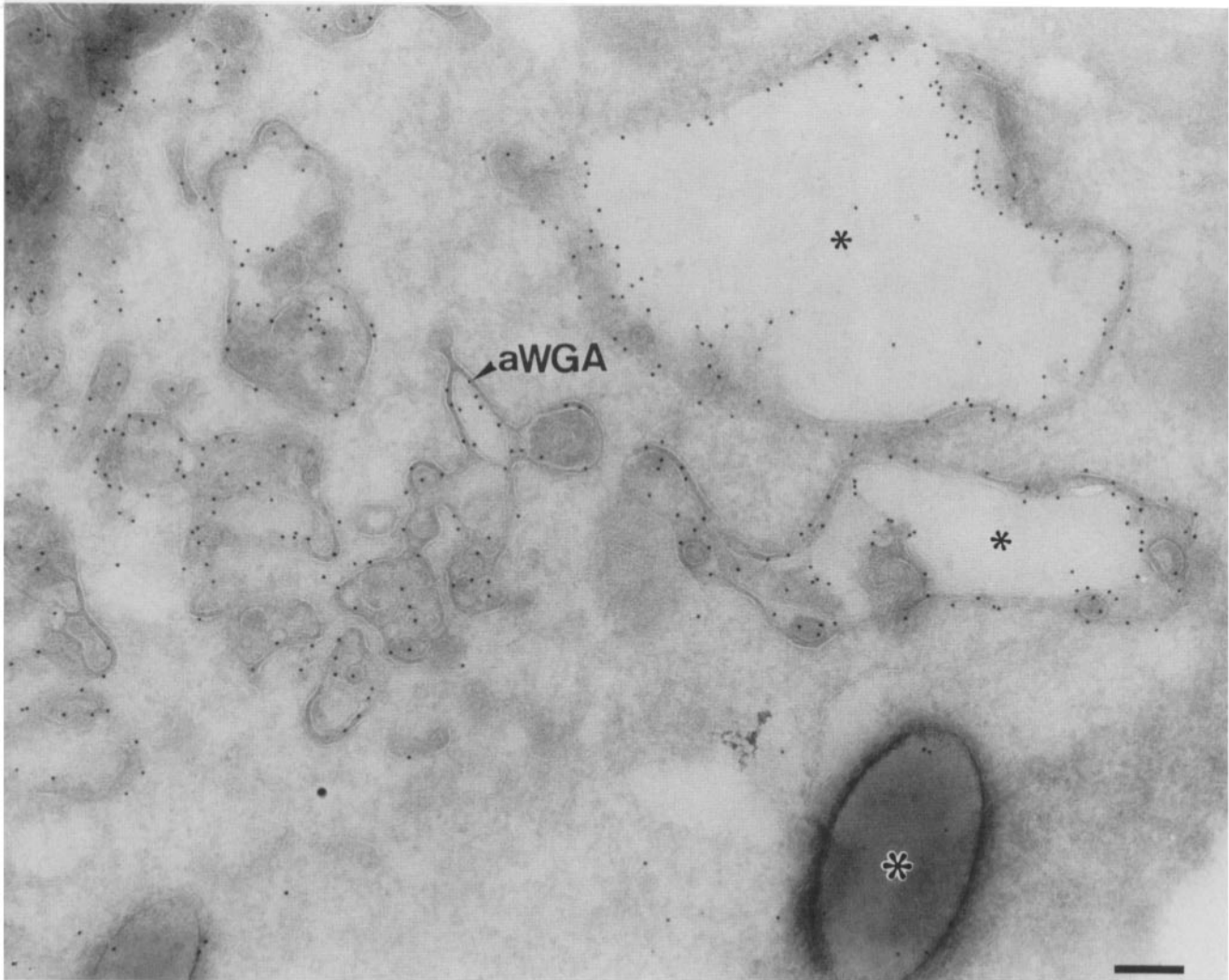


Figure 12. Morphological alteration of the TC induced by WGA. A sections of a WGA treated M ϕ labeled with anti-WGA and 9-nm protein A-gold (arrowheads). Note the extensive flattened cisternae of the TC as well as vacuolated areas (black asterisks). These cells had internalized latex particles (black and white asterisk) with an overnight chase before the WGA treatment. They also were exposed to 5-nm gold (8 min, 22-min chase), but none is evident in these TC elements. Bar, 200 nm.

ized gold particles, as well as to show quantitatively that they were kinetically before the TC.

The late endosomal TC showed a distinct antigenic profile (enriched for rab 7 CI-MPR, Lamp 1, and Lamp 2) and a complex tubulo-vesicular structure. This compartment was similar in every respect to the CI-MPR-enriched PLC we

have described in other cells (Griffiths et al., 1988, 1990) except that the localization of the CI-MPR was restricted to certain parts of the structure. It received significant amounts of endocytic tracers by 15 min of internalization, and thus also corresponded kinetically to the structures referred to as tubular lysosomes that had been visualized in M ϕ with fluo-

Figure 11. Retardation of delivery of 5-nm gold into TC after WGA treatment. The large arrow indicates the 16-nm gold (overnight chase) and the black and white asterisk indicates the latex particle, both internalized before the application of WGA. The 5-nm gold (small arrows) was internalized for 8 min followed by a 22-min chase subsequent to the WGA treatment. Note the accumulation of this marker in three endosome carrier vesicles (V) directly adjacent to the enlarged TC vacuole (black asterisk) that contains only five small gold particles (all indicated). The membrane of this vacuole is probably continuous with that around the latex. Note the complete lack of labeling of the Golgi complex (G). In B, treated identically to A, significant amounts of 5-nm gold has not "chased" out of typical early endosome elements (E) that show low but specific labeling with anti-WGA. Although small amounts of these small gold particles are often found in early endosomes under normal conditions for as long as 60 min of chase, the relatively large amounts seen here are due to the WGA "block." No 5-nm gold particles are evident in this profile of the TC (black asterisk) that labels heavily with anti-WGA (9-nm gold). In C, after 45-min chase the 5-nm gold is found closely packed in a typical endosome carrier vesicle (V) that is evidently in contact with (large arrowhead) the membrane of the TC that is heavily labeled for α WGA (9-nm gold). A large aggregate of 16-nm gold (overnight chase) is visible on the luminal side of the TC (large arrow). Bars: (A and B) 200 nm; (C) 100 nm.

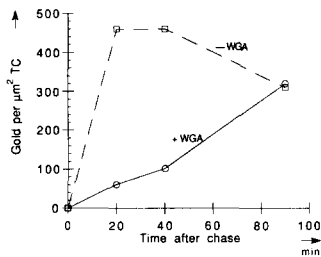


Figure 13. Quantitation of the delay in the passage of internalized 5-nm gold-BSA conjugate into the TC when cells had been pretreated with WGA, as described in Materials and Methods. We presume that the drop in the number of gold particles between 40 and 90 min in the absence of WGA reflects passage of significant amounts of gold into the spherical lysosomes.

rescent markers by Swanson and co-workers (Swanson et al., 1987; Knapp and Swanson, 1990). The complex morphology of this compartment is also consistent with the morphological description of tubular lysosomes by the latter group. The TC was, in parts, in close vicinity to the Golgi complex, which mostly lacked these antigenic markers and did not receive detectable amounts of internalized gold tracers. The TC also contained coated bud structures whose composition and function are presently unclear.

In $M\phi$ the CI-MPR was restricted to juxta-Golgi elements of the TC, and is therefore not a universal marker for this compartment, in contrast to the LAMP proteins and the late endosome-specific, GTP-binding protein rab7, which uniformly labeled the membranes of this structure. Our obser-

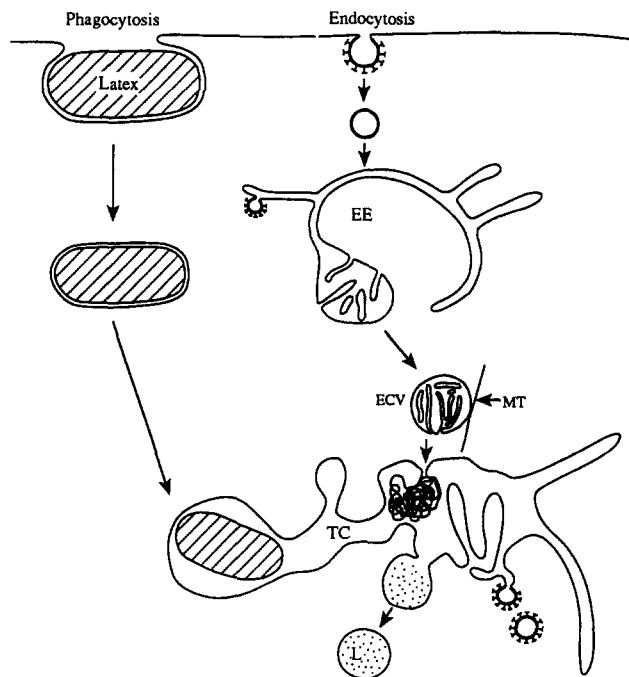


Figure 14. Schematic model of the phagocytic and endocytic pathways in macrophages. The endocytic pathway is depicted as four different structures distal to the clathrin-coated vesicle step, the early endosome (EE), the endosome carrier vesicle (ECV), the tubular compartment (TC), and the spherical lysosome vesicle (L). The idea of transport of ECV along microtubules is based on analogy with data from other cells (see Gruenberg and Howell, 1989). The latex bead, taken up by phagocytosis is shown to fuse with the TC. We emphasize that this is not shown directly in this paper but is the model that best fits all the available data (see Discussion).

variations are consistent with the recent immunofluorescent study of Tassin et al. (1990), who showed that CI-MPR labeling was restricted to "a cluster of a few granules located on one side of the nucleus." Assuming the TC in $M\phi$ is equivalent to the PLC in other cells, the $M\phi$ would be the first cell type in which we have observed an apparent restriction of the CI-MPR to distinct regions of the PLC/late endosome. While the significance of this is not obvious, it points out the danger of using the CI-MPR as a universal marker of late endosome structures. As in other cell types, endocytic markers persisted in the PLC even after prolonged chase; this may simply reflect incomplete clearing, or may arise from a retrograde fusion between terminal lysosomes and the PLC, as previously postulated (Griffiths et al., 1990; Griffiths, 1991).

Lysosomes were identified as spherical structures containing densely packed BSA-Au₁₆ after an overnight chase. As expected, they were enriched for lysosomal antigens (LAMP 1, LAMP 2) and lacked the CI-MPR as well as rab 7. Since the CI-MPR does not label the TC uniformly the lack of this marker in the lysosomes is admittedly a weak criterion for their identity in these cells. These structures may correspond to the vesicular lysosomal structures described recently in macrophages by Tassin et al. (1990). These workers could distinguish two different kinds of "lysosomes" in the same cell at the light microscopic level. Both of these structures were late compartments of the endocytic pathway, both accumulated the amine cytochemical reagent DAMP, and both labeled similarly for lysosomal enzymes and Lamp-like membrane glycoproteins. The first class of structures were tubular elements that probably correspond to the typical tubular lysosomes of Swanson et al. (1987), and to the TC in our study. The second class of structures were, however, small vesicles in which non- or poorly degradable material taken in by endocytosis or phagocytosis preferentially accumulated after periods longer than 3 h.

Lectins and Intracellular Trafficking

Several lectins have been described which perturb normal membrane traffic in $M\phi$ and result in the formation of large, persistent vacuoles (Goldman et al., 1976); the best studied is Con A (Edelson and Cohn, 1976a,b). We chose to work with WGA, which has similar functional effects while binding a smaller subset of $M\phi$ glycoproteins (Rabinowitz and Gordon, 1989). Our data show that internalized WGA accumulates predominantly in the TC and causes gross alteration in the morphology of this compartment: lectin-induced vacuoles evident by phase contrast microscopy were found by EM to represent hugely dilated portions of TC cisternae. In other regions extensive flattened cisternae were seen, possibly due to the lectin, which is tetravalent, cross-linking binding sites on the TC membrane. This WGA treatment blocked the delivery of subsequent internalized markers to this compartment. Tracer was held up in the spherical endocytic carrier vesicles, which clustered nearby and often appeared in close contact with the TC without fusing. These data suggest that the build up of WGA in the TC somehow delays the fusing of the incoming spherical endosome vesicles with this compartment.

Macrosialin as a Marker for PLC

Macrosialin is a membrane glycoprotein confined to $M\phi$

and dendritic cells (Rabinowitz and Gordon, 1991). We found that this antigen was highly expressed in the TC, including phagolysosomes; it was undetectable, or present at low levels, in the plasma membrane, early endosomes, carrier vesicles, and lysosomes. This distribution resembles that of a 57-kD membrane glycoprotein recently reported to be predominantly in the PLC/late endosomes of bovine cells (Park et al., 1991).

Phagosomal Structures

Early phagosomes, which are known to be functionally distinct from early endosomes, were not examined in this study. Newly formed phagosomes, unlike both early and late endosomal compartments, become transiently alkaline before acidifying (Geisow et al., 1981). Our data show that phagosomes must directly or indirectly fuse with the TC. First the antigenic profiles of phagolysosomal and TC membranes were essentially indistinguishable. Second, they received endocytic traces simultaneously, and third, their membranes were often seen to be directly continuous. We favor the view that the pathway from early phagosome to the TC is a direct one that bypasses the early endosomes (Fig. 14). This would be the simplest explanation for the contrasting effects of lectins and polyanionic compounds; the lectins selectively block the pathway from endosomes to TC (this study; Kielian and Cohn, 1981) without affecting the phagosome to lysosome route (Goldman et al., 1976; Kielian and Cohn, 1981) while the polyanionic compounds such as dextran sulphate inhibit phagosome-lysosome fusion without affecting endocytosis (Kielian et al., 1982). Collectively, these data argue strongly for two distinct pathways, one from early phagosomes and one from early endosomes to later stages of the pathway. The notion that early phagosomes fuse directly in the TC would also agree with the recent double-label immunofluorescence studies of Knapp and Swanson (1990) who observed that the tubular lysosomes, identified by prelabeling with Lucifer yellow, fused with Texas red-labeled phagosomes, a process that could first be seen 15 min after the initiation of phagocytosis. In contrast to this interpretation, Mayorga et al. (1991) recently postulated that phagosomes could fuse with both early and late endosomes. In their study endosomes and phagosomes were labeled in vivo with colloidal gold particles of different sizes and mixing of the two markers was first detected 9 min after the administration of the first gold marker. In this time, however, a significant amount of this marker might be expected to have moved into late endosome/TC. In our study the bulk of internalized gold markers had moved into the TC after a 5-min pulse and a 10-min chase (Fig. 8). Furthermore, although their study included an in vitro reconstitution of fusion between phagosomes and endocytic vesicles, this fusion only occurred under nonphysiological conditions.

Phagolysosomes and Antigen Processing

Immunocytochemical data indicate that class II molecules can be detected in both early and late endosomes (Guagliardi et al., 1990; Peters et al., 1991; Pieters et al., 1991). Since some hydrolase activity is present in both compartments (see Kornfeld and Mellman, 1989), antigen processing, binding to class II molecules, and recycling to the cell surface could, in principle, be possible (and may indeed occur) from both

compartments. However, there are two sets of data that at least from a theoretical point of view make the late endosome/PLC a more attractive candidate. First, late endosomes/PLC have significantly higher concentrations of hydrolases than the early endosomes (Kornfeld and Mellman, 1989). Second, in macrophages, antigens presented from phagocytic particles are unlikely to be processed in early endosomes. As we have argued above, the early phagosome appears to be distinct from early endosome. It is not clear whether any recycling is possible from the early phagosome or whether these structures contain significant concentrations of acid hydrolases, but its alkaline pH (Geisow et al., 1981) would be expected to inhibit lysosomal enzyme activity and, therefore, processing of antigens.

Lysosome Terminology

The PLC is generally considered to be a late (low density) compartment in the endocytic pathway from which receptors such as the CI and CD MPRs (Kornfeld and Mellman, 1989), as well as some of the lgp/lamp molecules (Lippincott-Schwartz and Fambrough, 1987) can recycle to the plasma membrane. Conversely, since the high density, MPR-negative lysosome vesicles usually contain the highest concentration of lysosomal hydrolases, it is widely thought that these represent the compartment where the bulk of degradation occurs along the endocytic pathway. In macrophages, however, a number of observations are difficult to reconcile with this model. The most striking is the long term accumulation of phagocytic particles such as latex or bacteria in the extended TC rather than in the spherical (MPR-negative) lysosome vesicles, which are tightly filled with BSA-gold aggregates after an overnight chase. The bacteria showed obvious morphological signs of degradation. An alternative hypothesis that must be seriously considered is that the TC may be the functional lysosome compartment where the bulk of internalized material is degraded but that recycling receptors, as well as the LAMP/lgp membrane proteins, are somehow spared. Accordingly, the term "tubular lysosome", first used by Swanson et al. (1987) may indeed be an appropriate name for this compartment. The MPR-negative terminal lysosome is densely packed with lysosomal enzymes and highly reminiscent of secretory granules. Thus, rather than being the site of degradation, a possibility to consider is that the lysosomes, defined as the terminal, high density vesicles of the endocytic pathway that are devoid of MPRs (Kornfeld and Mellman, 1989), may be a storage site for lysosomal enzymes (analogous to the classical concept of primary lysosomes), which can be delivered by fusion to the PLC/TC. Unlike the idea of primary lysosomes, however, these structures can clearly also concentrate nonspecific, nondegradable markers such as colloidal gold, as well as being accessible to significant concentrations of slowly degradable markers, such as HRP (Tassin et al., 1990; Ludwig et al., 1991).

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