Plasticity of the tubular lysosomal compartment in macrophages

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Summary

Because bone marrow-derived macrophages differentiate in culture, their lysosomal compartment is largely devoid of the undigested particles that are common in macrophages removed from tissues. The morphology of this nearly vacant lysosomal compartment was observed, after labeling with fluorescent endocytic tracers such as Lucifer Yellow, to be an extensive, tubuloreticular network, which underwent extensive rearrangements in accommodating endocytic loads. It was converted to spherical organelles when the lysosomal compartment was loaded with osmotically active solutes such as sucrose or Acridine Orange. Enzymatic degradation of intravacuolar sucrose by pinocytosed invertase resulted in the shrinkage of vacuoles and the re-formation of the tubular network. After phagocytosis of opsonized erythrocytes or latex beads, tubular lysosomes wrapped around the phagosomes, then merged to form phagolysosomes. The disappearance of tubules was proportional to the total surface area of particles ingested. Degradation of the phagocytosed contents permitted shrinkage of the phagolysosome and concomitant re-formation of the tubuloreticular network. Non-degradable contents such as latex beads prevented re-formation of the tubular network. These rearrangements of the lysosomal compartment indicate that the organelle exhibits considerable plasticity and interconnectedness, and that maturation of lysosomes after endocytosis does not necessarily entail irreversible morphological changes.

Key words: macrophage, lysosome, phagocytosis.

Introduction

It has long been known that the lysosomal compartment can expand to accommodate content, and that material entering the compartment *via* endocytosis can determine its shape. The most conspicuous example of this occurs during macrophage phagocytosis, in which small lysosomes fuse with larger, particle-containing phagosomes and conform to the shape of the ingested particle (Cohn and Wiener, 1963; Cohn *et al.* 1966). In addition, membrane-impermeant solutes, which accumulate in lysosomes either through endocytosis or by pH-dependent trapping, can expand that compartment *via* osmotic effects (Cohn and Ehrenreich, 1969; Ohkuma and Poole, 1981).

The shape of the lysosomal compartment is also influenced by cytoplasmic factors. In some macrophages, lysosomes stained for acid phosphatase or labeled *via* pinocytosis of soluble tracers appear elongate and tubular (Nichols, 1982; Swanson *et al.* 1985). This shape is achieved in part through an association between lysosomes and microtubules (Swanson *et al.* 1987). Moreover, both the shape and the distribution of macrophage lysosomes can be altered by conditions that change cytoplasmic pH (Heuser, 1989).

The tubular morphology has been observed in some macrophages but not others. Lysosomes labeled by pinocytosis of the fluorescent dye Lucifer Yellow are tubular in cultured human monocytes, phorbol ester-treated peritoneal macrophages, J774 macrophages and bone marrow-derived macrophages, but those of unstimulated peritoneal or alveolar macrophages are not tubular (Swanson *et al.* 1987). Lysosomes labeled in living macrophages using the fluorescent probe Acridine Orange have not been described as tubular.

Acridine Orange is membrane permeant at neutral pH, but less so at lower pH; and therefore it accumulates in acidic intracellular compartments such as lysosomes. Cells incubated in low concentrations of Acridine Orange quickly accumulate the dye in lysosomes to high enough concentrations for the fluorescence to appear more red. Although lysosomes labeled with Acridine Orange for 10 (Hart and Young, 1975) or 20 (Kielian *et al.* 1982) min appear vesicular, we wondered if this might be because the concentrations of Acridine Orange necessary for the red shift in its fluorescence emission spectrum might also be high enough to expand the compartment osmotically.

The purpose of this investigation was to determine how endocytosed particles, endocytosed solutes or accumulated Acridine Orange affect lysosome morphology in bone marrow-derived macrophages. Labeling with low concentrations of highly fluorescent tracer molecules

permitted light microscopic observation of lysosomes during and after phagocytosis or osmotic expansion. Our results indicate that macrophage lysosomes are tubular when they lack any endocytic load, but lose their tubular morphology as their luminal content increases. Moreover, they exhibit considerable plasticity in their movements and morphological rearrangements following endocytosis.

Materials and methods

Cells

Bone marrow-derived macrophages were obtained from the femurs of adult, female, ICR mice (Trudeau Institute, Saranac Lake, NY) or C3H/HeJ mice (Jackson Labs, Bar Harbor, ME) as described previously (Swanson, 1989*b*). Bone marrow exudate was cultured for 6 or 7 days in growth medium consisting of Dulbecco's minimal essential medium (DME) with 30% L cell-conditioned medium, 20% heat-inactivated fetal bovine serum and 2p.p.m. (parts per million) of β-mercaptoethanol at 37°C, 5% CO_2, with 10 ml additional growth medium added on day 3. After this growth period dishes were washed and cells removed in cold divalent cation-free phosphate-buffered saline (PD: 137 mM NaCl, 3 mM KCl, 7 mM phosphate buffer, pH 7.4), and were plated onto 12 mm, no. 1 circular coverslips at 10^5 cells per coverslip or onto 22 mm square coverslips at 3×10^5 cells per coverslip. After 45 min at 37°C PD was replaced with DME containing 10% heat-inactivated fetal bovine serum (DME-10F), and returned to 37°C, 5% CO_2 for 24 h.

Fluorescent labeling of lysosomes

Macrophages were incubated in Lucifer Yellow CH, potassium salt (LY; Molecular Probes, Eugene, OR) at 0.5 mg ml^{-1} in DME-10F at 37°C, 5% CO_2 for 1–2 h and then washed and incubated in phosphate-buffered saline with 10% heat-inactivated fetal bovine serum for 30 min. This protocol was also employed to label lysosomes with fluorescein–dextran (1.0 mg ml^{-1}; Sigma Chem. Co., average M_r 10 000) Texas Red–dextran (1.0 mg ml^{-1}, Molecular Probes, Inc., average M_r 10 000) and Texas Red-labeled ovalbumin (40 μg ml^{-1}; Molecular Probes). Lysosomes were labeled with Acridine Orange by incubating cells in 5 μg ml^{-1} Acridine Orange in DME-10F for 5–20 min before rinsing and observing. To monitor lysosome expansion and collapse, cells on coverslips were incubated overnight in DME-10F±20 mg ml^{-1} sucrose. This medium was replaced with DME-10F containing 0.5 mg ml^{-1} LY±sucrose, and incubated for another 3 h at 37°C. Cells were then either washed in PBS and observed, or were incubated in DME-10F with 0.5 mg ml^{-1} invertase (Sigma Chem. Co., St Louis, MO) for an additional 3 h before microscopic observation.

Phagocytosis

Sheep red blood cells (SRBC; Cappell) were washed twice by centrifugation (1200 revs min^{-1}, 10 min) and resuspension in PBS (137 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 7 mM phosphate buffer, pH 7.4) at 4°C. They were opsonized by resuspending 5×10^8 cells in 1 ml PBS containing 2 μl anti-SRBC IgG (Cappell), 30 min at 37°C, and washing three times in cold PBS. SRBC ghosts were prepared by osmotic rupture. A total of 3×10^8 cells were suspended and lysed in 1 ml of 20 mosM sodium phosphate, pH 7.0, 5 mM $MgCl_2$ and 10 mg ml^{-1} Texas Red–dextran, average M_r 10 000 (TrDx) for 10 min at 4°C. Sodium chloride (0.25 ml, 4.5%) was then added to restore physiological osmolarity and cells were allowed to reseal for 45 min at 37°C. They were then washed twice in cold PBS by centrifugation (1100 revs min^{-1}, 3 min) and opsonized as above. Polystyrene microspheres (latex beads), 7.0 μm diameter (±0.04 μm) and 3.98 μm diameter (±0.03 μm) (Duke Scientific, Palo Alto, CA), were suspended in 10 mg ml^{-1} bovine serum albumin (BSA) in PBS for 1 h, washed in 1 mg ml^{-1} BSA in PBS, and finally resuspended in warm PBS.

Particles for phagocytosis were introduced to macrophages by adding 1 ml PBS with 1×10^6 to 3×10^6 particles into a 16 mm plastic well containing macrophages on circular coverslips, then centrifuging at 1000 revs min^{-1} for 5 min. Cells were then incubated for various times in DME-10F (37°C).

Microscopy

All quantitative scoring experiments were performed using macrophages on 12 mm diameter, circular no. 1 coverslips mounted on glass slides and sealed with Valap as described previously (Swanson, 1989*a*). Micrographs were taken of macrophages either on 12 mm circular coverslips or on 22 mm square coverslips mounted on slides in the same manner. Observations were made using a Zeiss Plan-Neofluar 63× lens (NA 1.25) in a Zeiss photomicroscope III equipped for epifluorescence with LY and rhodamine filter sets. Use of the LY filter set allowed simultaneous observation of LY and TRDx. Color photographs were taken with Kodak Ectachrome film, ASA 400, which was push processed. Black and white photos were taken with Kodak T-Max 400, which was also push processed.

Results

Osmotic expansion of the lysosomal compartment

Lysosomes observed in living cells after labeling *via* pinocytosis of LY appeared as a branched tubular network extending throughout the cytoplasm (Fig. 1A). The network could also be labeled with a variety of other fluorescent probes including Texas Red-conjugated ovalbumin and fluorescein- or Texas Red-conjugated dextrans of various sizes (data not shown). Under these conditions tubules labeled by pinocytosis were observed in more than 95% of the cells. Some cells displayed web-like interconnections between tubules (Fig. 1D).

Acridine orange altered the compartment morphology. After a 5-min incubation in 5 μg ml^{-1} Acridine Orange, red fluorescent macrophage lysosomes appeared tubular (Fig. 2A). However, after 10 min, the lysosomes began to appear discontinuous and vesicular (Fig. 2B). After 20 min in Acridine Orange the lysosomes were enlarged and distended (Fig. 2C). The observed change in morphology indicates that the accumulation of Acridine Orange in lysosomes for as little as 10 min caused the osmotic expansion from their initial elongated morphology.

Osmotic expansion of the lysosomal compartment can also be achieved by endocytosis of the membrane-impermeant solute sucrose (Cohn and Ehrenreich, 1969; Swanson *et al.* 1986). Incubation of bone marrow-derived macrophages in 20 mg ml^{-1} sucrose (60 mM) for 3 (Fig. 1B) or 15 h (Fig. 1C) resulted in a shape change in the LY-labeled tubular lysosomal network. Tubules appeared first to blister (Fig. 1B), and then those blisters expanded into spherical organelles. Incubation of su-

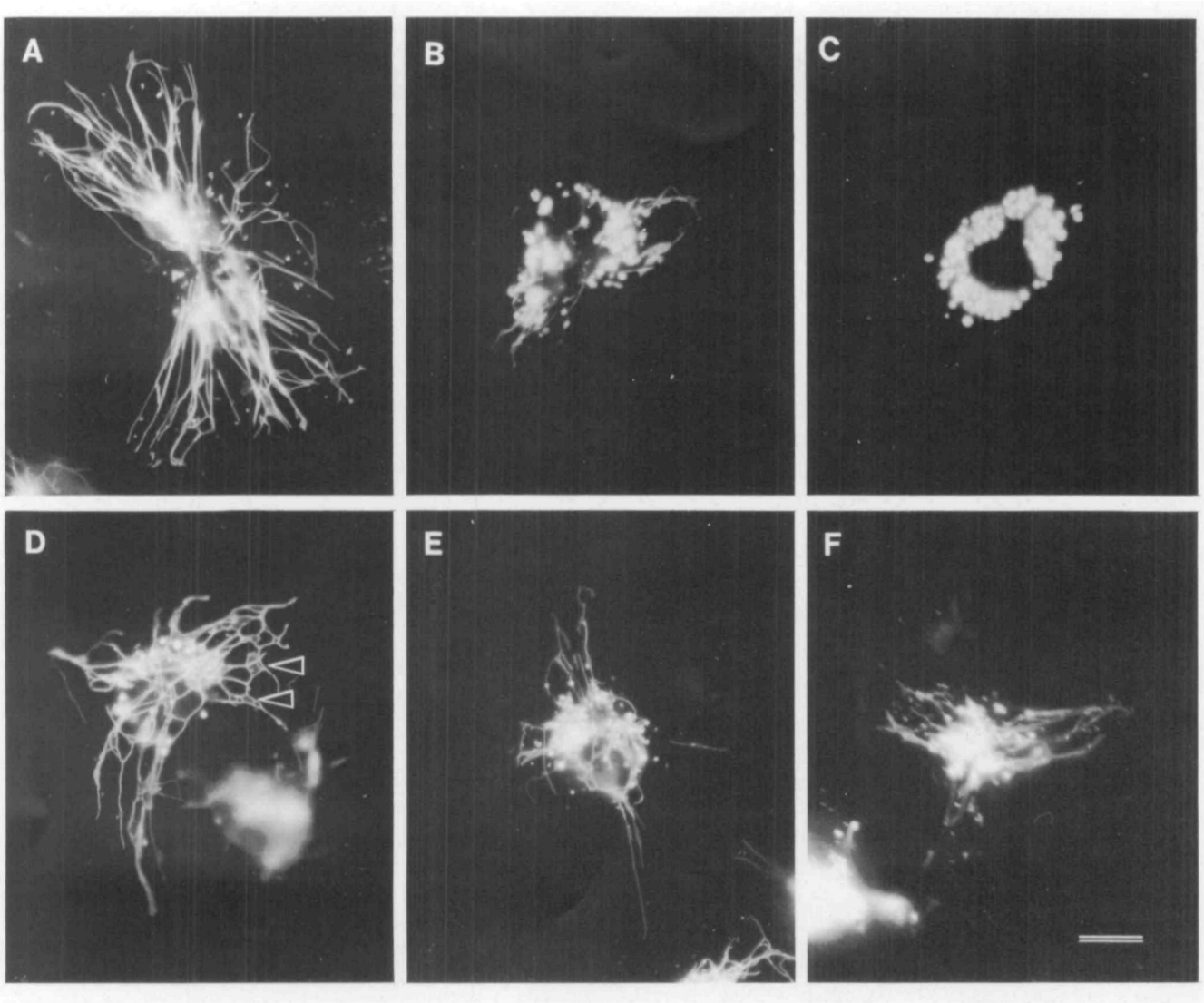

Fig. 1. Reversible expansion of lysosomes by sucrose. Macrophages were first incubated overnight in DME-10F (A,B,D,E) or in DME-10F plus 20 mg ml^{-1} sucrose (C,F). They were then incubated 3 h in DME-10F plus 0.5 mg ml^{-1} LY (A,D) or in DME-10F, LY and 20 mg ml^{-1} sucrose (B,C,E,F). Cells were then either rinsed in PBS and observed by fluorescence microscopy (A–C) or incubated 3 h in DME-10F containing 0.5 mg ml^{-1} invertase (D–F) before being rinsed in PBS and observed by fluorescence microscopy. Arrows indicate thin, sheet-like connections between several tubules. Bar, 10 μm.

crose-vacuolated macrophages in 0.5 mg ml^{-1} invertase allowed delivery of that enzyme into sucrose vacuoles, the conversion of sucrose to component monosaccharides, and the collapse of the spherical organelles (Cohn and Ehrenreich, 1969). After incubation in invertase-containing medium for 3 h, vacuoles containing sucrose regained a tubular morphology (Fig. 1E,F). Thus, the distention of tubular lysosomes by osmotic expansion was reversible.

Morphological rearrangements accompanying phagocytosis

Macrophages whose lysosomes had been labeled *via* pinocytosis of LY were allowed to phagocytose sheep erythrocyte ghosts that had been opsonized with goat anti-sheep erythrocyte antibody and loaded *via* osmotic rupture with Texas Red-labeled dextran (TRDx). Sufficient TRDx was loaded into the erythrocyte ghosts to allow its detection with the LY filter set. This permitted simultaneous morphological observation of both lysosomes (LY) and phagosomes (TR) during phagosome–lysosome fusion (Fig. 3). Shortly after phagocytosis, LY-labeled tubules surrounded phagosomes containing TRDx-labeled ghosts. After membrane fusion the two fluorophores mixed, creating orange phagolysosomes. Thirty minutes to one hour after phagocytosis the tubular lysosomal network disappeared (Fig. 3C), as the membrane previously associated with the network was incorporated into the combined phagolysosomal compartment. This compartment appeared as one or a few large, orange vesicles. Three hours after phagocytosis, the tubular network reappeared, labeled with a mixture of LY and TRDx fluorescence, thus indicating that the new network arose from the phagolysosome.

After phagocytosis of latex beads, the lysosomal network disappeared, as it did after phagocytosis of

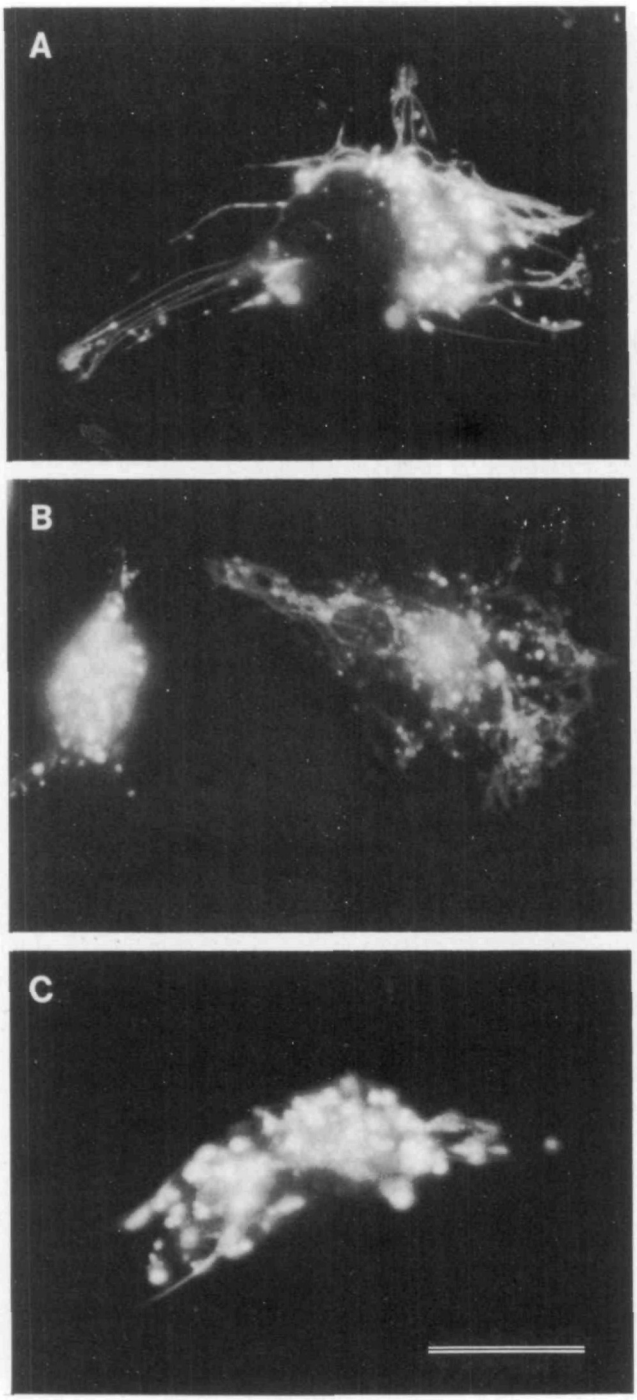

Fig. 2. Acridine Orange alters macrophage lysosome morphology. Macrophages were incubated in 5 μg ml^{-1} Acridine Orange in DME-10F for 5(A), 10(B) or 20(C) min at 37°C, then were quickly washed and observed by fluorescence microscopy (rhodamine filter set). These representative micrographs show the rapid conversion of tubular lysosomes into a more distended vesicular compartment. Bar, 10 μm.

erythrocyte ghosts. But in this case LY fluorescence remained surrounding the indigestible beads for many hours after disappearance of the tubules. A network did not reappear. Fig. 4 shows macrophages that were labeled by pinocytosis of LY before phagocytosis, but that also remained in the LY solution throughout phagocytosis of latex beads and the subsequent 5 h chase period. Continued pinocytosis would have labeled any newly formed lysosomes. Although pinosomes were labeled by this procedure, no new labeled tubular organelles appeared, indicating that the fluorescence surrounding the ingested beads represents the entire lysosomal compartment. The re-formation of a tubular network after phagocytosis of ghosts, but not after phagocytosis of latex, indicates that the re-formation of tubular morphology requires the phagolysosome shrinkage that accompanies digestion.

To quantify the depletion and reappearance of tubules following phagocytosis, macrophage lysosomes were labeled by pinocytosis of LY, and then cells were incubated with latex beads or opsonized erythrocytes for phagocytosis. The percentage of cells containing tubules was determined for each condition at various times after phagocytosis. As shown in Fig. 5, phagocytosis of opsonized erythrocytes depleted the tubular network transiently, whereas phagocytosis of latex beads led to irreversible depletion.

Using two sizes of beads we determined the extent of tubule disappearance with increasing phagocytic loads. For each number of beads ingested 10–20 macrophages were scored for the presence or absence of tubules. The percentage of cells with tubules was plotted in respect of the number of beads per cell (Fig. 6A), the total amount of bead surface area internalized (Fig. 6B) or the total amount of bead volume internalized (Fig. 6C). Phagocytosis of 4 μm diameter beads depleted the tubular network to the same extent as phagocytosis of 7 μm diameter beads when the quantity ingested was expressed as the total surface area of latex ingested (Fig. 6B). Complete distributions for beads of both sizes were determined on three separate occasions. There was variation between experiments in the number of beads required to deplete the cell of tubules. For each of the three trials, however, the total ingested surface area provided the closest correspondence between phagocytic load and tubule depletion.

Discussion

Because bone marrow-derived macrophages grow and differentiate in culture, their lysosomal compartment contains very little phagocytosed indigestible or undigested matter. The experiments described here demonstrate that in this relatively vacant condition lysosomes exist as an extensive tubular network. This network disappears as the lysosomal volume expands, then reappears as volume decreases.

The re-formation of the tubular morphology after shrinkage of sucrose vacuoles or phagolysosomes indicates that the compartment exhibits considerable plasticity at late stages in its maturation. The tubular network formed hours after phagocytosis of erythrocytes appears very similar to that labeled by a brief incubation with LY. This complicates morphological discrimination between early and late lysosomes in the macrophage. It also

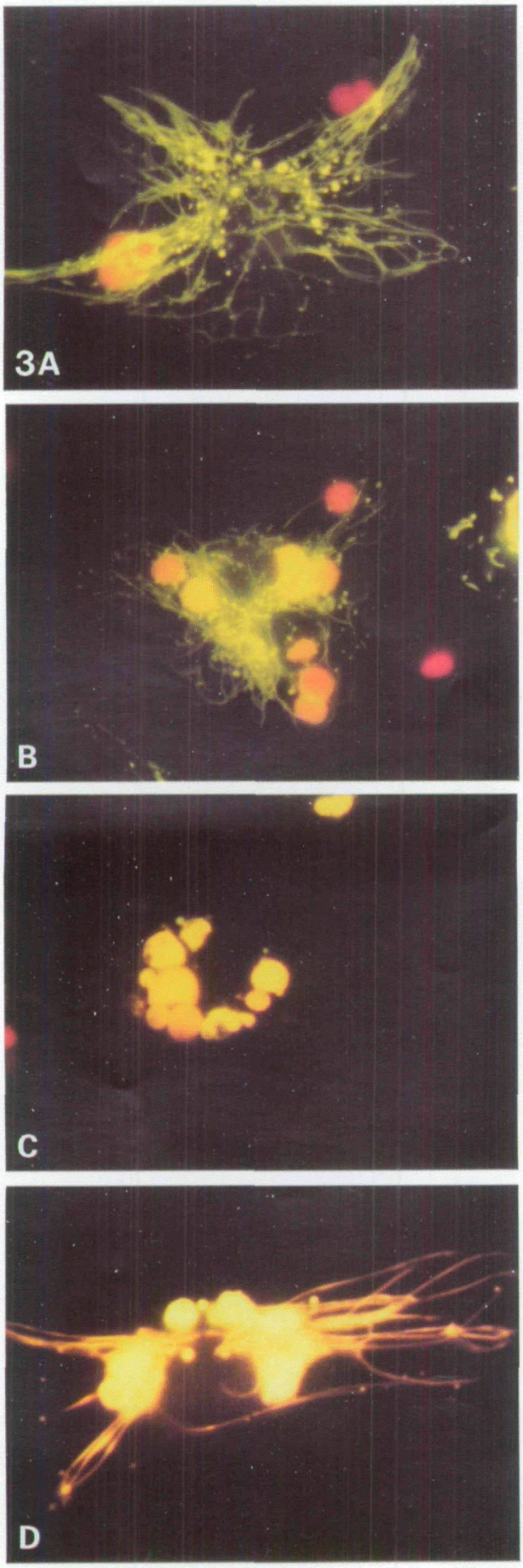

Fig. 3. Reorganization of lysosomes following phagocytosis. Macrophage lysosomes were first labeled by pinocytosis of LY (yellow fluorescence). They were then provided with opsonized erythrocyte ghosts that had been preloaded by osmotic rupture with TRDx (red fluorescence). A. Eight min after phagocytosis, red phagosomes appear independent of the yellow tubular lysosomal network. B. Fifteen min after phagocytosis, tubular lysosomes appear closely apposed to some phagosomes. Some mixing of the fluorophores is evident, indicating phagosome–lysosome fusion. C. Sixty min after phagocytosis, the two fluorophores are completely mixed inside phagolysosomes, and the tubular network has disappeared. D. Three hours after phagocytosis, a new tubular lysosomal network has re-formed from shrunken phagolysosomes.

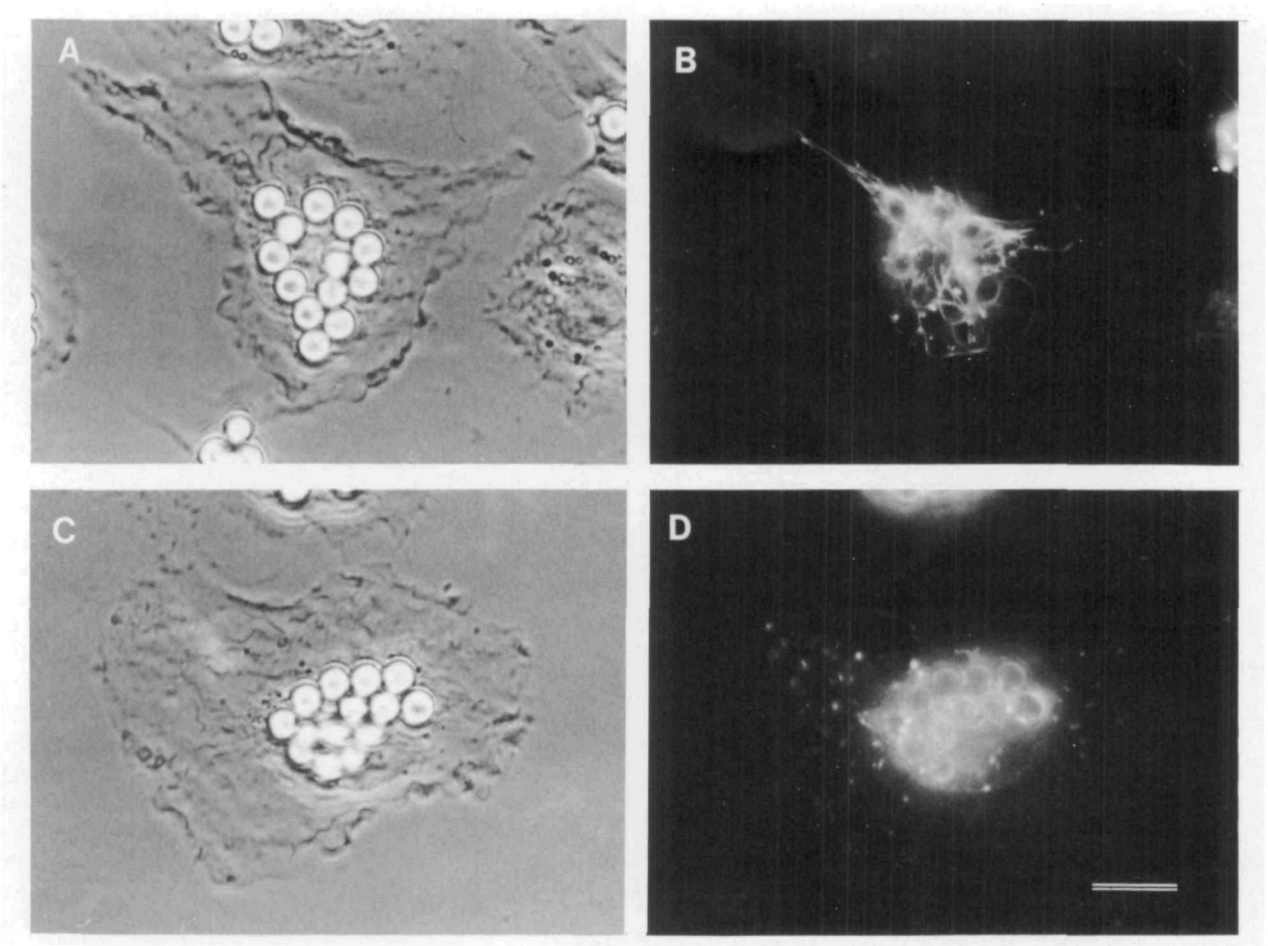

Fig. 4. Phagocytosis of latex beads causes irreversible redistribution of the tubular lysosomal compartment. Macrophages preloaded with LY were incubated with latex beads and LY for 10 min (A,B) or 5 h (C,D). Phagosome–lysosome fusion depleted the tubular network. Despite continued incubation in LY for 5 h, no tubular endocytic compartment reappeared. A and C, phase micrographs; B and D, corresponding fluorescence images. Bar, 10 μm.

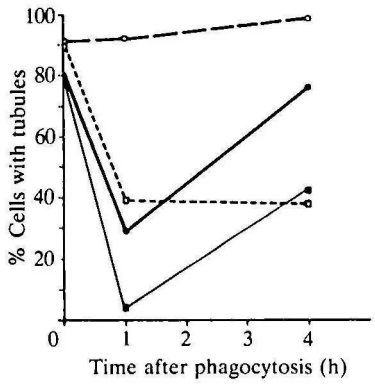

Fig. 5. The tubular lysosomal network reassembles after phagocytosis of digestible particles. Macrophages preloaded with LY were provided with latex beads or opsonized erythrocytes for phagocytosis, then were incubated in DME-10F for the times shown. Cells were scored for the presence of tubular lysosomes. (○) No phagocytosis; (□) 9.1±5.5 latex beads per cell; (●) 8.4±5.6 erythrocytes per cell; (■) 15.9±4.7 erythrocytes per cell. Similar results were obtained on three separate occasions. Variations in the number of particles phagocytosed precluded pooling of the data.

demonstrates that the tubular compartment is not simply an intermediate shape in the morphogenesis of a spherical lysosome. Instead, the tubular morphology is that of a stable, interconvertible organelle that can reversibly accommodate endocytic loads.

The traditional view of the lysosomal compartment as a population of discrete vesicles should be reconsidered now in light of the continuity we observe here. The anastomosing network, in its form and in its movements, indicates a considerable traffic of lysosomes in cytoplasm. Microtubules contribute to this traffic, possibly *via* microtubule-dependent motors such as kinesin (Swanson *et al.* 1987; Vale *et al.* 1985). However, microtubule-dependent lysosome movement is not rate-limiting for either phagosome–lysosome fusion or hydrolytic degradation of phagocytosed content, as microtubule-depolymerizing drugs have little or no effect on these rates (P.E.K., data not shown; Pesanti and Axline, 1975*a*,*b*). Microtubule association may be more important during the reorganization of the lysosomal structure following degradation of phagolysosomal contents. We have observed that the re-formation of tubular lysosomal networks after phagocytosis is prevented by depolymerization of microtubules with nocodazole (P.E.K., data not

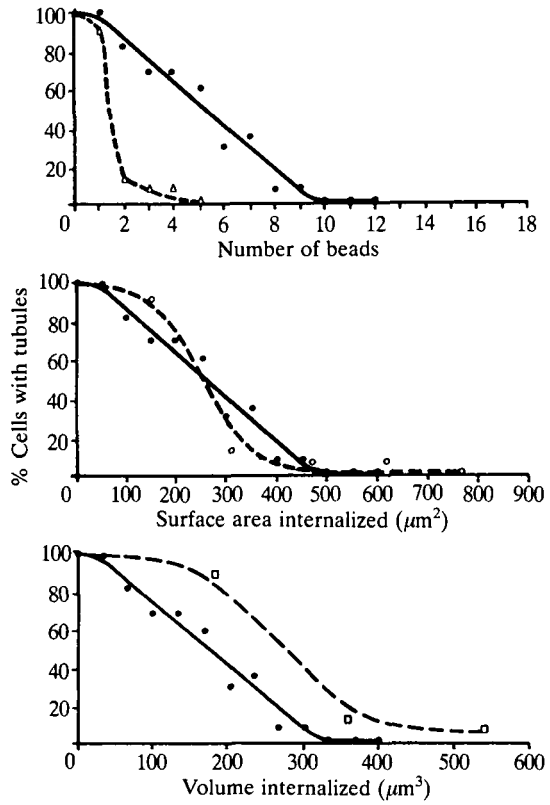

Fig. 6. Tubular lysosome disappearance correlates with the total surface area ingested by phagocytosis. Macrophages preloaded with LY were allowed to phagocytose latex beads, then the percentage of cells containing tubular lysosomes greater than 10 μm in length were scored for each bead load. A. Fewer 7 μm diameter beads (△) were required to deplete the tubular lysosomal network than 4 μm diameter beads (●). B. The data of A expressed in terms of total surface area ingested: (○) 7 μm diameter beads, (●) 4 μm diameter beads. C. The data of A expressed in terms of total bead volume ingested: (□) 7 μm diameter beads, (●) 4 μm diameter beads.

shown). Under these conditions, the absence of microtubule tracks along which to extend the lysosome may permit other morphological arrangements of that membrane (e.g. see Swanson *et al.* 1986).

Lysosomes left distended for long periods of time may lose some plasticity. In J774 macrophages, sucrose vacuoles formed by prolonged incubation in sucrose collapse upon invertase treatment into whorled residual bodies, instead of reverting to the tubular morphology of non-vacuolated cells (Swanson *et al.* 1986). A similar rearrangement was detectable in some of the bone marrow-derived macrophages observed by fluorescence after prolonged sucrose vacuolation and subsequent invertase-induced collapse (J.S., data not shown). It indicates that loss of lysosome plasticity, and the formation of residual bodies, is a maturation event that occurs only after prolonged occupancy of the compartment.

The usual morphology of lysosomes

It is possible that the fluorescent probes delivered by endocytosis or pH-dependent accumulation themselves induce the tubular morphology. However, tubular lysosomes can be recognized ultrastructurally using acid phosphatase histochemistry in macrophages fixed without any prior endocytosis of tracer molecules (Swanson *et al.* 1987).

Tubular lysosomes are difficult to preserve in fixed cells, and macrophage lysosomes often appear by light or electron microscopy as vesicles or rows of vesicles (Phaire-Washington *et al.* 1980; Swanson *et al.* 1987). Such difficulties in preservation, together with the fact that Acridine Orange disrupts tubular lysosome morphology in living macrophages, may explain why the extensive networks were not described until recently (cf. Buckley, 1973; Nichols, 1982). This is not to say that tubular lysosomes are ubiquitous. Many cultured cell lines, when labeled with fluorescent tracers, display spherical lysosomes (Miller *et al.* 1983). Tubular lysosomal networks in macrophages probably result from an increased content of lysosomal membrane relative to other cells. This membrane remains in the high surface-to-volume tubular morphology until its volume increases by accumulation of either solutes or phagocytosed particles. Tubule depletion is proportional to the amount of particle surface area ingested.

That lysosomes appear tubular even after considerable endocytosis of LY or TRDx suggests that these molecules do not reach concentrations that expand the compartment osmotically. Although LY is highly water-soluble (Stewart, 1978) and therefore potentially capable of osmotic expansion of lysosomes, it is added in these experiments at a concentration of only 1 mM, much lower than that of sucrose (60 mM), which does cause osmotic vesiculation. Therefore, LY does not appear to accumulate to disruptive concentrations during the incubation times used here. Rapid expansion of lysosomes by Acridine Orange is achieved at even lower extracellular concentrations (3 μM). Its pH-dependent concentration inside lysosomes, then, must occur much more rapidly than the endocytosis-dependent concentration of sucrose.

Why are tubular lysosomes evident in some macrophages but not others? Lysosomes of unstimulated, thioglycolate-elicited, mouse peritoneal macrophages seldom appear tubular. These macrophages normally contain internalized agar from the thioglycolate broth, which creates a permanently enlarged phagolysosomal compartment. Treatment with the tumor promoter phorbol myristate acetate rapidly leads to the appearance of tubular lysosomes (Swanson *et al.* 1987), suggesting that despite the partial expansion of the lysosomal compartment by agar, some membrane is available to form tubules. It also indicates that in the absence of stimulation these cells do not normally promote lysosome extension along microtubules. Similarly, the lysosomes of cultured cells often cluster in the vicinity of the nucleus, away from peripheral regions of cytoplasm (Zelenin, 1966; Herman and Albertini, 1984). Centrifugal movement of lysosomes may require activation of cytoplasmic motors on microtubules, and the activity of such motors may vary with the physiological state of the cell. The dramatic redistributions of lysosomes elicited by changing cytoplasmic pH are consistent with this hypothesis (Heuser, 1989).

Thus, two factors contribute to the formation of the tubular lysosomal compartment: the contents of the lumen and the interactions of the organelle with cytoplasmic microtubules. In bone marrow-derived macrophages, the lysosomal compartment is large, relatively vacant, and extended along microtubules. Conversely, lysosomes of thioglycollate-elicited macrophages are partly distended by indigestible agar and interact poorly with microtubules until the cell is stimulated with phorbol esters.

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