

# Tubular Lysosomes Accompany Stimulated Pinocytosis in Macrophages

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**Abstract.** A network of tubular lysosomes extends through the cytoplasm of J774.2 macrophages and phorbol ester-treated mouse peritoneal macrophages. The presence of this network is dependent upon the integrity of cytoplasmic microtubules and correlates with high cellular rates of accumulation of Lucifer Yellow (LY), a marker of fluid phase pinocytosis. We tested the hypothesis that the efficiency of LY transfer between the pinosomal and lysosomal compartments is increased in the presence of tubular lysosomes by asking how conditions that deplete the tubular lysosome network affect pinocytic accumulation of LY. Tubular lysosomes were disassembled in cells treated with microtubule-depolymerizing drugs or in cells that had phagocytosed latex beads. In unstimulated peritoneal

macrophages, which normally contain few tubular lysosomes and which exhibit relatively inefficient transfer of pinocytosed LY to lysosomes, such treatments had little effect on pinocytosis. However, in J774 macrophages and phorbol ester-stimulated peritoneal macrophages, these treatments markedly reduced the efficiency of pinocytic accumulation of LY. We conclude that a basal level of solute accumulation via pinocytosis proceeds independently of the tubular lysosomes, and that an extended tubular lysosomal network contributes to the elevated rates of solute accumulation that accompany macrophage stimulation. Moreover, we suggest that the transformed mouse macrophage cell line J774 exhibits this stimulated pinocytosis constitutively.

THE uptake of solutes by pinocytosis is a constitutive process in nearly all eukaryotic cells, and is particularly active in macrophages (15, 18). In these cells a significant proportion of pinocytosed solute is rapidly returned to the extracellular medium (2, 18). This is presumed to occur in vesicles that return membrane proteins from the endosomal compartment to the cell surface (15). Another portion of pinocytosed solute is delivered to the lysosomal compartment, where it accumulates. Macrophages have the capacity to regulate the relative proportions of pinocytosed solute entering each of these pathways (1, 18). For instance, mouse peritoneal macrophages return 80% of pinocytosed solute to the medium via the recycling pathway. When these cells are stimulated with the tumor promoter PMA they markedly increase the proportion of solute shunted to lysosomes, thereby increasing the efficiency and extent of solute accumulation. The mechanisms by which macrophages regulate the proportion of pinocytosed solute delivered to lysosomes are unknown. One clue to the mechanisms involved is the capacity of phorbol esters to promote the extension of tubular lysosomes into the cells' peripheral cytoplasm (11, 16). A second is the inhibitory effect of microtubule depolymerizing drugs on the capacity of phorbol esters to stimulate tubular lysosome formation and solute accumulation. These microtubule inhibitors have only a small effect on solute accumulation by unstimulated macro-

phages. These findings suggested to us that the availability of lysosomes in the cells' periphery, and the close association of these organelles with microtubules, might enhance both the accessibility of lysosomes to pinosomes and the efficiency with which lysosomes and pinosomes encounter one another. The experiments reported here were designed to test this hypothesis. The results provide substantial support for it.

## Materials and Methods

### Materials

Female mice of the ICR strain were obtained from Trudeau Institute, Saranac Lake, NY. Colchicine, nocodazole, and PMA were obtained from Sigma Chemical Co. Lumicolchicine was prepared by ultraviolet illumination of 30  $\mu$ M colchicine in PD, according to the methods of Wilson and Friedkin (21).

### Cells

Thio-macrophages were prepared as described previously (18).  $7.5 \times 10^5$  peritoneal cells in minimum essential medium with 10% fetal bovine serum (M10F) were plated into each well of a 24-well tissue culture dish (Costar, Cambridge, MA). After 2 h at 37°C during which the macrophages were allowed to settle, nonadherent cells were removed by rinsing with ice-cold divalent cation-free phosphate-buffered saline (PD). The adherent macrophages were then incubated overnight in M10F (37°C, 5% CO<sub>2</sub>) before experimentation.

J774.2 macrophages (13) were cultured in suspension at 37°C, in Dulbecco's modified essential medium containing 10% heat-inactivated fetal bo-

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vine serum with penicillin and streptomycin (DM10F). They were plated for experiments into tissue-culture dishes at  $1\text{--}1.5 \times 10^5$  cells/well.

## Phagocytosis

Latex beads (Duke Scientific Corp., Palo Alto, CA) were washed and stored in PD with 0.02%  $\text{NaN}_3$  and 1 mg/ml BSA. The BSA reduced bead clumping. Before addition to the cells the beads were washed three times in PD, then resuspended in M10F to a final concentration of  $2.5 \times 10^8$  beads/ml (1.1- $\mu\text{m}$  diameter beads),  $4 \times 10^7$  beads/ml (3.0- $\mu\text{m}$  diameter beads), or  $1 \times 10^7$  beads/ml (4.1- $\mu\text{m}$  diameter beads). The bead suspensions were then diluted to varying concentrations in M10F to provide different phagocytic loads to the thio-macrophages. 0.5 ml of bead suspension was added to each well, then the dish was incubated undisturbed for 2 h to allow beads to adhere to the cells and be phagocytosed. After this incubation the bead suspensions were replaced with bead-free M10F and the cells were reincubated for 1–2 h to complete phagocytosis.

## Pinocytosis

Fluid phase pinocytosis was measured using Lucifer Yellow CH (LY) (Sigma Chemical Co., St. Louis, MO) according to procedures described previously (18). After bead phagocytosis thio-macrophages were incubated for different times in M10F containing 1.0 mg/ml LY. Incubations were timed such that all ended at the same moment. LY was shaken out of each dish, then washed away by immersing dishes consecutively in two 1-liter vol of ice-cold PD with 1 mg/ml BSA, then two 1-liter vol of ice-cold PD. Wells were then aspirated dry and 0.60 ml of Triton X-100 (0.05% in water) was added to each well. Wells were scraped with rubber policemen, and 50  $\mu\text{l}$  of lysate removed for bead counts. The remaining lysate was centrifuged (3000 g, 8 min) to remove the beads, 0.10 ml of supernatant was taken for protein determination, and 0.35 ml taken and combined with 1.25 ml Triton X-100 (0.05% with 0.1 mg/ml BSA) for determination of LY fluorescence. Fluorescence of lysates was measured with a spectrofluorometer (Perkin-Elmer 650–40, excitation 430, emission 540; Perkin-Elmer Corp., Eden Prairie, MN). Standards of LY were prepared in Triton X-100 (0.05% with 0.1 mg/ml BSA). Protein was measured by the method of Lowry et al. (6). LY content was calculated for each well and expressed as nanograms LY per milligrams protein.

For bead counts, samples of lysate were diluted with water and counted in a hemocytometer. Concentrations were converted to number of beads per cell (200  $\mu\text{g}$  protein/ $10^6$  thio-macrophages, 230  $\mu\text{g}$  protein/ $10^6$  J774 cells).

Microtubule-depolymerizing drugs (colchicine, nocodazole) and their congeners (lumicolchicine) were added to cells at 5  $\mu\text{M}$ . Cells were preincubated for 60 min in drug-containing medium before LY solutions ( $\pm$  microtubule drugs and  $\pm$  10 ng/ml PMA) were added for pinocytic measurements.

## Microscopy

Macrophages were plated onto 12-mm diameter, No. 1 circular coverslips, then maintained at 37°C in M10F (thio-macrophages) or DM10F (J774 macrophages). Cells were incubated with 2- $\mu\text{m}$  diameter latex beads as described above to allow phagocytosis, then washed and incubated 2 h in medium containing 1 mg/ml LY.

In separate experiments, J774 macrophages on coverslips were incubated for 1 h in medium containing 1 mg/ml LY. They were then incubated for 1 h in medium with both LY and nocodazole (5  $\mu\text{M}$ ), then washed and incubated 15–25 min in dye-free medium, plus or minus nocodazole. Cells were examined by fluorescence microscopy using a Zeiss Photomicroscope III, equipped for epifluorescence (fluorescein filter set). Cells were photographed using Kodak Tri-X film (ASA 400).

## Results

### Thioglycollate-elicited Mouse Peritoneal Macrophages

Macrophages take up LY by fluid phase pinocytosis. A portion of the pinocytosed LY is returned to the extracellular

1. *Abbreviations used in this paper:* DM10F, Dulbecco's modified essential medium containing 10% heat-inactivated fetal bovine serum with penicillin and streptomycin; LY, Lucifer Yellow; M10F, minimum essential medium with 10% fetal bovine serum; PD, divalent cation-free phosphate-buffered saline.

medium, presumably within recycling membrane vesicles. The remainder is delivered to lysosomes (2, 18). In thioglycollate-elicited mouse peritoneal macrophages (thio-macrophages) the pinocytic accumulation of LY is initially very rapid, but slows during the first hour of measurement to a linear rate of accumulation that reflects both influx and efflux of LY. The linear rate of LY accumulation in thio-macrophages, which begins  $\sim$ 60 min after addition of LY to the medium (Fig. 4 and reference 17), is approximately one-fifth the rate of pinocytic influx and indicates a relatively inefficient delivery of pinocytosed fluid to lysosomes (18). Tumor-promoting phorbol esters, such as PMA, stimulate pinocytic accumulation of LY by thio-macrophages four to sevenfold (12, 18). This is due to both a small (60%) increase in LY influx and a much more efficient delivery of LY to lysosomes (18).

Thio-macrophages stimulated with PMA display an interconnecting network of tubular cisternae which extends nearly to the margins of these flattened and well-spread cells. These tubular cisternae are lysosomes, as evidenced by their content of cathepsin L and acid phosphatase (16). In contrast, the lysosomes of unstimulated thio-macrophages are rarely tubular and do not extend into the cell periphery as they do in PMA-treated thio-macrophages (16). We questioned whether the enhanced rate of pinocytosis in PMA-stimulated thio-macrophages corresponds with their increased content of tubular lysosomes. Tubular lysosomes disassemble when cytoplasmic microtubules are depolymerized with colchicine. Such treatment leaves small, spherical lysosomes dispersed through the cytoplasm (16). Treatment of thio-macrophages with 5  $\mu\text{M}$  colchicine or nocodazole had a relatively minor (10–20%) inhibitory effect on basal pinocytic accumulation of LY (Fig. 1), but completely blocked PMA-induced tubular lysosome formation (16) and inhibited by more than 75% the PMA-stimulated pinocytic accumulation of LY (Fig. 1). Lumicolchicine, an analog of colchicine that does not depolymerize microtubules, but that exhibits colchicine's effect on membrane transport (8), inhibited neither constitutive nor stimulated pinocytosis. Removal of nocodazole from the medium, which allows microtubule and tubu-

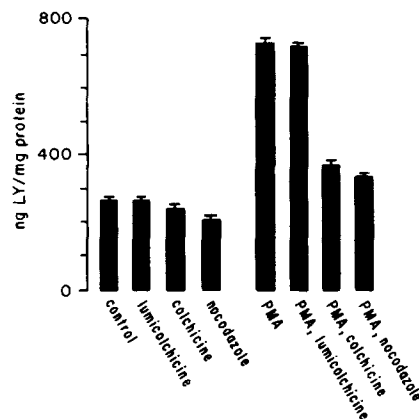
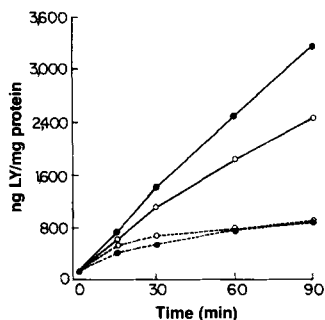
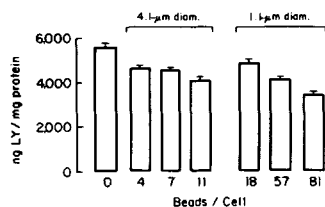


Figure 1. Agents that depolymerize microtubules inhibit PMA-stimulated pinocytosis in thio-macrophages. Cells were preincubated for 60 min with 5  $\mu\text{M}$  colchicine, nocodazole, or lumicolchicine, then incubated for 2 h in 1 mg/ml LY  $\pm$  PMA  $\pm$  microtubule-depolymerizing drugs. Bars indicate LY accumulation.



**Figure 2.** The effect of beads and PMA on pinocytic accumulation by thio-macrophages. Cells were fed 3- $\mu\text{m}$  diameter beads as described in Materials and Methods, then incubated in 1 mg/ml LY  $\pm$  10 ng/ml PMA for the indicated times. Solid lines represent LY accumulation in the presence of PMA, dotted lines indicate control (no PMA) rates. (●) No Beads; (○) 30 beads/cell.



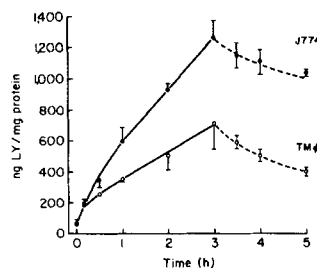
**Figure 3.** The effect of different diameter beads on the rate of PMA-stimulated pinocytic accumulation by thio-macrophages. Cells were fed 1.1- or 4.1- $\mu\text{m}$  diameter beads as described, then PMA-stimulated pinocytic accumulation was

measured (120 min). The rate of constitutive accumulation, unaffected by the latex beads, was subtracted from the stimulated rates. The points therefore represent the inhibition by beads of the stimulated pinocytosis only.

lar lysosome re-assembly (16), returned pinocytosis of PMA-treated macrophages to pretreatment levels (data not shown).

Tubular lysosomes are also diminished after phagocytosis. Thio-macrophages can internalize 30 or more 3- $\mu\text{m}$  diameter latex beads, and since the bead-containing phagosomes fuse with cellular lysosomes these beads can occupy a considerable portion of the lysosomal compartment. After phagocytosis of latex beads, PMA-stimulated thio-macrophages have many fewer tubular lysosomes (data not shown).

To examine the effect of phagocytosis on pinocytosis, thio-macrophages were allowed to phagocytose latex beads for 2 h, then were incubated for another hour in bead-free



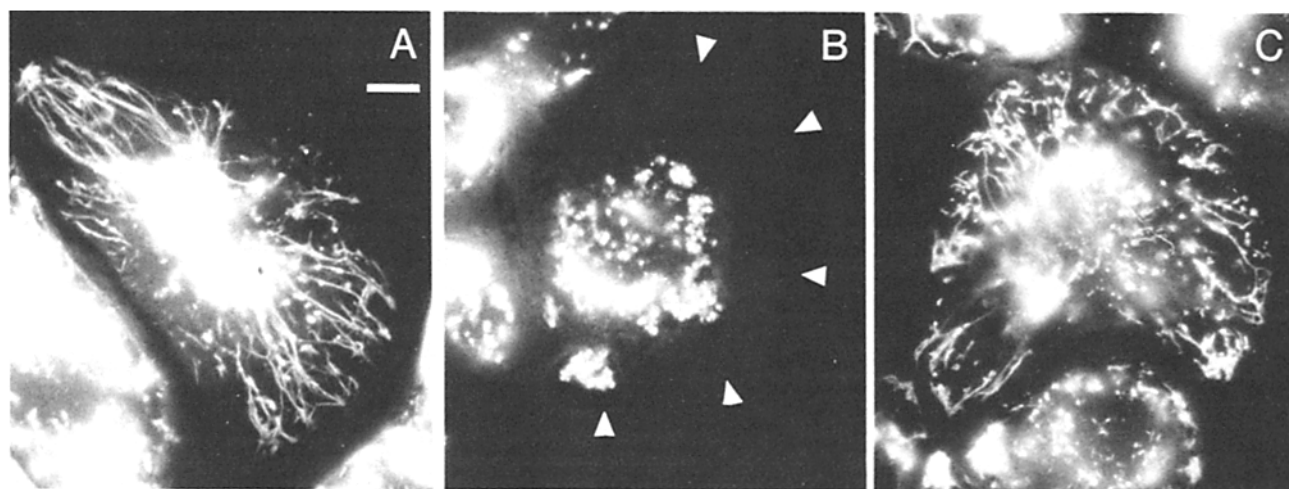
**Figure 4.** Accumulation and efflux of LY in J774.2 and thio-macrophages. Adherent macrophages were incubated in LY-containing medium as described in Materials and Methods. Solid circles indicate J774 macrophages; open circles represent thio-macrophages. Solid lines indicate accumulation. Broken lines show efflux at 37°C from cells loaded 3 h with LY.

medium. In the experiment shown in Fig. 2, the thio-macrophages contained an average of 30 beads per cell. These cells were then tested for their capacity to pinocytose LY in the presence or absence of PMA. As shown in Figure 2, basal pinocytosis was the same in cells with and without latex beads inside. However, the PMA-elicited increase in pinocytic accumulation of LY was inhibited 38% in cells that had ingested latex beads. Furthermore, the more beads phagocytosed by cells, the greater the inhibition of pinocytosis. The reduction in PMA-stimulated pinocytosis was not simply a function of the number of phagocytic events. If this were so one would expect a small bead to reduce accumulation by the same amount as a large bead. Instead we found that 57 1.1- $\mu\text{m}$  diameter beads were required to reduce pinocytosis as much as 11 4.1- $\mu\text{m}$  diameter beads (Fig. 3).

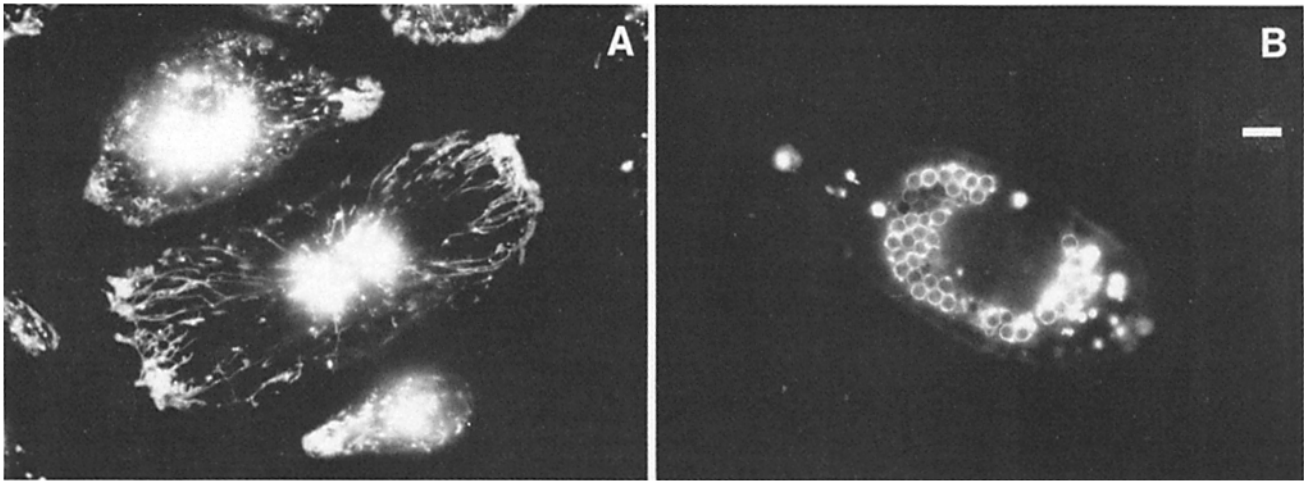
In summary, the enhancement of LY accumulation that is observed in PMA-stimulated thio-macrophages is prevented by conditions that disrupt the tubular lysosomal network. Unstimulated thio-macrophages have few tubular lysosomes and a much lower rate of LY accumulation. Phagocytosis of latex beads or drugs that cause disassembly of microtubules have little effect on LY accumulation by these cells.

#### J774.2 Macrophages

J774.2 designates a continuous cell line that expresses membrane receptors and phagocytic activity characteristic of



**Figure 5.** Reversible disassembly of tubular lysosomes in J774 macrophages by nocodazole. Lysosomes were labeled by pinocytosis of LY. (A) Control macrophages, not treated with nocodazole. (B) Macrophages incubated for 60 min in 5  $\mu\text{M}$  nocodazole, washed, then observed in nocodazole-containing medium. Arrowheads indicate the margin of the cell, identified by phase microscopy. (C) Macrophages incubated for 60 min in nocodazole, washed, and reincubated for 25 min in nocodazole-free medium. The tubular lysosomes shown in A and C extend to the cell margins. Bar, 5  $\mu\text{m}$ .



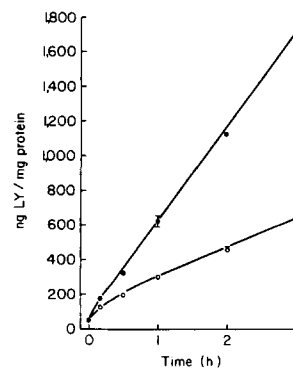
**Figure 6.** Tubular lysosomes are absent from J774 macrophages after phagocytosis of latex beads. Cells on coverslips were exposed to latex beads (B), or left unfed (A) for 2 h, then incubated in medium with LY for 2 h, washed, and examined by fluorescence microscopy. Bar, 5  $\mu$ m.

mouse macrophages. It was derived from a "lymphoma" in a BALB/c NIH mouse (13). The kinetics of pinocytosis in unstimulated J774 cells resembled those of PMA-stimulated pinocytosis in thio-macrophages in several respects. Although the initial rates of LY accumulation of J774 and in unstimulated thio-macrophages were very similar (Fig. 4), the J774 cells exhibited a linear rate of LY accumulation twofold greater than that of thio-macrophages. This suggested that the J774 cells delivered a greater percentage of their pinocytosed LY to lysosomes than did thio-macrophages. Indeed, comparative measurements of LY efflux from J774 cells and from thio-macrophages indicated that J774 cells returned a smaller percentage of pinocytosed LY to the medium than did thio-macrophages (Fig. 4).

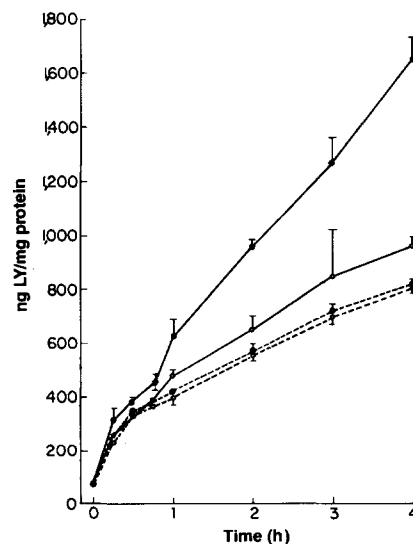
In addition to constitutively expressing a high rate of pinocytosis, J774 macrophages constitutively express tubular lysosomes (Fig. 5 and reference 17). These tubules can be observed as a fluorescent interconnecting network after the J774 cells have pinocytosed LY or other fluorescent fluid phase markers (16, 17). As in PMA-stimulated thio-macrophages, the networks extend to the margins of flat, adherent cells. Treatment of J774 cells with PMA does not increase their tubular lysosome content.

The tubular lysosomes of J774 cells could be disassembled by the same conditions that affect tubular lysosome morphology in PMA-stimulated thio-macrophages. Thus, treatment with 5  $\mu$ M colchicine disassembled microtubules and disrupted tubular lysosomes; 5  $\mu$ M nocodazole did so reversibly (Fig. 5). Phagocytosis of latex beads markedly reduced the number of tubular lysosomes visible in J774 macrophages (Fig. 6). These conditions, which caused the disappearance of tubular lysosomes, also inhibited pinocytosis of LY by 40–60% in the J774 cells (Figs. 7 and 8). Pinocytosis by J774 was also stimulated by PMA, but the extent of stimulation was much less than that seen after PMA-treatment of thio-macrophages. Although LY accumulation by thio-macrophages was stimulated three to sevenfold by PMA, pinocytosis by J774 cells was stimulated only 40% by PMA treatment (data not shown).

Thus, macrophages that contain an extensive tubular net-



**Figure 7.** Colchicine inhibits constitutive pinocytic accumulation in J774.2 macrophages. Colchicine-treated cells (○) were incubated 60 min in 5  $\mu$ M colchicine before being incubated for various times with LY plus colchicine. Colchicine-free control pinocytosis is indicated by the solid circles.



**Figure 8.** The effect of prior phagocytosis on subsequent pinocytosis in macrophages. J774.2 cells (solid lines) and thio-macrophages (dotted lines) were fed 3- $\mu$ m diameter latex beads as described in Materials and Methods, then incubated in 1 mg/ml LY (in DMIOF) for various times. Points represent accumulation in the presence (○) and absence (●) of beads. J774.2 had ingested 52 beads/cell, thio-macrophages had ingested 25 beads/cell.

work exhibit high rates of LY accumulation; and conditions that disassemble that network inhibit pinocytosis. In contrast, unstimulated thio-macrophages exhibit few tubular lysosomes and low pinocytic accumulation rates, and those rates are not markedly affected by agents that would disassemble the tubular lysosomal network. We suggest that a pinocytosis like that elicited by PMA in thio-macrophages is active constitutively in unstimulated J774 cells.

## Discussion

Pinocytosis occurs by a series of discrete, and possibly dissociable, steps. Extracellular fluid and solutes are first internalized in vesicles derived by invagination of plasma membrane. Next, the internalized pinosomes fuse with other intracellular vesicles, such as endosomes, other pinosomes, or lysosomes. Finally, internalized membrane is returned to the cell surface. Recycling can occur from lysosomes or from intermediate compartments (2, 15). This intracellular circulatory system is subject to regulation at several points. Ligands for plasma membrane receptors and phorbol esters have been shown to increase pinocytic influx (4, 18). Nutritional state, and, again, phorbol esters can increase the rate of solute delivery to lysosomes (2, 18). Insulin increases solute efflux from 3T3-L1 adipocytes (4).

Our studies suggest that changes in cellular microanatomy may contribute to the regulation of solute flow. Specifically, we have asked whether the spatial organization of lysosomes influences the rate and/or extent of solute accumulation. Our query was prompted by the observation that high rates of accumulation of pinocytosed solute by macrophages are accompanied by a striking extension of the lysosomal system into the peripheral cytoplasm, the region of pinosome formation. This network of tubular lysosomes is associated with cytoplasmic microtubules and appears dependent upon microtubule integrity for its form (Fig. 5 and reference 16). Disassembly of the tubular lysosome network, either by the use of drugs that depolymerize microtubules (16) or by phagocytosis of latex beads (Fig. 6), inhibited the high rates of solute accumulation via pinocytosis that are constitutive in J774 cells and induced by PMA in thio-macrophages. Unstimulated thio-macrophages, which have few tubular lysosomes, exhibit high rates of pinocytic influx of LY and inefficient transfer of pinocytosed LY to lysosomes (18). We suggest that their modest rate of LY accumulation results from the lack of an extended tubular lysosomal system. Our finding that pinocytic accumulation of LY in these cells was relatively unaffected either by drugs that depolymerize microtubules or by phagocytosis of latex beads is consistent with this hypothesis. However, despite this correlation between tubular lysosomes and high pinocytic accumulation rates, we cannot say whether the process is dependent on the structure or vice versa.

Previous studies of the effects of microtubule depolymerization on pinocytosis are consistent with the observations reported here. Pesanti and Axline (10) found that pinocytic accumulation of solute in stimulated macrophages was inhibited by colchicine. Phaire-Washington et al. (12) reported that microtubule depolymerizing drugs inhibit PMA-elicited pinocytosis, but not basal pinocytosis, in thio-macrophages. In contrast, Melmed et al. (7) observed that basal pinocytosis in the J774 macrophage cell line was inhibited 40% by col-

chicine, and we have confirmed this finding here (Fig. 7). The present work clarifies this apparent contradiction by showing that J774 cells exhibit many of the structural and functional characteristics of PMA-stimulated primary macrophages.

The association of lysosomes with microtubules and the saltatory movement of lysosomes within the cytoplasm have been documented in several cell types (3, 5) in addition to macrophages (16). We suggest that the association between these two organelles has both structural and functional consequences for the delivery of pinocytosed solutes to lysosomes. Transport of pinocytic vesicles along microtubules (14) could increase the frequency of their encounters with the tubular lysosomes, and consequently increase the delivery of pinocytosed solutes to the lysosomal compartment. Moreover, microtubule-associated translocation proteins such as kinesin (19) may contribute directly to the fusions between pinosomes and lysosomes.

Other interpretations of the correlation observed here are plausible. To maintain an elevated rate of pinocytic accumulation, macrophages must recycle some of their incoming membrane (2, 15, 18). Tubular lysosomes may form as a consequence of lysosomal membrane recycling to the cell surface (9, 20). Disassembly of the tubular lysosomal network could interfere primarily with this efferent pathway.

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