PHAGOLYSOSOME FORMATION IN NORMAL AND COLCHICINE-TREATED MACROPHAGES*

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It has been suggested that intact microtubules promote contact of lysosomes with endosomes in phagocytic cells and that microtubular disruption caused by colchicine (1–6) or vinblastine (7) would inhibit fusion of lysosomes with phagosomes or pinosomes. We have previously shown (8) that in the macrophage no inhibition of lysosomal fusion resulting from microtubular disruption could be demonstrated by evaluation of the functional consequences of fusion namely degradation of ingested materials. However, those results, like virtually all published data on the subject, relied on methods which gauged intracellular lysosomal fusion indirectly and thus provided only suggestive evidence that microtubular disruption did not significantly alter lysosomal fusion. Since methods which allow direct evaluation of transfer of lysosomal enzymes to phagocytic vesicles have been described (9–12), it was of interest to employ such a procedure to evaluate quantitatively the effects of colchicine on lysosomal fusion. In this communication, we describe the relationship between particle uptake and extent of lysosomal enzyme transfer to phagolysosomes in normal macrophages and in macrophages which have been treated with colchicine.

Materials and Methods

Mononuclear Phagocytes. Unstimulated peritoneal macrophages were harvested from male mice of the NCS/PA strain using methods previously described (13, 14). Monolayers of macrophages were maintained in Leighton tubes or glass 30 cm^2 T-flasks in medium 199 containing 30% heat-inactivated newborn calf serum (NBCS)[1] (Grand Island Biological Company, Grand Island, N. Y.) and 1,000 U/ml penicillin G for 24–48 h before use. Medium was changed daily.

Colchicine. Stock solutions of colchicine (Sigma Chemical Co., St. Louis, Mo.) were made up weekly as 10^{-3} M solutions in phosphate-buffered saline (PBS), pH 7.4, and stored at −20°C. Colchicine, at a final concentration of 10^{-6} M, was added to the medium 2 h before initiation of experiments for determination of phagocytosis or lysosomal enzyme transfer, and was present until the cells were harvested for assay of uptake or enzyme transfer.

Quantitation of Phagocytosis. Polyvinyltoluene (PVT) spherules (2-μm diameter) (Particle Information Service, Grants Pass, Oreg.) which had been rinsed repeatedly in PBS were added to macrophage monolayers in Leighton tubes such that the final concentration of PVT varied from 10 μg to 1,300 μg/ml. The tubes were incubated at 37°C and duplicate samples from control and

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[1] *Abbreviations used in this paper:* NBCS, newborn calf serum; PBS, phosphate-buffered saline; PVT, polyvinyltoluene.

colchicine-treated groups were harvested 20, 40, 60, and 120 min after addition of particles. Additional experiments were carried out at 4°C to evaluate the contribution of nonspecific adherence to measured uptake. Monolayers were harvested by rinsing five times with warm PBS and the cell layers digested with 0.5 ml of 0.1 N NaOH solution.

Assay for protein was carried out on 0.1-ml portions using the method of Lowry et al. (15) with crystalline egg white lysozyme as the standard. Filtration through Millipore filters with 0.45 μm pore size was performed to eliminate turbidity due to suspended PVT. Another 0.25-ml portion of the digested monolayers was placed in 12 × 75 mm tubes and evaporated to dryness. To each tube 0.5 ml *p*-dioxane (Matheson, Coleman & Bell, Norwood, Ohio) was added and the tubes allowed to stand for at least 2 h. Absorbance of each specimen was measured in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 267 nm, as described by Weisman and Korn (9), to yield a quantitative estimate of PVT concentration. Uptake of PVT, normalized for cell protein, was determined for the period during which uptake was linear (between 20 and 60 min after addition of particles) and was corrected for nonspecific adherence.

Quantitation of Transfer of Lysosomal Enzymes to Phagolysosomes. Macrophage monolayers were allowed to ingest varying concentrations of PVT particles for 15 min, 45 min, 2 h, or 18 h in T-30 flasks. At the termination of the phagocytic pulse, the medium was decanted and the cells rinsed five times with warm PBS. In several experiments, the cells were rinsed with warm medium 199 and reincubated with fresh medium 199 and 30% NBCS for 16–18 h, after which the cells were rinsed with PBS. After PBS was removed, flasks were placed in an ice bath and ice-cold 10% sucrose containing 10^{-3} M di-sodium EDTA (Fisher Scientific Co., Fair Lawn, N. J.) was added. Cells were removed by scraping with a rubber policeman, the contents of two to three flasks were pooled, and the vol adjusted to 5 ml. After homogenization in a Dounce homogenizer (16) sufficient to disrupt 75% of the cells, the suspension was centrifuged at 2,500 *g* for 10 min at 4°C to sediment intact cells. The supernatant fraction was mixed with cold 50% sucrose such that the final sucrose concentration was 27.5%. A discontinuous sucrose gradient was formed by layering 6.0 ml of the 27.5% sucrose solution containing disrupted cells and PVT over 2.0 ml 50% sucrose in a 14 x 90 mm cellulose nitrate ultracentrifuge tube. 2.0 ml of 10% sucrose followed by 2.0 ml of 5% sucrose were then carefully layered over the 27.5% sucrose layer. The sucrose gradient was centrifuged in a swinging bucket rotor at 27,000 *g* for 45 min at 4°C in an International Model B-60 preparative ultracentrifuge (International Equipment Company, Needham Heights, Mass.).

The ultracentrifuge tubes were then pierced with an ISCO Model 184 tube piercer (Instrumentation Specialties Co. (ISCO), Lincoln, Nebr.), cold 50% sucrose infused through the bottom of the tubes with an infusion pump, and 0.5-ml portions of the gradient collected with an LKB-7000 fraction collector (LKB-Produkter AB, Stockholm, Sweden).

Portions of the original cell suspension, the 2,500 *g* pellet, and the 2,500 *g* supernatant fractions were assayed for protein, PVT, and acid phosphatase. Portions of the fractions collected after 27,000 *g* centrifugation were assayed for PVT and acid phosphatase. Assays for protein and PVT were as described above. Assay for acid phosphatase was based on hydrolysis of alpha naphthyl acid phosphate at pH 5.0 using methods previously described (17).

Uptake of PVT was normalized for cell protein and the acid phosphatase activity in the phagolysosome fraction was normalized for total acid phosphatase activity recovered from the gradient. The amount of PVT recovered from the gradients was determined directly from the sums of the concentrations in each fraction. The amount of protein in the gradient was calculated from the measured concentration of protein in the 2,500 *g* supernatant fractions. "Phagolysosome fraction" refers to fractions of the gradient at the 5–10% sucrose interface in which the majority of the PVT was found. Transfer of acid phosphatase was thus calculated:

$$\frac{\text{acid phosphatase activity in phagolysosome fraction/total acid phosphatase activity in gradient}}{\text{PVT in gradient/calculated protein in gradient.}}$$

Electron Microscope Quantitation of Microtubules. Cytoplasmic microtubule content of cultivated macrophages was quantitated by Eve P. Reaven, Department of Medicine, Veterans Administration Hospital and Stanford University, Palo Alto, Calif., using methods previously described (18). After in vitro cultivation for 48 h, untreated macrophages and macrophages which had been exposed to colchicine for 2 h or 24 h were prepared for electron microscopy. Cell monolayers were rinsed with unbuffered normal saline (22°C) and fixed for 10 min in 1.0%

glutaraldehyde in 0.1 M cacodylate buffer (300 mosM, pH 7.2, 22°C). The cells were scraped from the glass, pelleted by centrifugation at 10,000 *g* for 1 min, placed in a vial with fresh glutaraldehyde, and fixed for 2 h. Cells were then postfixed in 1% osmium tetroxide in Palade's veronal buffer (pH 7.2) for 1 h and stained en bloc for 1 h with 0.5% uranyl acetate in veronal buffer (pH 5.5). Subsequently, cells were dehydrated in graded alcohols and embedded in epon-araldite plastic.

Cells in which the nucleus and Golgi apparatus were visible were randomly selected from thin sections and photographed at a magnification of 16,000. Microtubules were identified on enlarged photographic prints (final magnification, 44,000) and the volume density of microtubules (i.e., the fractional volume of cytoplasm occupied by microtubules) was estimated by stereologic methods (18).

Data Analysis. Straight lines were generated by least mean squares analysis and significance of slope was determined by Student's *t* test. Significance of difference between rates of uptake of PVT in control and colchicine-treated cells was determined by use of Student's *t* test for paired samples. Results are generally expressed as mean ± standard error.

Results

Electron Microscope Quantitation of Microtubular Disruption. Although previous experiments (8) had indicated that 10^{-6} M colchicine produced morphological alterations in macrophages which were compatible with those which would have been expected to result from microtubular disruption, we had no direct evidence that both the early rounding and the later bizarre forms which followed the addition of colchicine were indicative of depolymerization of microtubules. As is illustrated in Fig. 1 A, electron micrographs showed readily demonstrable microtubules in untreated peritoneal macrophages cultivated in vitro for 48 h whereas in vitro exposure to 10^{-6} M colchicine for 2 h was sufficient to abolish formed microtubules (Fig. 1 B). By use of quantitative stereologic evaluation of 16 cells, microtubules were found to occupy 0.09 ± 0.02% of the cytoplasmic volume of untreated macrophages. In contrast, after either 2 or 24 h of treatment with 10^{-6} M colchicine, no formed microtubules were identifiable by this method.

Uptake of PVT. Since it was expected that transfer of lysosomal enzymes to phagolysosomes would be directly related to the numbers of particles ingested, it was first necessary to quantitate the effect of colchicine on ingestion of PVT. If necessary, adjustments in quantities of particles added to the medium could then be made to result in similar accumulation of particles intracellularly in both control and colchicine-treated macrophages. In eight separate experiments in which paired comparisons of uptake were made at varying concentrations of PVT, 10^{-6} M colchicine was found to inhibit uptake of particles by 13.5 ± 6.0% ($P = 0.05$). These data indicated that although colchicine inhibited uptake of PVT by macrophages, the effect was slight and would not markedly interfere with measurements of enzyme transfer.

Transfer of Acid Phosphatase to Phagolysosomes. After centrifugation at 27,000 *g*, all of the PVT was found in the 10% sucrose layer, with the bulk (approximately 85%) just below the interface between 10% sucrose and 5% sucrose. The remainder of the PVT was found at the 10–27.5% sucrose interface (Fig. 2). Since PVT centrifuged in discontinuous gradients without cell material being present also separated into two fractions, the two populations did not reflect alterations in buoyancy that could have been attributable to presence of

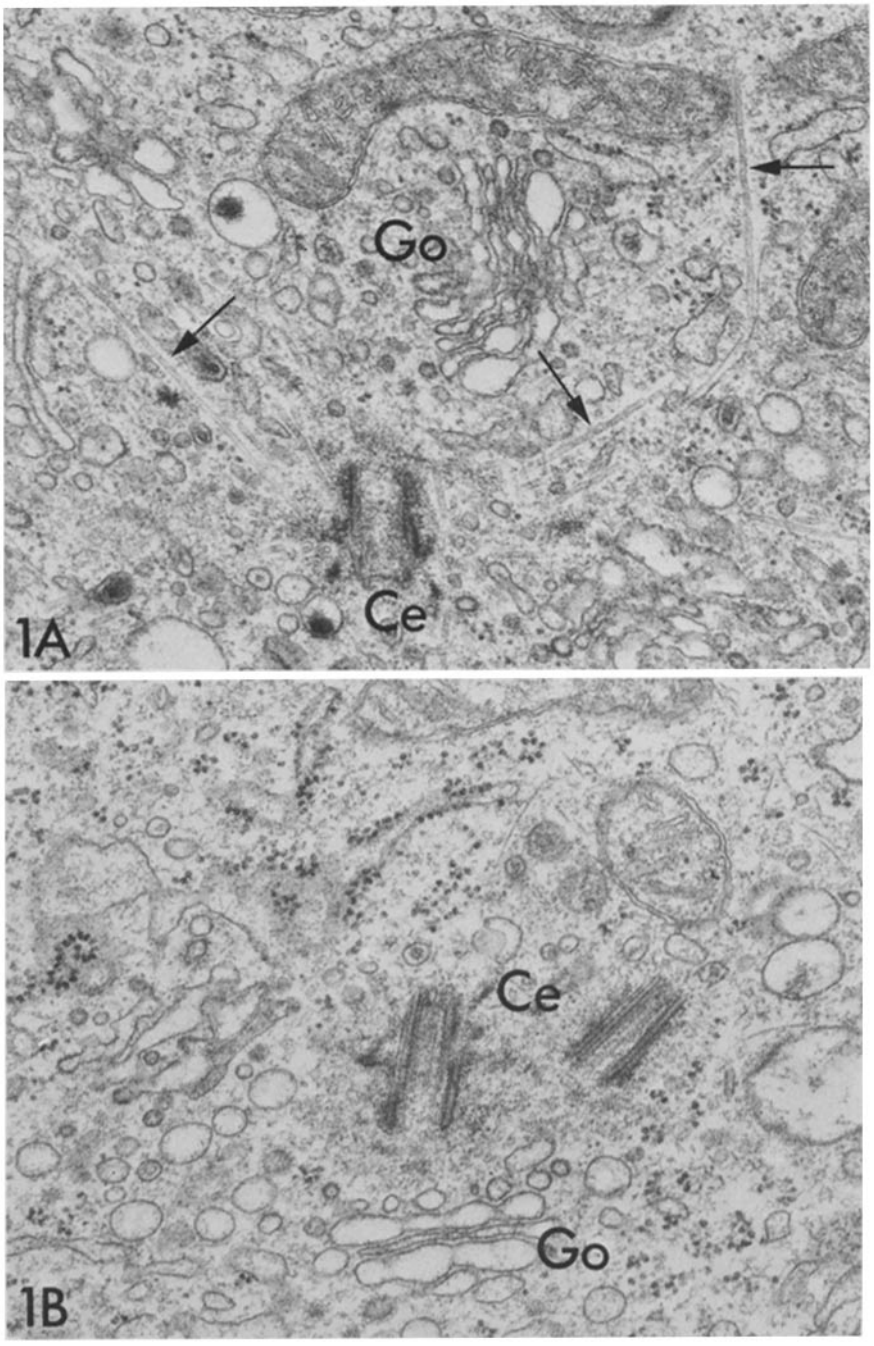

FIG. 1. Golgi (*Go*) and centriolar (*Ce*) regions of mouse peritoneal macrophages cultivated in vitro for 48 h. Glutaraldehyde fixation. × 44,000. (A) Untreated control macrophage. Longitudinal segments of microtubules are shown by *arrows*. (B) Macrophage treated with 1×10^{-6} M colchicine for 2 h. No microtubules are present.

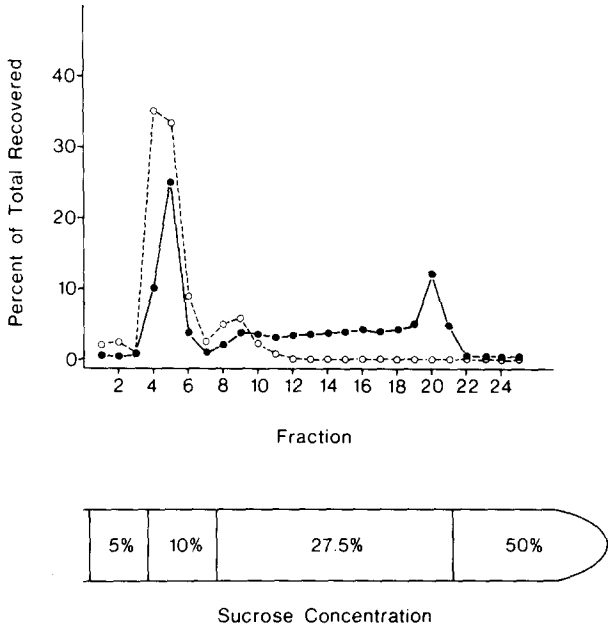

FIG. 2. Phagolysosome separation by discontinuous sucrose gradient fraction of macrophage homogenate. (●), acid phosphatase activity and (○), PVT. The discontinuous sucrose gradient is diagrammatically represented at the bottom of the illustration.

cellular material. Because the less buoyant fraction was found to contain acid phosphatase activity which merged indistinguishably with the enzyme activity in the 27.5% sucrose layer, the enzyme activity associated with this layer was not considered in calculations of extent of transfer of lysososmal enzyme activity to phagolysosomes. Thus, we slightly underestimated the magnitude of the actual transfer, and "phagolysosome fraction" will refer only to the PVT and acid phosphatase found at the 5–10% sucrose interface.

Data for distribution of acid phosphatase activity and PVT in representative gradients in which uptake of PVT per microgram cell protein was low (0.38 μg PVT/μg protein), intermediate (1.41 μg PVT/μg protein), and high (3.35 μg PVT/μg protein) are graphically illustrated in Fig. 3. Acid phosphatase activity associated with phagolysosomes was clearly separated from acid phosphatase activity contained in unfused lysosomes. As is evident in Fig. 3, the fraction of total acid phosphatase activity recovered from the gradient in the phagolysosome fraction increased as PVT uptake increased, while that portion in the unfused lysosomal fraction decreased with increasing PVT uptake. The portion of acid phosphatase activity which did not sediment into either of the two major peaks was not affected by the amount of PVT ingested.

When PVT was added at the time of homogenization of cells less than 4% of total acid phosphatase activity was found in phagolysosome fractions. As the uptake of PVT increased, the fraction of acid phosphatase activity found in phagolysosome fractions increased in a linear fashion. Transfer of acid phosphatase activity to phagolysosome fractions was significantly related to the extent of ingestion of PVT particles but was unaffected by prior treatment of macro-

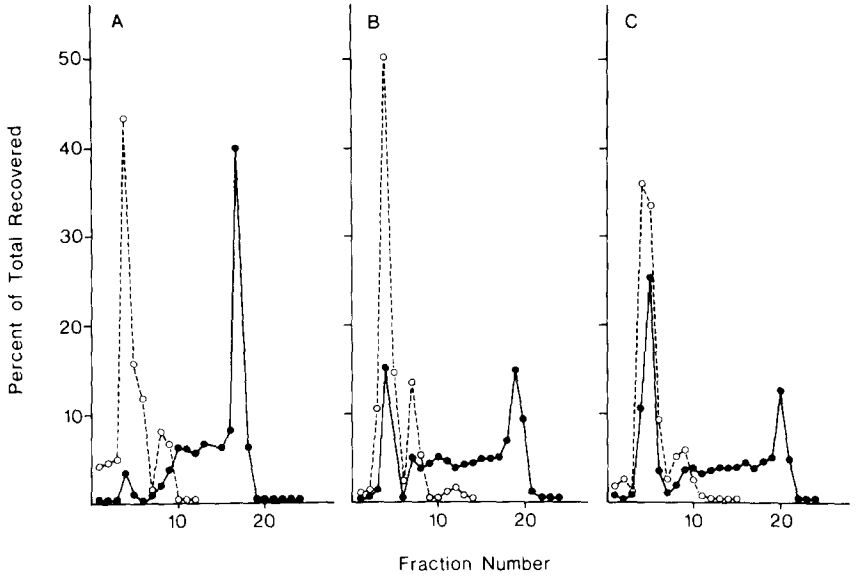

FIG. 3. Effect of particle uptake on transfer of acid phosphatase (●) to PVT-containing phagolysosomes (○). (A) Low PVT uptake (0.38 μg PVT/μg protein), (B) intermediate PVT uptake (1.41 μg PVT/μg protein), and (C) high PVT uptake (3.35 μg PVT/μg protein).

phages with 10^{-6} M colchicine or by duration of the phagocytic pulse (Fig. 4 and Table I). Although the line in Fig. 4 which summarizes the best fit relationship between lysosomal enzyme transfer and PVT uptake was calculated from all data points, it can be seen in Table I that the coordinates of the lines which were generated using data from the various subgroups were virtually identical. If the phagocytic pulse was varied between 15 min and 2 h in both control and colchicine-treated cells, approximately 10% of the total acid phosphatase activity recovered from the gradient was found in the phagolysosome fractions for each unit increment in PVT uptake. In addition, prolongation of the incubation to 18 h, either in the presence of PVT in the medium or in fresh medium after a 2 h phagocytic pulse, did not alter the direct relationship between uptake and enzyme transfer in control macrophages.

Although acid phosphatase activity recovered from 27,000 *g* gradients was less than the total activity applied to the gradient in all experiments, the efficiency of recovery was not different in control (60.0 ± 2.9%) and colchicine-treated (57.6 ± 3.1%) cells. Recovery of PVT from the gradient was almost complete in both groups (89 ± 2%). In addition, efficiency of recovery of acid phosphatase was not related to the uptake of PVT ($0.4 > P > 0.3$).

Discussion

These experiments approached directly the role of microtubules in the fusion of lysosomes with endocytic vesicles. With the exception of one previous statement that colchicine did not inhibit formation of phagolysosomes in polymorphonuclear leukocytes (19), all other data concerning the possible microtubular mediation of lysosomal fusion have relied on indirect measures of fusion or have

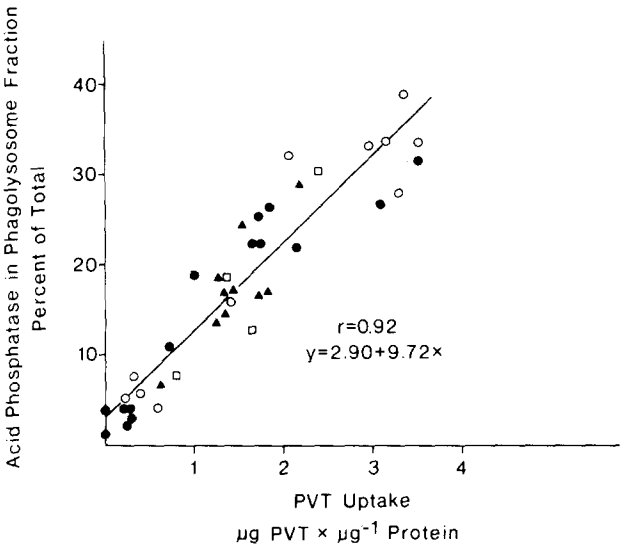

FIG. 4. Relationship between acid phosphatase transfer to phagolysosomes and uptake of PVT particles for control (○, ●, □) and colchicine-treated (▲) macrophages. Time allowed for phagolysosome formation in untreated macrophages was 15 min or 45 min (□), 2 h (●), or 18 h (○), and for colchicine-treated macrophages was 2 h (▲).

TABLE I

Transfer of Acid Phosphatase to Phagolysosome Fraction

	Time with PVT	Y intercept	Slope	P*	n‡
Control	15 or 45 min	1.38	10.70	<0.01	4
	2 h	3.57	9.14	<0.001	13
	18 h	2.40	9.50	<0.001	11
Colchicine	2 h	1.04	10.39	<0.005	10

* P, significance of relationship between acid phosphatase transfer (Y) and PVT uptake (X).

‡ n, number of experiments.

been based on processes which were presumed to be analogous to intracellular fusion (1–8, 20). The first suggestion that microtubules might play a role in lysosomal fusion in phagocytic cells was based on morphologic evidence showing that colchicine inhibited degranulation in phagocytosing polymorphonuclear leukocytes (1). However, using macrophages, Bhisey and Freed (21, 22) could find no morphologic evidence that colchicine inhibited fusion of lysosomes with endosomes.

A number of studies designed to test the role of microtubules in lysosomal fusion have been based on the assumption that mechanisms underlying the exocytosis of lysosomal enzymes into the medium, which can be induced in a variety of ways, are identical to those which promote intracellular fusion of lysosomes with endosomes. In these models, phagocytic cells attempt to ingest

objects such as aggregated globulin-treated surfaces (4, 5, 20) which are too large to be interiorized, or ingestion of inert particles is blocked by treatment of phagocytes with cytochalasin B (4, 6). In either case, the phagocytes secrete lysosomal enzymes into the medium. Although the majority of these studies have concluded that colchicine inhibited fusion of lysosomes with cell membranes (4–6) no inhibitory effect was noted in at least one study (20). In addition, in some studies, little attention seems to have been paid to dose of colchicine. Although we and others (23) have found that concentrations as low as 10^{-6} M are sufficient to disrupt microtubules, experiments demonstrating inhibition of exocytosis have been conducted with colchicine concentrations as high as 5×10^{-4} M (6) and no data were presented to show that such high concentrations were necessary to disrupt microtubules in the cell type under study. It has been shown that colchicine is able to interact with cell membranes and affect membrane-mediated functions as well as to bind tubulin and prevent polymerization of microtubules (24, 25). Thus, it is not clear that experiments in which colchicine concentrations of 10- to 1,000-fold in excess of those required to disrupt microtubules have been used demonstrated effects of colchicine that were related to microtubular disruption.

Although fusion between lysosomes and plasma membrane is required for both exocytosis and phagolysosome formation, it is quite possible that the two processes have differing requirements. That colchicine inhibits exocytosis in many systems but does not inhibit phagolysosome formation in either macrophages or polymorphonuclear leukocytes (19) makes this a likely possibility.

The lack of inhibition of phagolysosome formation found in this study confirms the results of our previous work (8). In those studies, we had reasoned that inhibition of lysosomal fusion should result in diminished degradation of materials ingested by endocytosis. However, we were unable to detect any alteration in the rate of degradation of heat-killed radiolabeled bacteria within colchicine-treated macrophages. In other experiments, macrophages were allowed to interiorize radiolabeled sucrose. Since the cells lack invertase, radiolabeled sucrose served as a relatively stable marker for secondary lysosomes. Addition of invertase to the medium was followed by interiorization of the enzyme within pinosomes and fusion of these pinosomes with sucrose-containing lysosomes. The resultant hydrolysis of sucrose to diffusible monosaccharides caused loss of radiolabel from the cells. Those experiments, too, showed no inhibitory effect of colchicine treatment on lysosomal function in macrophages.

The data in this publication confirm and extend the observations of Stossel et al. (11, 12) that lysosomal fusion occurs within a very short time after particle interiorization, and that prolonged intracellular residence is not required for and does not increase transfer of lysosomal enzymes to phagocytic vesicles. In alveolar macrophages (12) and in polymorphonuclear leukocytes (11), transfer of enzyme to phagolysosomes paralleled particle uptake during the first 1–2 h after initiation of phagocytosis. Enzyme transfer ceased when the medium was changed and the cells incubated in particle-free medium. With addition of more particles, enzyme transfer again resumed. Our experiments with mouse peritoneal macrophages confirm these observations and, in addition, extend the time range during which enzyme transfer is related only to numbers of interiorized

particles through 18 h after initiation of phagocytosis. These results suggest that microtubule-mediated directed flow of endosomes and lysosomes is not required for normal lysosomal fusion, and that interiorization of inert particles is alone a sufficient stimulus to promote fusion of lysosomes with endocytic vesicles.

It remains possible, however, that intracellular fusion of lysosomes with endosomes is susceptible to modulation. Boxer et al. (26) have suggested that microfilaments act to limit the extent of fusion of lysosomes with phagosomes and that defects in microfilament structure or function could enhance the fusion process. Edelson and Cohn (27) have noted apparent inhibition of pinolysosome formation after treatment of macrophages with concanavalin A. In addition, electron-microscope studies (28–31) have indicated that fusion of lysosomes with phagosomes does not always follow ingestion of certain parasites which are capable of intracellular replication. These data suggest that both inhibition and facilitation of lysosomal fusion are possible, but that agents active on the membrane of the endocytic vesicle or on immediately adjacent structures, such as microfilaments, would more likely be responsible. Available data indicate that microtubules are unlikely to play a critical role in intracellular fusion of lysosomes with endosomes in either polymorphonuclear leukocytes (18) or peritoneal macrophages.

Summary

Intracellular lysosomal fusion has been evaluated in cultivated mouse peritoneal macrophages by measurement of transfer of acid phosphatase to polyvinyltoluene (PVT)-containing phagolysosomes. Enzyme transfer was found to be directly and significantly related to the uptake of PVT and to be independent of time allowed for phagolysosome formation over time periods of 15 min to 18 h. In addition, the extent of transfer of lysosomal enzyme to phagolysosomes was unaffected by treatment of the cells with 10^{-6} M colchicine, a dose which eradicates morphologically identifiable microtubules in this cell type within 2 h. The data indicate that intracellular fusion of lysosomes with phagosomes in the macrophage does not require formed microtubules and suggest that fusion occurs promptly after interiorization of inert particles.

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