(24) W. I. Higuchi and C. T. O'Konski, *J. Colloid Interface Sci.*, **15**, 14 (1960).

(25) G. M. Pound, L. A. Madonna, and C. M. Sciulli, "Proceedings of the Conference on Interfacial Phenomena and Nucleation," Vol. 1, p. 85 (1955). Office of Technical Services, U.S. Dept. of Commerce, U.S. Research and Development Command, Cambridge, Mass.

(26) M. J. D. Powell, in "Numerical Methods for Nonlinear Algebraic Equations," P. Rabinowitz, Ed., Gordon & Breach Science Publishers, London, 1970.

(27) P. Jarratt, in "Numerical Methods for Nonlinear Algebraic Equations," P. Rabinowitz, Ed., Gordon & Breach Science Publishers, London, 1970.

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Ultrastructural Alterations in Macrophages after Phagocytosis of Acrylic Microspheres

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Abstract □ The effect of microparticles on the survival of cultured mouse peritoneal macrophages was investigated using doses of 0.01–0.1 mg of lyophilized particles/ml of medium and 5×10^5 cells, corresponding to ~4000–40,000 particles per cell. The lowest dose did not significantly change the survival time as compared with the controls, while ~75% of the cells were lost during the first 48 h on exposure to the highest dose. High doses of particles induce cellular damage. The morphology and stability of the lysosomal apparatus was followed with electron microscopy, acid phosphatase cytochemistry, and acridine orange uptake. Alteration of the lysosomal vacuome was characterized by a greatly enhanced rate of autophagocytosis, the formation of huge secondary lysosomes containing microparticles, and labilization of the vacuome with loss of acidity and a tendency to leak acid phosphatase into the cell sap.

Keyphrases □ Microspheres—polyacrylamide, phagocytosis by cultured mouse peritoneal macrophages, ultrastructural cellular alterations □ Macrophages, peritoneal—cultured from mice, effect of phagocytosis of microparticles, ultrastructural cellular alterations □ Phagocytosis—microparticles, by cultured mouse peritoneal macrophages, ultrastructural cellular alterations

Acrylic polymer microparticles have been introduced recently as carriers for enzymes and other macromolecules (1). When such particles are injected intravenously into mice and rats they are eliminated rapidly from the circulatory system, mainly by the fixed macrophages of the reticuloendothelial system (RES) (2), and accumulate intracellularly within the lysosomal vacuome of these cells (3). This lysosomotropic character of the particles has been utilized in an experimental animal model to effect an artificial storage disease (4).

When massive doses of particles (160 mg/kg of body weight) were injected intravenously into mice, some adverse reactions occurred (3). The first general reaction was a megaly of the liver and spleen. This phenomenon, detected by light and electron microscopy, was due to initial cell damage with mitochondrial swelling, rupture of the cristae, and cellular edema. This was later followed by cellular necrosis and invasion of inflammatory cells (3). The megaly was thus due to both cellular swelling and accumulation of inflammatory cells in the affected organs. The microscopic study also showed that the megaly was reversible and that the normal anatomical structure of the tissues was restored in ~4 weeks. The effects detected are dose dependent and are not seen in mice after injection of moderate doses (40 mg/kg) (3).

The ultrastructural changes detected in the liver, spleen, and bone marrow after injection of large amounts of acrylic microspheres are of general interest, as they can be expected to occur on injection of any small-sized particles. Even injection of relatively rapidly degradable liposomes give rise to ultrastructural changes in the liver and spleen (5).

The relationship between the dose of particles and the cellular effect is not conveniently studied in animal models, where the relationship between particles and affected cells cannot be quantitatively controlled. It is therefore, necessary to develop a method in which the number of parti-

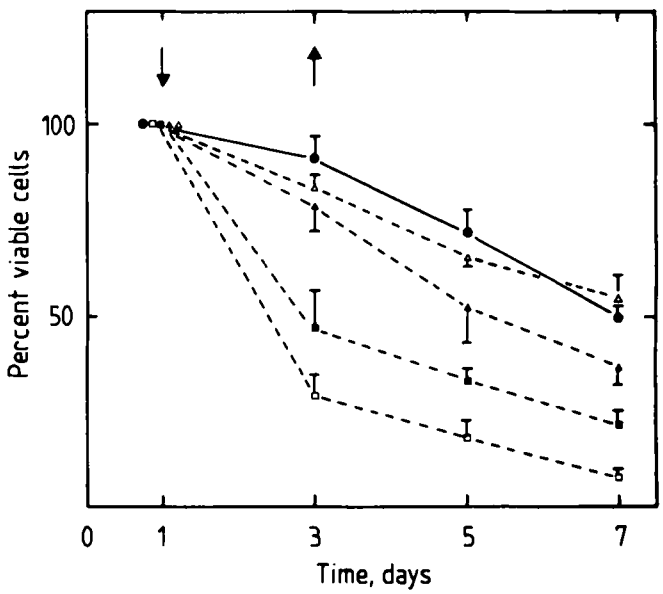

Figure 1—*Survival rates of cultured mouse peritoneal macrophages after exposure to microparticles at dose levels of 0.01 (△), 0.02 (▲), 0.05 (■), and 0.1 mg/mL (□). The normal survival rate (●) was followed during a period of 7 d. The arrows indicate the time of exposure to microparticles. Each point represents the mean ±SD from four experiments.*

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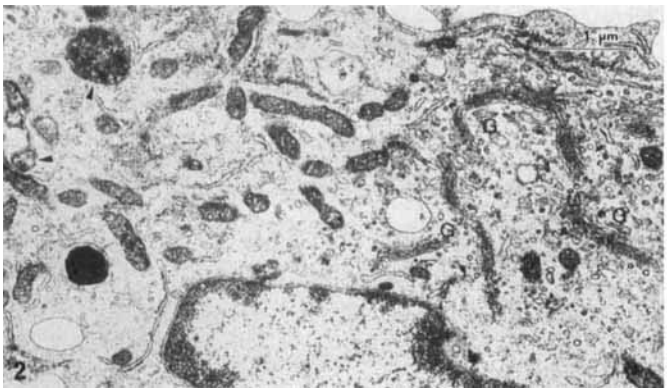

Figure 2—*Normal mouse macrophage cultured* in vitro, *showing elongated mitochrondria and secondary lysosomes (arrowheads). G denotes the Golgi apparatus.*

cles and phagocytic cells can be easily determined. The present paper describes a system in which mouse peritoneal macrophages in culture have been used to semiquantitatively study the effects of particles on cellular ultrastructure. The cellular alterations in the macrophages after exposure to particles were further correlated with biochemical and cytochemical changes in the cells.

EXPERIMENTAL

Acrylamide[1], *N,N'*-methylenebisacrylamide[1], cationized ferritin[2], *N,N,N',N'*-tetramethylethylenediamine[2], and other chemicals were of analytical grade. Pathogen-free male mice[3], weighing 20 g, were used throughout.

Preparation of Microparticles—Microparticles with immobilized cationized ferritin were prepared using a reported method (1, 3). The cationized ferritin (104 mg), acrylamide (0.3 g), and *N,N'*-methylenebisacrylamide (0.1 g) were dissolved in 5 mL of 0.005 M sodium phosphate buffer, pH 7.4, while maintaining a nitrogen atmosphere. The catalyst, ammonium peroxodisulfate (100 µL of a 0.5-g/mL solution in water) was added, the solution was poured into 200 mL of toluene–chloroform (4:1) containing 0.5 g of a detergent[4], and the resulting mixture homogenized to produce an oil-in-water emulsion. The accelerator *N,N,N',N'*-tetramethylethylenediamine (1.0 mL) was added to the emulsion, and after a few minutes polymerization started. The suspension was magnetically stirred for 20 min, and the microparticles were isolated by centrifugation and washed several times with buffer. After the last washing the particles were suspended in sterile physiological saline supplemented with antibiotics. Using this method, the microparticles had diameters ranging from 0.3–0.5 µm.

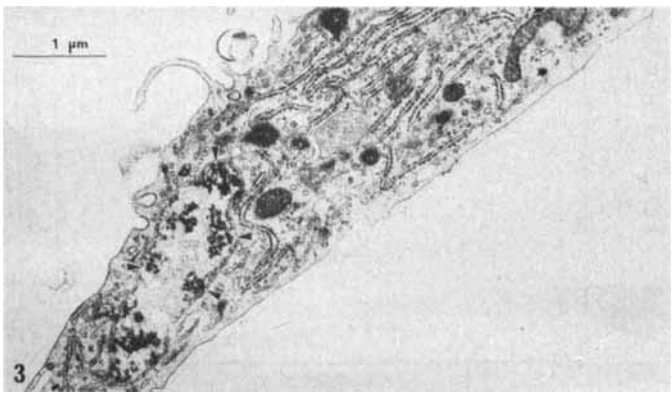

Figure 3—*Macrophage 72 h after exposure to ferritin-labeled microparticles (0.1 mg/mL of medium) showing a large vacuole containing ferritin-labeled particles (arrowheads).*

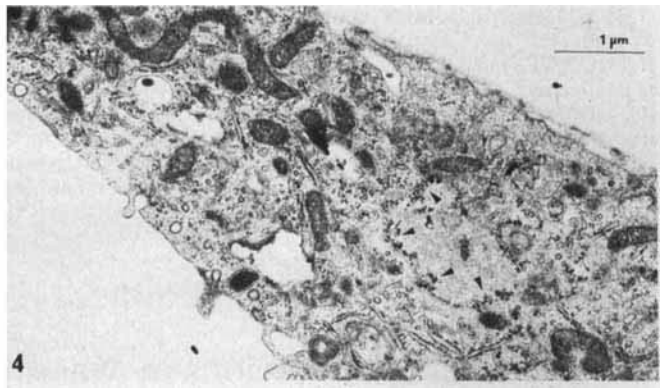

Figure 4—*Macrophage 72 h after exposure to ferritin-labeled microparticles (0.1 mg/mL). Note the localization of ferritin particles to the periphery of a large secondary lysosome (arrowheads).*

Preparation of Peritoneal Macrophage Cultures—Mouse peritoneal macrophages were collected by washing the unstimulated peritoneum of adult male mice with 3–4 mL of warm (37°C) phosphate-buffered saline, as described previously (3). The peritoneal exudate cells were suspended in 10 mL of cold (4°C) phosphate-buffered saline and centrifuged at 180× *g* for 10 min. The pellet was resuspended in a nutrient medium[5] (6) containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4. The medium was supplemented with penicillin G (100 U/mL), streptomycin (10 µg/mL), and 20% fetal calf serum. The cells were plated into 35-mm plastic dishes[6] at a concentration of 5×10^5 cells per dish and cultured at 37°C in air (85% relative humidity) containing 5% CO_2. After incubation for 2 h at 37°C, nonadherent cells were removed by washing with complete culture medium and fresh medium was added. The cultures were then incubated for 24 h. After that time, the medium was again changed and microparticles were added. During the ensuing cultivation period the medium was changed every 48 h.

Macrophage cultures were exposed to 0.01–0.1 mg/mL of microparticles containing ferritin as a marker. The cells were exposed to these particles for 24 or 48 h. To follow the number of viable and stretched macrophages in the dishes during the cultivation period, the mean number of cells counted in 10 different parts of the dishes was estimated every second day (before the medium was changed). With the aid of a stencil, 10 circles (diameter 3 mm) were drawn on the bottom of each dish in such a way as to ensure even distribution, but avoiding the exact center and the extreme periphery. In the center of each of these circles the cells were counted on a circular area of 0.24 mm^2 using an inverted phase-contrast microscope[7] and a 32× objective lens.

Preparation of Macrophages for Transmission Electron Microscopy—For morphological studies, the cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, pH 7.2 (total osmotic pressure 510 mOsm, effective osmotic pressure 300 mOsm) (7, 8) for 15 min at 37°C and for 45 min at 4°C. They were then rapidly rinsed in 0.15 M cacodylate buffer and postfixed in 1% OsO_4 in 0.15 M cacodylate buffer (pH 7.2) for 90 min at room temperature. The cells were then dehydrated *in situ* in an ethanol series, counter-stained *en block* for 12 h in 1% uranyl acetate in 50% ethanol, and embedded in plastic as described earlier (9). After polymerization, thin sections for transmission electron microscopy were cut with diamond knives, stained with lead citrate according to Reynolds (10), and examined at 60 kV in an electron microscope[8].

Demonstration of Acid Phosphatase—Coverslips with attached macrophages were fixed for 1 h at 0°C in 2% glutaraldehyde in 0.1 M sodium cacodylate–HCl buffer with 0.1 M sucrose (pH 7.2; total osmolality 510 mOsm, vehicle osmolality 300 mOsm), rinsed for 2 h in several changes of 0.1 M cacodylate buffer with 0.1 M sucrose (pH 7.2; 300 mOsm, 0°C), and incubated in a Gomori-type medium for acid phosphatase without the addition of sucrose or dimethyl sulfoxide to the incubating medium (7, 11, 12). The cells were incubated for 30, 45, and 60 min, and the secondary reaction product (lead phosphate) was converted to lead sulfide with a 1% solution of ammonium sulfide. After a light counterstaining with hematoxylin, the cells were dehydrated, cleared, and mounted in a synthetic resin.

[1] Eastman Kodak Co.
[2] Sigma Chemical Co.
[3] NMRI-mice; Anticimex, Stockholm, Sweden.
[4] Pluronics F-68 (polyoxyethylene-derived polyoxypropylene).
[5] F-10 medium; GIBCO BIO-CULT.
[6] Nunc. petri dishes.
[7] Leitz microscope.
[8] Philips 201.

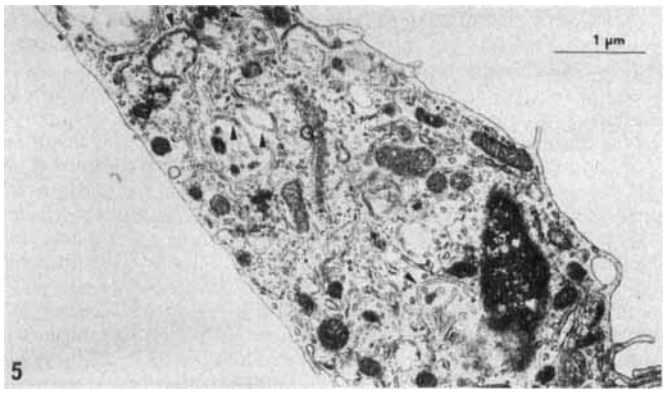

Figure 5—*Macrophage 72 h after exposure to microparticles. Note (arrowheads) the excessive formation of multilayer membranes wrapping around cytoplasmic areas (formation of autophagic vacuoles). G denotes a Golgi area.*

Visualization of the Lysosomal Vacuome with Acridine Orange—Coverslips with attached macrophages were cultured in plastic dishes[6]. Acridine orange was added to the medium to give a final concentration of 10 μg/mL. The cells were exposed to the dye for 10 min, whereupon the medium was changed twice. The cells were kept in fresh medium for 30 min in the dark in the incubation cabinet; the coverslips were then inverted on a drop of medium on an objective glass and rapidly examined under a fluorescent microscope[7] equipped with a high-pressure mercury lamp, a 5-mm BG-12 emission filter, and a 510-nm barrier filter. Acridine orange in lysosomes gives a bright red granular fluorescence, but in cells with a damaged lysosomal vacuome the nuclei and the cytoplasm show a diffuse green fluorescence with few remaining red granules. Micrographs were taken using black and white film[9].

RESULTS

Effect of Polyacrylamide Microspheres on the Survival of Cultured Mouse Peritoneal Macrophages—The effect of various doses (0.01, 0.02, 0.05, and 0.1 mg/mL of medium, total culture medium 2 mL) of polyacrylamide particles on the survival of macrophages is shown in Fig. 1. The cells were exposed to the particles over a 48-h period. As seen in the figure, the difference in survival between controls and macrophages treated with 0.01–0.02 mg/mL of microspheres is not significant, while concentrations of >0.02 mg/mL cause a rapid decline in survival rate. The significance of the difference was verified by variance analysis, $p < 0.01$. The highest dose (0.1 mg/mL) initiates a rapid decline in the number of viable and stretched macrophages. After an exposure time of 48 h, only ~30% of the initial cells remained, while the controls showed 90–100% viability.

Transmission Electron Microscopy—The control macrophages showed a normal ultrastructural appearance with extensive surface projections, many of which folded back on the plasma membrane under formation of macropinocytotic vacuoles. The nuclei were bean shaped with a euchromatic pattern and closely localized to them were several well-developed Golgi areas. The mitochondria were elonged, the endoplasmic net was regular, and the cells contained an abundance of secondary lysosomes with a moderate electron-dense, irregular matrix without any ferritin-like structures. Many of the lysosomes were large, and liquid droplets were observed (Fig. 2).

Following ingestion of ferritin-labeled microparticles, most secondary lysosomes were moderately enlarged and showed accumulations of ferritin particles in clusters (Fig. 3). The ferritin particles were usually localized near the periphery of the secondary lysosomes and sometimes formed a rim along the whole circumference (Fig. 4). In the cells exposed to acrylic microspheres there was a greatly enhanced rate of autophagocytosis with many flattened saccules wrapping around cellular structures during formation of autophagosomes (Fig. 5.)

Acid Phosphatase (Gomori Staining)—Control cells showed a distinct granular reaction product pattern with almost no diffuse staining and no nuclear impregnation. The cells had the same appearance 48 and 72 h after initiating the cultures.

Cells exposed to spheres in various concentrations (0.01, 0.05, and 0.1 mg/mL of cell medium) deviated from the control cells by a reduction

[9] Agfa, black and white 27 Din 400 ASA film.

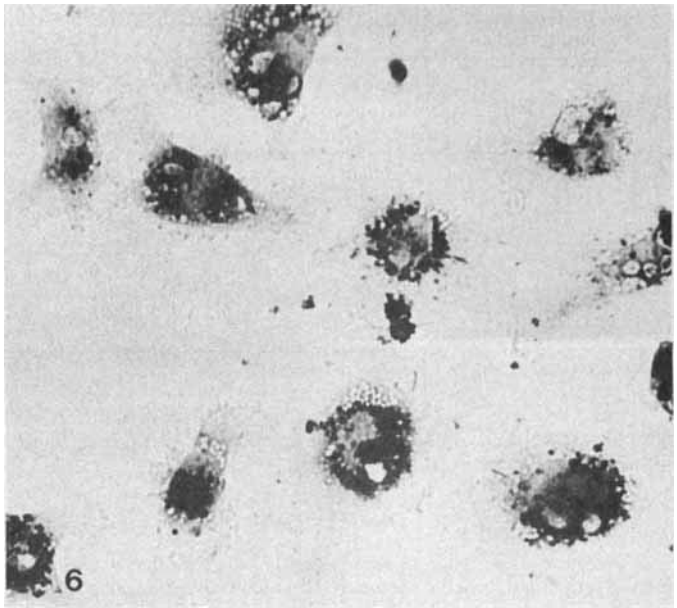

Figure 6—*Macrophages 48 h after exposure to microparticles (0.01 mg/mL). Reaction product, indicating acid phosphatase activity, is localized in granular sites in a way similar to control cells. Original magnification 640×.*

of granularity and an increase of diffuse cytoplasmic staining. The effect was dose dependent, with little or no effect at the 0.01-mg/mL dose (Fig. 6) and pronounced effect at the 0.1-mg/mL dose (Fig. 7). After 48 h many cells exposed to the high dose showed few remaining granular reaction sites and intense diffuse cytoplasmic staining. Many of these cells were becoming round and were obviously degenerating and dying. A number of cells in the 0.05- and 0.1-mg/mL groups contained no reaction product at all, neither granular nor diffuse.

Acridine Orange Uptake—Control cells showed distinct, red cytoplasmic granular fluorescence and weak nuclear green fluorescence. The red granular fluorescence corresponded well with the Gomori lysosomal pattern.

After treatment with high doses (0.05 and 0.1 mg/mL) of microparticles for 24 h, several cells, still being well stretched as detected by phase-contrast microscopy, had lost their granulated red fluorescence. As seen in Table I, this effect was dose dependent. Thus, 17% of the remaining

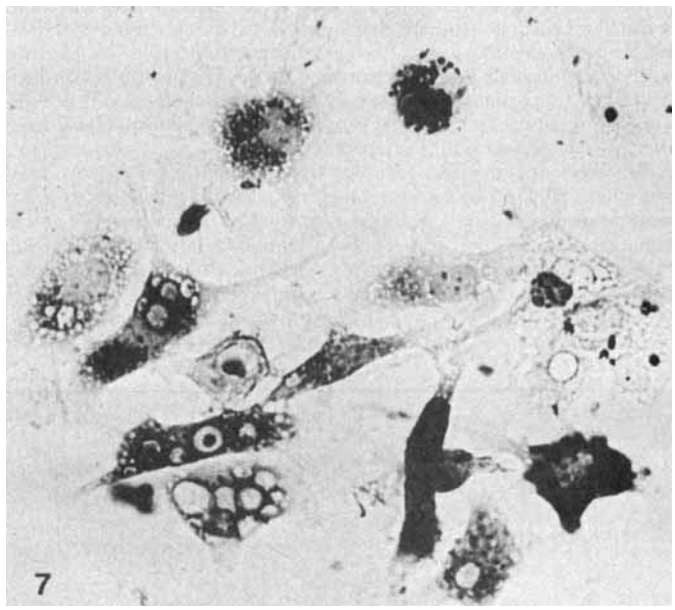

Figure 7—*Macrophages 24 h after exposure to microparticles (0.1 mg/mL). Compare with Fig. 6 and note cells with nongranular, diffuse reaction product as well as several almost unreactive cells. Original magnification 640×.*

Table I—Uptake of Acridine Orange by Macrophages Exposed to Microparticles

Exposure Time, h	Dose of Microparticles, mg/mL	Well-Stretched Cells with Granular Red Fluorescence, %[a]
24	Control	98.6 ± 0.0
	0.01	94.3 ± 3.6
	0.05	78.2 ± 5.5
	0.1	83.6 ± 4.2
48	Control	100 ± 0.0
	0.01	98.3 ± 1.1
	0.05	93.0 ± 2.1
	0.1	84.2 ± 2.3

[a] Mean ± *SE*; $n = 10$.

stretched cells exposed to a 0.1-mg/mL dose for 24 h contained no red granules at all and almost no green cytoplasmic or nulcear fluorescence.

DISCUSSION

The results presented in this study clearly show that the adverse effects on the reticuloendothelial system (RES) of massive doses of acrylic microspheres, previously demonstrated in mice (3), can be studied in greater detail in cultures of mouse peritoneal macrophages *in vitro*. The toxic reactions detected in these culture cells are strictly dose related and detectable only when the cells (5×10^5 cells/dish) are exposed to >0.01 mg of lyophilized particles/mL of medium (total culture medium per dish is 2 mL). From a particle density of 1.14 mg/mL (13) and a mean diameter of 0.25–0.3 μm, it can be estimated that 0.01 mg contains $\sim 1 \times 10^9$ particles. This means, that the ratio between the number of particles and cells is ~4000 when the cellular alterations can first be detected. In a previous study (3), it was shown that an intravenous injection of 1 mg of particles to mice (weighing ~20 g) produced only insignificant changes in the liver, spleen, and bone marrow. It was also shown that 50% of the injected dose was localized to the liver. This means that $\sim 50 \times 10^9$ particles were distributed to and taken up by the macrophages of the liver. It is reported by Knook and Sleyster (14) that the liver from a young rat contains 10×10^7 Kupffer cells per gram of tissue, and if we assume that the mouse liver is commensurable, it means that ~5000–6000 particles were taken up per phagocytosing cell before any reaction was detected in the tissue. The results of the present study thus indicate that cultured peritoneal macrophages are more sensitive to exposure of particles than fixed macrophages of the RES and that they may serve as a sensitive tool for studying the toxicity of microparticles.

The microspheres were phagocytosed by the macrophages and accumulated within large secondary lysosomes. Since the acrylic polymer itself has very little electron density, the particles had to be labeled with ferritin to allow visualization in the electron microscope. The uneven distribution of the ferritin particles within the secondary lysosomes (mostly peripheral) is probably due to partial resolution of the microspheres during dehydration and embedding in plastic[10].

The most drastic effect seen was the enormously enhanced rate of autophagocytosis seen in macrophages exposed to the largest concentration of microspheres. Autophagocytosis is a normal process and is enhanced as an unspecific response to a broad variety of cell-damaging processes (15). As judged from the cytochemical demonstration of the lysosomal enzyme acid phosphatase, there are indications of enhanced fragility of secondary lysosomes. Many exposed cells exhibited diffuse activity and few granular reaction sites. This may reflect alterations that occurred in a more pronounced way in the exposed cells than in control cells during fixation and the cytochemical procedure. It may, however, also indicate leakage of lysosomal enzymes from dilated secondary lysosomes already in culture as a response to the heavy loading of the lysosomal vacuome with microparticles. Such a conclusion is supported by the congruent result obtained with acridine orange showing that many exposed cells have few or no secondary lysosomes with the normal low pH. If so, such a leakage of hydrolytic lysosomal enzymes may contribute to the cell damage and stimulate the demonstrated autophagocytosis.

After exposure to high doses of microparticles some cells did not reveal any acid phosphatase at all with the Gomori method. About the same number of cells failed to accumulate the weak base acridine orange, which normally accumulates in the acidic milieu of the lysosomal vacuome (16). These findings suggest that some cells have completely lost the normal content of their lysosomal vacuome as a result of particle phagocytosis. Macrophages exposed to a variety of stimuli are subject to escape of the lysosomal enzymes during phagocytosis. This is referred to as regurgitation during feeding (17–19). Such regurgitation may possibly become extreme and lead to complete disappearance of lysosomal control, resulting in cells without the positive reaction for acid phosphatase and without uptake of acridine orange. Whether such cells will regain normal lysosomal content or will die and detach from their substratum is unknown.

[10] Epon.

REFERENCES

(1) P. Edman and I. Sjöholm, *J. Pharmacol. Exp. Ther.*, **211**, 663 (1979).

(2) I. Sjöholm and P. Edman, *J. Pharmacol. Exp. Ther.*, **211**, 656 (1979).

(3) P. Edman, I. Sjöholm, and U. Brunk, *J. Pharm. Sci.*, **72**, 658 (1983).

(4) P. Edman and I. Sjöholm, *Life Sci.*, **30**, 327 (1982).

(5) T. de Barsy, P. Devos, and F. van Hoof, *Lab. Invest.*, **34**, 273 (1976).

(6) R. G. Ham, *Exp. Cell Res.*, **29**, 515 (1963).

(7) B. Arborgh, P. Bell, U. Brunk, and V. P. Collins, *J. Ultrastruct. Res.*, **56**, 339 (1976).

(8) V. P. Collins, B. Arborgh, and U. Brunk, *Acta Path. Microbiol. Scan. A*, **85**, 157 (1977).

(9) U. Brunk, J. L. E. Ericsson, J. Pontén, and B. Westermark, *Exp. Cell Res.*, **67**, 407 (1971).

(10) E. S. Reynolds, *J. Cell Biol.*, **17**, 208 (1963).

(11) U. Brunk and J. L. E. Ericsson, *Histochem. J.*, **4**, 349 (1972).

(12) T. Barka and P. J. Anderson, *J. Histochem. Cytochem.*, **10**, 741 (1962).

(13) B. Ekman and I. Sjöholm, *J. Pharm. Sci.*, **67**, 693 (1978).

(14) D. L. Knook and E. C. Sleyster, *Biochem. Biophys. Res. Commun.*, **96**, 250 (1980).

(15) J. L. E. Ericsson, in "Lysosomes in Biology and Pathology," Vol II, J. T. Dingle and H. B. Fell, Eds., 1969, p. 345.

(16) D. de Duve, B. Poole, A. Trouet, P. Tulkens, and F. van Hoop, *Biochem. Pharmacol.*, **23**, 2495 (1974).

(17) B. C. Page, P. Davies, and A. C. Allison, *Arch. Oral. Biol.*, **18**, 1481 (1973).

(18) N. R. Acherman and J. R. Beebe, *Nature (London)*, **247**, 475 (1974).

(19) H. U. Schlorlemner, B. Burger, W. Hylton, and A. C. Allison, *Br. J. Exp. Pathol.*, **58**, 313 (1977).

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