

CHANGES IN CELLULAR ENZYME LEVELS AND EXTRACELLULAR
RELEASE OF LYSOSOMAL ACID HYDROLASES IN
MACROPHAGES EXPOSED TO GROUP A
STREPTOCOCCAL CELL WALL SUBSTANCE*

BY PHILIP DAVIES, ROY C. PAGE,† AND A. C. ALLISON

(From the Division of Cell Pathology, Medical Research Council, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, and the Departments of Pathology and Periodontics and the Center for Research in Oral Biology, University of Washington, Seattle, Washington 98105)

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Infection by group A streptococci may lead to chronic inflammatory lesions of the myocardium, heart valves, joints, and skin. The pathogenesis of these lesions remains uncertain (1), and several experimental animal models have been used to study chronic inflammation induced by group A streptococci and their extracellular products (2). Cell wall preparations containing only type-specific polysaccharide and peptidoglycan (PPG)¹ have the capacity to induce chronic-recurring granulomatous lesions in the skin of rabbits (3), lesions resembling those of rheumatic fever in the hearts of mice (4) and rabbits (5), and severe polyarthritis in rats (6). Available evidence indicates that PPG induces inflammatory lesions by direct interaction with infiltrating leukocytes, rather than by an immunopathologic mechanism (7). In experimental lesions, PPG becomes associated with macrophages (3), and it appears that these cells lack the ability to digest the material (5, 8–11). These observations have led us to investigate the effects of PPG on mouse peritoneal macrophages maintained in culture. We have found that PPG, at concentrations which do not appear to be cytotoxic, induces marked morphologic and biochemical changes in these cells. They undergo a three- to fourfold increase in size, and there is a significant elevation in the levels of acid hydrolases and other cellular enzymes. Furthermore, there is a selective release of the hydrolases into the extracellular environment with no detectable loss of cell viability.

Materials and Methods

Materials.—Bovine pancreatic ribonuclease (type I-A), twice crystallized pancreatic trypsin (type III), phenolphthalein glucuronic acid 0.01 M, pH 7.0; *o*-nitrophenyl- β -D-galactopyranoside, leucine-2-naphthylamide, α -naphthyl acid phosphate and glycogen type II (from

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¹ Abbreviations used in this paper: PPG, type-specific polysaccharide and peptidoglycan; PBS, phosphate-buffered saline.

oyster) were from Sigma Chemical Co., London, England; Medium 199 and L-15 were from Grand Island Biological Co., Grand Island, N. Y.; newborn calf serum was from Tissue Culture Supplies Ltd., Slough, Bucks, England; Triton X-100 was from British Drug Houses, Poole, Dorset, England; LDH UV-Test, Biochemica Test Combination 15948 TLAS was from Boehringer Corporation (London) Ltd., Ealing, London, England; hemoglobin substrate for protease assay was from Worthington Biochemical Corp., Freehold, N. J.; *o*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside was from Koch-Light Laboratories, Colnbrook, Bucks, England; heparin (5,000 U/ml) was from Boots Pure Drug Co., Nottingham, England; lipopolysaccharide B, *Escherichia coli* 055, B5, latex particles and Todd-Hewitt broth were from Difco Laboratories, Detroit, Mich., and fluorescein dibutyrate was from Nutritional Biochemicals Corp., Cleveland, Ohio.

Preparation of PPG and Other Bacterial Substances.—A freeze-dried culture of strain D-58 group A streptococci was kindly provided by Dr. John H. Schwab (University North Carolina, Chapel Hill). The culture was placed in 25 ml Todd-Hewitt broth and incubated for 18 h at 37°C. Purity of the culture and identity of the microorganisms were established by subculture on blood agar, microscopic examination of Gram-stained preparations, and reaction with type-specific antiserum kindly provided by Dr. W. R. Maxted (Streptococcus and Staphylococcus Reference Laboratory, Colindale, London).

Microorganisms were grown in 32-liter batches and cell walls prepared as described by Schwab (4, 12). 5-liter Erlenmeyer flasks containing 4 liters of sterile Todd-Hewitt broth were inoculated with 1.0 ml of an 18-h culture of microorganisms. The flasks were incubated with stirring for 18 h at 37°C. The microorganisms were harvested in a continuous-flow centrifuge (Measuring Scientific Instruments, London, England) operating at 16,000 *g* with a flow rate of 100 ml/min. Subsequently, the cells were washed four times by suspension in 1 liter of phosphate-buffered saline pH 7.4 (PBS) followed by centrifugation at 12,000 *g* for 15 min. The washed cells were suspended in 200 ml of PBS and ruptured in a Braun MSK Homogenizer (Shandon, London, England). Portions of 25-ml bacterial suspension were placed in 75-ml glass Braun bottles along with 25 ml of no. 12 ballotini glass beads and treated in the homogenizer for 3 min. The extent of cell rupture was monitored by periodic microscopic examination of Gram-stained smears of the material. The material was kept cool by a continuous flow of carbon dioxide through the homogenizer. After 3 min in the homogenizer, cell rupture was greater than 99% complete. The unbroken cells and residual glass beads were removed from the preparation by centrifugation at 1,000 *g* for 10 min and discarded. Cell walls were harvested by centrifugation at 12,000 *g* for 30 min and the resultant soluble supernatant discarded. The cell walls were washed four times by resuspension in 1 liter of PBS followed by centrifugation at 12,000 *g* for 30 min. As described by Schwab (12), only approximately the surface two-thirds of the pellet was recovered after each centrifugation and the remaining material was discarded. The material was resuspended each time by treatment for 1 min in 30 ml of PBS in an Ultrasonic Disintegrator (Measuring Scientific Instruments) operating at 20 kcycle followed by dilution to 1 liter.

The washed cell walls were suspended in 100 ml of sterile PBS containing 0.025% wt/vol ribonuclease and digested for 4 h at 37°C. The residual material was collected by centrifugation at 75,000 *g* for 30 min followed by one wash in PBS. The preparation was resuspended and incubated for 4 h at 37°C in 100 ml sterile PBS containing 0.025% wt/vol pancreatic trypsin. Following trypsin digestion, the residual material was collected by centrifugation and washed twice in 250 ml of sterile PBS followed by four washes in the same volume of deionized distilled sterile water. In each case, the material was collected by centrifugation at 75,000 *g* for 30 min, the pellet resuspended in 30 ml of buffer or water by sonication for 1 min, and subsequently diluted to 250 ml. After the final wash the material was suspended in water and freeze dried.

The nature of the cell wall substance prepared by this method has been studied extensively (12-15) using several different techniques. It is made up of type-specific polysaccharide and peptidoglycan (or mucopeptide) from the cell wall and its is free of exotoxins and soluble

cytoplasmic constituents. The two components have been separated by hot formamide extraction and studied separately. Neither the polysaccharide component nor the peptidoglycan is pathogenic alone. Electron microscopic examination of negatively stained preparations of the particles (Fig. 2 a) shows that the PPG preparation consists of fragments of empty cell walls with maximum diameters ranging from 0.4 to 1.2 μm .

Preparation of Lysosomes from Rabbit Polymorphonuclear Leukocytes.—A 0.1% wt/vol glycogen solution in 0.9% wt/vol NaCl was incubated at 37°C for 24 h before intraperitoneal injection of 250 ml per rabbit. Harvesting of polymorphonuclear leukocytes was carried out as previously described (16). The cells were washed and suspended in 0.34 M sucrose containing 50 U of heparin/ml. The cells were disrupted by repeated passage through a Swinny filter assembly (16). Nuclear debris was sedimented by centrifugation at 400 g for 10 min and the milky supernate was used for the measurement of lysosomal sedimentability.

Effect of PPG on Isolated Polymorphonuclear Leukocyte Lysosomes.—Portions of the 400 g postnuclear supernate were incubated with increasing concentrations of PPG ranging from 2 to 100 $\mu\text{g}/\text{ml}$. After 30 min incubation at 37°C samples were centrifuged for 20 min at 15,000 rpm in a SS-34 head of a Sorvall S-3 centrifuge. The supernates were retained for enzyme assay. Total enzyme release was measured after addition of 0.1% vol/vol Triton X-100 to portions of postgranule supernate.

Macrophage Collection and Culture.—Macrophages were obtained by peritoneal lavage of Swiss mice (I.O. strain) with Medium 199 containing 10% newborn calf serum as described by Cohn and Benson (17). The medium contained 100 U/ml of penicillin and streptomycin. 5-ml aliquots of the collected fluid containing $1-1.5 \times 10^6$ cells/ml were distributed into 48-mm culture dishes (Nunc, Jobling Laboratories Division, Stone, Staffs., England), and incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C for 4 h to allow attachment of adherent cells. Nonadherent cells were removed from the cultures by washing four times with warm PBS. Further incubation under the same conditions gave a regular sheet of spread cells within 24 h. The medium was then replaced with fresh medium containing various amounts of PPG and further incubated for 24–72 h. PPG-containing medium was prepared by sonicating the material in serum-free medium for 3 min at 20 kcycycle. The appropriate volume of serum was then added. For morphologic observation cells were allowed to adhere to glass coverslips (22 mm²) but otherwise they were cultured in the same way. In all experiments quadruplicate cultures were used and the biochemical data are expressed as the mean and standard deviation. At the end of the incubation period, the medium was removed and the adherent cells washed once with warm saline. The cells were released by adding saline containing 0.1% vol/vol Triton X-100. The protein content of the cell-containing fraction was measured by the method of Lowry et al. (18).

Morphologic Observations of Macrophages.—Cover slips with cultured macrophages were observed in the living state using phase-contrast optics. Additional unfixed cover slip preparations were incubated at 37°C with acridine orange at a concentration of one part per 400,000 (wt/vol) in L-15 culture medium for 5 min, transferred to the same medium without acridine orange for 15 min and examined in the living state by fluorescence microscopy. Other cover slip preparations were fixed in methanol and stained by the Giemsa method.

Hydrolysis of Fluorescein Dibutyrate.—Fluorescein dibutyrate was dissolved in acetone (5 mg/ml) and diluted in Medium 199 containing 10% newborn calf serum to a final concentration of 15 $\mu\text{g}/\text{ml}$. The cells were incubated in this medium at 37°C for 15 min, washed once in fresh medium and placed in fresh medium for observation. Fluorescence microscopy was carried out using a Zeiss Universal microscope using a HB0200 mercury lamp with BG38 and BG12 primary filters and Zeiss secondary filter no. 47.

Enzyme Assays.—The levels of various enzymes both in the cells and culture media were measured as a function of time and PPG dose. All assays were conducted under conditions giving linear release of product in relation to the amount of sample used and the time of incubation.

Lactate dehydrogenase (EC 1.1.1.27 L-lactate: NAD oxidoreductase) was assayed by determining the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm following the instructions issued with the Biochemica LDH Test Combination.

β -glucuronidase (EC 3.2.1.31 β -D-glucuronide glucuronohydrolase) activity was assayed by the method of Talalay et al. (19). β -galactosidase (EC 3.2.1.23, β -D-galactoside galactohydrolase) was assayed by the method of Conchie et al. (20), using *o*-nitrophenyl- β -D-galactopyranoside as substrate. Leucine-2-naphthylamidase activity was measured by the method of Goldberg and Rutenberg (21), as described by Davies et al. (22). Acid protease was assayed by the method of Anson (23) and the tyrosine content of the trichloroacetic acid supernate determined with Folin Ciocalteu's reagent. *N*-acetyl- β -D-glucosaminidase was assayed by the method of Woollen et al. (24) using *o*-nitrophenyl-2-acetamido-2-deoxy- β -D-glycopyranoside as substrate.

Acid phosphatase activity was measured using α -naphthyl acid phosphate as substrate. Incubation mixtures contained 0.2 ml of enzyme and 0.2 ml of substrate solution (1.234 mg/ml in 0.1 M acetate buffer pH 5.0). A blank containing enzyme only was prepared for each assay sample. After incubation for 5 h at 37°C, the assay was terminated and color developed by the addition of 0.6 ml of diazotizing mixture used for assaying leucine-2-naphthylamidase, and substrate was added to the blank tubes. The incubation tubes were shaken vigorously and allowed to stand for 1 h followed by measurement of the optical density at 560 nm. All samples were assayed immediately after removal from culture plates.

RESULTS

Morphology.—Peritoneal lavage of mice yields a heterogeneous cell population from which actively phagocytic cells can be selected by utilizing their property of adherence to the surface of the culture vessel. The adherent cell population is not homogeneous (25), but is made up predominantly of cells that spread on the surface of the culture vessel, phagocytose and exhibit the morphologic features of macrophages (17). After 24-h incubation, the cells are polarized with two or more long cytoplasmic processes terminating in ruffled membranes (Fig. 1 A). The nucleus, which is indented, is frequently located to one side of the cell, and discrete granules, which are lysosomes, are seen in a perinuclear position.

After exposure to PPG the cells undergo rapid and marked morphologic changes which develop by 6–8 h and persist for at least 72 h. These include a two- to fourfold increase in cell size, an increase in the number of lysosomes as shown by acridine orange uptake, and a marked increase in ruffled membrane activity (Fig. 1 B). These morphologic changes are brought about by PPG concentrations in the range of 1–10 μ g/ml. When cultured in the presence of 25–50 μ g/ml of PPG, the cells show comparable increases in size, they tend to become more rounded, the cytoplasm exhibits prominent large vacuoles and fewer lysosomes are visualized (Figs. 1 C, D). These doses of PPG do not affect cell viability, since the cells remain attached to the culture vessels and they do not release lactate dehydrogenase. Furthermore, in cultures where the cell wall-containing medium is replaced by fresh control medium after 72 h, the cells remain viable for at least another week. Changes of the type described were not induced by incubation of the cells with particles of latex or anatase at concen-

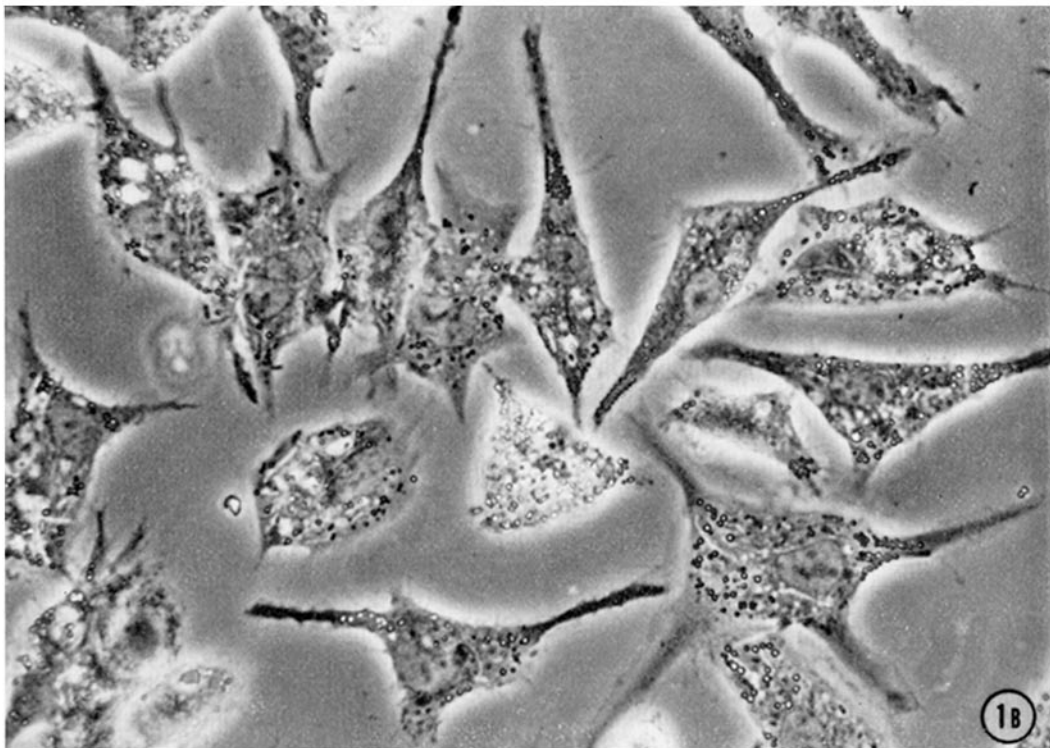
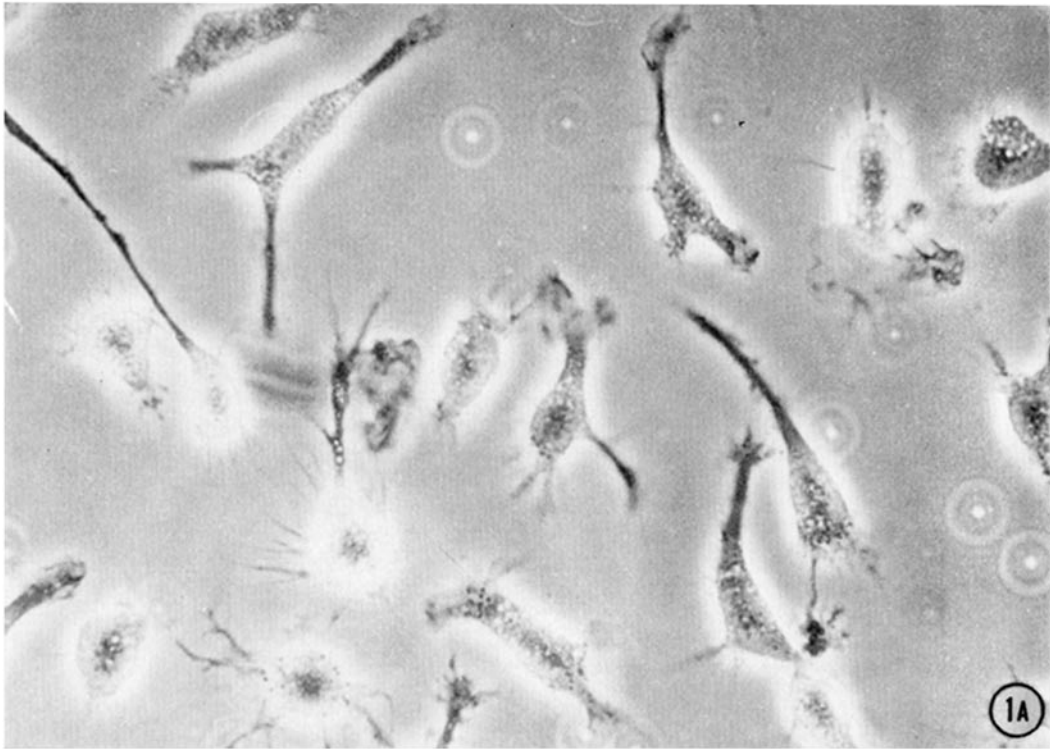
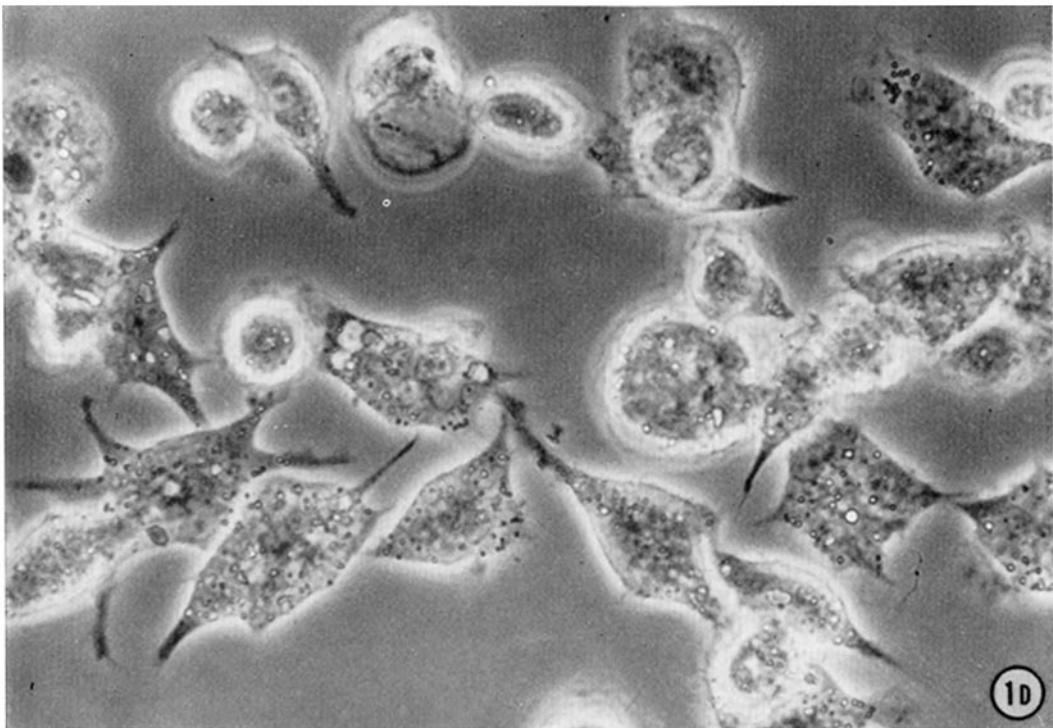
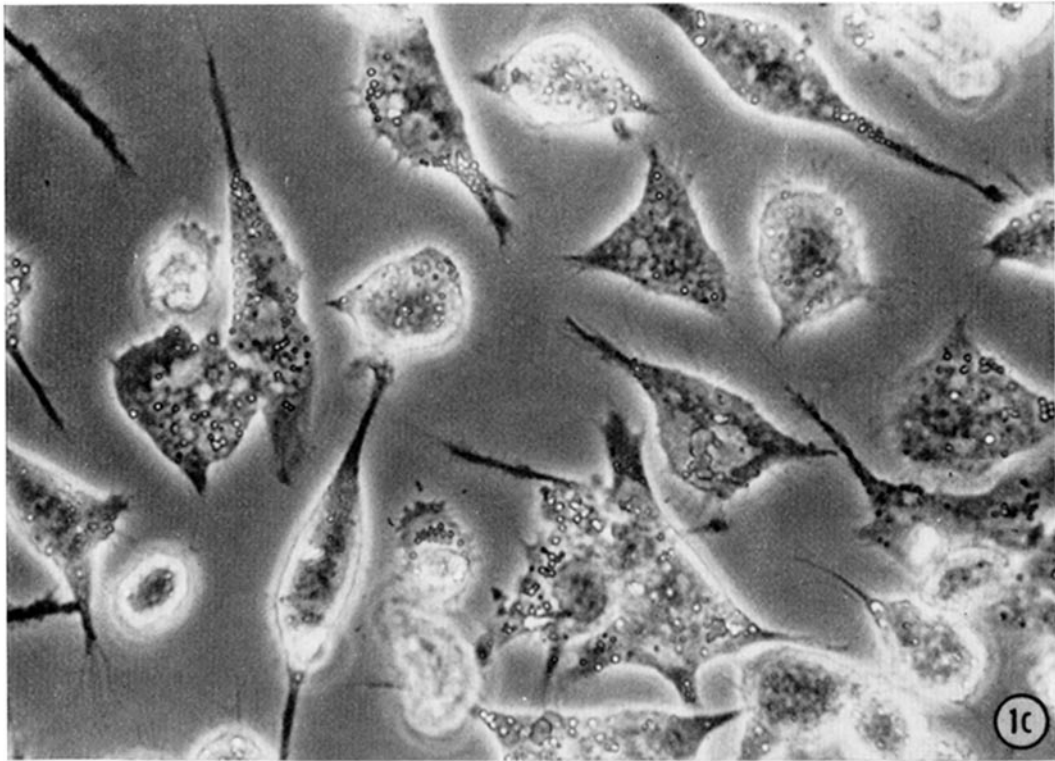


FIG. 1. Morphologic features of macrophages maintained on coverslips in Medium 199 with 10% calf serum in the absence and presence of various concentrations of PPG for 72 h and viewed in the living state by phase-contrast microscopy ($\times 840$). Note the remarkable increase in cell size and the accumulation of phase-dense granules and vacuoles especially in B. Cells are seen to be vacuolated and rounded at 50 μg PPG/ml. (A.) Control, (B.) PPG 5 μg /ml, (C.) PPG 25 μg /ml, (D.) PPG 50 μg /ml.



trations of 50 $\mu\text{g}/\text{ml}$. Cells exposed to endotoxin showed only slight increases in size and granularity.

Hydrolysis of Fluorescein Dibutyrate.—Macrophages retain the ability to hydrolyse fluorescein dibutyrate after exposure to 50 $\mu\text{g}/\text{ml}$ PPG for 72 h (Fig. 2 *b, c*). In untreated control cultures 99.0% of the cells showed fluorescence, indicating the release of product by the nonspecific esterase activity of the macrophage, while in treated cultures 98.5% of cells hydrolysed substrate. In marked contrast only 1.0% of cells incubated in the presence of 2% fresh rabbit serum (a source of complement which is fixed by the natural macroglobulin of newborn calf serum after binding with antigenic determinants on the plasma membrane of the mouse macrophages leading to cell lysis) can cause fluorescein formation (Fig. 2 *d*). Biochemical estimations made in the same experiment showed control cultures to contain 35.4 ± 8.5 mU lactate dehydrogenase per cover slip, PPG-treated cells contained 28.6 ± 8.2 mU per cover slip, while those incubated with rabbit serum had only 11.7 ± 14.3 mU/cover slip. Control cover slips retained $81.0 \pm 15.4\%$ of total β -glucuronidase within cells but PPG and rabbit serum-treated cells retained only $15.1 \pm 3.6\%$ and $12.0 \pm 1.9\%$ of total activity respectively. Total activities for control, PPG, and rabbit serum-treated cultures were 20.3 ± 2.9 , 24.1 ± 2.9 and 23.0 ± 4.4 nmol product/h/culture respectively.

Protein Content.—The increase in cell size seen after culture in the presence of PPG is reflected by the cellular protein levels. As seen in Fig. 3, the adherent cells, cultured for 72 h in the presence of 10–50 $\mu\text{g}/\text{ml}$ of PPG, exhibit two- to threefold increases in protein content.

Enzyme Levels and Distribution.—Marked changes in the levels and distribution of the activities of acid hydrolases and other cellular enzymes accompany the morphologic alterations induced in macrophages by PPG. Two types of experiments were performed. Firstly the effect of various concentrations of PPG on the level and distribution of enzyme activity after 24 and 72 h was measured. Secondly, the effect of a single concentration of PPG on the time course of changes in enzyme level and distribution was determined.

Exposure to concentrations of 1–50 μg of PPG/ml for a period of 24 h did not significantly alter the cellular levels of lactate dehydrogenase (Fig. 4), while in experiments carried out for 72 h (Fig. 5) there was a statistically significant increase ($P < 0.01$) in cellular lactate dehydrogenase levels at concentrations of PPG of 1 $\mu\text{g}/\text{ml}$ and above. This dose-dependent increase in activity was observed at concentrations up to 15 μg PPG/ml, above which it leveled off. The time course of the increase in lactate dehydrogenase concentration in response to a single dose of 15 $\mu\text{g}/\text{ml}$ is shown in Fig. 6. There was a lag period of approximately 24 h. Enzyme levels increased linearly during the second day, and leveled off by the beginning of the third day. The maximum increase over control values during the 72-h culture period was approximately twofold. In the dose range studied, lactate dehydrogenase release into the culture medium was not detectable.

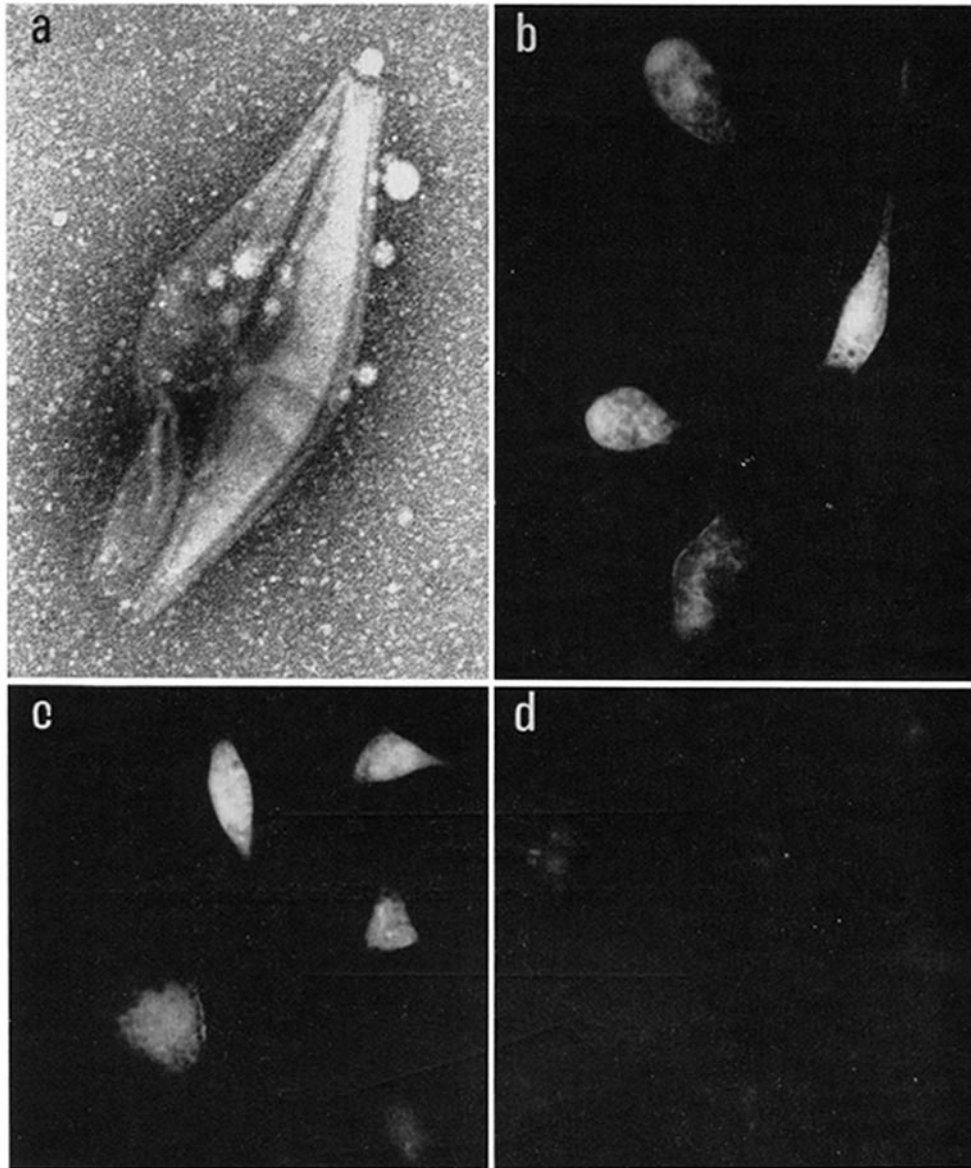


FIG. 2. (a) Morphologic features of negatively stained PPG preparation after sonication as seen in the electron microscope. Particles ranged in size from 0.4 to 1.2 μm \times 40,000. (b-d) The hydrolysis of fluorescein dibutyrate by macrophages in culture for 72 h in Medium 199 with 10% newborn calf serum. (b) control cultures, (c) cultures containing PPG, 50 $\mu\text{g}/\text{ml}$, and (d) cultures containing 2% fresh rabbit serum as a source of complement. Note the bright fluorescence in (b) and (c) and its almost total absence in (d) indicating that the esterase responsible for hydrolysis is still present in the control cells and those exposed to PPG, while the complement-exposed cells no longer hydrolyze the reagent. \times 640.

Large increases in the cellular levels of leucine-2-naphthylamidase were also observed, although the response of this enzyme to the presence of PPG differed in several respects from that of lactate dehydrogenase. The maximum effect obtained was greater and it occurred at an earlier time with a lower PPG con-

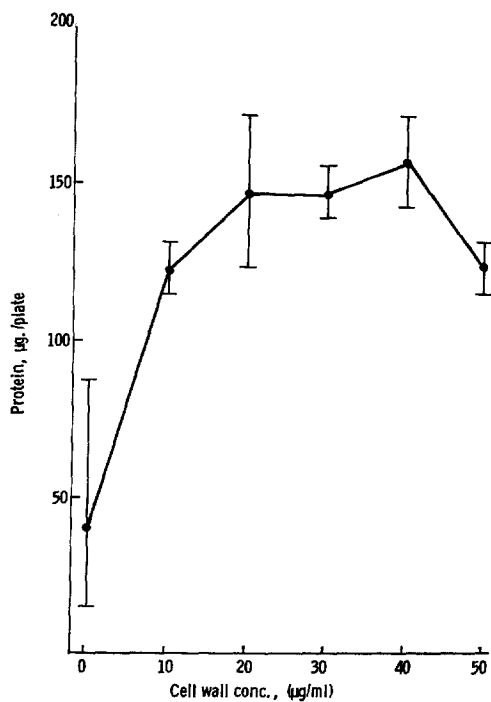


FIG. 3. The protein content of macrophages cultured for 72 h in the absence and presence of various concentrations of PPG.

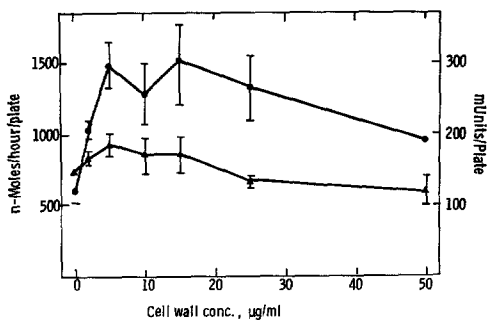


FIG. 4. Changes in the activities of macrophage leucine-2-naphthylamidase (●—●), left hand ordinate, and lactate dehydrogenase (▲—▲), right hand ordinate, after incubation for 24 h in the presence of various concentrations of PPG.

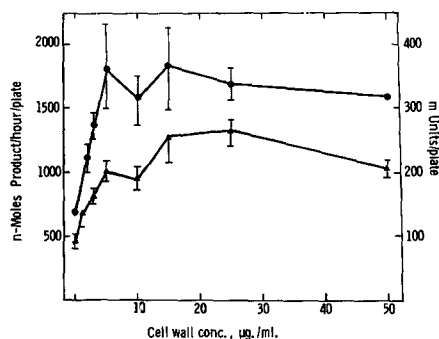


FIG. 5. Changes in the activities of macrophage leucine-2-naphthylamidase (●—●), left hand ordinate, and lactate dehydrogenase (▲—▲), right hand ordinate, after incubation for 72 h in the presence of various concentration of PPG.

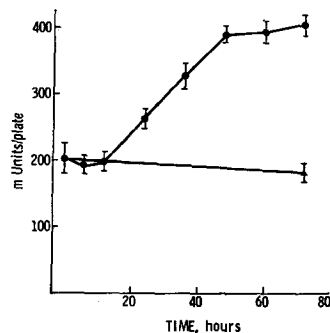


FIG. 6. The effect on cellular levels of lactate dehydrogenase of incubation of macrophages in the presence (●—●) and absence (▲) of PPG (15 µg/ml) over a period of 72 h.

centration than was the case with lactate dehydrogenase (Figs. 4 and 5). The leucine-2-naphthylamidase doubled during the first 24 h in response to 1 µg/ml PPG and the maximum enzyme levels achieved, which were approximately fourfold greater than normal control values, were observed within 24 h (Fig. 7). The elevation in leucine-2-naphthylamidase activity began soon after exposure to PPG and continued in a linear fashion for the first 24 h, after which a plateau was reached and maintained for the remainder of the experimental period (Fig. 7).

Marked alterations in both the levels and distribution of activity of acid hydrolases were induced in the cells by the presence of PPG. In cultures continued for 24 h in the presence of various concentrations of PPG, there was a dose-dependent and selective release of acid hydrolases from the cells into the culture medium (Fig. 8). In some experiments this release amounts to approximately 80% of the total enzyme present (Fig. 9). While data are presented only for β-glucuronidase, the same pattern of release was observed

for other acid hydrolases measured including acid protease, acid phosphatase, *N*-acetyl- β -D-glucosaminidase and β -galactosidase. The time course of the release in the case of β -glucuronidase is illustrated in Fig. 9. Release begins within an hour after exposure of the cells to PPG and is virtually complete within about 4-6 h.

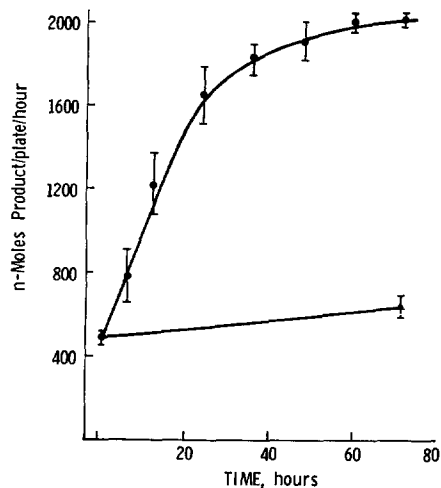


FIG. 7. The effect of PPG (15 μ g/ml) (●—●) on macrophage levels of leucine-2-naphthylamidase over a period of 72 h compared to control (▲).

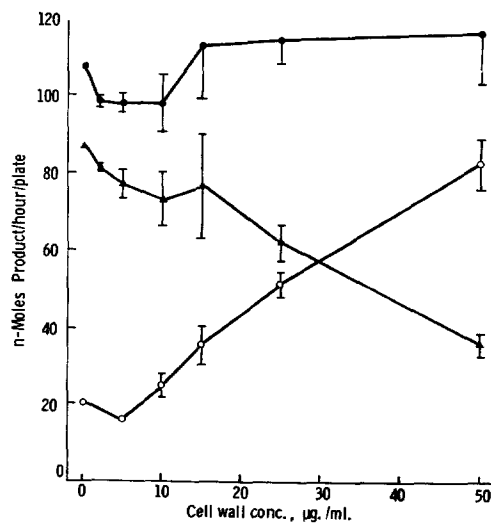


FIG. 8. The effect of increasing concentrations of PPG (2-50 μ g) on levels of β -glucuronidase activity in the total culture (●—●), in cells (▲—▲), and in the medium (○—○). Cultures were assayed after incubation with PPG for 24 h.

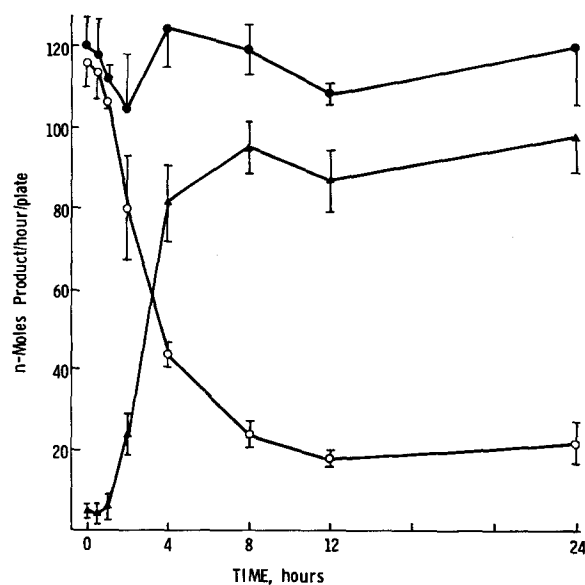


FIG. 9. The effect of PPG (15 $\mu\text{g}/\text{ml}$) on β -glucuronidase activity in the total culture (●—●), in the cells (▲—▲) and in the culture medium (○—○) at time intervals over a period of 24 h.

The presence of PPG in macrophage cultures induces large increases in the levels of lysosomal acid hydrolase activity. The magnitude of these increases range from 2.0- to 2.5-fold for β -glucuronidase and acid protease respectively to almost sixfold in the case of acid phosphatase (Figs. 10 and 11). The enzyme levels were statistically significantly greater than control values ($P < 0.01$) in the cells exposed to a PPG concentration of 1 $\mu\text{g}/\text{ml}$ for acid protease and 10 $\mu\text{g}/\text{ml}$ for β -glucuronidase. The time course of the increase in lysosomal enzyme was not the same for all of the enzymes measured. Levels of β -glucuronidase remained constant for the first 24 h then began to increase (Fig. 11). A comparable lag phase was noted in the case of β -galactosidase and acid protease, while acid phosphatase activity began to increase immediately after exposure and reached maximum levels within about 12 h.

The specificity of the lysosomal enzyme release induced by PPG was investigated further by comparing its effects on macrophages with those induced by comparable exposure of other cells. Human gingival fibroblasts were selected for study since these cells can be maintained easily in culture. They phagocytose PPG and they contain sufficient amounts of lysosomal enzymes for accurate measurement. Concentrations of PPG up to 50 $\mu\text{g}/\text{ml}$ induced statistically significant increases in the levels of lactate dehydrogenase and leucine-2-naphthylamidase, but neither the levels nor distribution of lysosomal hydrolases were affected (Engel and Page, unpublished observations), and the material was not cytotoxic.

The effects of PPG on macrophages were also compared to those induced in the cells by exposure to several other endocytosable materials. As seen in Fig. 12, endotoxin, in concentrations ranging from 0.5 to 10 $\mu\text{g}/\text{ml}$, did not increase levels of β -glucuronidase activity, nor did it induce release of the enzyme from

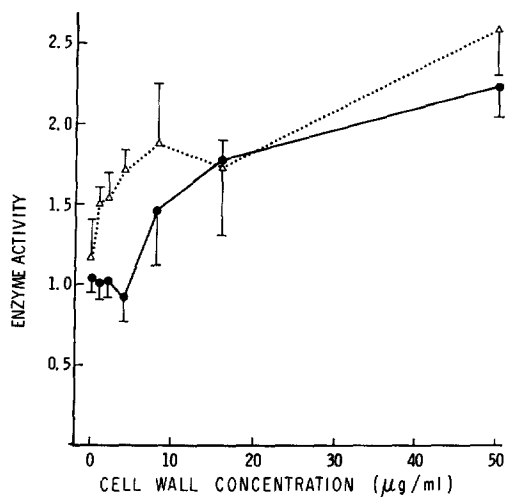


FIG. 10. The effects of various concentrations of PPG (1–50 $\mu\text{g}/\text{ml}$) on the total levels of β -glucuronidase ($\Delta \dots \Delta$) (nmol tyrosine/plate/h $\times 10^2$) and acid cathepsin ($\bullet \dots \bullet$) (nmol tyrosine/plate/h $\times 10^3$) in macrophage cultures over a period of 72 h.

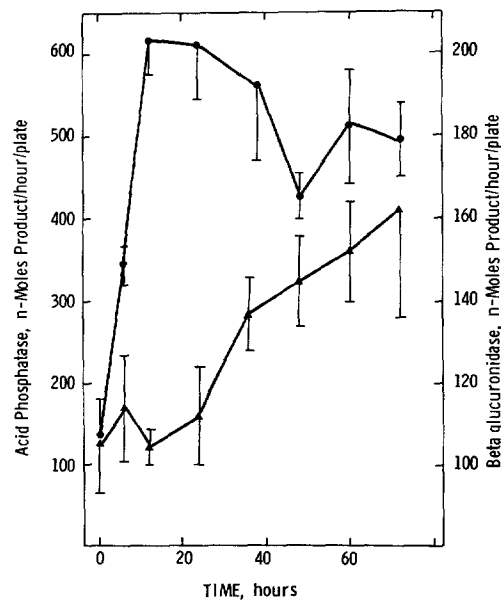


FIG. 11. Changes in total acid phosphatase ($\bullet \dots \bullet$) and β -glucuronidase ($\blacktriangle \dots \blacktriangle$) activities in cultures maintained for 72 h in the presence of PPG (15 $\mu\text{g}/\text{ml}$).

the cells into the culture medium. The endotoxin-treated cells did release small amounts of acid phosphatase and *N*-acetyl- β -D-glucosaminidase. Purified protein derivative at concentrations of 50 and 100 $\mu\text{g}/\text{ml}$ and phagocytosable inert particles were ineffective in inducing enzyme release (Table I).

The Effect of PPG on Isolated Polymorphonuclear Leukocyte Lysosomes.—The mechanism by which PPG induces lysosomal hydrolase release was investigated further by exposure of isolated lysosomal granules, obtained from rabbit polymorphonuclear leukocytes, to the material, followed by assay of lysosomal enzymes in the granules and in the supernate. Concentrations of PPG up to 100 $\mu\text{g}/\text{ml}$ failed to induce enzyme release in this system (Table II). Thus, it appears unlikely that the material exerts direct effects on lysosomal membranes.

DISCUSSION

A component of group A streptococcal cell walls induces chronic inflammatory lesions lasting several months when injected into experimental animals (3, 4, 6, 26-28). The physical, chemical, and biologic properties of the particles have

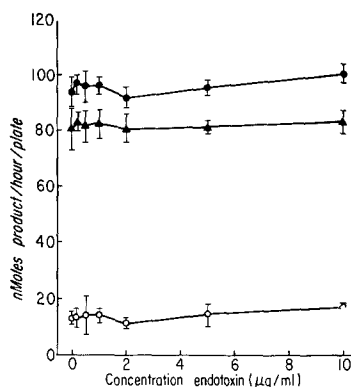


FIG. 12. The effects of the presence of various concentrations of endotoxin on the levels of β -glucuronidase activity in the total culture (●—●), in cells (▲—▲) and in the culture medium (○—○) of macrophage cultures maintained for 72 h.

TABLE I

Effects of Various Bacterial Substances and Inert Particles on the Distribution of Acid Hydrolases in Mouse Peritoneal Macrophage Cultures

Substance added	Percent activity in media	
	β -glucuronidase	α -naphthyl-phosphatase
Control cultures	5.3	10.6
Latex (50 $\mu\text{g}/\text{ml}$)	7.1	13.5
Anatase (50 $\mu\text{g}/\text{ml}$)	10.9	11.7
Purified protein derivative (50 $\mu\text{g}/\text{ml}$)	4.3	7.3
Purified protein derivative (100 $\mu\text{g}/\text{ml}$)	3.6	13.0
PPG (50 $\mu\text{g}/\text{ml}$)	65.0	41.0

TABLE II
Effect of PPG on Enzyme Release by Lysosomes In Vitro

PPG	β -glucuronidase*	<i>N</i> -acetyl- β - <i>D</i> -glucosaminidase*
$\mu\text{g/ml}$		
0	17.4 (1.0)	39.2 (1.3)
2	17.4	38.0
10	17.8	37.6
50	14.9 (0.8)	37.1 (1.3)
100	13.2 (0.4)	34.6 (0.8)

* Percent of the total enzyme (that released by exposure to 0.1% Triton X-100) released by exposure to various concentrations of PPG. Mean of three incubations (\pm SD) except at 2 and 10 $\mu\text{g/ml}$ concentrations where only two incubations were done. 100% activity released by Triton X-100 corresponded to 16.8 nmol phenolphthalein/h/mg post granule supernatant for β -glucuronidase and 166.8 nmol *o*-nitrophenol/h/mg post granule supernatant for *N*-acetyl- β -*D*-glucosaminidase. Each ml of postgranule supernatant contained 3.325 mg protein.

been studied (12-15). The material is made up of pieces of bacterial cell wall ranging in size from 0.4 to 1.2 μm . These contain type-specific polysaccharide and peptidoglycan (or mucopeptide). Separation of these two components by hot formamide extraction results in loss of pathogenic activity.

The lesions induced by injection of the material into experimental animals are made up almost exclusively of macrophages (26, 28) and they appear to be free of an immunopathologic component (7, 28). Macrophages exposed in vitro to the substance become enlarged and granular (Fig. 1) and produce large amounts of lysosomal hydrolases, lactate dehydrogenase, and leucine-2-naphthylamidase. Lysosomal acid hydrolases are selectively released from the cells into the culture medium.

A primary consideration is the possibility that the changes described result in loss of cell viability. With the doses of PPG (1-50 $\mu\text{g/ml}$) and time of exposure (up to 72 h) that we used, the cells seem to remain viable as indicated by the several criteria. They remain adherent to the surface of the culture vessels and retain marker cytoplasmic enzymes such as lactate dehydrogenase which readily leak from damaged cells. Cells exposed to PPG (50 $\mu\text{g/ml}$) for 72 h also retain the ability to hydrolyse fluorescein dibutyrate (Fig. 2) while those which have been damaged by action of complement do not. In addition, cultures exposed to high doses of PPG for prolonged periods of time show increased cellular levels of protein (Fig. 3) and both lysosomal and nonlysosomal enzymes (Figs. 5 and 10). All these observations provide evidence that PPG is not toxic to macrophages in the doses and for the time of exposure used in the experiments described in this paper. Further experiments not detailed here have shown that macrophages maintained in culture for periods of 2 wk after an initial exposure to PPG for 72 h remain viable.

The observations described provide new information on the role of endocytosed substances in the stimulation of enzyme production by macrophages,

the selective release of lysosomal hydrolases and the possible role of lysosomal hydrolase secretion in the evolution of chronic inflammatory lesions.

The times required after exposure to PPG for the macrophages to attain significant increases in activity of the various enzymes did not correlate one with another nor with the time needed for the morphologic changes to appear (Fig. 1). During the first 6–8 h following exposure to PPG, prominent morphological changes were apparent and the levels of leucine-2-naphthylamidase, acid phosphatase, and *N*-acetyl- β -D-glucosaminidase were elevated, but the levels of activity of the energy producing enzymes, as exemplified by LDH, and the remaining acid hydrolases, remained constant. During this period there was a major redistribution of the acid hydrolases, in some cases amounting to about 80%, from the cells to the medium. Enhanced levels of activity of lactate dehydrogenase, β -glucuronidase, β -galactosidase, and acid protease were observed only after a lag period of 24 h. Thus, when macrophages were exposed to PPG, the initial events appeared to include alterations in cell morphology, rapid production of leucine-2-naphthylamidase (Fig. 4), and some, but not all, of the acid hydrolases (Fig. 11), and exocytosis of the acid hydrolases. During the ensuing time periods, conditions appear to stabilize. The cells acquire elevated levels of lactate dehydrogenase (Fig. 5) and leucine-2-naphthylamidase (Fig. 5), and they continue to produce and secrete large amounts of lysosomal enzymes (Fig. 10), at least for the 72-h period which we studied.

In spite of intensive investigation (29–33), the relationship between endocytosis by macrophages and increased lysosomal enzyme production remains unclear. Mouse peritoneal macrophages phagocytosing sheep erythrocytes, aggregated bovine gamma globulin, or L-amino acid homopolymers exhibit a dose-dependent, puromycin-inhibitable increase of two–threefold in the levels of acid phosphatase activity and a very small increase in the levels of β -glucuronidase and cathepsin (29). These levels reach maximum values at about 24 h and decrease by 48–72 h. Endocytosis of nondigestible particles such as polyvinyl toluene, polystyrene, starch, and D-amino acid homopolymers does not stimulate elevation of enzyme levels. Based on these observations, it has been suggested that the quantity and digestibility of endocytosed material may control acid hydrolase production (29). PPG stimulated acid hydrolase production greatly, but it is not digestible by macrophages (9, 11, 34). Group A streptococcal cell walls can be digested partially by *N*-acetyl- β -D-glucosaminidase, an enzyme found in certain soil bacteria (10). A similar enzyme with a high apparent affinity for model substrates is present in macrophages, but the phagocyte enzyme is essentially ineffective in digestion of the polysaccharide component of group A streptococcal cell walls (10). In spite of this indigestibility, endocytosis of PPG increases the production of lysosomal and other cellular enzymes. Indeed, nondigestible disaccharides appear to have a similar effect (30, 35).

The magnitude and time course of the activation of hydrolase production by

various endocytosable substances differ remarkably. Substances of at least three categories are apparent: (*a*) indigestible substances such as polyvinyl toluene, latex, anatase, and starch which are endocytosed but stimulate no acid hydrolase production; (*b*) digestible substances such as serum proteins, sheep erythrocytes, and aggregated proteins which stimulate low levels of enzyme production on a temporary basis; and (*c*) indigestible substances such as PPG, sucrose, and possibly certain microorganisms which stimulate a greatly enhanced level of enzyme production which endures for long time periods. Endocytosis of the latter category may be accompanied by hydrolase secretion. Since dose-dependency is a feature in all cases where stimulation of hydrolase production is observed, the quantity of endocytosed substance may be a controlling factor. However, the observations described above lead to serious questions of the postulated role of digestibility.

Up to 80% of the total β -glucuronidase present in macrophages can be released by exposure to relatively small doses of PPG. The releasing cells appear to be otherwise healthy and viable. Three different mechanisms, all of which are considered to be indicative of cell damage or abnormal function, have been described to account for the release of acid hydrolases from cells (32). These include (*a*) loss of integrity of the plasma and lysosomal membranes resulting in enzyme leakage and cell death, (*b*) endocytosis of substances which perturb lysosomal and plasma membranes leading to fusion and selective release, and (*c*) regurgitation of acid hydrolases during aborted or incomplete phagocytosis. Cells exposed to PPG do not release LDH, and PPG does not have the capacity to induce enzyme release from isolated lysosomes (Table II). Thus direct membrane-lytic effects are not likely to be involved in the observed release. Likewise, regurgitation due to aborted phagocytosis does not appear to account for the release. Macrophages phagocytosing zymosan particles release acid hydrolases, and regurgitation has been shown to be an important factor (32). However, in this system only 10–25% of the enzyme is released while PPG induces release of the bulk of the total enzyme present. The more probable mechanism of enzyme release by PPG is by exocytosis. This is a process similar to that seen in secretory cells where the contents of the lysosomes are discharged after the fusion of the organelle with the plasma membrane of the macrophage. Presumably interaction of the cell wall PPG with membranes provides the stimulus to lysosomal hydrolase release, but whether this occurs at the surface of the cell or in secondary lysosomes containing PPG (28) is not yet known.

The phenomenon of selective release of acid hydrolases in response to various stimuli has been widely described in cell types other than macrophages. Polymorphonuclear leukocytes release acid hydrolases when exposed to immune complex (36, 37) and fibroblast and limb bone cultures behave similarly when exposed to complement-sufficient antiserum or to indigestible sugars (35, 38, 39). Vaes (40) has shown that hydrolases are released under physiologic conditions by bone cells exposed to parathyroid hormone. Release of lysosomal

enzymes from macrophages appear to occur under a variety of conditions. Cohn and Wiener (33) demonstrated release from alveolar macrophages exposed to *E. coli* and, more recently, a similar release was induced by exposure to cytochalasin B (41, 42).

The behavior of macrophages exposed to PPG in vitro may reflect certain aspects of the pathogenesis and evolution of the lesions induced in vivo. In rabbits a single intradermal injection of PPG initiates a chronic inflammatory granulomatous lesion characterized by remissions and exacerbations and persisting for several months (3, 7, 26). Administration by an appropriate route results in the development of inflammatory lesions in the joints and rheumatic-like lesions in the heart (4, 6, 7, 27, 28). The injected material becomes associated with macrophages where it persists for the entire duration of the lesion (3, 4, 27, 28). As noted above, macrophages do not have the capacity to digest PPG (8-11, 34) even though they contain large quantities of the enzyme *N*-acetyl- β -D-glucosaminidase. Injection into the peritoneal cavity of mice results in the development of lesions in the heart and joints (4, 27) and fluorochrome-labeled streptococci injected into the tonsils of rabbits with experimental arthritis accumulate within phagocytic cells in the inflamed joints. Monocytes and macrophages may be responsible for the transport of PPG to various sites. Furthermore, the spreading and exacerbation of the skin lesions (26, 28) may be accounted for, at least in part, by sequestration of PPG inside macrophages.

The chronicity of the lesions and the characteristic damage to the connective tissues may be a consequence of long-term hydrolase production and release induced in macrophages by PPG. Histopathologic analysis of the skin lesions (28) indicates that PPG-containing macrophages are the most prominent cell type present. If these cells behave in a manner similar to macrophages in vitro, a situation would exist in which viable long-lived cells would be producing and releasing over long time periods acid hydrolases with a known capacity for degradation of constituents of the connective tissues (43-45). An event of this sort would be expected to create damage of the type observed. Indeed, hydrolase production and secretion by macrophages may account, in part, for the extensive tissue damage accompanying many forms of chronic inflammatory and immunopathologic disease.

SUMMARY

Mouse peritoneal macrophages exposed to type-specific polysaccharide and peptidoglycan (PPG) from group A streptococci undergo marked morphologic and biochemical changes. The cells show increases in size and an increased number of lysosomes as demonstrated by vital staining with acridine orange. There are significant elevations in the levels of both lysosomal and nonlysosomal enzymes. Higher doses of PPG cause selective release of the hydrolases into the extracellular environment with no detectable loss of cell viability. The in vitro

phenomena may be relevant to understanding the role of macrophages in chronic inflammation.

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