SECRETION OF LYSOSOMAL HYDROLASES
BY STIMULATED AND NONSTIMULATED MACROPHAGES

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In recent years, numerous studies have documented that macrophages have important secretory activities. Activated macrophages have been shown to secrete plasminogen activator (1), collagenase (2), and an elastase-like enzyme (3), in addition to lysozyme (4), which is also secreted by nonactivated macrophages. Macrophages have also been shown selectively to release β-glucuronidase and other acid hydrolases when they are stimulated by undigestible particles (5), lymphokines (6), or complement products (7). In this paper, we show that macrophages synthesize and secrete considerable amounts of lysosomal enzymes over long periods of culture, independently of external stimuli.

Materials and Methods

Macrophage Cultures. Macrophages from male OF1 mice (Sandoz Ltd., Basel, Switzerland) weighing 20-24 g were collected by peritoneal lavage with ice-cold complete medium (see below) containing 20 U of heparin per ml, according to a standard method (8). The mice were either untreated, or treated 4 days before cell harvesting by an intraperitoneal injection of 0.75 ml of one of the following agents: Brewer's thioglycollate (TA)[1] medium, a 10% proteose-peptone (PP) medium (both from Difco Laboratories, Detroit, Mich.), or a suspension of streptococcus A cell wall material (SA) in phosphate-buffered saline (PBS). Washed type A_4 streptococci were disrupted by a modification of the method of Colman and Williams (9). The cells were shaken for 20-30 min in a Braun cell disintegrator (Apparateben Braun, Melsungen, W. Germany) with 0.1-mm glass beads at −60°C. The contents of the disintegrator were suspended in water, filtered through glass wool to remove the beads, and the bacterial fragments were pelleted by centrifugation at ≅ 50,000 g·min. The pellets were washed with water and lyophilized. This material was suspended in PBS (0.6 mg/ml) before use.

The medium, a minor modification of that used by Cohn and Benson (8), was made up as follows. To 89 ml of a solution of 140 mg of sodium bicarbonate in double-distilled water was added 10 ml of 10-fold concentrated medium 199 (Hanks' salts; Grand Island Biological Co., Grand Island, N. Y.), and then 1 ml of 1 M Hepes. Penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and mycostatin (30 U/ml) were added, and the mixture was sterilized by filtration through Millipore filters, type GSWP, with a pore diameter of 0.22 μm (Millipore AG, Kloten, Switzerland). This medium was stored at 4°C for up to 2 wk. Before use, 1 ml of a sterile 200 mM solution of L-glutamine and serum, usually 5 ml of sterile acid-treated (1) fetal bovine serum (FBS; Flow Laboratories, Rockville, Md.) per 100 ml was added.

The peritoneal cell suspensions were centrifuged at 120 g for 5 min at room temperature, the cells were resuspended in culture medium and plated on 60-mm plastic culture dishes (Falcon Plastics, Div. Becton, Dickinson & Co., Cockeysville, Md.) at a density of 4-6 × 10⁶ cells per dish in 3 ml of medium. The cultures were kept for 2-3 h at 37°C, and nonadherent cells were then

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FBS, fetal bovine serum; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PP, proteose peptone medium; SA, streptococcus A cell wall material; TA, Brewer's thioglycollate medium; TCA, trichloroacetic acid.

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removed by aspirating the medium and washing three times with 5 ml of PBS. Three ml of fresh medium were then added and the cultures were kept for up to 2 wk at 37°C in an atmosphere of 5% CO_2 in air. The medium was changed at different intervals, but at least every 3rd day.

Cell Counting. Peritoneal lavage cells were counted in suspension and differentiated after May-Grünewald-Giemsa staining. Adhering cells were counted under an inverted microscope using an ocular equipped with a counting lattice. Ten randomly-selected areas measuring 8,760 μm^2 were counted on each dish by two observers independently, and the counts were averaged.

Samples for Biochemical Analysis. Cells from peritoneal wash-outs were collected by centrifuging at 120 g for 5 min at room temperature, and they were then lysed in 0.05% (wt/vol) digitonin. Adherent cells were washed three times with PBS on the dishes and then lysed by the addition of 2.4 ml of 0.05% (wt/vol) digitonin. Culture media were freed from nonadherent cells and possible debris by centrifuging at 1,300 g for 5 min.

Lactate dehydrogenase (LDH) and plasminogen activator were always assayed immediately. The other enzymes, which were found to be stable in frozen samples (−20°C), and protein were determined between 1 and 7 days after sampling.

Subcellular Fractionation of Thioglycollate-Elicited Peritoneal Cells. Cells were harvested with medium and centrifuged as described above. Erythrocytes were lysed by hypotonic shock: cell pellets were resuspended in 10 ml of 0.225% KCl and, after 2 min, 20 ml of 1.8% KCl was added to restore isotonicity. The remaining cells were pelleted as above, resuspended in 0.25 M sucrose buffered with 5 mM Tris-HCl, pH 7.4, at a density of 20 × 10^6 cells/ml, and homogenized in a Dounce homogenizer (Kontes Co., Vineland, N. J.) by 15 strokes with the B-pestle. A post-nuclear supernate was obtained at 1,000 g for 3 min, and then fractionated by differential centrifugation in two subsequent steps at the integrated average centrifugal forces of 250,000 and 6 × $10^6 g \cdot$ min, respectively. The two pellets obtained were resuspended in portions of the medium used for homogenization. All fractions were assayed for protein content and enzyme activities in the presence of 0.05% (wt/vol) digitonin.

Incorporation of [³H]Leucine. Macrophages were cultured for 3 days in minimal essential medium (Earls' Salts; Grand Island Biological Co.) containing 1/100th of the usual amount of leucine. Cycloheximide (0.25 μg/ml) was then added to the cultures, and 2 h later the cultures were supplied with new medium containing in addition 5 μCi/ml of [³H]leucine. Control cultures were treated identically, with the omission of cycloheximide. At appropriate times, the media were collected and the cultures were washed once with PBS containing 0.6 mg/ml of cold leucine. Media and washing solutions were combined, and the protein was precipitated in 10% trichloroacetic acid (TCA). After centrifugation at 20,000 $g \cdot$ min and washing with 10% TCA, the precipitate was dissolved in 85% formic acid, and portions were counted in Riasolve (Koch-Light Laboratories Ltd., Colnbrook, England) using a Beckman LS 233 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). The channel ratio method was used for quenching correction. The monolayers were washed three more times with PBS and then scraped off with a rubber policeman in PBS containing 0.1% EDTA, 0.6 mg/ml cold leucine, and 0.5 mg/ml bovine serum albumin (BSA). The cells were disrupted in a Dounce homogenizer and then processed for counting as described for the media.

Biochemical Assays. N-acetyl-β-glucosaminidase (EC 3.2.1.30), β-glucuronidase (EC 3.2.1.31), and α-mannosidase (EC 3.2.1.24) were determined by incubating 100 μl of sample with 100-μl aliquots of the following substrates: 10 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 0.1 M sodium citrate buffer, pH 4.5, containing 0.2 M NaCl, 2 mM 4-methylumbelliferyl-β-D-glucuronide trihydrate or 8 mM 4-methylumbelliferyl-α-D-mannopyranoside in 0.2 M sodium acetate buffer, pH 4.5. Incubation was carried out at 37°C for 10–60 min and was stopped with 3 ml of 50-mM glycine-NaOH buffer, pH 10.4, containing 5 mM EDTA. The 4-methylumbelliferone formed was measured fluorometrically (10) in a Hitachi 203 spectrofluorimeter (Perkin-Elmer Corp., Norwalk, Conn.). Standard assays contained 2 or 4 nmol of 4-methylumbelliferone. Acid phosphatase was assayed by incubating 100 μl of sample with 100 μl of substrate containing 5 mM 4-nitrophenyl phosphate in 0.1 M sodium acetate buffer, pH 4.5, for 2 h at 37°C. The reaction was stopped by the addition of 1 ml of 1 M NaOH. The 4-nitrophenol formed was measured photometrically at 405 nm, using an Eppendorf photometer (Eppendorf Gerätebau, Netheler & Hinz GmbH, Hamburg, W. Germany). 4-Nitrophenyl phosphate was used in this assay after establishing that the activity measured was inhibited by >85% by 2 mM sodium fluoride, which is characteristic for the lysosomal acid phosphatase (11). Lysozyme (EC 3.2.1.17)

was assayed by incubating 300 μl of sample with 700 μl of a freshly prepared suspension of *Micrococcus lysodeikticus* in 50 mM sodium citrate-sodium phosphate buffer, pH 6.0. The decrease in absorbance from the initial absorbance of 0.430 was measured at 546 nm in an Eppendorf photometer equipped with a cuvette holder which was thermostatized at 25°C, and it was recorded on a W+W lin-log recorder (W+W Electronic AG, Münchenstein, Switzerland) with a fourfold scale expansion. With every assay series, a calibration curve was made with crystalline hen egg-white lysozyme. Cathepsin D (EC 3.4.4.23) was assayed according to Hille et al. (12), using iodinated hemoglobin (13). Plasminogen activator was determined as described by Unkeless et al. (1), using small plastic culture dishes coated with ^{125}I-fibrin. 200-μl samples were added to 1.8 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 5–7 μg of purified human plasminogen (14). The radioactivity released was measured after 2 and 4 h in a Beckman Biogamma II counter (Beckman Instruments, Inc.). LDH (EC 1.1.1.27) was assayed at 25°C in thermostatized cuvettes. 200-μl samples were mixed with 500 μl of 0.1 M sodium phosphate-HCl buffer, pH 7.5, and with 100 μl of 0.8 mM $NADH_2$. After 1 min, the reaction was started with 100 μl of 4 mM sodium pyruvate. Decrease in absorbance was recorded at 334 nm using an Eppendorf photometer and a W+W lin-log recorder. Protein was assayed in cell lysates (0.05% digitonin in water) according to Miller (15). BSA served as a standard.

Enzyme Units. One unit of plasminogen activator is defined as the quantity that produces solubilization of 10% of the initial radioactivity in 1 h at 37°C using Petri dishes coated with fibrin at a concentration at 15 μg per cm^2. As an analogue to plasminogen activator, 1 unit of cathepsin D is defined as the amount that induces the release of 10% of initial radioactivity in 1 h at 45°C using 0.9% denaturated hemoglobin. The activity of lysozyme is expressed in μg equivalents of hen egg-white lysozyme. In all other cases, 1 unit of activity is defined as the amount of enzyme that transforms 1 μmol of substrate in 1 min under the conditions given above.

Materials. The following special reagents were used: BSA (Armour Pharmaceutical Co., Chicago, Ill.); hen egg-white lysozyme (C. Boehringer & Soehne, GmbH, Mannheim. W. Germany); 4-methylumbelliferyl-substrates of the glycosidases (Koch-Light Laboratories Ltd.); 4-nitrophenol, 4-nitrophenyl phosphate, disodium salt $5H_2O$, digitonin crystals, streptomycin sulfate (E. Merck AG, Damstadt, W. Germany; L-[4.5-^{3}H(N)]-leucine, iodine-125, protein iodination grade (New England Nuclear, Boston, Mass.); heparin (F. Hoffmann-La Roche & Co. AG, Basel, Switzerland); hemoglobin (Sigma Chemical Co., St. Louis, Mo.); penicillin G (SPECIA, Paris, France); mycostatin (E. R. Squibb & Sons, New York); fibrinogen (Swiss Red Cross Laboratory, Berne, Switzerland).

Results

Enzyme Activities in Adherent Cells. Some of the biochemical properties of adherent cells from untreated and treated mice are listed in Table I. All three treatments used increased the macrophage yield. In this respect, the most effective agent was TA, followed by SA and PP. TA-elicited cells contained twice as much protein and LDH as the other cells studied. A distinguishing feature of TA cells was their content of plasminogen activator, which was nearly 20 times higher than in the other types of macrophages. The contents and the specific activities of lysosomal hydrolases were higher in cells from treated mice than in those from untreated mice. The largest difference was found with acid phosphatase. Compared to the other acid glycosidases, α-mannosidase behaved anomalously; its activity in normal cells was higher than in elicited cells.

Subcellular Localization of Enzymes. A simple fractionation scheme was used to gain some information about the subcellular localization of the hydrolases studied. These experiments were performed with peritoneal cells from TA-treated mice. As shown in Fig. 1, all hydrolases tested, except α-mannosidase, were largely sedimentable at the moderate centrifugal force of 250,000 $g \cdot$ min. They appear, therefore, to be bound to particles that are similar in size to liver

TABLE I

*Numbers and Specific Activities of Adherent Mouse Peritoneal Cells Obtained by Various Pretreatments**

Parameter	Unit		Protein content and specific activities‡		
			Pretreatment of mice		
		None	TA	SA	PP
Number of cells per mouse × 10⁶		2.31 ± 0.12 (12)	11.32 ± 1.04 (7)	7.63 ± 0.84 (5)	4.62 ± 0.58 (3)
Protein	μg (A)	66.42 ± 1.70 (20)	102.94 ± 2.43 (20)	46.90 ± 0.47 (3)	54.68 ± 3.20 (11)
β-Glucuronidase	mU (A)	1.10 ± 0.04 (25)	2.63 ± 0.11 (21)	1.44 ± 0.17 (9)	1.73 ± 0.09 (11)
	(B)	17.25 ± 0.48 (24)	26.73 ± 0.63 (17)	20.39 ± 1.25 (7)	31.86 ± 0.64 (11)
N-acetyl-β-glucosa-minidase	mU (A)	12.79 ± 0.36 (25)	28.03 ± 1.05 (21)	19.69 ± 1.88 (5)	27.74 ± 1.38 (11)
	(B)	192.36 ± 5.30 (24)	287.12 ± 13.09 (17)	242.00 ± 22.89 (4)	510.64 ± 9.85 (11)
α-Mannosidase	mU (A)	2.41 ± 0.09 (15)	0.98 ± 0.04 (20)	1.52 ± 0.16 (9)	1.46 ± 0.09 (9)
	(B)	37.79 ± 1.20 (14)	10.14 ± 0.14 (16)	23.69 ± 1.90 (7)	31.77 ± 1.65 (6)
Acid phosphatase	mU (A)	0.90 ± 0.07 (8)	4.50 ± 0.17 (6)	2.77 ± 0.06 (3)	
	(B)	14.00 ± 0.96 (8)	43.70 ± 3.00 (6)	59.07 ± 1.12 (3)	
Cathepsin D	U (A)	17.35 ± 0.35 (2)	40.00 ± 0.30 (3)		
	(B)	276.00 ± 14.5 (2)	425.00 ± 7.30 (3)		
Plasminogen activator	U (A)	0.91 ± 0.40 (4)	16.24 ± 3.60 (5)	0.91 ± 0.24 (3)	
	(B)	14.15 ± 6.20 (4)	188.00 ± 49.0 (5)	19.34 ± 5.10 (3)	
Lactate dehydrogenase	mU (A)	60.07 ± 2.23 (21)	124.80 ± 4.08 (17)	75.85 ± 5.87 (9)	58.99 ± 4.39 (11)
	(B)	936.25 ± 18.38 (19)	1331.54 ± 37.09 (13)	1273.29 ± 96.84 (7)	1072.18 ± 28.78 (11)

* Cells were collected from the peritoneal cavity and allowed to settle on dishes for 2–3 h at 37°C. Nonadherent cells were eliminated by washing (Materials and Methods) and the adherent cells were counted and used for biochemical assays.

‡ Mean values ± SEM from experiments performed over a period of 2 yr (numbers of determinations are given in parentheses). Enzyme activities are expressed per 10⁶ cells (A), or per mg of cellular protein (B).

lysosomes (16) or human polymorphonuclear leukocyte granules (17). A comparatively large proportion of α-mannosidase was found in the soluble fraction. Since nonlysosomal α-mannosidases are known to exist in other tissues (17, 18), the fractions obtained were examined further. Fig. 2 shows that the soluble and the particulate α-mannosidase activities differ in their pH-dependency, suggesting the existence of more than one α-mannosidase in macrophages. By contrast, β-glucuronidase, which was tested for comparison, showed the same pH-optimum curve in both fractions, suggesting that the soluble enzyme originated from disrupted lysosomes.

Specific Activities. Specific activities of enzymes within the cells and in the culture media may be related either to actual cell numbers or to the amount of cellular protein. Both specific activities are given in Table I. We prefer to express enzyme activities per 10⁶ cells, since total cellular protein may drop as a consequence of enzyme release, and increase considerably in the course of cell activation.

Stability of Enzymes in the Culture Medium. Data on enzyme release must account for possible inactivation in the culture medium. Fig. 3 shows that most of the enzymes assayed in this study remain fully active under culture conditions for at least 3 days. One notable exception is plasminogen activator, which is inactivated relatively rapidly after release. Since this enzyme was not
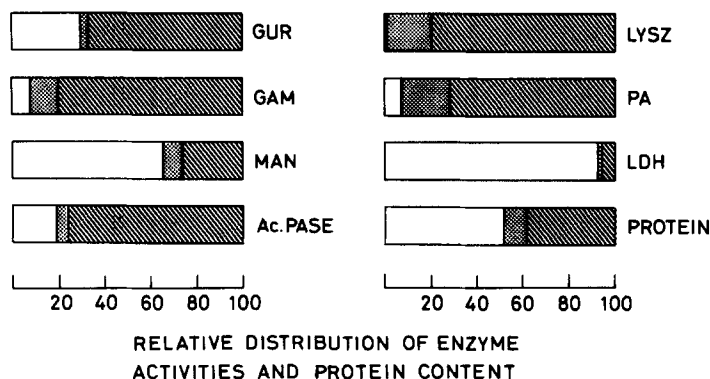
GUR

LYSZ

GAM

PA

MAN

LDH

Ac. PASE

PROTEIN

20 40 60 80 100   20 40 60 80 100

RELATIVE DISTRIBUTION OF ENZYME
ACTIVITIES AND PROTEIN CONTENT

Fig. 1. Fractionation of subcellular components of TA-elicited marcophages by differential centrifugation. Relative distribution of enzyme activities and protein content in the 250,000 g · min pellet (hatched area) and in the 6 × 10^6 g · min pellet and the corresponding supernate (dotted and white areas, respectively). GUR, β-glucuronidase; GAM, N-acetyl-β-glucosaminidase; MAN, α-mannosidase; Ac. PASE, acid phosphatase; LYSZ, lysozyme; PA, plasminogen activator.

RELATIVE ACTIVITY

100
80
60
40
20

α-MANNOSIDASE β-GLUCURONIDASE

3 4 5 6 7   3 4 5 6 7

pH
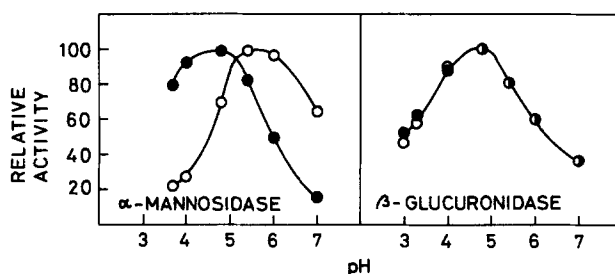
Fig. 2. pH-dependence of α-mannosidase and β-glucuronidase activities in the 250,000 g · min pellet (●) and the corresponding supernate (○).

of primary importance in the present study, no corrections were made. Cathepsin D was also found to be inactivated and, therefore, was not used as a lysosomal marker. In TA-elicited cells, some inactivation of acid phosphatase was also observed.

Properties of the Cultured Macrophages. The main properties of macrophages from untreated and TA-treated mice are shown in Fig. 4. In both types of culture, cell numbers decreased with time. Only slight cell loss was observed in cultures of TA-elicited macrophages. In cultures of nonelicited macrophages, cell loss was relatively high during the first 2 days, but subsided thereafter. In confirmation of the original observations by Unkeless et al. (1), TA-elicited macrophages secreted large amounts of plasminogen activator. Both elicited and nonelicited macrophages secreted similar amounts of lysozyme, which is in accord with the results of Gordon et al. (4).

We found, unexpectedly, that both normal and elicited macrophages released considerable amounts of lysosomal hydrolases into the medium. As shown in the case of β-glucuronidase, the amount of enzyme accumulating in the medium during culture periods of 10–13 days exceeded the intracellular level which remained constant or increased slightly. This release process was not dependent
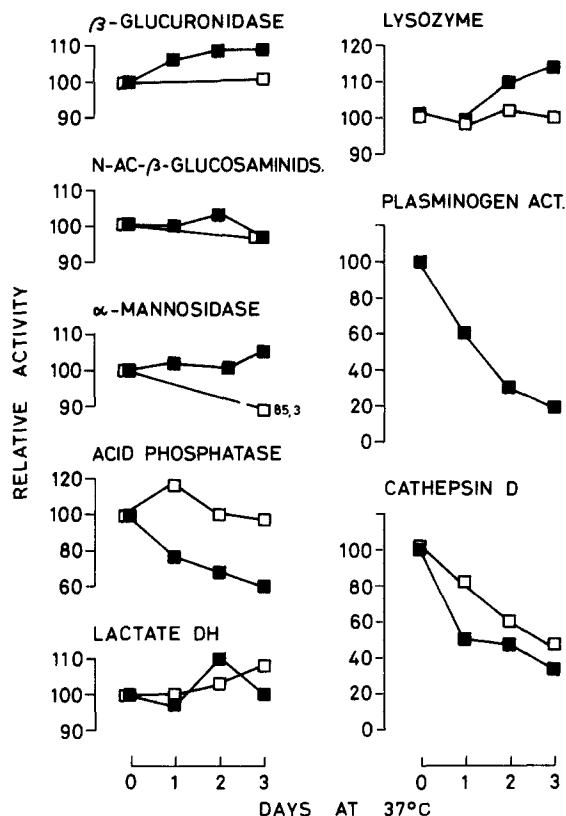
Fig. 3. Stability of enzymes from nonelicited (□) and TA-elicited (■) macrophages in the conditioned culture media at 37°C.

on phagocytosis since the cells were not provided with particles and, therefore, it appears to represent true secretion.

The Process of Lysosomal Enzyme Secretion. The average secretion of β-glucuronidase and N-acetyl-β-glucosaminidase from macrophages of untreated and TA-treated mice, obtained in several experiments performed over a period of about 2 yr, is shown in Fig. 5. In both types of cultures, large amounts of glycosidases accumulated in the medium. Their secretion curves crossed the curves representing the respective intracellular enzyme levels between days 4 and 6. In macrophages from untreated mice, secretion became apparent after an initial lag period of about 2 days. LDH activity was also measured. In both types of culture, intracellular LDH increased with time to about twice the initial level after 10 or 13 days of culture, as shown in Fig. 5. LDH release is usually considered to be an indication of cell damage or death. A correlation appears to exist between LDH accumulation and cell loss in the case of TA-elicited macrophages (Fig. 4). In the cultures of macrophages from untreated mice, however, the initial cell loss was not reflected by an accumulation of LDH. This suggests that the cells detaching from the plates retained their soluble cytoplasmic constituents. We found that LDH was inactivated up to 100% in the media and to a lesser extent in the cell lysates by freezing.
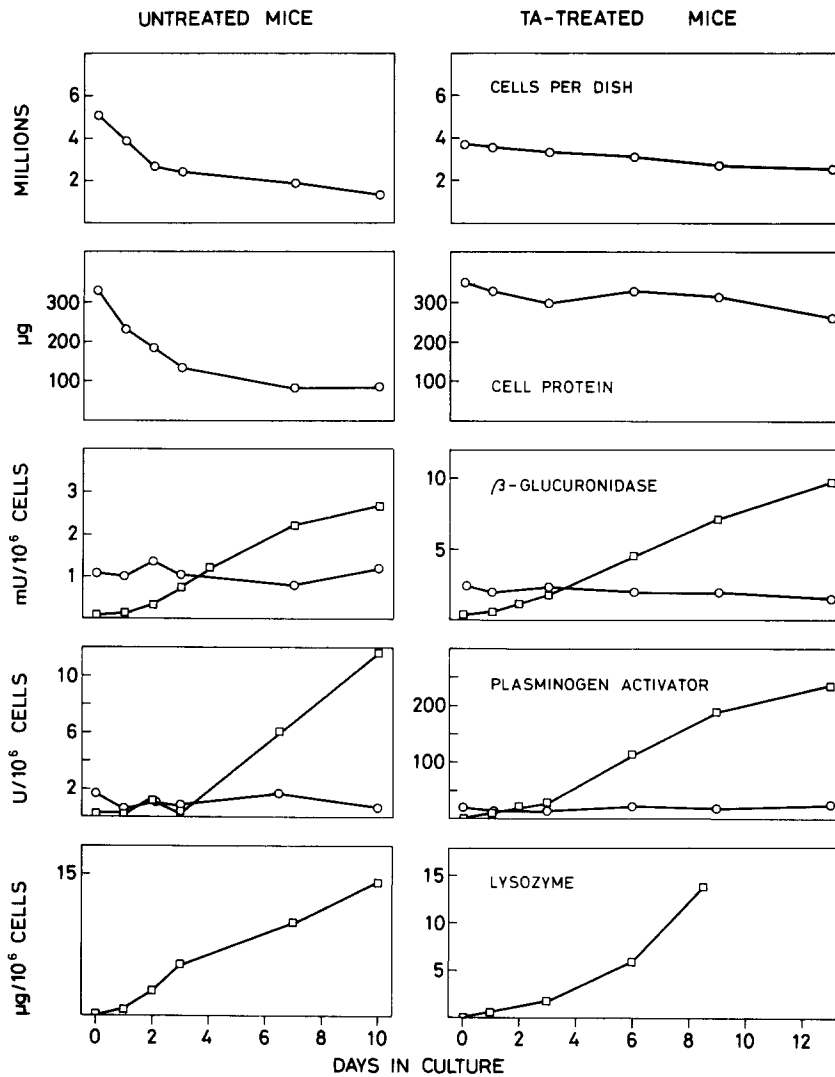
PERITONEAL CELLS FROM

UNTREATED MICE   TA-TREATED   MICE

FIG. 4. Biochemical properties of nonelicited and TA-elicited macrophages. (O), cell numbers or intracellular levels of enzyme or protein. (□), levels of enzymes released into the media.

As shown in Fig. 6, acid hydrolases were secreted by all types of macrophages studied. The acid hydrolase contents and the rates of their secretion were highest (e.g., up to 4 times the rate of the controls) in TA-elicited macrophages. In these cultures, the total amounts of β-glucuronidase, N-acetyl-β-glucosaminidase (Fig. 5), and α-mannosidase (not shown) accumulating in the media exceed the intracellular levels four- to sixfold. Acid phosphatase showed a similar trend, but the amounts found in the media after 12–13 days were only twice the intracellular levels. It must be kept in mind, however, that acid
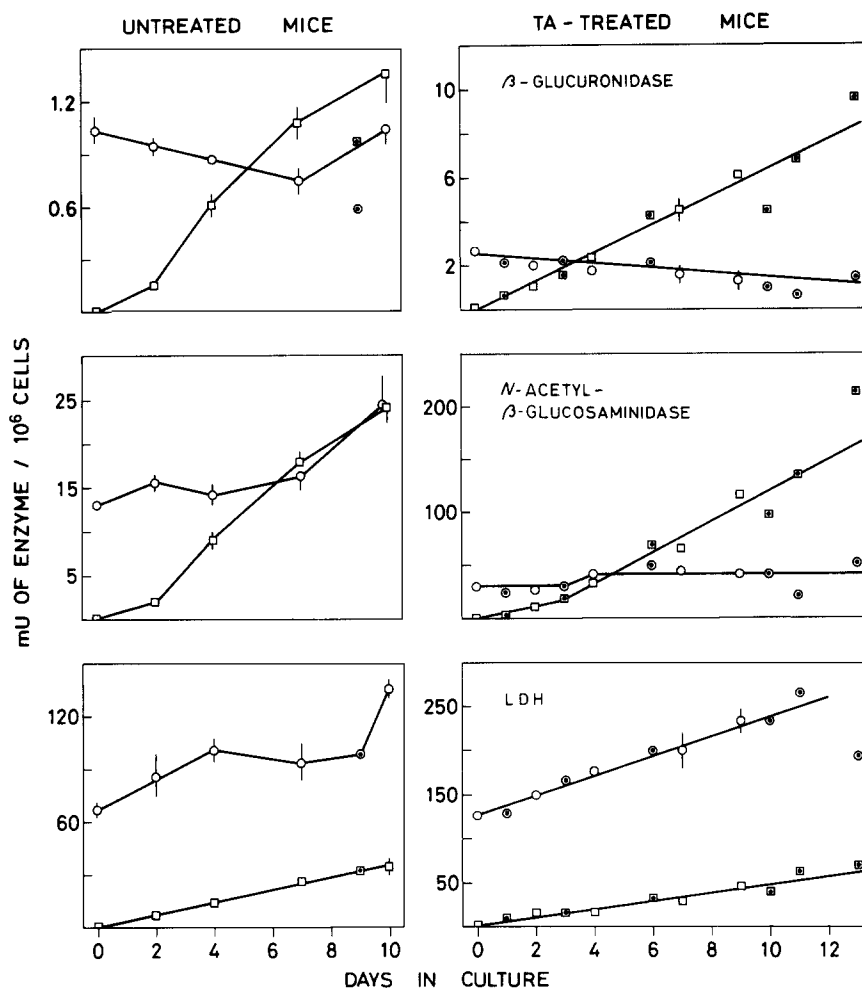
PERITONEAL CELLS FROM

FIG. 5. Selective secretion of β-glucuronidase and N-acetyl-β-glucosaminidase by macrophages. (O), intracellular enzyme levels, (□), extracellular enzyme levels. White symbols represent mean values from 5 to 7 experiments. When larger than the symbol's width, the SEM is represented by a vertical bar. Symbols with a dot represent values obtained in single experiments (triplicate cultures).

phosphatase is inactivated upon release (Fig. 3). The amounts of acid hydrolases secreted and the rates of secretion were similar in control macrophages and in SA- or PP-elicited macrophages.

Factors Influencing Lysosomal Enzyme Secretion. In the experiments reported above, the cells were cultured with acid-treated serum to permit the determination of plasminogen activator (1). As shown in Fig. 7, the secretion of β-glucuronidase and LDH were virtually identical in macrophages that were cultured with acid-treated FBS, normal FBS, or without serum. This figure also shows that the presence of 10 mM Hepes, which we used in combination with
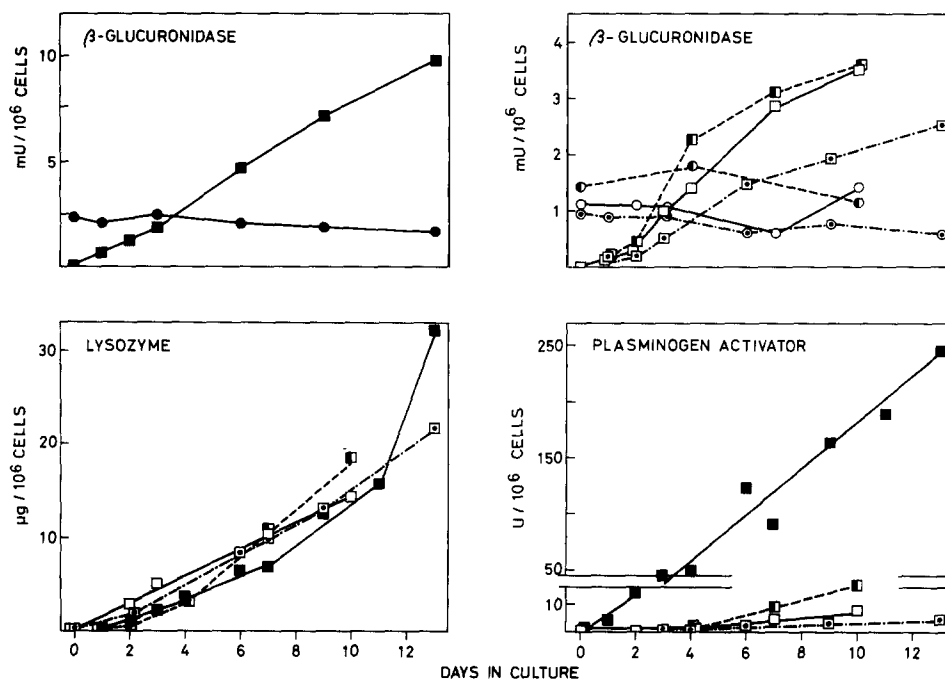
FIG. 6. Effects of pretreatment on the secretory activity of macrophages. Intracellular (circles) and extracellular (squares) enzyme activities in macrophages from untreated mice (white) and mice treated with PP (half-black), SA cell walls (dotted), and TA (black) are shown.

the carbonic acid-bicarbonate buffer, did not influence the secretory activity of the cells.

Fig. 8 illustrates the effects of inhibition of protein synthesis on lysosomal enzyme secretion by nonelicited macrophages. Low concentrations of cycloheximide added to the medium at the onset of culture almost totally prevented β-glucuronidase secretion, while remaining without effect on the intracellular enzyme content. Similar results were obtained in TA-elicited macrophages. The secretion of both β-glucuronidase and plasminogen activator was inhibited by noncytotoxic concentrations of cycloheximide. In a separate set of experiments, the effect of cycloheximide on the incorporation of tritiated leucine into macrophage protein was studied. As shown in Fig. 9, cycloheximide at a concentration of 0.25 μg/ml inhibited the incorporation of the label into intracellular protein. Under these conditions, virtually no radioactively labeled protein was secreted. The control experiments (cycloheximide omitted) show an interesting difference between nonelicited and TA-elicited macrophages. In nonelicited cells, a large proportion of the labeled (i.e., newly synthesized) protein appeared as secretory product, whereas in TA-elicited cells, only a minor proportion of the labeled protein was secreted.

In Vivo Secretion of Lysosomal Enzymes by Macrophages. To establish whether peritoneal macrophages secrete lysosomal enzymes under in vivo conditions, we have assayed acid glycosidases and LDH in the cells and the
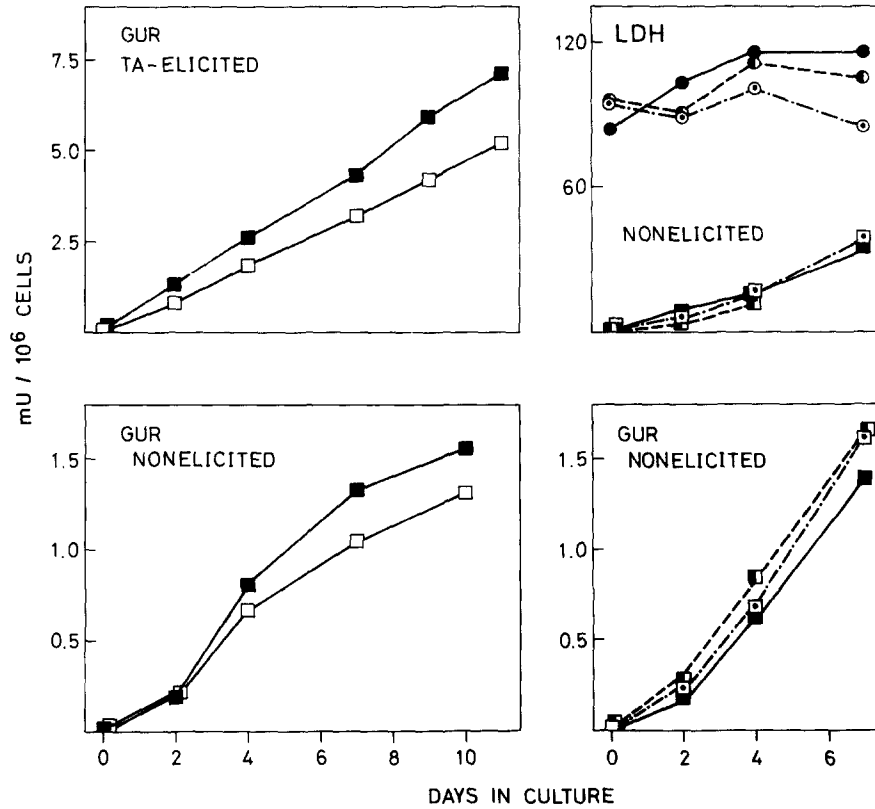
FIG. 7. Effect of medium composition on the secretion of β-glucuronidase (GUR) from TA-elicited and nonelicited macrophages. The symbols represent the following conditions: black, standard medium containing 5% acid-treated FBS; white, standard medium without serum; dotted and half-black, standard medium without Hepes containing 5 and 20% heat-inactivated FBS, respectively. Intracellular (circles) and extracellular (squares) levels of LDH are also shown.

media immediately after harvesting from the mouse peritoneal cavity and after the adherence period of 2 h at the beginning of culture. The mice were untreated or treated in the usual way (see Materials and Methods) with either TA or SA. The cells were harvested with 2 ml of cold medium and either kept for biochemical analysis or plated on dishes as described in Materials and Methods. After 2 h of culture, the medium was collected and freed of nonadherent cells. Adherent cells and the media were kept for biochemical analysis. The results are presented in Table II. In all instances, measurable amounts of lysosomal glycosidases were found both in the lavage fluid and in the medium used during the first 2 h of culture, when macrophages were allowed to adhere to the culture dishes. By contrast, LDH was undetectable in the extracellular media, thus indicating that lysosomal contents were released selectively by fully viable cells. The amounts of glycosidases found in the peritoneal fluids and in the adherence-phase media were very high (25-74% of the intracellular levels) in TA-treated mice, compared to SA-treated or control animals. This is in
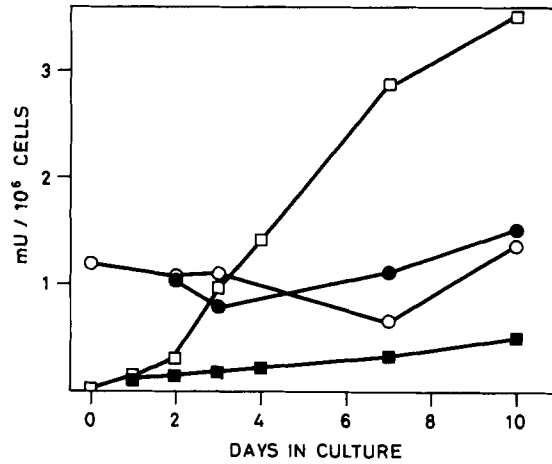
FIG. 8. Effects of cycloheximide (black symbols) at the concentration of 0.25 μg/ml on the intracellular (circles) and the extracellular (squares) levels of β-glucuronidase in non-elicited macrophages. The values obtained in the absence of cycloheximide are represented by white symbols.
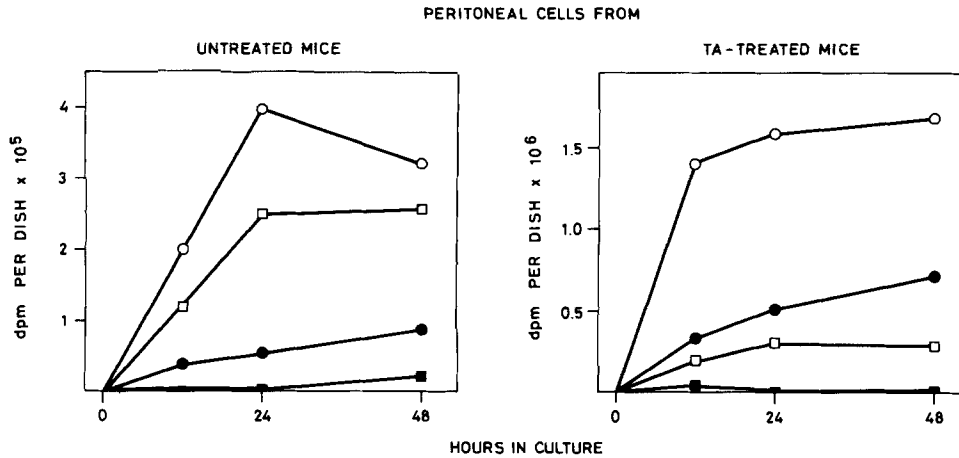

FIG. 9. Effects of cycloheximide, 0.25 μg/ml (black symbols) on the incorporation of [^{3}H]leucine into intracellular (circles) or secreted (squares) TCA-precipitable material. The corresponding values obtained in the absence of cycloheximide are represented by white symbols.

accordance with the observations made in vitro that TA-elicited macrophages secreted lysosomal enzymes at maximum rate from the onset of culture, whereas in cells elicited by other stimuli or obtained from untreated mice, secretion increased progressively from very low rates during the first 2 days of culture. The lavage medium of TA-elicited macrophages contained, on the average, ≅68 U/10^6 cells of plasminogen activator, which corresponds to the amount produced during 4–6 days in culture. No plasminogen activator was found in the lavage medium of nontreated mice.

TABLE II

Secretion of Lysosomal Hydrolases by Mouse Peritoneal Macrophages in Vivo

Pretreatment of mice	Enzyme	Activity per 10^6 cells*			
		Lavage cells	Lavage medium	Adherent cells	Culture medium
		mU			
TA	GAM‡	30.13 ± 5.6	8.65 ± 1.26	23.85 ± 3.11	12.3 ± 1.59
	GUR§	4.49 ± 0.95	1.38 ± 0.26	3.07 ± 0.51	2.0 ± 0.31
	LDH	200.00 ± 38.4	0	138.60 ± 16.16	0
SA	GAM	15.91 ± 1.32	0.97 ± 0.06	14.57 ± 2.45	1.73 ± 0.2
	GUR	1.12 ± 0.22	0.07 ± 0.01	1.00 ± 0.16	0.13 ± 0.01
	LDH	72.61 ± 5.83	0	58.59 ± 6.21	0
none	GAM			8.85 ± 0.64	1.83 ± 0.17
	GUR	not determined		1.13 ± 0.11	0.08 ± 0.01
	LDH			47.37 ± 3.72	0

* Mean values ± SEM from 5 to 10 determinations.
‡ GAM, *N*-acetyl-β-glucosaminidase.
§ GUR, β-glucuronidase.

Discussion

We have shown that mouse peritoneal macrophages release lysosomal hydrolases into the culture medium at nearly constant rates for up to 2 wk. The amounts of enzyme released largely exceed the intracellular levels which remain nearly constant. Enzyme release is not a consequence of cell damage, as indicated by the comparatively low amounts of LDH found in the medium and by the steady secretion of lysozyme (4). The release process is not related to phagocytosis. We think, therefore, that lysosomal enzymes are released from cultured macrophages by true secretion. This process is dependent on protein synthesis, as suggested by the inhibitory effect of cycloheximide, and it does not require the presence of serum in the culture medium.

In most cells and particularly in phagocytes, lysosomal enzymes are released mainly into endocytic or autophagic vacuoles, and they serve the purpose of intracellular digestion of engulfed or segregated material. During phagocytosis, lysosomal enzymes may escape from the cell when lysosomes fuse with incomplete vacuoles. This phenomenon has been amply documented for polymorphonuclear leukocytes (19–21). Extracellular release of lysosomal contents in association with phagocytosis has been described in macrophages (5, 22, 23). In these studies, macrophages were shown to release β-glucuronidase and *N*-acetyl-β-glucosaminidase when they phagocytose certain nondigestible particles. Release of lysosomal enzymes from macrophages can also be induced in the absence of phagocytosable particles. Pantalone and Page (6) induced the release of four acid hydrolases with supernates of mitogen-activated lymphocytes. Schorlemmer and Allison (7) obtained similar effects by supplying macrophage cultures with C3b. Our results show that large quantities of lysosomal hydrolases are secreted from both normal and elicited macrophages under conventional culture conditions. The addition of external stimuli, such as soluble or particulate agents which activate complement by the alternative pathway (24),

does not appear to be essential. The secretory activity is virtually identical in the presence of normal (i.e. not heat-inactivated), or acid-treated serum, or when serum is omitted.

Secretion by macrophages that were obtained from untreated mice was initially very low, and reached the maximum rate only during the 2nd or 3rd day in culture. This may explain why in the short-time experiments (5, 22, 23) only low secretion rates were observed in nonstimulated macrophages. The lag in the secretory activity of nonelicited macrophages may indicate that these cells become activated during the first days in culture. The molecular rearrangements within the plasmalemma and/or the ectoplasmic zone, which are likely to occur during attachment and spreading of the cells on the culture dishes, could facilitate the exocytotic discharge of lysosomes. Secretion may occur exclusively on the area of the plasmalemma which is in contact with the dishes. It is also conceivable that macrophages may become activated by their own products (24, 25). Lysosomal enzyme secretion, however, is not likely to be a mere artefact of culture since sizable amounts of acid hydrolases are found in the peritoneal fluid of the mice. This is particularly striking in TA-treated mice whose peritoneal cell population consists essentially of macrophages.

The secretion of lysosomal hydrolases as described in this paper appears to be an important property of macrophages. By comparison, secretion of acid hydrolases by cultured fibroblasts is very low (26, 27). Macrophages that are activated by inflammatory stimuli such as thioglycollate, secrete, both in vitro and in vivo, enormous amounts of these digestive enzymes. This suggests that lysosomal hydrolases from macrophages must be regarded as a potential pathogenetic factor in chronic inflammation. Acid hydrolases are thought to act at nearly optimum pH in secondary lysosomes. Their activity is generally low at the pH of the extracellular fluid. In the microenvironment of the secreting macrophage, however, the pH may be lower than in serum or in exudates, and the concentration of the released enzymes may be sufficiently high for the induction of lytic damage. This problem has been discussed in detail by Dingle (28) and by Poole et al. (29, 30) in connection with the possible role of cathepsin D from synovia or cartilage in arthritic tissue destruction.

In nonelicited macrophages, secretion of lysosomal enzymes can be differentiated on a time-course basis from that of other products such as lysozyme and plasminogen activator. Lysozyme is secreted from the onset of culture while the lysosomal enzymes appear in the medium after an average lag period of 2 days. Secretion of plasminogen activator, which is very low but measurable in these cells, starts much later, on days 4 or 5.

In physiological terms, the secretory process described may be viewed as a mechanism for the replacement, by Golgi-derived membrane, of plasmalemma that becomes internalized in the course of pinocytosis. Extensive membrane recycling by a shuttle mechanism is thought to occur in secretory cells and nerve terminals (31). Another membrane-recycling principle involving the reutilization of the membrane of pinocytic vacuoles has been proposed for macrophages by Steinman et al. (32), and for fibroblasts by Tulkens et al. (33).

Summary

Peritoneal macrophages were obtained from untreated mice and from mice

treated with thioglycollate medium (TA), proteose peptone medium (PP), or a suspension of streptococcus A cell wall material (SA). The biochemical and secretory properties of these cells in long term cultures (up to 2 wk) were compared.

TA-elicited macrophages contained more protein, lactate dehydrogenase, lysosomal hydrolases, and in particular, more plasminogen activator than the other cells studied. All types of macrophages studied were found to release considerable amounts of lysosomal hydrolases (β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and acid phosphatase) into the medium. Release was independent of phagocytosis and must, therefore, be regarded as true secretion. In both elicited and nonelicited macrophages, the rates of lysosomal enzyme secretion were virtually identical in the presence and in the absence of serum, and they were not enhanced by increasing serum concentrations. Lysosomal enzyme secretion in macrophages appears to depend on protein synthesis, since it was blocked by low concentrations of cycloheximide which neither affected cell viability nor lowered the intracellular enzyme levels. The amounts of lysosomal hydrolases secreted were highest in TA-elicited macrophages. The rates of secretion of PP- or SA-elicited and of nonelicited macrophages were about one-fourth of that of the TA-elicited cells. This difference, although significant, is much smaller than that observed for the secretion of plasminogen activator which was 20-50 times higher in TA-elicited cells.

Acid glycosidases were also found in the peritoneal lavage media used for cell harvesting from both treated and nontreated mice. This indicates that active secretion of lysosomal hydrolases may be an in vivo property of the macrophage.

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