

IN VITRO SYNTHESIS AND SECRETION OF LYSOZYME BY MONONUCLEAR PHAGOCYTES*

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In 1922 Alexander Fleming described the remarkable bacteriolytic activity of an enzyme, lysozyme, which was widely distributed in tissues and secretions (1). Lysozyme (muramidase) is a cationic enzyme, mol wt 14,307, which hydrolyses *N*-acetyl muramic β -1, 4 *N*-acetyl glucosamine linkages in the bacterial cell wall (2). Although a great deal is known about its structure and enzymology its function other than in host defence is still poorly understood.

High concentrations of lysozyme are found in leukocytes, especially the polymorphonuclear leukocyte (PMN)¹ and rabbit alveolar macrophage (3). Fractionation studies of the rabbit PMN show that 70% of its intracellular lysozyme is sedimentable and, unlike other hydrolases, it is found in both the azurophil and specific granules of the cell (4). The BCG-induced rabbit alveolar macrophage is able to release a large fraction of its intracellular lysozyme into the medium during phagocytosis (5) and may secrete lysozyme during cultivation in vitro (6). Large amounts of lysozyme accumulate in the serum and urine of patients (7) and animals (8) bearing monocytic leukemia.

In this paper we report that mouse peritoneal macrophages and human monocytes synthesize and secrete substantial amounts of lysozyme in culture. We also study factors which influence the rate of lysozyme production and examine the effect of phagocytosis on its secretion.

Materials and Methods

Cell Cultures.—

Mouse peritoneal macrophages: Female mice of the NCS (Rockefeller) strain, weighing 25–30 g were used. Peritoneal macrophages were harvested, without anticoagulants, by standard procedures (9); the cells were obtained either 4 days after stimulation by intraperitoneal injection of 0.75 ml thioglycollate medium (10) or from control, unstimulated mice. The cell yield from unstimulated mice was $5-8 \times 10^6$ cells, of which 30–40% were macrophages and the remainder lymphocytes; thioglycollate-stimulated mice yielded $15-20 \times 10^6$ cells, consisting of 75–90% macrophages and 10–25% lymphocytes.

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¹ *Abbreviations used in this paper:* CM, conditioned medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LH, lactalbumin hydrolysate; LZM, lysozyme; NBCS, newborn calf serum; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte.

Cells were routinely cultured in MEM or Dulbecco's medium supplemented with 5% fetal calf serum (FCS) that had been heated at 56°C for 30 min. Horse and newborn calf serum (NBCS) were also used, as specified. Peritoneal cell suspensions were plated in Falcon plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at a density of 2×10^5 thioglycollate stimulated cells per square centimeter or 1×10^6 unstimulated cells per square centimeter. The cultures were incubated at 37°C in the presence of 5% carbon dioxide. The monolayers were washed vigorously with Hanks' balanced salt solution (HBSS) to remove nonadherent cells within 2–24 h.

Human monocytes: Monocytes were obtained from 20–50 ml blood using either heparin (20 U/ml) or EDTA, final concentration 1.5 mM, as anticoagulant. The blood was layered on a mixture of Ficoll-Hypaque (24 vol 9% Ficoll:10 vol 34% Hypaque; 5 vol blood:4 vol Ficoll-Hypaque) and centrifuged at 300 g for 45 min at room temperature (11). The mononuclear band was resuspended in PBS containing 0.3 mM EDTA and centrifuged at 1,250 g for 10 min. The platelet-rich supernatant was then discarded and the cells were washed and centrifuged three times in PBS-EDTA (100 g for 10 min). The cells were resuspended in MEM containing 5% FCS, and plated in tissue culture plastic or glass dishes at a density of $2-4 \times 10^6$ mononuclear cells per square centimeter. Differential cell counts were obtained from cytocentrifuge preparations which had been air dried and stained by a Wright-Giemsa procedure. The yield of mononuclear cells averaged $0.5-2.5 \times 10^6$ cells/ml blood and consisted of 30–50% monocytes, 50–70% lymphocytes, and fewer than 2% PMN. Variable numbers of platelets and erythrocytes were present. The mononuclear cell cultures were incubated in 5% CO₂ at 37° and were washed vigorously within 24 h to remove nonadherent lymphocytes. Monocyte cultures were re-fed every 2–3 days. In some experiments lactalbumin hydrolysate (LH 0.02% wt/vol) was added to the above medium.

Human PMN's: Human PMN's were purified according to Edelson et al. (12). This method includes dextran sedimentation, Ficoll-Hypaque centrifugation and lysis of erythrocytes by ammonium chloride. Final preparations contained more than 98% PMN. The PMN were resuspended and plated in the same way as mononuclear cell preparations.

Mouse peritoneal lymphocytes: Lymphocytes were purified by passing cell suspensions over Sephadex G-10, according to the method described by Chen (13).

Cell morphology: Cell morphology was studied by phase contrast microscopy of living or glutaraldehyde-fixed material, and cell viability was measured by trypan blue exclusion.

Cell lysates and conditioned medium, (CM): Lysates and CM were prepared as follows. Conditioned medium was collected from monolayer cultures and centrifuged at 1,000 g for 10 min to remove cellular debris. Triton X-100 was added to the supernate, final concentration of 0.2% wt/vol, as required. The cell monolayer was washed twice with normal saline and scraped in 0.2% Triton X-100 with the aid of a plastic policeman. Cell lysates were also prepared in normal saline, as required. Harvested materials were kept on ice or stored at –20°C.

Serum-free CM was prepared in the same way by incubating 1-day old macrophage cultures in MEM or Dulbecco's medium supplemented with 0.02–0.05% lactalbumin hydrolysate. Macrophages were viable in this medium for at least 3 days.

To prepare ¹⁴C-labeled CM 2-day old macrophage cultures were first washed three times in HBSS and then incubated in medium consisting of HBSS, 5 μCi/ml reconstituted [¹⁴C]algal hydrolysate, $\frac{1}{100}$ MEM, and 5% FCS previously dialyzed against 0.01 M sodium bicarbonate 0.15 M sodium chloride, pH 7.4. After 4 h of incubation the medium was discarded, and the cultures were washed three times with HBSS and then incubated for 2 days in Dulbecco's medium containing 0.05% (wt/vol) of lactalbumin hydrolysate. This serum-free CM was centrifuged at 1,000 g for 15 min and stored at –20°C. ¹⁴C-labeled CM was prepared for polyacrylamide gel electrophoresis (cationic proteins) by dialysis against 0.1 M sodium carbonate, pH 8.0, and was lyophilized. Other samples were prepared for SDS-polyacrylamide gel electrophoresis, as described elsewhere (14).

Lysozyme: Lysozyme was assayed by measuring the initial rate of lysis of a suspension of *Micrococcus lysodeikticus* (15), with the aid of a Gilford 240 spectrophotometer (Gilford In-

strument Laboratories, Inc., Oberlin, Ohio) fitted with an automatic recorder. Standards or lysozyme-rich materials were diluted in MEM containing 5% FCS. Reaction conditions were as follows: 1 ml of sample was mixed at room temperature with 2 ml of a suspension of *M. Lysodeikticus*, 0.25 mg/ml 1/15 M phosphate buffer, pH 6.3. The rate of decrease in turbidity was measured at 540 nm, using a ratio switch position of 0.5 and with full recording scale equal to 0.3 OD units. The initial reaction rate was linear for at least 1 min and was directly proportional to egg lysozyme concentration in the range 0.2–2 $\mu\text{g/ml}$. Rat and human lysozyme standards showed 2.0- and 3.3-fold higher initial rates of lysis, respectively, than a hen lysozyme standard. In this paper we report mouse macrophage lysozyme activity in terms of the rat standard and human monocyte lysozyme in terms of the human standard. Appropriate reagent controls were included as required. Triton X-100, 0.2% wt/vol, had no significant effect on lysozyme activity.

The inhibition of lysozyme by antisera was measured as follows. Lysozyme was adsorbed from control and specific rabbit antisera with activated charcoal, which was in turn removed by centrifugation. Macrophage conditioned medium, containing 1.0 μg lysozyme/ml, was incubated for 30 min at 37° with an equal volume of a serial dilution of the rabbit serum. The mixture was then centrifuged and the supernatant fraction assayed for residual lysozyme activity.

Other enzymes were measured as follows: acid phosphatase, substrate α -naphthyl acid phosphate, according to Allen and Gockerman (16). *N*-acetyl β -glucosaminidase, substrate *p*-nitrophenyl-*N*-acetyl β -glucosaminide, β -glucuronidase, substrate phenolphthalein β -glucuronide, β -galactosidase, substrate *p*-nitrophenyl- β -galactoside and cathepsin D, substrate denatured hemoglobin, all according to Bowers et al. (17). Suitable controls for CM, cell lysates, and all reagents were included.

Protein: Protein was assayed using egg lysozyme as standard (18).

Polyacrylamide gel electrophoresis: Electrophoresis for cationic proteins was performed in 10% polyacrylamide gels (19). SDS-polyacrylamide gel electrophoresis and autoradiography were carried out as described elsewhere (14).

Phagocytosis: The effect of phagocytosis on the production and secretion of lysozyme was studied in unstimulated mouse peritoneal macrophages which had been previously cultivated for 48 h in MEM with 10% horse serum. Latex particles, 1.1 μm in diameter, were washed seven times by centrifugation and sterilized with ultraviolet light. Stock suspensions, about 1×10^9 particles/ml, were stored at 4° C in MEM. Macrophage cultures were washed twice with HBSS and placed in fresh MEM with 10% horse serum, 2.5 ml per 60 mm dish. The latex was resuspended vigorously and 0.1 ml added per dish. The cultures were then incubated at 37°C and the course of phagocytosis followed by phase contrast microscopy. After more than 95% of macrophages had taken up at least 20 latex particles per cell, usually within 2 h, excess latex was removed, and the cells were washed twice and incubated in MEM with 10% horse serum. Control cultures, not given latex, were handled in parallel.

The medium was removed at intervals, the cells washed twice in PBS and then scraped from the dish with a policeman in 0.2% Triton. Cell lysates and medium were centrifuged at 1,000 g for 15 min and the supernate was assayed for lysozyme.

Reagents.—

Media: MEM, FCS, horse serum, and NBCS were obtained from Grand Island Biological Co., Grand Island, N. Y.; thioglycollate medium from BBL, Division of BioQuest, Cockeysville, Md.; lactalbumin hydrolysate from ICN Nutritional Biochemicals Div., Cleveland, Ohio; and polystyrene latex particles, 1.1 μm , from Dow Diagnostics, Indianapolis, Ind.

Chemicals: Ficoll was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; Hypaque (sodium diatrizoate) from Winthrop Laboratories, Sterling Drug Co., N. Y.; and Colchicine from Sigma Chemical Co., St. Louis, Mo. Cycloheximide was a gift from Dr. S. Silverstein, The Rockefeller University. Cytochalasin B was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Biological reagents: *Micrococcus lysodeikticus*, spray dried, was obtained from Miles Laboratories, Inc., Kankakee, Ill.; and egg white lysozyme, 2 × crystalline, from Worthington Biochemical Corp., Freehold, N. J.; rat lysozyme and rabbit antirat lysozyme antiserum were provided by Dr. E. Osserman, College of Physicians and Surgeons of Columbia University, New York. Rabbit antimouse lysozyme was a gift from Dr. R. Riblet, Salk Institute, La Jolla, Calif.

Isotope: Reconstituted algal hydrolysate, [¹⁴C]amino acids was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

All other reagents were purest grade available from standard commercial suppliers.

RESULTS

General Considerations.—Homogeneous, nondividing cultures of murine and human mononuclear phagocytes were routinely produced by the methods described. The morphologic appearance of unstimulated (9) and thioglycollate-stimulated (20) mouse peritoneal macrophages in culture has been described previously. The human monocytes differentiated into macrophages after 3 days in culture, after which they remained stable for at least 2 wk, commonly giving rise to multinucleated giant cells. Monocytes displayed active membrane ruffling and were rich in homogeneous, peroxidase-positive granules, which disappeared during the first 2 days in culture. The human macrophages contained a more heterogeneous population of cytoplasmic organelles, including peroxidase-negative granules and lipid droplets.

The use of lactalbumin hydrolysate to replace or supplement serum resulted in well-spread, more spindly macrophages and was especially useful in maintaining human monocyte cultures.

Lysozyme production in vitro: The production of lysozyme by mononuclear phagocytes in culture was first demonstrated by assaying cell lysates and conditioned medium at daily intervals. Fig. 1 and Table I illustrate such an experiment using unstimulated and thioglycollate stimulated mouse macrophages as

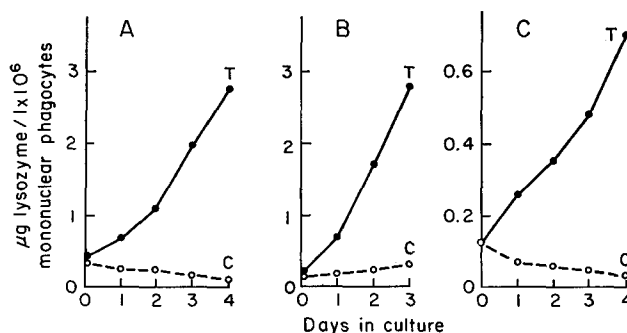


FIG. 1. Lysozyme production by mononuclear phagocytes in culture. (A) Unstimulated mouse peritoneal macrophages. (B) Thioglycollate stimulated mouse peritoneal macrophages. (C) Human blood monocytes. C, cell lysate; T, total cell lysate and cumulative medium content. Each point is the average of replicates which differed by less than 10%.

well as human monocytes. Mononuclear phagocytes containing 0.12–0.35 μg lysozyme, depending on cell type, produced a 4- to 11-fold increase in total lysozyme during 3 days. The intracellular content remained relatively constant whereas extracellular lysozyme increased daily, representing 87–90% of total production after 3 days. All three types of cell showed a similar pattern of lysozyme accumulation.

Similar results were obtained in repeated experiments in which total lysozyme increased up to 25-fold in 3 days. Thioglycollate-stimulated macrophages consistently produced 140–250% more lysozyme per cell than unstimulated macrophages in which lysozyme specific activity based on cell protein was, however, twofold higher. Lysozyme production per day represented 0.5–2.5% of total cell protein per day. Because of the net increase in total lysozyme and its continued

TABLE I
Lysozyme Production by Mononuclear Phagocytes in Culture

	Before culture	After 3 days' cultivation			LZM production ($\mu\text{g}^*/\text{mg}$ cell protein)	Percent LZM secreted
	LZM content ($\mu\text{g}^*/1 \times 10^6$ mononuclear phagocytes)	μg^* LZM/ 1×10^6 mononuclear phagocytes		Total		
		Intra-cellular	Extracellular			
Unstimulated mouse peritoneal macrophages	0.35	0.2	1.8	2.0	35	90
Thioglycollate-stimulated mouse peritoneal macrophages	0.25	.35	2.45	2.8	18	87
Human blood monocytes	0.12	0.05	0.43	0.48	14	90

* A rat LZM standard was used for mouse cells, a human LZM standard for human cells.

extracellular accumulation we concluded that mononuclear phagocytes secreted lysozyme into the medium.

Human polymorphonuclear leukocytes, which contained 0.6 μg lysozyme per million cells, were next cultivated under similar conditions. Lysozyme was released into the medium, but these cells died after several hours in culture without further production of lysozyme. Cell lysates and conditioned medium obtained from several other cell types were tested and found to be uniformly lacking in lysozyme. Cells examined include: rat thoracic duct lymphocytes, human lymphoid cell lines 8866 and WIL-2, human D98 and VA₂ epithelioid cell lines, mouse L cell fibroblasts, primary chick embryo fibroblasts, Rous sarcoma virus-transformed fibroblasts from chick embryos, primary mouse embryo fibroblasts, murine sarcoma virus mouse embryo fibroblasts, mouse neuroblastoma, and Chinese hamster ovary cells.

Characterization of mouse macrophage lysozyme: The nature of the lysozyme secreted by mouse macrophages was next studied. The enzyme was stable to

boiling for 1 min at pH 3.5. Specific rabbit antisera against purified rat or mouse lysozyme inhibited mouse macrophage lysozyme whereas control rabbit sera had no effect (Fig. 2).

The biosynthesis and secretion of lysozyme during *in vitro* culture were confirmed by identifying ^{14}C -labeled lysozyme in culture media obtained from both unstimulated and thioglycollate-stimulated macrophages which had been previously incubated in radioactive amino acids. SDS-polyacrylamide gel electrophoresis revealed a large radioactive peak, 14,000 mol wt, which coelectrophoresed with a lysozyme standard (14). The macrophage medium also contained a radiolabeled cationic molecular species which coelectrophoresed with a

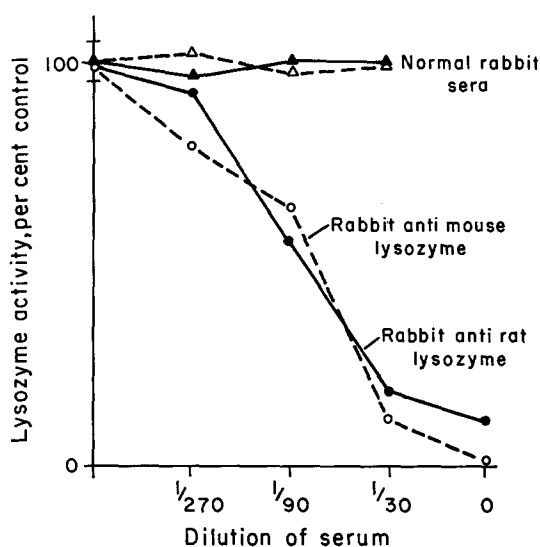


FIG. 2. Inhibition of mouse macrophage lysozyme by specific rabbit antisera.

lysozyme standard (Fig. 3). The mouse macrophage enzyme thus resembled lysozyme in antigenicity, size, and charge and was clearly synthesized and secreted *in vitro*.

Quantitation of lysozyme secretion. The above experiments provided strong evidence that lysozyme is a cell-specific macrophage secretory product and since lysozyme itself is stable and well-characterized, further experiments were designed to explore the usefulness of this culture system as a model for macrophage secretion.

In order to measure lysozyme secretion accurately the following criteria were met. More than 95% of cells were viable; intracellular lysozyme was fully released by both treatment with 0.1–0.2% Triton X-100 and by five cycles of freezing and thawing in the absence of detergent; CM-containing lysozyme, removed from cells and incubated further at 37°, showed no loss of activity, and lysozyme was also stable after pro-

longed storage at -20°C and upon repeated freezing and thawing; mixing experiments, using cell lysates and conditioned media, showed no evidence for inhibitors or activators of lysozyme activity.

Fetal calf serum contained no endogenous lysozyme and background activity in horse or NBCS was subtracted when necessary. Replicate or triplicate cultures were analyzed in all experiments and showed less than 20% variation. Only the average values are therefore given for most experiments.

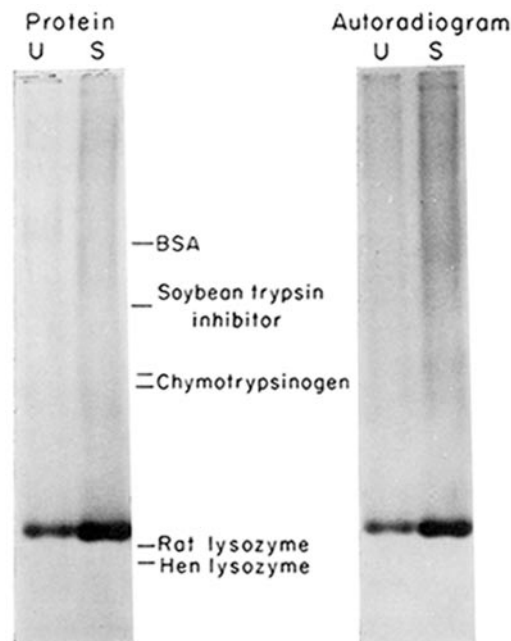


FIG. 3. Electrophoresis of labeled products obtained from the culture medium of unstimulated (U) and thioglycollate stimulated (S) macrophages. 3 ml of culture medium from U and 2 ml from S cells (see text for details of culture) were dialyzed against 0.1 M ammonium carbonate buffer and lyophilized. 30 μl of the solubilized residue was applied to 10% cationic polyacrylamide slab gels. The pattern on the left was stained with Coomassie blue and that on the right developed with X-ray film for 4 days.

Control of Lysozyme Production and Secretion.—Various aspects of macrophage cultivation were examined to identify those factors which influence lysozyme synthesis and secretion in vitro.

Cell density: Different numbers of mononuclear phagocytes were plated and lysozyme secretion measured under standard conditions. Fig. 4 shows that there was a linear relationship between lysozyme secretion and the number of (A) thioglycollate-stimulated macrophages, or (B) human monocytes cultured per

dish. Lysozyme content is therefore a useful measure of macrophage number and cell density has no effect on lysozyme production.

The kinetics of lysozyme secretion: The rate of lysozyme production and secretion was examined in mononuclear phagocyte cultures of variable duration. Fig. 5 shows that (A) unstimulated mouse macrophages and (B) human monocytes continued to secrete lysozyme over a period of at least 11 and 17 days, respec-

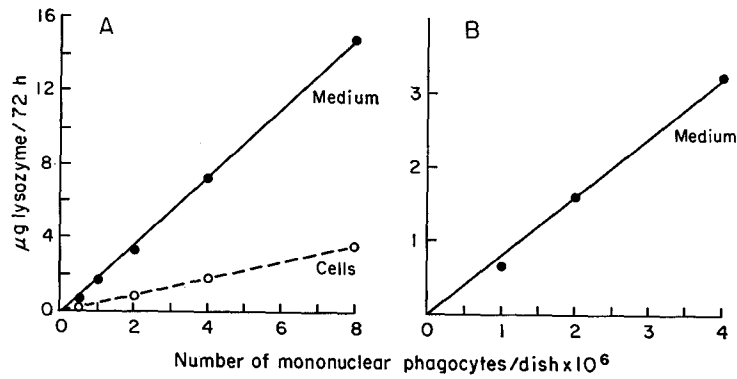


FIG. 4. Lysozyme production in relation to the number of mononuclear phagocytes. (A) Thioglycollate-stimulated mouse macrophages (60 mm dish). (B) Human monocytes (35 mm dish).

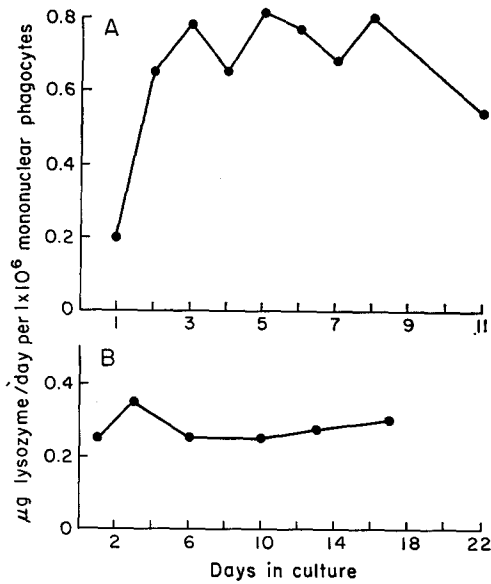


FIG. 5. Rate of lysozyme secretion by mononuclear phagocytes. (A) Unstimulated mouse macrophages. (B) Human monocytes.

tively. Unstimulated mouse macrophages showed a low initial rate of secretion followed by a threefold rise on the 2nd day in culture and a relatively stable rate of subsequent secretion, $0.75 \mu\text{g}$ lysozyme/ 1×10^6 cells per day. The rate of secretion by human monocytes ($0.3 \mu\text{g}/\text{day}$) changed little throughout this period, in spite of a dramatic morphologic change to mature macrophages after the 3rd day in culture. An increased rate of secretion after continued cultivation could also be seen in short-term experiments with thioglycollate-stimulated macrophages (Fig. 6). Freshly explanted, stimulated macrophages only started

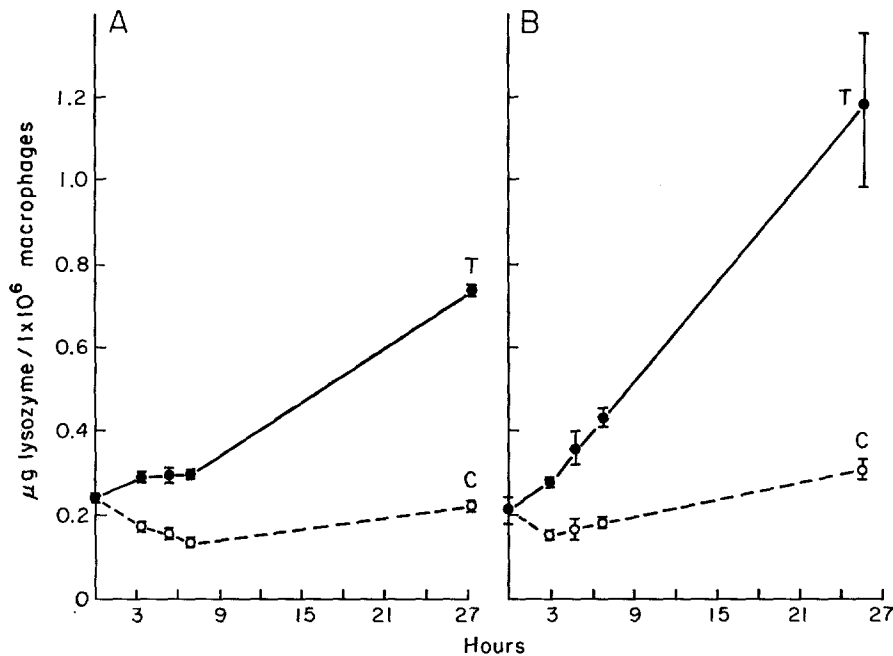


FIG. 6. Lysozyme production by thioglycollate-stimulated macrophages 0.5 h (A) and 48 h (B) after start of cultivation. C, cell lysate, T, total medium and cell lysate. Average of triplicate assays.

to produce lysozyme after a lag period of about 7 h (6 A). After 48 h in culture, a higher secretion rate had become established and total lysozyme production proceeded linearly with time. (Fig. 6 B).

The increased secretion by mouse macrophages on their 2nd day in culture was investigated further. No inhibitor of lysozyme production was detected in mouse peritoneal fluid, which did contain free lysozyme ($0.12 \mu\text{g}/1 \times 10^6$ macrophages). The harvest medium and plating procedure did not influence the initial lag period. Increased lysozyme secretion in vitro could also have been due to the removal of a lymphoid cell inhibitor. A cocultivation experiment with purified populations of well-washed, adherent macrophages and peritoneal

lymphocytes which had been freed of all adherent cells by passage over a Sephadex G-10 column, is illustrated in Table II.

Lymphocyte recovery after fractionation was 27%, all of which were viable, and 41-50% of these lymphocytes remained viable after 3 days cultivation. 5×10^{-5} M mercaptoethanol was added to some cultures to improve the survival of lymphocytes. Medium, adherent, and nonadherent cell fractions were collected, the adherent cells consisting entirely of macrophages, the nonadherent cell fraction containing lymphocytes and fewer than 10% macrophages.

Lysozyme was detected in all of the media which had been exposed to macrophages (*A, B, C*), but not in lymphocyte-conditioned medium (*D*). The lysates of adherent cells (*A, B, C*) contained lysozyme, whereas nonadherent lysates

TABLE II
Peritoneal Lymphocytes and Macrophage Lysozyme Production

	A	B	C	D
Culture system				
Macrophages*	+	+	+	-
Lymphocytes†	-	-	+	+
Mercaptoethanol, 5×10^{-5} M	-	+	+	+
Lysozyme‡				
Medium	1.51	1.57	1.69	0
Adherent cells	0.16	0.20	0.18	0
Nonadherent cells	0	0	0	0
Total	1.67	1.77	1.87	0

* 1.5×10^6 per dish.

† 2×10^6 per dish.

‡ Micrograms per dish.

showed no detectable activity. Total lysozyme production was comparable in all cultures which contained macrophages. This experiment therefore showed that only the macrophages secreted lysozyme, and the lymphocytes, under these conditions, had no significant effect on macrophage production and secretion.

The influence of serum: Since the production of acid hydrolases can be stimulated by cultivating macrophages in the presence of high concentrations of newborn calf serum (21), the influence of serum on lysozyme production and secretion was examined in unstimulated mouse macrophage cultures. Fig. 7 shows that lysozyme production rose with increased NBCS, reaching a maximum at 5%, without increase at higher serum concentrations. 82-88% of the total lysozyme was recovered in the medium at all times and lysozyme specific activity was also constant, 58 $\mu\text{g}/\text{mg}$ cell protein except at the lowest serum concentration when it fell to 42 $\mu\text{g}/\text{mg}$. Other sera were tested in the same way and the results are listed in Table III. The optimal concentration of FCS was also 5% and

lysozyme production was actually inhibited at higher concentrations. The fall in specific activity from 40 to 14 μg lysozyme/mg protein at higher serum concentrations (Table III A) excludes a nonspecific toxic effect. Dialysis reversed the inhibition of lysozyme production by 50% FCS (Table III B). In the case of horse serum, Table III C, a relatively small further increase in lysozyme production was seen at serum concentrations above 10%, without change in specific activity.

Although the effects of different sera were complex, it is clear that high rates of lysozyme production could be achieved at a serum concentration of 5–10%.

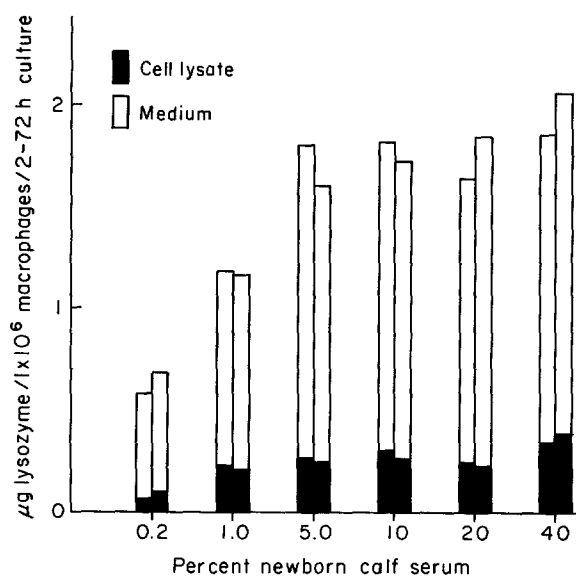


FIG. 7. Lysozyme production and secretion by unstimulated mouse macrophages cultivated in newborn calf serum.

The influence of serum-free media: Lysozyme production was also studied in the absence of serum to evaluate the importance of serum constituents. Lactalbumin hydrolysate served as a useful serum substitute in which macrophages remained healthy and maintained a high rate of lysozyme production and secretion (Table IV). Optimal lysozyme production and secretion were observed at an LH concentration of 0.05% wt/vol. The combined use of 10% horse serum and 0.05% LH resulted in only marginal superiority over either alone. The replacement of serum lactalbumin hydrolysate did not alter the increased rate of lysozyme secretion observed on the 2nd day in culture (not shown).

Short-term experiments with 2-day old thioglycollate-stimulated macrophages were used to explore the ability of defined basal media to support lysozyme secretion (Table V). These studies were terminated at 8 h since cell viability was

TABLE III
The Effect of Serum on Lysozyme Production and Secretion by Unstimulated Macrophages

Serum	Conc. vol/vol	Total LZM* in $\mu\text{g}/1 \times 10^6$ macrophages	Medium/ medium/ + cell	Total μg LZM*/mg cell protein	Collection period
			%		<i>h</i>
(A) FCS	1	1.21	84	31	2-46
	5	1.81	78	40	"
	10	1.68	80	37	"
	20	1.32	88	29	"
	50	0.98	83	14	"
(B) FCS	5	1.80	80	35	2-72
	50	1.05	88	18	"
Dialyzed‡ FCS	50	2.15	83	40	"
(C) Horse	0.2	1.20	85	57	2-72
	1	1.30	96	53	"
	5	1.98	90	69	"
	10	2.38	90	76	"
	20	2.48	89	70	"
	40	3.38	87	70	"
FCS	5	1.70	85	70	"

* Serum control subtracted.

‡ Dialysis for 2 days against 0.01 M bicarbonate, 0.15 M sodium chloride, pH 7.4.

TABLE IV
*Lysozyme Production by Unstimulated Macrophages Cultured in Lactalbumin Hydrolysate**

Medium	Total LZM	Percent in medium	Percent control‡
	$\mu\text{g}/\text{dish}$		
Dulbecco's + 10% horse serum	10.42	92	
" + 0.2% LH	9.62	90	92
" + 0.05% LH	11.18	92	106
" + 0.02% LH	10.17	91	97
" + 0.01% LH	10.29	92	99
" + 0.005% LH	9.43	93	90
" + 10% horse serum and 0.05% LH	12.18	91	116

* 4×10^6 macrophages plated in Dulbecco's + 10% horse serum for 4 h, washed three times and incubated in LH medium for 72 h.

‡ Dulbecco's + 10% horse serum.

affected by more prolonged incubation in basal media. Cultures in MEM with 5% horse serum served as control. A net increase of 30% lysozyme, compared with control, occurred after incubation in PBS. The addition of glucose or divalent cations to PBS had little effect on total lysozyme, but the medium con-

tent rose from 30% to about 40%. The use of HBSS and MEM brought about a further increase in lysozyme production, 40% and 57% of control, respectively. These experiments showed that macrophages continued to produce and secrete lysozyme, at a reduced rate, in the absence of serum.

The release of macrophage acid hydrolases into the medium: We next examined mouse macrophage cell lysates and conditioned medium to compare secretion of lysozyme with that of acid hydrolases. An experiment with thioglycollate-stimulated macrophages is shown in Table VI. The enzymes chosen for this comparison all showed an acid pH optimum, and were readily measurable in macrophage-conditioned media without interference from medium constituents.

TABLE V
The Effect of Basal Culture Media on Lysozyme Production and Secretion by Thioglycollate-Stimulated Macrophages

Medium*	Total medium + cell LZM <i>μg/dish</i>	Medium/medium + cell lysate %	Net increase† of LZM <i>μg/dish</i>	Net increase/net increase of control
MEM + 5% horse serum (= control)	2.24	50	1.26	100
MEM	1.69	41	0.71	57
HBSS	1.48	45	0.50	40
PBS + Ca, Mg, and glucose	1.40	43	0.42	34
PBS + Ca, Mg	1.35	40	0.36	29
PBS + glucose	1.37	44	0.39	31
PBS	1.36	30	0.38	30

* PBS contains 8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na₂HPO₄, and 0.2 g/liter KH₂PO₄·CaCl₂, 100 mg/liter, MgCl₂·6H₂O, 100 mg/liter and glucose, 1 g/liter, added as indicated.

† 48-h macrophages containing 0.98 μg LZM per dish were washed three times in isotonic saline and incubated for 8 h in appropriate medium. Cell viability, in all cases, >95%.

Lysozyme production and secretion exceeded that of all other hydrolases tested. After 24 h in culture, lysozyme secretion represented 76% of total enzyme whereas 25% or less of all other enzymes was found in the medium. After 72 h in culture, the percentage of extracellular lysozyme, cathepsin D, and β-glucuronidase was 79, 42, and 35%, respectively. β-galactosidase and N-acetyl β-glucosaminidase release was less than 15% of total activity. Only lysozyme production increased significantly (+211%) during cultivation in 5% FCS, whereas the other enzymes showed either a net loss (−24 to −33%) or only a small increase, in the case of cathepsin D (+21%). The production and secretion of lysozyme clearly differed from that of macrophage acid hydrolases under these culture conditions.

The influence of phagocytosis: During phagocytosis the macrophage internalizes a substantial portion of its plasma membrane and delivers a large frac-

tion of its lysosomal enzymes into phagocytic vacuoles. It was therefore of interest to determine whether a defined, massive pulse of latex particles, for example, would cause any alteration in lysozyme production or secretion. Fig. 8 shows an experiment in which more than 95% of the macrophages in culture had ingested more than 20 latex particles per cell. No measurable lysozyme was detected in the medium during the 2 h period of phagocytosis. Latex-laden macrophages contained more than 90% of the lysozyme of control cells at all times tested. The distribution of extra- and intracellular lysozyme did not differ significantly from control at 8 or 22 h after phagocytosis, 71 vs. 71% and 78 vs. 84%, respectively. Similar results were obtained over longer periods of observa-

TABLE VI
*Enzyme Distribution after Cultivation of Thioglycollate-Stimulated Macrophages**

	β -glucuronidase†	β -galactosidase†	<i>N</i> -acetyl β -glucosaminidase†	Cathepsin D‡	Lysozyme μ g/dish
T ₂ cell lysate	3.4	2.08	182	0.22	4.5
T ₂₄ cell lysate	2.1	1.30	112	0.20	2.1
Medium	0.68	0.23	15	0.066	4.9
Total	2.8	1.53	127	0.27	7.0
Medium, % total	25	16	13	25	76
T ₇₂ cell lysate	1.8	1.15	106	0.17	3.0
Medium	0.91	0.27	19	0.12	11
Total	2.7	1.42	125	0.29	14
Medium, % total	35	15	13	42	79
% Net change total (T ₇₂ - T ₂)/T ₂	-24	-33	-31	+21	+211

* 8×10^6 macrophages per dish in MEM + 5% FCS.

† Micromoles per minute per dish $\times 10^{-3}$.

‡ Chromogenic equivalents 1 mg/ml albumin per min per dish.

tion and after use of different particles like formalin-treated sheep or rabbit erythrocytes. A pulse of *M. lysodeikticus*, a source of lysozyme substrate, had no significant effect on lysozyme production and secretion.

The effect of inhibitors: The effect of inhibitors on lysozyme production and secretion was next investigated. Cycloheximide, an effective inhibitor of protein synthesis, prevented the synthesis of new lysozyme in the concentration range, 0.1–2.0 μ g/ml. Fig. 9 illustrates an experiment in which lysozyme production by thioglycollate-stimulated macrophages was completely blocked by 0.4 μ g/ml cycloheximide. Some release into the medium continued as the cell content fell. Cycloheximide treatment longer than 8 h brought about a loss in cell viability.

The effect of treatment with colchicine was also examined since secretion by other cells can be affected by this reagent. Fig. 10 shows that 10^{-6} M colchicine abolished lysozyme production after a lag period of at least 8 h. Under these

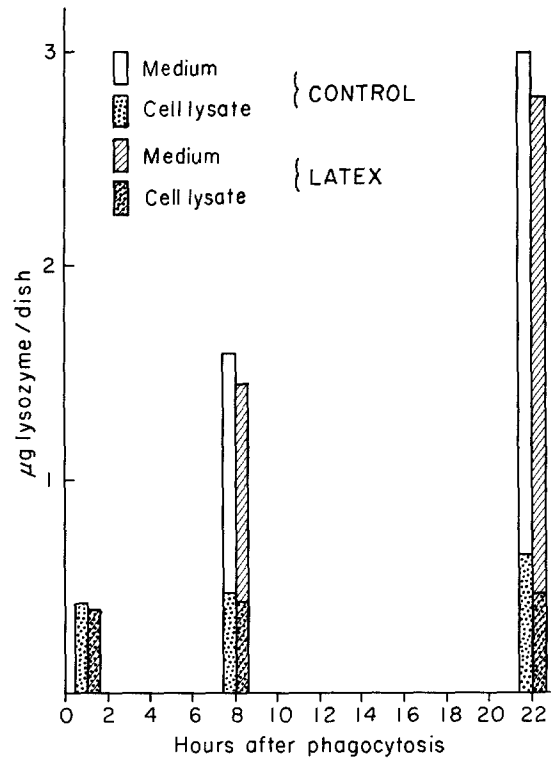


FIG. 8. Latex phagocytosis and lysozyme production and secretion by unstimulated mouse macrophages (13×10^6 per dish).

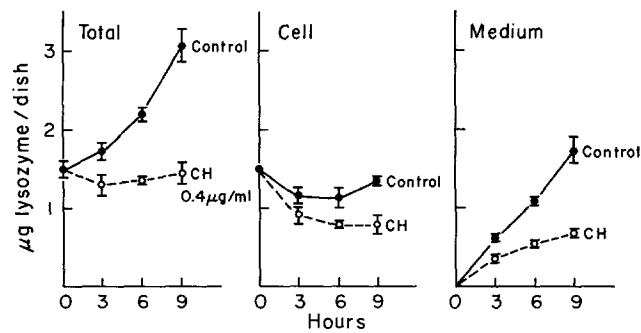


FIG. 9. The effect of cycloheximide (CH) treatment ($0.4 \mu\text{g/ml}$) on lysozyme production and secretion by thioglycollate-stimulated macrophages.

conditions the macrophages became drastically altered in morphology, without loss in cell viability or in total cell protein. Other experiments, using both stimulated and unstimulated mouse macrophages, showed that 10^{-7} – 10^{-6} M colchicine reproducibly diminished intracellular lysozyme and then reduced its secre-

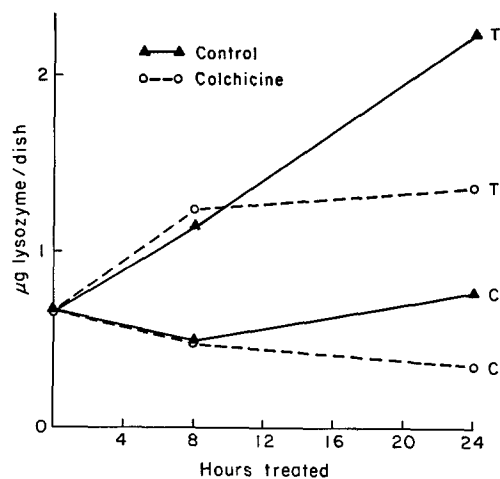


FIG. 10. The effect of 10^{-6} M colchicine on lysozyme production and secretion. 10×10^6 unstimulated mouse macrophages were exposed to colchicine after 2 days in culture. (—), control; (---), colchicine, C, cell lysate, T, total medium and cell lysate.

tion into the medium. The effect of colchicine on lysozyme production was always secondary to a lag period during which normal production rates were maintained.

Cytochalasin B, another inhibitor implicated in secretion, had no effect on lysozyme production or secretion.

DISCUSSION

These studies establish that mononuclear phagocytes are active secretory cells with lysozyme as a major secretory product. The macrophage lysozyme is indistinguishable from lysozyme standards by several criteria, rapid lysis of *M. lysodeikticus*, heat stability at pH 3.5, inhibition by specific antisera, a size of 14,000, and cationic charge. Lysozyme secretion is associated with substantial net synthesis, a relatively constant intracellular concentration and continued accumulation in the medium. ^{14}C -labeled lysozyme is the most prominent product secreted by both unstimulated and thioglycollate-stimulated macrophages in culture (14). Although human monocytes and mouse macrophages differ in maturity these mononuclear phagocytes show a similar pattern of lysozyme secretion. BCG-stimulated rabbit alveolar macrophages, in contrast, contain a high level of intracellular lysozyme ($6.7 \mu\text{g}/1 \times 10^6$ cells), and net synthesis in culture is relatively low so that most of the lysozyme which is released into the medium by these cells represents preformed enzyme (unpublished observations). The human PMN, another rich source of lysozyme, releases only preformed lysozyme into the medium during cultivation, unlike the continued production and secretion by mononuclear phagocytes.

Lysozyme production by mononuclear phagocytes remained remarkably

constant under a wide variety of culture conditions. No specific inducer molecules could be identified in these experiments since lysozyme production remained relatively high in basal media and could only be blocked by inhibiting the synthesis of all proteins. The apparent stability of lysozyme production and secretion can be contrasted with the inducibility of other macrophage enzymes like acid phosphatase (22) and heme oxygenase (23).

Several observations make it likely that lysozyme secretion is independent of endocytosis and the production of acid hydrolases. Lysozyme production was close to maximum when the concentration of serum in the culture medium did not favor the accumulation of acid hydrolases in the cell (21) and no comparable net increase or release of other enzymes was observed. A heavy phagocytic load of latex particles had no effect on overall lysozyme production and secretion. A minor increase in lysozyme release into the medium during active phagocytosis cannot be excluded, however, since the intracellular pool in the mouse macrophage is small relative to the high rate of continuous secretion.

Macrophages secrete the equivalent of its intracellular lysozyme pool in 5–8 h, but further pulse-chase labeling studies are required to establish how soon newly synthesized molecules reach the medium. The factors which control the release of lysozyme from the cell are also not known, although a temperature-dependent step may be involved (unpublished observations). In all other situations diminished secretion of lysozyme was associated with a reduced level within the cell. Treatment with colchicine, for example, inhibited lysozyme production, rather than its release, and the lag in its effect suggested an indirect mechanism of action. Release of lysozyme into the medium could continue, to a limited extent, when new production was inhibited by cycloheximide, but the macrophages could not be totally depleted in this way. A genetic block in lysozyme secretion could also not be demonstrated in macrophages from beige mice (24), which may accumulate other intracellular hydrolases (unpublished observations).

This study raises several unanswered questions concerning the intracellular pathway of lysozyme secretion. Pancreatic zymogens are packaged in vesicles derived from the Golgi apparatus and are stored in condensing vacuoles until secretagogues stimulate their discharge (25). The Golgi system also plays a role in the secretion of proteins by the hepatocyte (26–28) although the mechanism of their release from the cell is not established. At present there is no information whether lysozyme secretion proceeds by vesicular transport or by a different mechanism. The relationship between the intracellular lysozyme compartment and the lysosomal system of the macrophage (29) is also obscure. Separate populations of lysosome-like granules could be designed for export from the cell, as in *Tetrahymena* (30). During the early stages of human monocyte differentiation the promonocytes contain granules, corresponding to primary lysosomes, which contain peroxidase, arylsulfatase, and acid phosphatase (31). A second peroxidase-negative population of granules appears during maturation. Further ex-

periments should establish whether the peroxidase-negative granules are involved in the packaging of secretory products, like lysozyme.

Plasma and urinary lysozyme levels are elevated in a variety of clinical conditions (32). Secretion by increased numbers of mononuclear phagocytes could contribute substantially to the high extracellular levels seen in monocytic leukemia and in chronic inflammation. Calculations on granulocyte turnover should therefore be reevaluated in the context of secretion by viable mononuclear phagocytes (33). Since lymphocytes lack lysozyme it serves as a useful marker of the phagocytic leukocytes. Other cells, e.g. chick oviduct and the lachrymal gland, may also secrete lysozyme, but its absence from fibroblasts and lymphoid and epithelial cell lines makes lysozyme a suitable marker of macrophage-specific function in cell hybrids (34) and other continuous cell lines derived from mononuclear phagocytes (35).

No evidence is, at present, available which suggests that lysozyme acts as an enzyme on any nonbacterial substrate, although interactions with cartilage (36) and cell membranes (37, 38) may be due to its unique polyelectrolyte properties. Even its role in extracellular killing of microorganisms is not fully understood, although the range of susceptible organisms could be expanded by synergistic action between lysozyme, antibody, and complement (39, 40). In addition to lysozyme the mononuclear phagocyte contributes other secretory products which allows it to exert a potent effect on its extracellular environment. Further studies on such secretory products will be reported elsewhere.

SUMMARY

Pure cultures of three types of mononuclear phagocytes—mouse peritoneal macrophages, unstimulated or after thioglycollate stimulation, and human monocytes—synthesize and secrete large amounts of lysozyme *in vitro*. The macrophage lysozyme is indistinguishable from authentic lysozyme in its ability to lyse *M. lysodeikticus*, inhibition by specific antisera, a similar size of 14,000 and cationic charge. Lysozyme secretion in culture is characterized by a large net increase in total lysozyme, 4–20-fold in 3 h, 75–95% of which is in the medium, and its continued extracellular accumulation over at least 2 wk in culture. Lysozyme is the major ¹⁴C-labeled protein secreted into the medium by both unstimulated and thioglycollate-stimulated macrophages and the 0.75–1 μg produced per 1×10^6 cells/day represents 0.5–2.5% of the total cell protein.

Lysozyme is a cell-specific marker for mononuclear phagocytes and the PMN, which contains preformed enzyme, since it is absent in lymphoid cells and a variety of fibroblast and epithelioid cell lines. Lysozyme production is also a useful measure of mononuclear phagocyte cell number.

The rate of lysozyme production and secretion is remarkably constant for all cell types under a variety of culture conditions. Production by the mouse macrophage increases threefold on the 2nd day in culture and then remains linear with time. Production is optimal at a relatively low serum concentration,

but can be maintained, in the absence of serum, in lactalbumin hydrolysate or, at a reduced level in basal media.

The production and secretion of lysozyme are independent of the production of macrophage acid hydrolases. Net increase and secretion of lysozyme occur under conditions where acid hydrolases like *N*-acetyl β -glucosaminidase, β -glucuronidase, β -galactosidase, and cathepsin D are neither accumulated nor secreted. Massive phagocytosis of latex particles has no effect on lysozyme production and secretion.

Lysozyme production can be rapidly inhibited by treatment with cycloheximide (0.4 μ g/ml) whereas inhibition of its production by colchicine (10^{-6} M) occurs only after a lag period of more than 8 h, and is probably due to a secondary effect.

These results show that mouse macrophages provide a simple in vitro system to measure lysozyme secretion and its control. These studies also indicate the possible importance of mononuclear phagocytes in the secretion of a variety of biologically active products and in the modification of their environment.

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