

SECRETION OF PLASMINOGEN ACTIVATOR BY STIMULATED MACROPHAGES*

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We have recently shown that, following transformation by oncogenic viruses or chemicals, avian and mammalian cells release an enzyme that functions as a plasminogen activator (1-3). The evidence obtained so far indicates that the increased activation of plasminogen determines, at least in part, several of the phenotypic properties of transformed cells; these include colony formation in semisolid media, and the characteristic changes in cell morphology and migration (4). Although the significance of plasminogen activation for cell growth remains to be determined, its association with transformation and neoplasia suggests that the formation of plasminogen activator may also occur during normal cellular proliferation, such as that in the lymphoid and hemopoietic systems.

In view of the role of macrophages in lymphocyte activation by lectins and antigens (5, 6), in the production of factors regulating granulocyte differentiation (7), and in chronic inflammation, we have examined pure populations of peritoneal macrophages for production of plasminogen activator. Mononuclear phagocytes are a rich source of acid proteases, such as cathepsins (8), which are thought to play a role in intracellular digestion, but proteases capable of acting extracellularly, at neutral pH, have not been characterized.

In this paper, we report that thioglycollate-stimulated mouse macrophages produce and secrete a plasminogen activator in vitro similar to that reported previously from transformed cells. Unstimulated peritoneal macrophages do not produce this enzyme. We have characterized the plasminogen activator secreted into the medium by thioglycollate-stimulated macrophages and have examined the production and release of the enzyme in culture. In addition, we have found that stimulated macrophages may also release a variety of proteases that are not formed by unstimulated cells.

Materials and Methods

Cell Cultures.—Female mice of the NCS (Rockefeller) strain, weighing 25-30 g, were used throughout. Peritoneal macrophages were always harvested without anticoagulants and cul-

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tured by standard procedures;¹ the cells were obtained either 4 days after stimulation by intraperitoneal injection of 0.75 ml thioglycollate medium (9), or from control, unstimulated mice. Use of thioglycollate medium which contains agar resulted in increased yields of macrophages; these cells adhered to the dish more rapidly and showed more membrane ruffling than unstimulated macrophages. The cell yield from control 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 80-90% macrophages and 10-20% lymphocytes. Variable numbers of fibroblasts, not exceeding 5% of the total, were found after thioglycollate stimulation, and small numbers of granulocytes and mast cells were also present in both preparations.

Cells were routinely cultured in MEM² or Dulbecco's medium with 5% of fetal bovine serum (FBS) that had been heated at 56° for 30 min. Other sera that were used included: (a) human serum depleted of plasminogen (10) by two cycles of affinity chromatography on L-lysine-Sepharose 4B (11); (b) acid-treated fetal bovine serum (AT-FBS). This serum was adjusted to pH 3.2 by the addition of 1 N hydrochloric acid, incubated at room temperature for 2 hours and then neutralized with 1 N sodium hydroxide. This acid treatment destroys the serum plasmin inhibitors.³ Cells were also cultured in serum-free medium consisting of MEM or Dulbecco's supplemented with 0.02-0.05% (wt/vol) lactalbumin hydrolysate.

Peritoneal cell suspensions were plated onto regular or [¹²⁵I]fibrin-coated petri dishes; unless otherwise specified the densities were 8×10^5 cells per cm² for cells from control animals and 2×10^5 cells per cm² for those from thioglycollate-stimulated mice. The cultures were washed vigorously with MEM to remove nonadherent cells within 24 h after plating. Conditioned serum-free medium was prepared from 2-day old cultures. The macrophages could be incubated in serum-free MEM containing 0.05% (wt/vol) lactalbumin hydrolysate for up to 4 days without loss of viability (measured by staining with trypan blue) or decrease in synthesis and secretion of lysozyme.¹ To prepare ¹⁴C-labeled conditioned medium, cultures were first washed three times in HBSS and then incubated in medium of the following composition: Hank's balanced salt solution (HBSS), containing 5 μCi/ml reconstituted [¹⁴C]algal hydrolysate, 1/100 MEM, and 5% of FBS previously dialyzed against 0.1 M sodium bicarbonate buffer, pH 7.4. This procedure was designed to render the cellular amino acid pools highly radioactive. After 4 h of incubation the medium was removed, the cultures were washed three times with HBSS, and incubated in fresh Dulbecco's medium containing 0.05% (wt/vol) of lactalbumin hydrolysate. The macrophages continued to secrete lysozyme at normal rates and appeared healthy by morphological criteria for at least 2 days. Cultures of mouse embryo fibroblasts transformed by murine sarcoma virus and SV101 mouse fibroblasts were used to prepare conditioned medium, as described previously (2).

Fibrin Plate Assays.—Plastic petri dishes (35 mm diameter) were coated with [¹²⁵I]fibrinogen (550 cpm/μg) at a concentration of 10 μg/cm² as described elsewhere (12). Before use dishes were first incubated at 37°C for 2 h with MEM containing 5% of FBS to convert fibrinogen to fibrin, and then washed twice with HBSS. Such dishes were used to measure fibrinolytic activity by cells plated on fibrin, by cell fractions, or conditioned serum-free medium.

¹ Gordon, S., J. Todd, and Z. Cohn. 1974. The in vitro synthesis and secretion of lysozyme by mononuclear phagocytes. Manuscript submitted for publication.

² *Abbreviations used in this paper:* AT-FBS, acid-treated fetal bovine serum; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; MEM, Eagle's minimal medium; NPGb, nitrophenyl-*p*-guanidinobenzoate; Plg, human plasminogen; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor.

³ Loskutoff, D. J., and E. Reich. 1974. Inhibitors of fibrinolysis in fetal bovine and chicken sera. Manuscript in preparation.

For cell-free fibrinolysis assays the conditions were as follows: a final volume of 1 ml contained 0.1 M Tris-HCl buffer, pH 8.0, 8 μ g of human plasminogen purified according to Deutsch and Mertz (11) and the fraction to be tested for plasminogen activator (usual volume 10–100 μ l). The dishes were incubated for 2–4 h at 37°C and the solubilized radioactivity was measured in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill). The reaction was proportional to enzyme concentration and was linear with time provided that fibrinolysis did not exceed 20% of the total on the plate. All experiments included appropriate controls for all reagents and media. Plasminogen activator content of conditioned media was measured after centrifugation (1,000 g, 15 min) to remove cells and debris. For measurement of intracellular plasminogen activator the cell layer was first washed with normal saline, then lysed by the addition of Triton X-100 (0.2% wt/vol in water; 1.5 ml per 60 mm petri dish) (Rohm and Haas Co., Philadelphia, Pa.) and scraped from the surface of the dish by means of a plastic policeman. The lysates were frozen and stored at –20°C until assayed; the activity was stable through several cycles of freezing and thawing. Increasing the concentration of Triton X-100 to 1% did not affect the observed level of enzyme activity. Addition of Triton X-100 (final concentration 0.01%) increased the activity of extracellular plasminogen activator by 80%. Triton X-100 was not routinely added to assays of conditioned medium. One unit of plasminogen activator is defined as the amount that stimulates the release of 10% of the initial radioactivity in 4 h under standard assay conditions.

Fibrinolytic activity of intact macrophages was measured after plating on [¹²⁵I]fibrin-coated dishes. To prevent fibrinolysis immediately after plating, soybean trypsin inhibitor (STI) (60 μ g/ml) was added to growth media. Fibrinolysis measurement was begun by washing cells free of inhibitor and substituting fresh media containing 5% of AT-FBS. Plasminogen-dependence of fibrinolysis was tested by use of plasminogen-depleted sera; control incubations without cells were always included. All assays were performed in duplicate, and the range of variation did not exceed 15%.

Lysozyme was assayed by using *Micrococcus lysodeikticus* as reported elsewhere.¹ Rat lysozyme was used as a standard.

Polyacrylamide Gel Electrophoresis.—Samples were prepared for electrophoresis by dialysis against 0.1–0.2% sodium dodecyl sulfate (SDS), and then lyophilized. When conditioned medium was concentrated, 0.1 volume of 1 M sodium sulfate was added, the solution was transferred to a dialysis bag and the volume reduced by packing in dry Sephadex. Plasminogen activator was not inactivated by this procedure.

SDS-polyacrylamide gel electrophoresis was performed according to Maizel (13). Polyacrylamide gradient slab gels were run at 25 mA for 6–8 h. The final sample buffer consisted of 0.0625 M Tris HCl, pH 6.8, 3% ethylene glycol, 2–5% SDS, and bromphenol blue. The samples were not reduced or boiled. Molecular standards, similarly prepared, were: bovine serum albumin (BSA), pepsin, chymotrypsinogen, and lysozyme. After electrophoresis, appropriate lanes were cut out, frozen, and sliced (1.1 mm). To assay plasminogen activator the slices were incubated in [¹²⁵I]fibrin dishes as described. To assay [³H]diisopropyl fluorophosphate ([³H]DFP), the slab was stained and destained, sliced and the slices swollen overnight at 37°C in a mixture of 4 M ammonium hydroxide: Protosol: Liquifluor 1:5:50. All of the radioactivity in a control sample of ³H-DFP labeled trypsin was recovered from the gel after electrophoresis. Samples were counted in a Packard scintillation counter (Packard Instrument Co., Inc.) with an efficiency of 30%. For autoradiography of ¹⁴C-labeled materials the slabs were dried, placed on X-ray film and exposed for 4 days.

³H-DFP Labeling of Conditioned Medium.—For preparation of conditioned medium freshly plated cells (7×10^7 control or 2×10^7 thioglycollate-stimulated macrophages) were first incubated for 2 days in Dulbecco's medium containing 5% of FBS, and then transferred to serum-free Dulbecco's medium supplemented with 0.05% of lactalbumin hydrolysate. The medium was collected after 2 days of incubation, centrifuged, and stored at –20°C until used. Medium conditioned by thioglycollate-stimulated cells (2,200 U) was concentrated eightfold

as described above to a final volume of 2 ml, made 0.1 M in Tris-sulfate pH 7.4, and incubated with 0.1 ml [^3H]DFP (0.21 mg/ml, 0.9 Ci/mmol) for 20 h at room temperature, in the presence or absence of nitrophenyl-*p*-guanidinobenzoate (NPGb) (8×10^{-6} M). Treatment with DFP alone inactivated 94% of the plasminogen activator. The samples were then dialyzed for 2 days against 3×100 volumes of 0.1% SDS and lyophilized. The residue was dissolved in 25- μl sample buffer and electrophoresed in an SDS slab gel containing an 8–15% polyacrylamide gradient, for 8 h at 20 mA. To identify plasminogen activator by catalytic activity a parallel sample of conditioned medium obtained from 3×10^6 thioglycollate-stimulated macrophages (380 U) was dialyzed against 0.1% SDS, lyophilized, and the concentrated material was analyzed by polyacrylamide gel electrophoresis in parallel with ^3H -DFP labeled samples. This lane was cut into slices and assayed for plasminogen activator.

Protein Assay.—Protein was routinely assayed using the method of Lowry et al. (14) with BSA as standard. Protein in conditioned medium was assayed after dialysis against 0.1 M NaHCO_3 .

Materials.—Reagents were obtained as follows: MEM, HBSS, and FCS (Grand Island Biologicals, Grand Island, N. Y.); Brewer thioglycollate medium (Difco Laboratories, Detroit, Mich.); *Micrococcus lysodeikticus*, spray dried, STL, component VI, and bovine fibrinogen, fraction I (Miles Laboratories Inc., Kankakee, Ill.); lactalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio); Sepharose 4B and Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.); DFP (Aldrich Chemical Co., Milwaukee, Wis.); outdated human plasma (New York Blood Center, New York); SDS (BDH Chemicals Ltd., Poole, England); Protosol and Liquifluor (New England Nuclear Corp., Boston, Mass.); NPGb (Dr. H. Wood, Drug Development Branch, National Cancer Institute). All other reagents were of the highest grade available from standard sources. Isotopes were obtained as follows: [^{125}I]sodium iodide, carrier-free and [^{14}C]reconstituted algal hydrolysate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.); [^3H]DFP, 0.9 Ci/mmol (New England Nuclear).

RESULTS

Fibrinolysis in Macrophage Cultures.—The fibrinolytic activity of macrophages was measured after plating on [^{125}I]fibrin-coated dishes, in which the cells attached and spread as effectively as they did in control dishes. As seen in Fig. 1, thioglycollate-stimulated macrophages showed rapid fibrinolysis; virtually all of this activity was plasminogen-dependent since it was not observed when the serum was depleted of plasminogen. In contrast to the high fibrinolytic activity of thioglycollate-stimulated cultures, that of the unstimulated cells was very low and barely exceeded the background level in cell-free control dishes.

To obtain an accurate estimate of the difference in fibrinolytic activity between thioglycollate-stimulated and unstimulated macrophages, serial dilutions of cells were assayed. The use of acid-treated FBS, in which at least 95% of the plasmin inhibitors have been destroyed³ increased the sensitivity of the assay; however, qualitatively similar results were obtained using FBS. As shown in Fig. 2, the rate of fibrinolysis by macrophages was approximately linear with time and also related to cell density. In this and other experiments the rate of fibrinolysis was proportional to stimulated macrophage number, in the range $1\text{--}6 \times 10^5$. Unstimulated macrophages (60×10^5) showed mini-

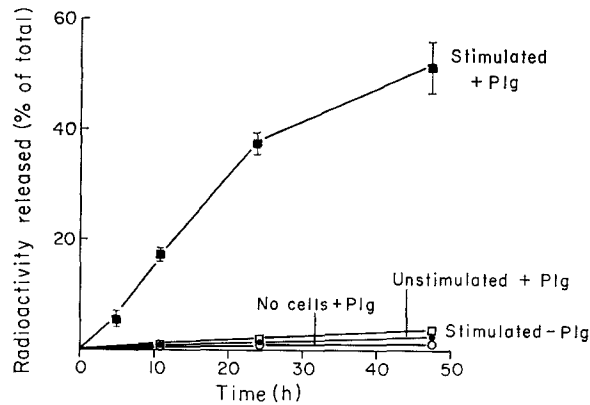


FIG. 1. Fibrinolysis by thioglycollate-stimulated and unstimulated macrophages. 1.5×10^6 stimulated, or 4×10^6 unstimulated macrophages were plated on 35 mm $[^{125}\text{I}]$ fibrin-coated dishes in MEM containing 5% of FBS and $60 \mu\text{g/ml}$ STI and incubated for 24 h. After washing, MEM containing 5% of human serum, or 5% of plasminogen depleted human serum (total volume 3 ml) was added, and 0.3-ml samples were removed as indicated. (■—■) stimulated cells, human serum; (□—□) stimulated cells, plasminogen depleted serum; (●—●) unstimulated cells, human serum; (○—○) control dishes without cells, human serum.

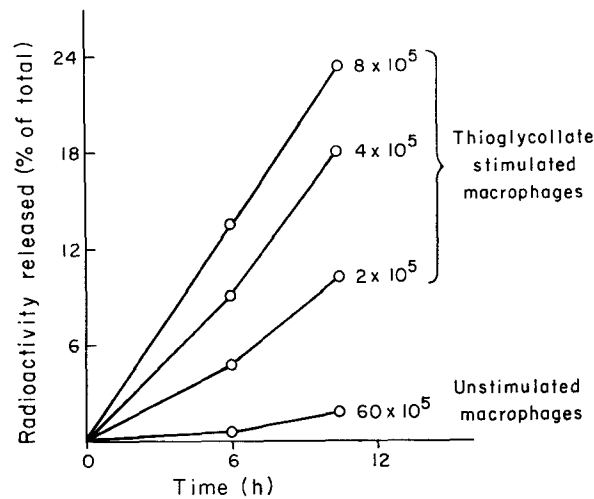


FIG. 2. Dose response curve of thioglycollate-stimulated and unstimulated macrophages. Cells on 35 mm $[^{125}\text{I}]$ fibrin-coated dishes were cultured in MEM containing 5% of FBS and $60 \mu\text{g/ml}$ STI for 2 days, washed, and incubated in MEM containing 5% of AT-FBS.

mal fibrinolysis. The activity per cell of thioglycollate-stimulated macrophages was therefore at least 100-fold higher.

Fibrinolysis by Conditioned Medium and Cell Lysates.—Both the conditioned serum-free medium and cell lysates from stimulated macrophages showed plasminogen-dependent fibrinolysis whereas the same preparations from un-

stimulated macrophages showed no comparable activity. Fresh lysates of unstimulated macrophages do show some fibrinolysis, (Table 1 A) but this is independent of plasminogen and is thus due to proteases that attack fibrin directly. The specific activity, plasminogen activator per unit protein, is 60 times higher in conditioned medium from stimulated macrophages than in cell lysates, suggesting that this enzyme is actively secreted by the cells (Table 1 B). These differences between stimulated and unstimulated macrophages have been confirmed by the results of at least seven independent experiments during a 5 mo period. No significant activity was ever observed in conditioned medium from unstimulated cells. Lysozyme production and secretion were similar to those of plasminogen activator, but lysozyme was secreted by both stimulated and unstimulated cells (Table 1 B).

TABLE I A
Fibrinolysis by Thioglycollate-Stimulated and Unstimulated Macrophages

	Fibrinolysis* (U per dish)			
	Stimulated		Unstimulated	
	Plasminogen: Present	Absent	Plasminogen: Present	Absent
Cell lysates				
24 h †	18	1.0	23	23
72 h	55	0.40	12	7.0
Conditioned medium				
24-72 h	800	26	3.4	2.8

* Appropriate background values were subtracted (blanks varied from 1.1 to 1.4% of total radioactivity). 25-50 μ l aliquots were assayed; all assays were performed in duplicate and the average is reported.

† Cells were plated for 24 h in Dulbecco's medium containing 10% of FBS, washed, and incubated for 2 days with Dulbecco's medium containing 0.05% (wt/vol) lactalbumin hydrolysate.

TABLE I B
Specific Activities of Plasminogen Activator and Lysozyme

	Total macrophage No.	Plasminogen-dependent fibrinolysis	Total protein	Plasminogen activator sp act	Lysozyme	Lysozyme sp act
		U/dish	mg	U/mg	μ g/dish	μ g lysozyme/mg protein
Stimulated macrophages	4×10^6					
Cell lysate (24 h)		17	0.60	28	0.43	0.70
Cell lysate (72 h)		55	0.75	74	1.3	1.7
Conditioned medium (24-72 h)		770	0.17	4,500	11	62
Unstimulated macrophages	12×10^6					
Cell lysate (24 h)		0	0.70	0	0.40	0.57
Cell lysate (72 h)		5.0	0.40	12.0	0.85	2.1
Conditioned medium (24-72 h)		0.60	0.22	3.0	11	50

To test for the production of inhibitors by unstimulated macrophages, cell lysates and/or conditioned medium were mixed with corresponding fractions from stimulated cultures; no inhibition was found in any combination, even when a fourfold excess of protein from unstimulated cultures was used.

The time course of plasminogen activator formation in cultures of stimulated macrophages is present in Fig. 3 A; also shown for comparison is the formation of lysozyme in the same cultures (Fig. 3 B). These cells, which had been transferred to serum-free lactalbumin hydrolysate medium after 24 h in culture, synthesized and released plasminogen activator over a period of at least 3 days. The intracellular activity remained relatively constant whereas the total activity in the medium continued to increase and greatly exceeded that present in the cells.⁴ This fact provides additional evidence for specific secretion of the enzyme. The kinetics of plasminogen activator secretion were similar to those of lysozyme secretion by the same cells.

Properties of Plasminogen Activator from Macrophages.—

Stability: The plasminogen activator in conditioned medium was stable at 37°C for at least 48 h. The pH stability has not been systematically investigated; however no loss of activity has been observed in the range pH 2–9 during incubation at 4°C for several hours.

Treatment of plasminogen activator with dithiothreitol (10 mM), followed by dialysis, resulted in complete loss of activity. The plasminogen activator probably does not contain any essential sulfhydryl groups since exposure to iodoacetamide (10 mM), iodoacetate (10 mM), *N*-ethylmaleimide (10 mM) or *p*-chloromercuribenzoate (0.1 mM) for 24 h at room temperature, followed by dialysis, did not affect its activity. Likewise, EDTA (50 mM for 28 h at room temperature) was without effect.

The plasminogen activator was stable at neutral pH in solutions containing concentrations of SDS as high as 2%. The enzyme activity could be measured in the fibrin plate assay provided that the final concentration of SDS did not exceed 0.006%; this concentration of SDS gave results identical to those of control samples.

Inhibitors: The plasminogen activator from mouse macrophages, like that from transformed chick fibroblasts (3), is irreversibly inhibited by DFP at a concentration of 2.5×10^{-4} M.⁵ The kinetics of inactivation are typically first-order (Fig. 4), with a half time of 10 min under these conditions.

The activator is also inhibited 50% by a low concentration (10^{-8} M) of the active site reagent NPGB (15), a compound known to acylate the active site of serine proteases. The inhibition by NPGB is reversible on prolonged dialysis, consistent with the expected slow hydrolysis of the acyl enzyme complex.

⁴The activity in the conditioned medium was probably greater than indicated, because Triton X-100, which is stimulatory (see Materials and Methods) was not added.

⁵There was no inhibition of plasmin by DFP carried over into the fibrinolytic assay (1.3×10^{-6} M final concentration).

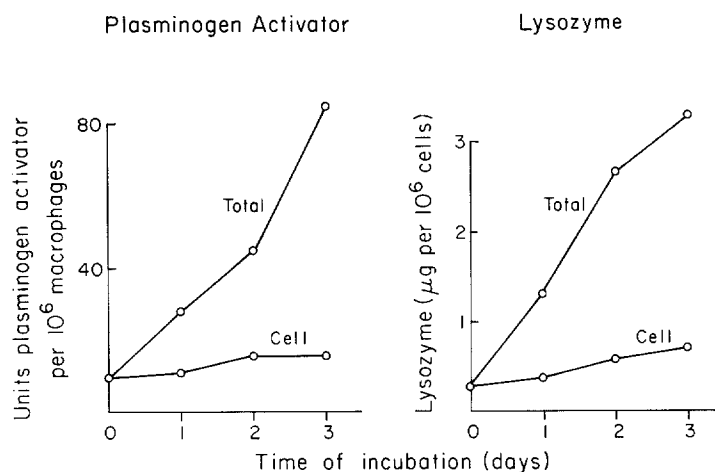


FIG. 3. Production and secretion of plasminogen activator and lysozyme. 4×10^6 thioglycollate stimulated macrophages on 60-mm dishes were cultivated for 24 h in MEM containing 5% of FBS, washed three times and incubated in MEM containing 0.02% of lactalbumin hydrolysate. Cell lysates and conditioned medium were prepared from replicate dishes daily for 3 days after transfer to lactalbumin hydrolysate medium. Samples (10–100 μ l) of cell lysates and conditioned medium were assayed for plasminogen activator and lysozyme.

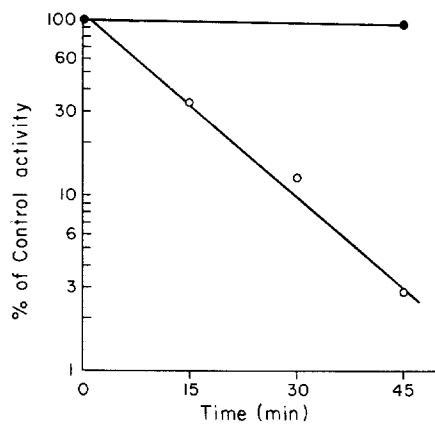


FIG. 4. Inactivation of the plasminogen activator by DFP. A sample of conditioned medium containing 320 U of plasminogen activator per ml, 0.1 M Tris-SO₄, pH 7.4, and DFP (0.25 mM final concentration) was incubated at room temperature. At the indicated times triplicate samples (5 μ l) were withdrawn and assayed directly for plasminogen activator. (O—O) DFP treated; (●—●) control.

SDS-Polyacrylamide Gel Electrophoresis.—To identify the molecular species of plasminogen activators released by macrophages, conditioned serum-free medium was concentrated (Materials and Methods) and analyzed by SDS-polyacrylamide gel electrophoresis. The gel was sliced and the fibrinolytic activity in the gel measured both in the presence and absence of plasminogen.

As seen in Fig. 5, a major symmetrical peak of activity was found at a mol wt of 48,000 with a smaller peak at 28,000; in addition, a very small amount of fibrinolysis was associated with heterogeneous material larger than the main active peak. All of the fibrinolysis was strictly dependent on plasminogen and the activity peaks in the gel therefore represent plasminogen activators. This electrophoretic pattern of plasminogen activators was reproduced exactly in four separate experiments using medium collected from independent cultures of stimulated macrophages. The mol wt of the major macrophage activator (48,000) is very close to that of the plasminogen activators released by trans-

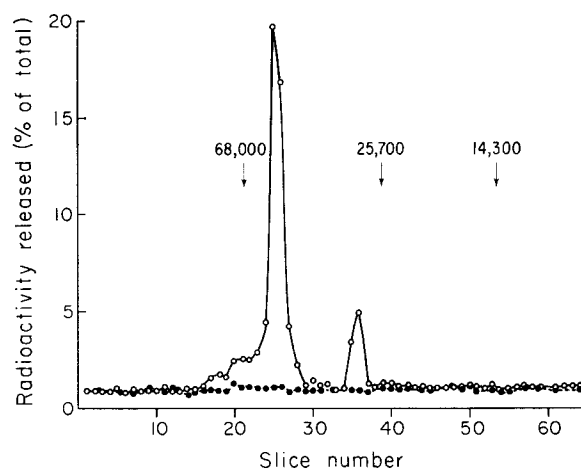


FIG. 5. Electrophoresis of plasminogen activator on an SDS-polyacrylamide gel slab. Duplicate aliquots of conditioned medium (2 ml, containing 310 U plasminogen activator) were dialyzed, lyophilized, dissolved in sample buffer and applied to a 10–20% polyacrylamide SDS slab gel. After electrophoresis, slices from the duplicate lanes were assayed in the presence (○—○) and absence (●—●) of plasminogen.

formed mouse fibroblasts. As seen in Fig. 6, mouse embryo fibroblasts transformed by the murine sarcoma virus, an RNA virus, and 3T3 mouse fibroblasts transformed by SV-40, a DNA virus, released plasminogen activators of mol wt 48,000–50,000, although SV-40 transformed cultures also produced an activator of mol wt 83,000.

SDS-Polyacrylamide Gel Electrophoresis of ³H-DFP Labeled Conditioned Medium.—The striking difference in plasminogen activator activity of thio-glycollate-stimulated and unstimulated cells prompted further attempts to extend the comparison of macrophage products in the respective conditioned media. Two independent but complementary approaches were used. In the first, samples of both cultures were incubated with [³H]DFP and the reaction products analyzed by SDS-polyacrylamide gel electrophoresis. At the pH in these experiments [³H]DFP reacts only with serine residues; the radioactivity

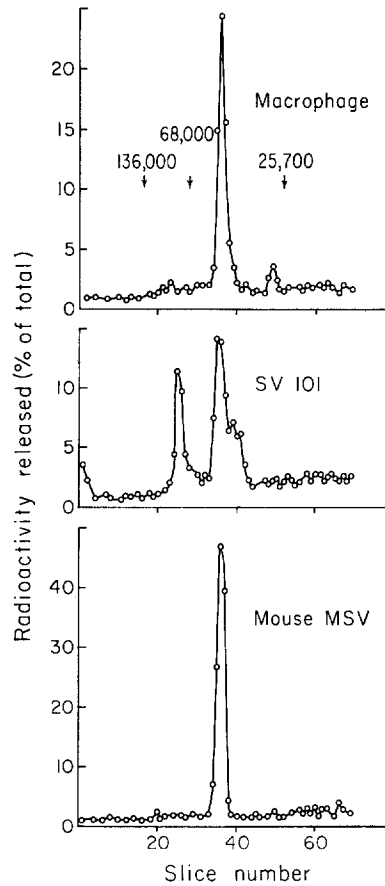


FIG. 6. Comparison of plasminogen activators from stimulated macrophages, SV-40 transformed 3T3 cells (strain SV-101), and murine sarcoma virus transformed mouse embryo fibroblasts. Conditioned medium (2-4 ml) was concentrated, electrophoresed in a 10-15% polyacrylamide SDS slab gel, and assayed as described. The plasminogen activator titer of the respective conditioned media was 320 U from stimulated macrophages, 130 U from SV-101 mouse fibroblasts, and 230 U from mouse sarcoma virus transformed mouse fibroblasts.

profile in the gel should therefore give an indication of the spectrum of serine enzymes formed by the two types of macrophages. As seen in Fig. 7 A conditioned medium from thioglycollate stimulated macrophages again contained two species of plasminogen activator of mol wt 48,000 and 28,000 respectively. The pattern in Fig. 7 B shows the radioactivity profile of ^3H -DFP labeled proteins. Multiple peaks were found in both samples. There were two DFP-labeled peaks in the medium from thioglycollate-stimulated macrophages which corresponded with plasminogen activator activity; both of these were absent from unstimulated macrophage conditioned medium. The ratio of

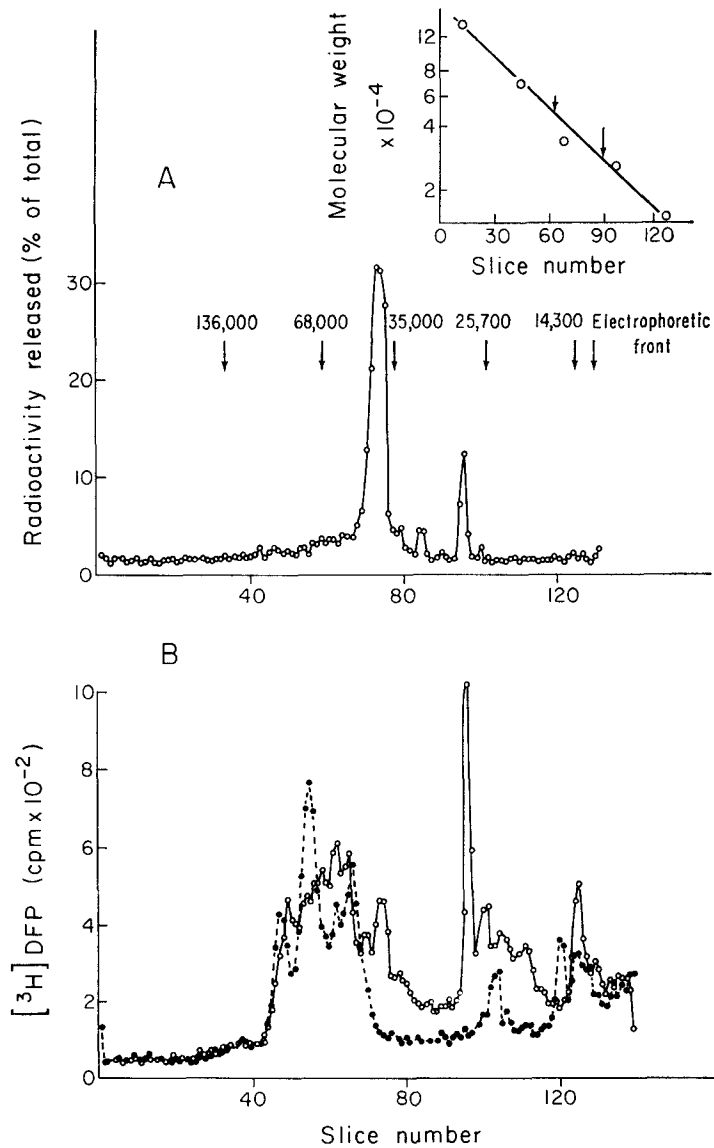


FIG. 7. Correlation of plasminogen activator and ^3H -DFP labeling in conditioned medium from stimulated and unstimulated macrophages. (A) plasminogen activator activity. (B) ^3H -DFP labeling of stimulated macrophage-conditioned medium, (O—O); unstimulated macrophage-conditioned medium (●—●).

plasminogen activator activity to ^3H]DFP was fivefold greater in the 48,000 mol wt peak. The main activator peak contained approximately 3 pmol of incorporated ^3H]DFP and, assuming 1 mol of DFP per mole of enzyme, this represents 0.15 μg of plasminogen activator released by 2×10^7 cells in 48

h. For comparison this culture produced 40 μg of lysozyme during the same period.

As a first step in characterizing the serine enzymes secreted by macrophages, conditioned media were labeled with [^3H]DFP in the presence of NPGB in order to identify trypsin-like enzymes. NPGB acylates the active site of enzymes with tryptic specificity, but the acyl enzyme is slowly hydrolyzed; thus the reaction with [^3H]DFP should be reduced but not completely blocked. NPGB also reacts with chymotrypsin, but the acyl intermediate is rapidly hydrolyzed (15) and should therefore not inhibit the ^3H -DFP labeling of chymotrypsin-like enzymes. As shown by the gel profiles in Fig. 8 A, the labeling of several peaks in medium from stimulated cultures was inhibited by NPGB; consistent with their sensitivity to NPGB, the labeling of the plasminogen activator peaks was particularly decreased (Fig. 8 A). In contrast, the labeling of all DFP reactive species released by unstimulated cultures was completely resistant to NPGB, whereas only a minority of the labeled peaks from stimulated cultures behaved in this way (Fig. 8 B). Thus the serine enzymes released by the two kinds of macrophages differed both in specificity and in electrophoretic mobility.

Biosynthetic Labeling of Secreted Macrophage Proteins with [^{14}C]Amino Acids.—In a second method of comparing secreted macrophage proteins the cultures were first incubated for 4 h in media containing a mixture of radioactive amino acids, and the conditioned media from a subsequent 48-h period were collected, concentrated and analyzed by SDS-polyacrylamide gel electrophoresis. The results are presented in Fig. 9; the gels stained for protein and the corresponding autoradiogram showed some protein species common to both types of macrophages, for example lysozyme (C_1) and a lower molecular weight protein, C_2 . Other proteins, such as S_1 , S_2 , and S_3 were present in stimulated conditioned medium only, whereas proteins U_1 and U_2 were restricted to the conditioned medium from unstimulated macrophages. All of these proteins were present both in the autoradiogram and the stained gel, whereas a stained band, common to both stimulated and unstimulated macrophages, which coelectrophoresed with BSA, was absent in the autoradiogram; this probably represents albumin remaining in the culture from the preincubation in serum supplemented medium.

DISCUSSION

Several aspects of this work deserve comment. The first concerns the formation of plasminogen activator by macrophage cultures. The data presented above show that thioglycollate-stimulated macrophages synthesize, accumulate, and secrete plasminogen activator. This conclusion is supported by the following facts: (a) Intact, stimulated macrophages promoted active plasminogen-dependent fibrinolysis in culture for periods of several days. (b) Conditioned media, as well as cell lysates from such stimulated cultures also catalyzed plasminogen-dependent fibrinolysis. (c) The major activator of

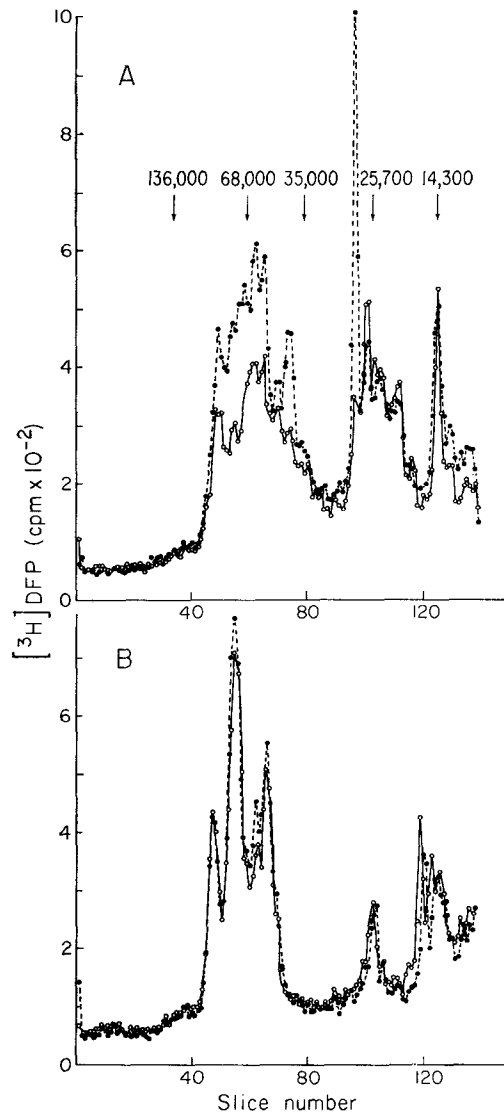


FIG. 8. Inhibition by NPGB of ^3H -DFP labeling of proteins in conditioned medium from stimulated and unstimulated macrophages. (A) stimulated macrophage conditioned medium (\bullet — \bullet) without NPGB; with $8 \times 10^{-5}\text{M}$ NPGB (\circ — \circ). (B) unstimulated macrophage conditioned medium (\bullet — \bullet) without NPGB; with $8 \times 10^{-5}\text{M}$ NPGB (\circ — \circ).

macrophages, which activates plasminogen by a specific hydrolytic cleavage, is an NPGB- and DFP-sensitive protease of mol wt 48,000 whose intracellular form is associated with the granule fraction (unpublished observation). Thus the enzyme from macrophages closely resembles and is probably homologous

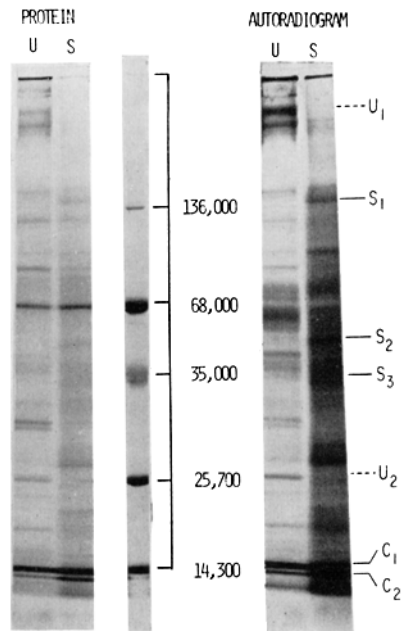


FIG. 9. Incorporation of [^{14}C]amino acids into products secreted by stimulated and unstimulated macrophages. ^{14}C -labeled conditioned medium from 1×10^7 unstimulated or 3×10^6 thioglycollate stimulated macrophages was electrophoresed on a 8–15% polyacrylamide SDS slab gel after concentration and dialysis (see Materials and Methods).

with the plasminogen activator produced by other murine cells such as transformed fibroblasts.

Although the function of the activator may not be limited to activation of plasminogen, the generation of plasmin by macrophages may be significant. The high level of circulating plasminogen acts as a large reservoir of potential proteolytic activity that could be rapidly recruited by cells releasing activator. Therefore, the interactions of plasmin with the enzymatic cascades involved in coagulation, (16) complement (17), and kinin formation (18) provide several pathways for generating peptides that are pharmacologically active, particularly in their effects on vascular permeability. Since the pH optimum of plasmin corresponds to that of tissue fluid, plasminogen activation may be a convenient mechanism for exercising cellular control of the local extracellular environment, and the role of plasmin in macrophage effector functions clearly requires further investigation.

With respect to plasminogen activator formation, the differences between thioglycollate-stimulated and unstimulated macrophages were substantial: (a) Plasminogen-dependent fibrinolysis by unstimulated cells, cell lysates, or conditioned media barely exceeded the background levels; (b) it was at

least 100-fold lower than the corresponding activities of stimulated cells; and (c) no peak of ^3H -DFP labeling was present in SDS-polyacrylamide gels of conditioned medium proteins from unstimulated cultures.

Although this contrast was striking, it appeared to be only one of many seen in the secretion products of the two kinds of macrophages. Both stimulated and unstimulated macrophages released a range of proteins that reacted with [^3H]DFP. However, the ^3H -DFP labeling of numerous species from stimulated cultures was reduced in presence of NPGB, indicating that they were probably proteases with tryptic specificity, whereas essentially none of the ^3H -DFP labeled peaks from unstimulated cultures was decreased by NPGB. The latter are thus more likely to be esterases or serine enzymes catalyzing other reactions.

The differences in proteins secreted by stimulated and unstimulated macrophages were not restricted to DFP-reactive serine enzymes; the gel patterns of biosynthetically labeled materials revealed further differences involving other protein species, and they showed in addition that certain proteins, such as lysozyme, were secreted in substantial amounts by both kinds of macrophages. All of these observations indicate that both the synthetic and secretory activities of macrophages are extensively modified by thioglycollate stimulation; the concentration of the individual components in conditioned media suggests that a number of these could be isolated in useful amounts.

Finally, it should be noted that the combination of biosynthetic labeling and ^3H -DFP labeling, both in the presence and absence of specific protease inhibitors, is a promising approach to the study of cellular secretion products and their regulation; it could be refined and applied to isolation and characterization of functionally significant enzymes from cell cultures, and to the study of macrophage effector functions.

SUMMARY

Cultured thioglycollate-stimulated peritoneal macrophages synthesize, accumulate, and continuously release high levels of plasminogen activators for at least 4 days whereas cultures of unstimulated macrophages do not; the higher specific catalytic activity of released vs. cell-associated enzyme suggests that the plasminogen activators are actively secreted. The major macrophage plasminogen activator is a serine protease of mol wt 48,000, and thus resembles the comparable enzyme released by virally transformed fibroblasts. Macrophages release a second plasminogen activator of mol wt 28,000 that is also a serine enzyme.

The secretion products released by stimulated and unstimulated macrophages have been compared by SDS-polyacrylamide gel electrophoresis after chemical labeling with ^3H -DFP or biosynthetic labeling with ^{14}C -amino acids. These procedures show that some proteins are formed in both cultures, whereas others are uniquely secreted by each type of macrophage. The serine enzymes

released by the two kinds of macrophages differ in specificity and electrophoretic mobility.

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