SECRETION OF A SPECIFIC COLLAGENASE BY
STIMULATED MACROPHAGES*

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Collagenases are specific neutral proteinases which initiate the breakdown of native collagen fibrils by cleavage at a unique site (1). The deposition and lysis of collagen play an important role in a variety of normal and pathologic conditions, including tissue remodeling and chronic inflammation, but the cellular sources and regulation of collagenase activity are still poorly understood.

Recent studies have shown that rabbit synovial fibroblasts secrete high levels of a specific collagenase in culture (2) and that polymorphonuclear leukocyte granules are also a source of the enzyme (3, 4). Since macrophages are often present in large numbers in conditions associated with rapid collagen turnover and since macrophages can be induced to secrete other specific neutral proteinases which act as plasminogen activators (5), it was important to establish whether macrophages also release a specific collagenase. Several brief reports have indicated that Kupffer cells and other mononuclear phagocytes may contain collagenolytic activity (6-8).

In this paper we show that purified populations of mouse peritoneal macrophages, obtained after stimulation with thioglycollate broth, also secrete a specific collagenase in vitro. The enzyme has been partially characterized and conditions for its secretion established. Unstimulated peritoneal macrophages secrete barely detectable levels of the enzyme, but these cells can be stimulated to secrete more collagenase after phagocytosis of indigestible latex particles. While this work was in progress, another report indicated that stimulated guinea pig peritoneal macrophages also secrete collagenase in vitro (9).

Materials and Methods

Reagents. Reagents were obtained from the following sources: Dulbecco's modification Eagle's medium, fetal bovine serum (FBS),[1] lactalbumin hydrolysate (LH), RPMI medium, Neumann and Tytell medium from Gibco-Biocult, Paisley, Renfrews., Scotland; Brewer's thioglycollate medium from Difco Laboratories, Detroit, Mich.; polystyrene latex particles, 1.1 μm in diameter from Micro-

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[1] *Abbreviations used in this paper:* AT-FBS, acid-treated fetal bovine serum; CM, conditioned medium; 4-CMB, 4-chloromercuribenzoate; Dip-F, diisopropyl phosphorofluoridate; EDTA, ethylenediaminetetraacetic acid; LH, lactalbumin hydrolysate.

bio Labs. Ltd., London, England; dextran sulfate 2000 was a gift of Pharmacia Fine Chemicals, Inc., London, England; α_2-macroglobulin and leupeptin-Pr were gifts of Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, England; penicillamine, trypsin inhibitor (soybean), 4-nitrophenyl 4'-guanidinobenzoate were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, England. All other reagents were laboratory reagent grade available from standard commercial suppliers.

Cell cultures. Female Swiss mice, 25–30 g in weight, were obtained from the colony maintained at the Dunn School of Pathology, Oxford, or from Strangeways Research Laboratory, Cambridge. Peritoneal macrophages were harvested from unstimulated control animals or from mice injected with 1 ml of thioglycollate medium 4 days earlier, as described previously (5, 10, 11). Cells were routinely cultivated in Dulbecco's modified Eagle's medium supplemented with glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml), as well as 15% (vol/vol) acid-treated FBS (AT-FBS). Acid-treated serum was prepared by adjusting serum to pH 3.2 with 2 M HCl in 0.15 M NaCl for 120 min and returned to pH 7.4 with 2 M NaOH in 0.15 M NaCl. Acid treatment destroys α_2-macroglobulin, a serum inhibitor of collagenase (5, 12).

Peritoneal cells were placed in medical flats of 50, 100, and 500 ml size and nonadherent cells removed by vigorous washing after 2–4 h. Fresh medium was added to the macrophage monolayer and replaced at 24 h. Conditioned medium (CM) was routinely prepared after 48 h cultivation. The cells were washed three times to remove serum and then incubated in serum-free Dulbecco's medium containing 0.2% (wt/vol) LH, usually for a period of 2–4 days. Macrophages have been incubated in such medium for up to 3 wk without loss of viability. All cultures were prepared in duplicate or triplicate. Other media used included Neumann and Tytell serumless medium, to prepare CM, and RPMI 1640.

For phagocytosis experiments latex particles were prepared and added to monolayers which had been cultivated for 24 h as described previously (11). After phagocytosis the cells were exposed to medium containing AT-FBS for 24 h before preparing CM.

For studies on the effects of pinocytosis, dextran sulfate 2000 was dissolved in Dulbecco's medium and sterilized by membrane filtration. Cells were exposed to 20 μg of dextran sulfate/ml, in the presence of serum, for 48 h.

Preparation of CM for Assay. Media decanted from the cultures were centrifuged (500 g, 20 min) to remove debris and nonadherent cells, then used directly for assay or dialyzed in size 8/32 Visking tubing against 100 vol of 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM $CaCl_2$ and freeze-dried. Generally, the freeze-dried CM was reconstituted with water at 5 or 10% of the original volume. Collagenase activity in the concentrated medium was stable to several cycles of freezing and thawing. In a few experiments media were concentrated by ultrafiltration with a Sartorius membrane filter (type SM 12133) fitted in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.).

Cell Lysates. Cell lysates were prepared by freezing and thawing cells in 0.1% Triton X-100. In some experiments the cell lysates were dialyzed against 10 mM Tris-HCl buffer, pH 7.6, containing 1 mM $CaCl_2$, then freeze-dried in parallel with the CM.

Optimal Conditions of Culture for Demonstrating Collagenase Activity. Thioglycollate-elicited macrophages were cultivated under a variety of conditions to determine the best conditions for maintaining the cells and for measuring the medium enzyme. Higher levels of activity were found in serum-free CM obtained after preincubation in AT-FBS than in FBS. The presence of 15% AT-FBS in the medium masked collagenase activity and was found to inhibit the enzymic activity of serum-free conditioned medium on reconsituted collagen fibrils. However, on dilution AT-FBS was only one-tenth as inhibitory as FBS. CM was therefore always collected as free of serum constituents as possible. Macrophages tolerated RPMI 1640 medium well but unidentified components in the medium interfered in the collagenase assay. Dulbecco's medium containing 0.2% LH maintained the macrophages for longer than medium with only 0.05% LH, and gave higher levels of enzyme activity. Neumann and Tytell serumless medium also yielded optimal cell survival and enzymic activity and was used in some experiments.

Preparation of Conditioned Media for Enzymological Studies. Cells were harvested from 10 mice which had been injected 4 days earlier with thioglycollate broth. About 1.5×10^8 macrophages could be obtained in this way. Cells were plated in three to four 32 oz medical flats in Dulbecco's medium containing 15% AT-FBS as before. Conditioned medium (about 100 ml) was prepared from 48 to 120 h. Media were dialyzed and lyophilized as described above and reconstituted in 10% of the original volume.

Other Cell Lines.   Primary mouse synovial fibroblasts were grown from explants of mouse synovium as described for rabbit synovium (2). Established strains of mouse cells derived from 3T3, L and melanoma cells were obtained from Dr. I. Craig, Genetics Laboratory, Oxford. For studies of collagenase secretion newly confluent monolayers in 8 oz culture bottles were exposed to serum-free Dulbecco's medium as described for macrophage cultures. Enzyme activity was assayed directly in CM or after the medium had been concentrated by ultrafiltration or freeze-drying as described for macrophage CM.

Assays.   For assay of collagenase activity [^{14}C] glycine-labeled rat skin collagen (17, 200 dpm/mg) (2) or [^{14}C] glycine-labeled rabbit skin collagen[2] prepared in the same way (7,092 dpm/mg) were used. Collagenolytic activity was analyzed with the radioactive substrate in the form of reconstituted fibrils with 10 mM $CaCl_2$ present during assay, as described previously (2, 13). Assays were standarized so that reconstituted fibrils from rabbit and rat collagens would give comparable results; accordingly, assays were performed at 37°C and 35°C for rabbit and rat collagens, respectively. 1 U of collagenase activity was defined as the hydrolysis of 1 μg of reconstituted fibrils/min under these conditions (2, 13). 4-Chloromercuribenzoate (1 mM) (4-CMB) was included in assays with unprocessed CM. This reagent has been shown to activate collagenase activity in the presence of culture medium components (2, 13). Incubation was usually for 18 h and all assays included appropriate blanks and trypsin controls to ensure that the collagen was not denatured. Trypsin (10 μg/tube) released less than 6.1% of the total releasable counts with rat collagen and less than 4.2% of the counts with rabbit collagen. Only values for collagenolytic activity exceeding these were taken as signficant. Only assays giving linear release of products with respect to enzyme concentration were used and all assays were made in duplicate or triplicate.

For maximal rates of collagen lysis viscometric assays were performed with collagen in solution at 35°C in the presence of arginine, by the method of McCroskery et al. (14). At this temperature both products of digestion of collagen by specific collagenase denature spontaneously to gelatin, which has negligble viscosity and thus initital rates of the decrease in viscosity give a measure of the rate of specific cleavage. Reaction mixtures (2 ml) contained 50 mM arginine, 600 μg collagen, 10 mM $CaCl_2$, 100 mM Tris-HCl buffer, pH 7.6, and 200 mM NaCl. Specific viscosities (η_{sp}) were determined in Ostwald U-tube microviscometers having water flow times of 60s and 25s. For analysis of the reaction products of collagenase, assays with collagen in solution at 24°C were made essentially as before (2, 13). Samples of conditioned medium (20 μl of 10–20 times concentrated CM) were reacted with 100 μg of collagen for 20 h, denatured by heating in urea and run on polyacrylamide gels at pH 3.5 (2, 13). SDS-acrylamide gels were generally unsuitable because of the relatively high level of protein in the macrophage CM.

Protein was determined by the method of Lowry et al. (15). Cathepsin D was determined using [^{3}H]acetyl-hemoglobin as substrate (16). Buffers used in biochemical determinations usually contained 0.02% sodium azide to prevent bacterial growth. Results are shown as ± SEM, and statistical tests of significance were made with Student's *t* test.

Results

Collagenolysis by Media from Unstimulated and Thioglycollate-Stimulated Macrophages.   Collagenolytic activity of the serum-free CM of thioglycollate-stimulated mouse macrophages was measured using the assay with reconstituted collagen fibrils. Although the activity directly detectable in the culture medium was small this could be measured accurately in particularly active preparations in the presence of 4-CMB. Even small amounts of enzymic activity in the CM were stable to dialysis and concentration by freeze-drying, and activity was nearly quantitatively recovered (Table I). In contrast very little activity could be measured in CM from unstimulated macrophages and even after concentration the values rarely exceeded the level of nonspecific lysis seen in control assay mixtures containing trypsin.

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[2] Werb, Z., and M. C. Burleigh. The degradation of rabbit collagens by rabbit collagenases. Manuscript in preparation.

TABLE I

Stability of Macrophage Collagenase to Processing

Treatment*	Collagenase activity	
	% Fibril lysis/ 100 µl of medium§	U/flask
Thioglycollate macrophages		
None‡	5.1	0.46
Dialysis	5.3 ± 0.2	0.47 ± 0.07
Lyophilized and reconstituted at 10 × concentration	46.2 ± 4.5	0.42 ± 0.04
Unstimulated macrophages		
None‡	0	0
Dialysis	1.60	‖
Lyophilized and reconstituted at 10 × concentration	1.72	0.02‖

* 2.2 × 10⁷ thioglycollate or unstimulated macrophages were plated; CM was collected from 48–120 h of culture.
‡ Assayed in presence of 1.0 mM 4-CMB.
§ Trypsin controls were 4.1% fibril lysis with the buffer blank subtracted.
‖ Not significant.

Comparison of Collagenolytic Activity in Conditioned Media and Cell Lysates. An experiment was made to test whether the collagenase was released selectively into the culture medium of mouse macrophages. Intra- and extracellular collagenase activity was compared for both unstimulated and thioglycollate macrophages with that of cathepsin D, a lysosomal enzyme. As shown in Table II the collagenase activity was found almost exclusively in the culture medium of stimulated macrophages and only neglible amounts within cells, in contrast with the predominantly intracellular distribution of cathepsin D in both types of macrophage. In other experiments it was found that cell lysates of thioglycollate macrophages prepared 2 h and 70 h after explanting also contained negligible levels of collagenase. Therefore the appearance of collagenase activity in the medium during cultivation probably represented net production and secretion of the enzyme.

In view of the marked difference between collagenase activity demonstrable in stimulated and unstimulated cultures the presence of inhibitors of the enzyme was tested in unstimulated cell lysates and CM by performing mixing experiments with CM from thioglycollate-stimulated macrophages. No evidence was found for such inhibitors, nor did activity appear in the inactive preparations after treatment with trypsin, a method used to activate mouse bone collagenase (17), although a systematic study of collagenase activation was not done.

Time-Course of Secretion of Collagenase from Thioglycollate-Stimulated Macrophages. The experiments described above suggested that collagenase was secreted from stimulated macrophages. The capacity of thioglycollate-stimulated macrophages to continue to produce and release the enzyme during in vitro cultivation was next examined. Macrophages were cultured in Dulbecco's medium containing 15% AT-FBS for 48 h, then placed in Dulbecco's medium

TABLE II

Distribution of Collagenase in Cells and Conditioned Medium*

Source of macrophage enzyme	Collagenase activity		Cathepsin D/(U/flask)
	% Fibril lysis‡	U/flask	
Cells			
Unstimulated	1.6§	<0.05	0.82
Thioglycollate	2.7§	<0.05	1.23
Medium			
Unstimulated	2.0§	<0.05	0.22
Thioglycollate	25.3	0.47	0.43

* 2.1 × 10⁷ unstimulated macrophages (0.78 mg cell protein at end of experiment) and 2.3 × 10⁷ thioglycollate-stimulated macrophages (1.55 mg cell protein) were plated in medical flats. Serum-free medium was conditioned between 48 and 120 h after explanting and processed as described in the text.

‡ Cells and medium were reconstituted in 2.0 ml and 100 μl aliquots assayed with reconstituted collagen fibrils for 18 h as described in the Materials and Methods section.

§ Values lower than trypsin control (4.3% lysis in this experiment) were not significant.

supplemented with 0.2% LH, with change of medium every 24 h. As shown in Fig. 1, the secretion of collagenase (0.012 U/24 h) remained nearly linear for 7 days. At the end of this period in the absence of serum the cells remained viable and well-spread.

In another experiment the effect of serum on the continued secretion of collagenase was examined. Thioglycollate-stimulated macrophages were cycled on alternate days between medium containing 15% AT-FBS and medium with 0.2% LH. When the CM from the period in serum-free medium was tested for collagenase it was found that the enzymic activity secreted (0.018 U/day) remained constant for four alternate cycles and was similar to the enzymic activity secreted from macrophages maintained in serum-free medium alone for the entire period (0.020 U/day).

The time when collagenase activity first appeared was also determined. 10 replicate cultures of thioglycollate-stimulated macrophages were plated in Dulbecco's medium containing 15% AT-FBS. Duplicate cultures were placed in Dulbecco's medium containing 0.2% LH for 48 h immediately after removing nonadherent cells (day 0) or subsequently, on days 1, 2, 3, or 4. The collagenase activities found in CM were 0.010, 0.010, 0.015, 0.022, and 0.010, for collection periods starting on day 0, 1, 2, 3, and 4, respectively. Hence, collagenase is secreted even by newly explanted macrophages. The continued and relatively constant level of release of collagenase suggested that stimulated macrophages produce and secrete the enzyme in vitro.

Requirement for Protein Synthesis. The effect of cycloheximide on the continued secretion of macrophage collagenase was tested. In the range of 1–2 μg/ml protein synthesis is inhibited but the cells remain viable for up to 24 h (10). When cycloheximide (0.4 μg/ml) was added to thioglycollate-stimulated macrophages at the beginning of the period in serum-free medium, the medium contained less than 8% of the control levels of collagenase at 11 h, and less than 2% of controls at 22 h.
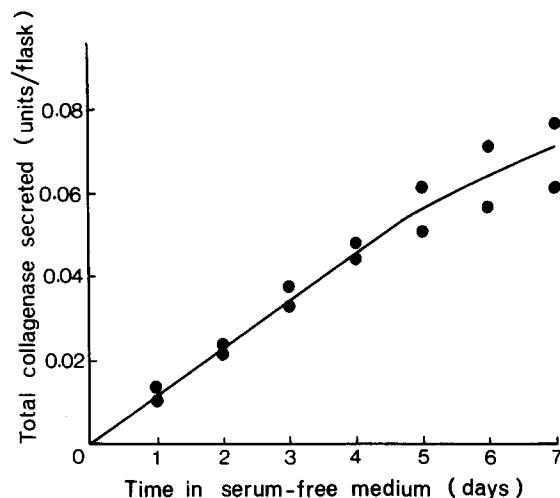
FIG. 1. Time-course of collagenase secretion from stimulated macrophages. Cumulative totals of the collagenase secreted from thioglycollate-stimulated macrophages ($1.5 \times 10^7/125$ ml flask) with daily medium changes are shown for two separate cultures. Conditioned media were processed then reconstituted at 5% of the original volume for assay.

Relationship of Collagenase Secreted to Macrophage Number. The relationship between the number of thioglycollate-stimulated macrophages plated and collagenase secretion was next examined. Up to 7×10^6 cells were plated in 50-ml medical flats with resulting cell densities of 0.2–0.8 mg cell protein per culture. The collagenase secreted during a 4-day collection period was proportional to cell protein with an average secretion of 0.061 ± 0.011 (SEM, $n = 11$) U collagenase/mg cell protein.

Characterization of Macrophage Collagenase

ASSAYS WITH RECONSTITUTED COLLAGEN FIBRILS. Release of radioactivity from collagen fibrils by macrophage collagenase was linear to almost 70% fibril lysis (Fig. 2). Solubilization of fibrils also progressed in a linear fashion with time between 6 and 24 h of incubation.

The collagenase in CM was tested for inhibition by a number of potential inhibitors (Table III). Metal-ion chelators including EDTA and 1,10-phenanthroline were efficient inhibitors. The inhibition by disulfide-reducing reagents including cysteine was probably due to their metal-ion chelating properties. Inhibitors of serine proteinases such as Dip-F and soybean trypsin inhibitor were without effect. The collagenase was effectively inhibited by α_2-macroglobulin. Inhibitors of thiol proteinases such as leupeptin and 4-chloromercuribenzoate (18) had no effect on the macrophage collagenase and 4-nitrophenyl 4′-guanidinobenzoate, an inhibitor of macrophage plasminogen activator (15) also had no inhibitory effect. Polyanions had little effect on the activity of this enzyme. Thus, the macrophage collagenase is a metal proteinase like other vertebrate collagenases (1).

VISCOMETRIC ASSAYS WITH COLLAGEN IN SOLUTION. The collagenolytic activity of CM from stimulated and unstimulated macrophages was assayed with collagen in solution at 35°C. This assay measures only the rate of cleavage of the collagen
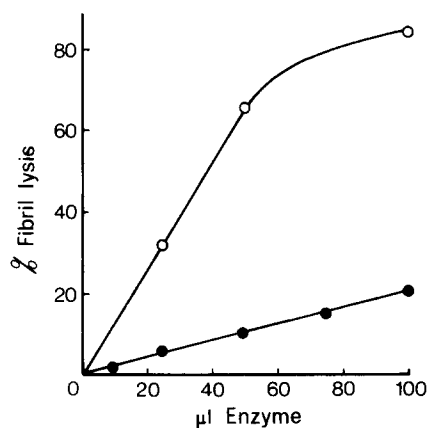
FIG. 2. Linearity of the release of ^{14}C-labeled peptides from reconstituted collagen fibrils by mouse macrophage collagenase. Two preparations of concentrated conditioned medium from thioglycollate-stimulated macrophages were used as source of enzyme. Assays contained 104 μg collagen and were incubated for 18 h at 37°C. ●, conditioned medium from 4 × 10^7 macrophages concentrated to 2.5 ml; ○, conditioned medium from 3 × 10^7 macrophages (1.4 mg cell protein) concentrated to 1.0 ml.

TABLE III

The Effect of Potential Inhibitors on Macrophage Collagenase

Compound	Concn. in assay*	Activity
		%
None		100
Trisodium EDTA	30 mM	2
	10 mM	5
Cysteine	10 mM	19
Penicillamine	1 mM	17
Dithiothreitol	1 mM	0
1, 10-Phenanthroline	1 mM	4
Leupeptin-Pr	0.1 mg/ml	97
Dip-F	1 mM	96
Soybean trypsin inhibitor (Kunitz)	1 mg/ml	108
α_2-Macroglobulin	250 μg/ml	3
4-Chloromercuribenzoate	1 mM	96
Iodoacetamide	10 mM	94
Heparin	0.5 mg/ml	123
Dextran sulfate 2000	0.1 mg/ml	100
	0.5 mg/ml	132
4-Nitrophenyl 4'-Guanidinobenzoate	5 × 10^{-5} M	102

* All assays were made in the presence of 15 mM CaCl$_2$; macrophage CM giving 34% fibril lysis in 18 h was added to each tube.

molecules independent of release from an insoluble fibrillar structure which might be aided by other proteinases, and gives a more accurate reflection of the collagenolytic activity present in CM from the two types of macrophages.

As shown in Fig. 3 there was considerable collagenolytic activity in the CM
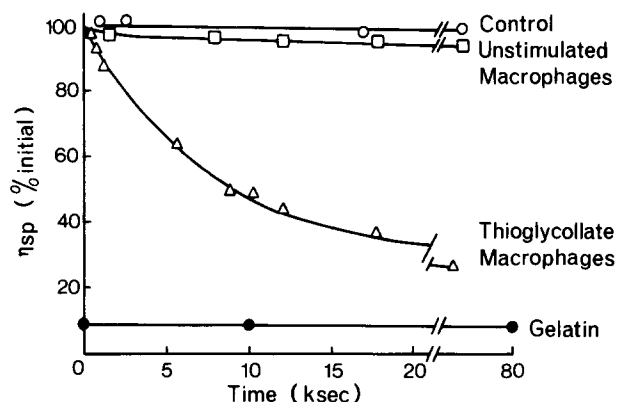
Fig. 3.   Reduction of the specific viscosity of collagen in solution at 35°C by collagenases from stimulated and unstimulated macrophages. Assays contained 600 μg collagen and: O, no enzyme; □, 300 μl of medium from unstimulated macrophages [conditioned medium (1.0 ml after processing) from 2.1×10^7 cells (1.6 mg protein) for 72 h]; △, 300 μl enzyme from thioglycollate-stimulated macrophages [conditioned medium (1.0 ml after concentrating) from 2.3×10^7 cells (2.1 mg protein) for 72 h]. ●, control containing 600 μg gelatin (made by heating collagen to 50°C for 30 min) instead of collagen.

from the stimulated macrophages, but very little in the medium from un-stimulated cells. The initial rates of hydrolysis of rabbit collagen at 35°C were 12.6 and <0.1 μg collagen/min/ml enzyme for the CM from stimulated and unstimulated macrophages respectively. This can be compared with the activities of the same enzyme preparations on reconstituted rabbit collagen fibrils (at 37°C), which were 0.64 and 0.02 μg collagen/min/ml enzyme. Therefore the true ratio in the collagenase activities of thioglycollate-stimulated and unstimulated macrophages is more than 100 :1.

CHARACTERIZATION OF THE REACTION PRODUCTS OF MACROPHAGE COLLAGENASE WITH COLLAGEN IN SOLUTION AT 24°C.   The reaction products of macrophage collagenase on collagen in solution were determined at 24°C where the fragments remain native and thus resistant to degradation by contaminating proteinases. As shown in Fig. 4 a the enzyme from the medium of stimulated macrophages gave the typical specific reaction products of mammalian collagenases. The bands derived from intact single subunits of collagen α, and the cross-linked dimers, β chains, gave rise to the approx. three-quarter length A fragments and the approx. one-quarter length B fragments (2). Although contaminating proteinases were present in the CM the cross-link region of the collagen molecule was not cleaved under these conditions (i.e. no β to α conversion was observed.)

The CM from unstimulated macrophages which had insignificant activity on assay with reconstituted collagen fibrils showed traces of specific products. Similarly a trace amount of specific products could be seen when collagen in solution was reacted with concentrated cell lysates from thioglycollate-stimulated macrophages although activity could not be detected in the assay with collagen fibrils. The formation of the specific products was inhibited by the presence of EDTA or 1,10-phenanthroline in the reaction mixture, and was unaffected by the presence of Dip-F (Fig. 4 b).
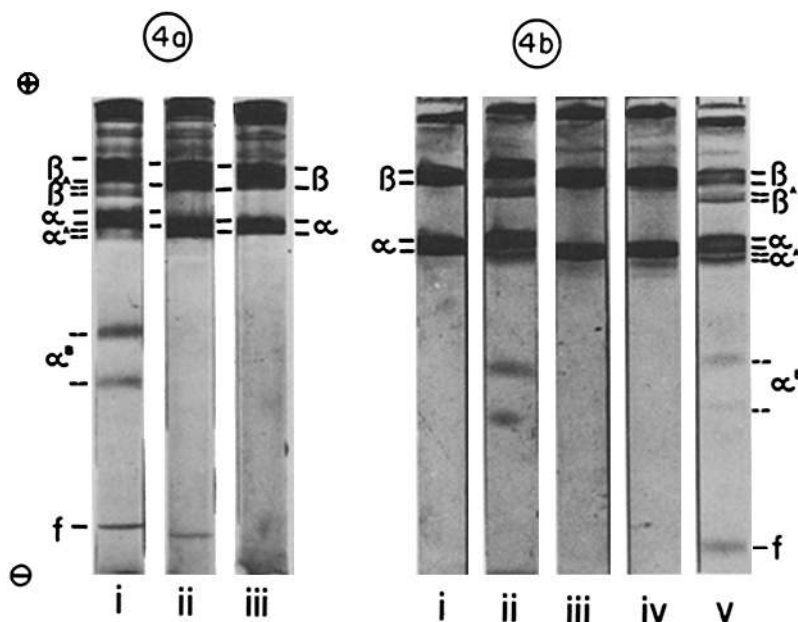
FIG. 4. Gel electrophoretic patterns of the denatured products of reaction of macrophage collagenases with collagen in solution at 24°C. Products were denatured by heating in 5 M urea and run on polyacrylamide gels at pH 3.5. Migration was downwards towards the cathode. Bands arising from single subunits of collagen (α) and cross-linked dimers (β) were degraded to approx. three-quarters length fragments (α^A and β^A) below their respective intact subunits and approx. one-quarter length fragments (α^B) further down the gel as described for other vertebrate collagenases (1). (a) Comparison of the products produced by incubating collagen with conditioned medium from (i) stimulated and (ii) unstimulated macrophages. (iii) control collagen. For details of enzyme preparations see legend for Fig. 3. (b) Effect of potential inhibitors on reaction products using the collagenase from thioglycollate-stimulated macrophages. (i) Collagen control; (ii) enzyme; (iii) enzyme + EDTA (30 mM in the presence of 20 mM CaCl$_2$); (iv) enzyme + 1,10 phenanthroline (1 mM); (v) enzyme + Dip-F (1 mM). f, buffer front.

Regulation of the Secretion of Collagenase from Macrophages

EFFECT OF STIMULATION. As noted already thioglycollate-stimulated macrophages secreted more collagenase than unstimulated macrophages. Since the secretion of plasminogen activator from thioglycollate-stimulated macrophages also exceeds that from unstimulated macrophages this difference was compared further.

Secretion of collagenase by thioglycollate-stimulated macrophages was observed in nine independent experiments over 6 mo. Although variable levels of stimulation were observed (0.03–0.18 U/10^7 cells/24 h), these values were appreciably higher than collagenase secretion observed for unstimulated cells (0.001 to 0.005 U/10^7 cells/24 h). This difference was highly significant ($P <$ 0.001, n $=$ 37), even taking into account a three- to fivefold higher protein content of the thioglycollate-stimulated macrophages.

EFFECT OF PHAGOCYTOSIS OF LATEX. Phagocytosis of nondigestible latex particles has been found previously to stimulate the secretion of collagenase from

rabbit fibroblasts (13) and of plasminogen activator from macrophages (11). Accordingly, the effect of ingestion and intralysosomal storage of latex particles by unstimulated macrophages was examined. As shown in Table IV macrophages exposed to latex particles secreted at least twice as much collagenase as the nonphagocytosing controls. However, the amount of enzyme secreted was still less than the levels observed for thioglycollate macrophages cultured at the same time.

The level of collagenase secreted by unstimulated cells after latex phagocytosis was related to the amount of material ingested. Unstimulated macrophages (4.5 × 10⁶/culture) were exposed to latex particles (2.5 mg/culture) for 0, 30, 60, or 120 min, then placed in 10% FBS in Dulbecco's medium for 24 h, followed by 6 days in Dulbecco's + 0.2% LH. The macrophage cultures which had ingested 0, 280, 690, and 1,030 µg latex secreted 0, 0.03, 0.13, and 0.34 U collagenase/6 days, respectively.

TABLE IV

Effect of Phagocytosis on Secretion of Collagenase from Macrophages

Macrophages	Cell number	Collagenase secreted	
		U/flask/72 h	Fibril lysis*
			%
Unstimulated	1 × 10⁷	0.016 ± 0.002	6.8
Unstimulated⁺ latex	1 × 10⁷	0.029 ± 0.002	11.4
Thioglycollate	0.8 × 10⁷	0.081	31.8

* Assay of 100 µl of 20 times concentrated (from 6 ml/flask) conditioned medium. Values shown are the average of two cultures and are ± range.
† Difference between unstimulated and latex-fed macrophages statistically significant (*P* < 0.05).

After ingestion of latex the secretion was maintained at the new increased level for more than 2 wk. In the experiment illustrated in Fig. 5 an eightfold stimulation was sustained for at least 10 days. Variation in the level of stimulation between experiments could have been due to differences in latex loading and/or the level of macrophage activation in control animals (11). For four experiments the increased rate of collagenase secretion in macrophages containing latex was significantly higher than the secretion from nonphagocytosing controls (*P* < 0.001).

EFFECT OF PINOCYTOSIS OF DEXTRAN SULFATE. The specificity of the stimulation of collagenase secretion after endocytosis was tested. Dextran sulfate greatly increases pinocytosis by macrophages and results in the accumulation of distended lysosomes (19). Although the net result of increasing the number and size of secondary lysosomes is the same for both latex and dextran sulfate, there are a number of differences. Detran sulfate is very anionic, is taken up by pinocytosis, and inhibits a number of lysosomal hydrolases (16, 19).

The cells exposed to dextran sulfate had 340% of the collagenase activity in their CM compared with controls. This was much higher than any direct effect of dextran sulfate on the enzymic activity (see Table III). Thus, the stimulation after endocytosis is not specific for the inducing particle. It is worth noting that
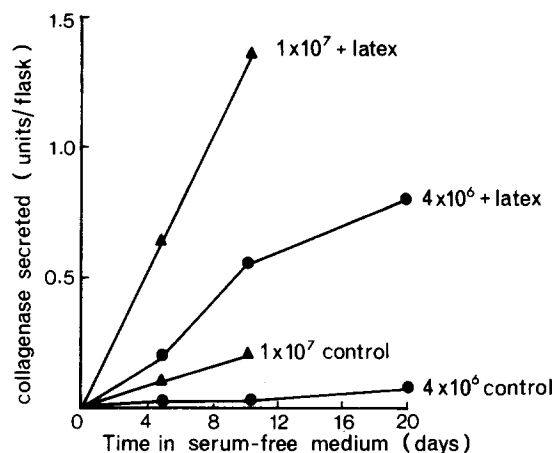
FIG. 5. Effect of phagocytosis on the secretion of collagenase from unstimulated macrophages. Macrophages were exposed to latex particles as described in the Materials and Methods section. In this experiment the conditioned media were concentrated by ultrafiltration and collagenase assays made in the presence of 1.0 mM 4-CMB (2, 13). The collagenase secretion achieved in this experiment was higher than usual for unstimulated macrophages. (▲——▲), 1 × 10⁷ macrophages; (●——●), 4 × 10⁶ macrophages.

thioglycollate-stimulated macrophages contain inclusions of agar and other undigested materials and thus the variable level of collagenase secreted may well be due to differences in the amounts of materials stored within cells from different batches of animals.

CELL SPECIFICITY OF COLLAGENASE SECRETION. Since plasminogen activators are secreted from stimulated macrophages as well as other cells (15) and since collagenase is also secreted from normal rabbit fibroblasts (13), other mouse cells were examined for their capacity to secrete collagenase. As shown in Table V stimulated mouse macrophages regularly secreted collagenase, but far less than those rabbit fibroblasts which secreted enzyme. Rabbit synovial fibroblast lines which did not secrete collagenase have also been isolated (Werb, unpublished observation). A single primary culture of mouse synovial fibroblasts was also shown to secrete a specific collagenase and the IR mouse fibroblast cell line at times secreted a little collagenolytic activity. Other established mouse endo-thelial and melanoma cell lines were inactive although the capacity of these cells to secrete collagenase under different conditions of cultivation was not investigated.

Since thioglycollate-stimulated macrophage cultures may contain up to 5% fibroblasts control experiments were made to ensure that the collagenase detected in macrophage CM was not due to a highly active subpopulation of contaminant fibroblasts. Macrophage cultures were treated with 0.25% trypsin-versene (30 min, 37°C) in the absence of serum and washed thoroughly. This procedure efficiently removes fibroblasts, whereas macrophages resist detach-ment by trypsin. After further incubation for 1 day in AT-FBS, CM was collected from trypsinized and untreated cultures and showed no reduction in the level of collagenase.

TABLE V

Collagenase Secretion from Various Cells in Culture

Cell type*	Collagenase secretion	
	U/24 h/10^7 cells	U/24 h/mg cell protein
Thioglycollate-stimulated mouse macrophages	0.15 (0.08–0.21)	0.11
Unstimulated mouse macrophages	0.01	0.03
Mouse endothelial cell strain (3T3 TK⁻)	0	0
Mouse fibroblast strain (1R HGPRT⁻)	0.02 (0–0.04)	0.013
Mouse melanoma cells (PG19 HGPRT⁻)	0	0
Primary mouse synovial fibroblasts (MS2/1)	0.6 (0–1.2)	0.3
Rabbit synovial fibroblasts	12 (0–31)	1.8

* Cells were cultured for 72 h in serum-free medium as follows: Macrophage CM as described in the methods section; 3T3 TK⁻, 1R HGPRT⁻, PG19 HGPRT⁻ were established lines developed from the parent lines 3T3, L-cells and PG19 respectively; MS2/1 was a primary outgrowth from normal mouse synovium. CM was prepared by incubating the confluent cells in Dulbecco's medium for 48 h; data for the rabbit synovial fibroblasts from Werb et al. (2, 13).

Discussion

These studies show that macrophages in culture can produce and secrete a specific collagenase which cleaves native, helical collagen into typical three and one-quarter fragments by limited proteolysis at neutral pH. The macrophage enzyme is a metalloproteinase which resembles other mammalian collagenases (1) since it is inhibited by chelating agents and it is unaffected by inhibitors of serine and thiol proteinases. It can be distinguished from the plasminogen activator also secreted by stimulated macrophages (5) by its inhibitor profile, including resistance to 4-nitrophenyl 4'-guanidinobenzoate, and its plasminogen independence. Other proteinases present in macrophage culture medium did not interfere with the collagenase assay, but may contribute to further degradation of collagen fragments to low molecular weight peptides at 37°C, though not at 24°C. There was no cleavage seen of the collagen cross-links located in the nonhelical region of the molecule, to produce the β- to α-conversion of collagen subunits observed in other studies (9). Further characterization of the mouse macrophage collagenase is necessary to compare it with the enzyme secreted by rabbit fibroblasts and the enzyme of human granulocytes.

The extracellular accumulation and low levels of activity in cell lysates suggest that active collagenase is not stored within macrophages, but is primarily a secretory product. Prolonged secretion over many days and the inhibition of secretion by cycloheximide provide some evidence for new synthesis of enzyme protein in culture, although the presence of an inactive precursor within the cell cannot be excluded (17, 20).

The requirement for thioglycollate stimulation and endocytosis to stimulate secretion of collagenase shows that its regulation in the macrophage closely parallels that of the plasminogen activator (11). The induced secretion of these specific neutral proteinases can be contrasted with the constant secretion of lysozyme (10), independent of cell stimulation and phagocytosis, and the

predominantly intracellular retention of other macrophage enzymes such as cathepsin D. Although we did not study the role of endotoxin in inducing secretion of collagenase by mouse macrophages (11), Wahl and co-workers (9) have demonstrated that oil-induced guinea pig peritoneal macrophages secrete collagenase after exposure to endotoxin in vitro. The transient release of enzyme observed in their studies is probably the result of endotoxin degradation by the macrophages since nondigestible particles such as latex and dextran sulfate stimulated prolonged secretion of the mouse macrophage collagenase, as noted previously for secretion of collagenase and neutral proteinase from rabbit fibroblasts (13) and for plasminogen activator from mouse macrophages (11).

The present studies suggest that macrophage collagenase could play an important role in collagen degradation in vivo. Macrophages are present in large numbers during wound repair (21, 22) and in granulomatous lesions caused by nondegradable irritants such as carrageenin (23), which are often associated with collagenolysis. During involution of the uterus macrophages may contain fragments of collagen fibrils in intracellular vacuoles (24) and macrophages may secrete specific collagenases found in these tissues at the same time as collagen degradation proceeds within lysosomes. Indeed, uptake of collagen fibrils by macrophages could stimulate further secretion of the enzyme.

Since fibroblasts and macrophages are often found together in granulation tissue and chronic inflammation, both cell types could contribute to collagen breakdown. In culture the fibroblasts secrete collagenase without further stimulation whereas secretion by macrophages requires an additional inducing stimulus. Since macrophages are also more avid phagocytic cells than fibroblasts, the extent of local phagocytic activity could determine the contribution by macrophages to collagenolysis in vivo.

The secretory activity of macrophages undergoes considerable modification after macrophage activation. Previous studies with [^{3}H]Dip-F suggested that several other serine proteinases can be secreted by stimulated macrophages (5). At least two other secreted proteinase activities have now been found which are regulated similarly to collagenase and plasminogen activator, an elastase (25) and a neutral proteinase(s)[3] active against azocasein, gelatin and denatured fibrinogen. Thus, collagenase is but one of a spectrum of induced proteinases, and perhaps other products, by which the stimulated macrophage modifies its extracellular environment.

Summary

Thioglycollate-stimulated mouse macrophages release a specific collagenase into their medium during in vitro culture. The macrophage collagenase has been characterized as a typical metal proteinase which catalyzes the cleavage of the native collagen molecule into three and one-quarter fragments. The extracellular accumulation and low activity in cell lysates suggest that collagenase is a secretion product of the stimulated macrophage. Prolonged secretion of the enzyme at a constant rate for more than 7 days in culture and its inhibition by cycloheximide provide evidence for biosynthesis in vitro. In contrast, secretion

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[3] Werb, Z., and S. Gordon. Studies on the secreted neutral proteinase of mouse macrophages. Manuscript in preparation.

of collagenase is barely detectable from unstimulated macrophages which can, however, be stimulated to secrete the enzyme by ingestion and intralysosomal storage of latex particles or dextran sulfate. Macrophages laden with latex, an undigestible particle, continue to release collagenase for at least 20 days. Several established mouse cell lines have also been examined for their capacity to secrete collagenase. Collagenase is one of a class of inducible neutral proteinases by which the activated macrophage can modify its extracellular environment.

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