Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3598–3601, September 1974

Collagenase Production by Endotoxin-Activated Macrophages

(lipid A/lipopolysaccharide/connective tissue)

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Communicated by Victor A. McKusick, June 27, 1974

ABSTRACT **Peritoneal exudate macrophages, when exposed to bacterial lipopolysaccharide in culture, were found to produce collagenase (EC 3.4.24.3). This enzyme was not detected in extracts of the macrophages or in media from nonstimulated macrophage cultures. Lipid-containing fractions of the lipopolysaccharide, including a glycolipid from the rough mutant of *Salmonella minnesota* (R595) and lipid A, were potent stimulators of collagenase production. The lipid-free polysaccharide fraction had no effect. Cycloheximide prevented the production of collagenase by endotoxin-treated macrophages, suggesting that it was newly synthesized.**

Increased levels of collagenase (EC 3.4.24.3) have been found in acutely and chronically inflamed tissues, including healing wounds, rheumatoid joints, and inflamed gingiva, in which connective tissue has been destroyed. Polymorphonuclear leukocytes, which predominate in acute inflammatory reactions, produce collagenase (1). However, after the acute inflammatory process and in chronic inflammatory lesions where collagen breakdown is most evident, relatively few neutrophils are present. Instead, macrophages are present in the tissue in large numbers, suggesting that these cells may play an active role in connective tissue degradation. Indeed, macrophages of alveolar origin have been reported to contain collagenase (2, 3).

In the present experiments we found that macrophages obtained from a peritoneal exudate did not contain or secrete significant amounts of collagenase *in vitro*. Since it is known that certain activities of macrophages, including respiration (4), phagocytosis (5, 6), and release of some lysosomal enzymes (7, 8), increase when macrophages are incubated with a variety of stimulants, we exposed macrophage-rich cell populations to endotoxin *in vitro* and then assayed the culture media for collagenase activity. Here we report that activation of peritoneal macrophages by endotoxin, particularly the lipid A component, induces these cells to produce an enzyme that lyses reconstituted collagen fibers.

MATERIALS AND METHODS

Male Hartley guinea pigs (500 g) were injected intraperitoneally with 20 ml of sterile Drakeol (6-VR, Pennsylvania Refining Co., Butler, Pa.) to induce a cellular exudate. Four days later, the peritoneum was lavaged with 150 ml of heparinized (2 U/ml) saline and the cells were concentrated by centrifugation at 250 g. The cells were resuspended and washed twice in Dulbecco–Vogt's medium supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and 2 mM glutamine. Ten milliliter of this serum-free medium containing 4×10^6 cells per ml were added to 75-cm² plastic flasks and incubated in an atmosphere of 5% CO_2 and air at 37°. After 4 or 24 hr the cell cultures were washed three times with Dulbecco's medium to remove nonadherent cells and 10 ml of fresh serum-free medium were added to each flask. The adherent cells obtained in this manner consisted of 94–96% macrophages, as determined by morphology and by the ability of the cells to phagocytize latex particles, and 4–6% lymphocytes. Such preparations will be referred to here as macrophage cultures.

Lipopolysaccharides (LPS) derived from *Escherichia coli* (055:B5 Difco Laboratories, Detroit, Mich.) or *Salmonella typhimurium* (Difco Laboratories, Detroit, Mich.) were added in the concentrations indicated in each experiment to macrophage cultures. Control macrophage cultures received no LPS. In additional experiments, the ability of a lipopolysaccharide from the smooth strain of *S. minnesota* or an *O*-polysaccharide-deficient glycolipid from the rough mutant of *S. minnesota*, R595, to induce collagenase was compared. LPS was further separated into lipid and polysaccharide fractions. The lipid fraction of LPS was isolated by heating 100 mg of *S. typhimurium* in 0.1 M hydrochloric acid at 100° for 45 min (9–11). This treatment produces an insoluble precipitate designated lipid A and a soluble polysaccharide fraction. Both of these preparations were dialyzed and lyophilized. Alkaline hydrolysis of *S. typhimurium* lipopolysaccharide was also used to isolate the polysaccharide portion (12, 13). Lipid A and R595 glycolipid were dispersed by sonication and added to macrophage cultures in the indicated concentrations.

Macrophage cultures with or without the various LPS fractions were given fresh media daily, and the harvested media were stored at −20° for up to 14 days. These samples were subsequently dialyzed against several changes of 30 μM Tris·HCl (pH 7.5) and lyophilized. These concentrates were assayed for collagenase activity by adding 200 μl of 50 mM Tris·HCl (pH 7.5) plus 5 mM $CaCl_2$ containing either 250 μg of media protein or the media products from two culture flasks to 300 μg of [^{14}C]glycine-labeled fibers reconstituted from the collagen extracted with acid from guinea pig dermis (14). The extent to which nonspecific proteases might degrade the collagen gel was estimated by adding trypsin (0.01%) to some of the gels. After incubation at 35° for 16 hr the insoluble collagen was removed by centrifugation and the amount of labeled material present in the supernatant fluid was measured by liquid scintillation counting.

The products from the cleavage of collagen by the macrophage preparation were separated from uncleaved components

Abbreviation: LPS, lipopolysaccharide.

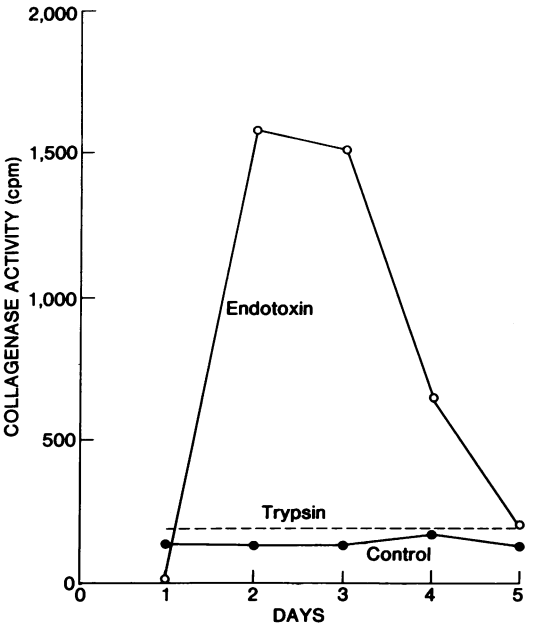

FIG. 1. Activation of peritoneal macrophages by endotoxin (LPS) to produce collagenase *in vitro*. The endotoxin- (*E. coli*) treated cultures received 30 μg/ml of LPS. Medium was removed daily from the cultures and assayed for collagenase activity with labeled collagen fibers (2200 cpm/substrate). Trypsin (0.01%) was added to certain of the collagen substrates to obtain a measure of the label that could be solubilized by nonspecific proteases. Higher concentrations of trypsin caused no further release.

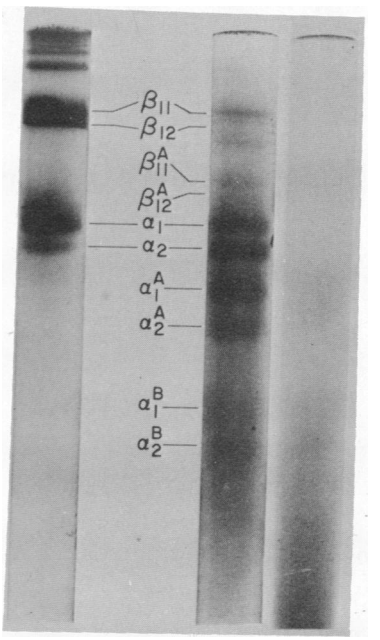

FIG. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the collagen–collagenase reaction mixture. The collagen control is on the left, the products from an enzyme–collagen reaction mixture incubated for 24 hr are shown in the center gel, and on the right is the enzyme alone. As judged by the migration of α_1 and α_2, β_{11} and β_{12}, the migration of the bands labeled α_1^A and α_2^A indicates they are 62–67% of their respective intact α-chain size. The proteins in the two faster moving bands were approximately 35% of the molecular weight of the α_1 and α_2 chains.

by electrophoresis on 5% sodium dodecyl sulfate polyacrylamide gels (15) after incubation of the reaction mixture in solution at 25° from 2 to 24 hr. Prior to electrophoresis, urea and sodium dodecyl sulfate were added to the reaction mixtures to give final concentrations of 2 M and 1%, respectively. These samples were than heated for 15 min at 100°.

RESULTS

No collagenase activity was detected in freshly isolated peritoneal macrophages after sonication or freeze–thawing. When these peritoneal exudate cells were cultured, 98% of the adherent cells were viable during 5 days of culture, as judged by the exclusion of trypan blue dye. Addition of LPS and the fractions derived from it at the indicated concentrations did not significantly alter cell survival in the treated cultures. Collagenase was not detectable in the media from control cultures in which cells were incubated without LPS, or when LPS was added to the harvested media, and was rarely detectable in the media of the first day of cultures treated with LPS. However, on the second day the LPS-activated macrophages secreted significant quantities of collagenase into the media (Fig. 1). The activated macrophages continued to produce similar quantities of the enzyme on the third day, but collagenase levels decreased on the fourth and fifth days of culture. The appearance of collagenase activity required protein synthesis, since it was blocked by cycloheximide. Addition of cycloheximide at any point after addition of endotoxin to the macrophage cultures inhibited the subsequent appearance of the enzyme (Table 1).

To establish the nature of the collagenase, we examined the products of the enzyme digestion. In these experiments, media from macrophage cultures on the second and third day of stimulation were incubated with collagen for 2–24 hr in solution. The products present in the reaction mixture were electrophoresed on 5% sodium dodecyl sulfate–polyacrylamide gels after denaturation. In addition to the α chains and β components of collagen, two faster moving bands, α_1^A and α_2^A, were observed in the samples of collagen incubated with the macrophage enzyme (Fig. 2). In several sodium dodecyl

TABLE 1. *Effect of cycloheximide on macrophage collagenase*

	Collagenase activity (cpm)* after macrophage culture for		
	24 hr	48 hr	72 hr
Endotoxin†	68	1001	908
Cycloheximide added at‡			
0 hr	19	11	7
24 hr	56	54	33
48 hr	22	1029	43

* Medium was removed daily and collagenase activity was determined in each day's media for 3 days after the addition of endotoxin with or without cycloheximide in the macrophage culture media. Collagenase activity is expressed as the radioactivity (cpm) solubilized from the labeled collagen fibers (1250 cpm/substrate) by the concentrated macrophage media.

† Macrophage cultures containing 40 × 10^6 cells received 30 μg/ml of endotoxin.

‡ Cycloheximide (1 μg/ml) was added to the macrophage cultures either simultaneously with the endotoxin or 24 or 48 hr after the addition of endotoxin.

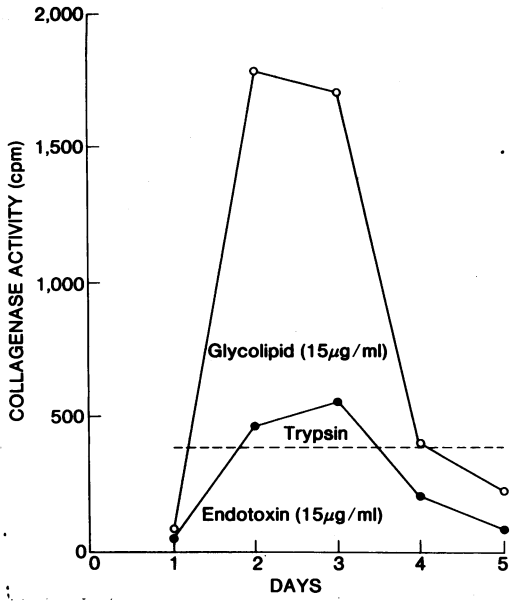

FIG. 3. Comparison of the ability of *S. minnesota* LPS (endotoxin) and the glycolipid produced by a mutant strain of *S. minnesota* (R595) to induce collagenase production by peritoneal macrophages (2200 cpm/substrate). Both were added in concentrations of 15 μg/ml.

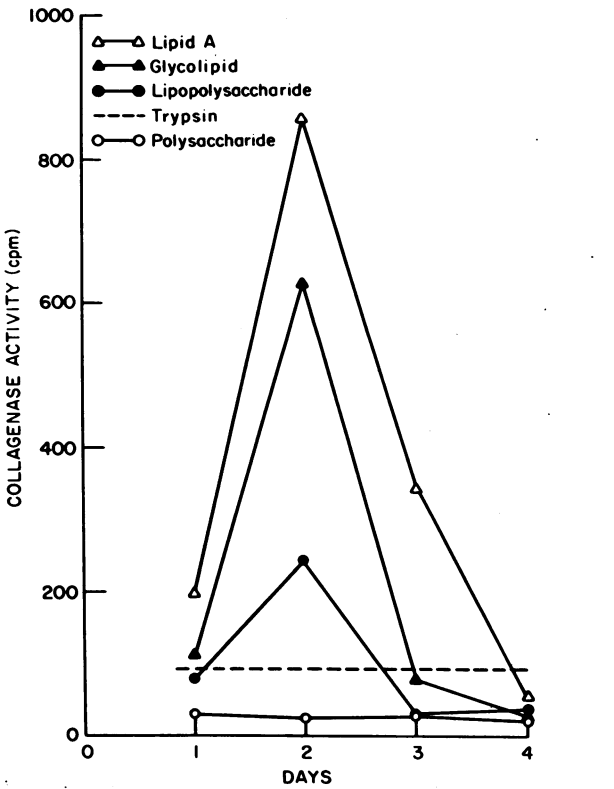

FIG. 4. Comparison of the effect of the various components of LPS on the production of collagenase by peritoneal macrophages. Five micrograms per ml of each component were added to the macrophage cultures and the media were assayed for enzyme activity (1250 cpm/substrate).

sulfate gel electrophoresis experiments these products were found to be 62–67% the size of α chains. Further studies utilizing the precipitation of the cleavage products into segment-long spacing aggregates indicated the predominant cleavage to be at a point 62% of the distance from the amino-terminal end of the collagen molecule (not shown). Some loss of β-components was observed when the reactions mixtures were run on dodecyl sulfate gels, suggesting the presence of nonspecific proteases in the macrophage preparation with the ability to cleave bonds in the nonhelical ends of the molecule.

Enzyme activity as judged by the fibril assay was inhibited by the addition of serum, or 10 mM ethylenediaminetetraacetate (EDTA), or 0.1 M cysteine to the reaction mixture.

We took two approaches to investigate which portion of the LPS molecule was active as the inducer. First, intact LPS from *S. minnesota* or a glycolipid, lacking *O*-polysaccharide, obtained from a mutant strain (R595) of *S. minnesota* was added to macrophage cultures. The glycolipid induced higher levels of enzyme activity than equal amounts of the intact LPS (Fig. 3). In the second series of experiments, the LPS molecule was fractionated after acid or alkaline hydrolysis and the isolated components were compared with the intact LPS and the glycolipid moiety for their ability to stimulate collagenase production. The lipid A fraction was the most active of the components in stimulating collagenase production (Fig. 4). The glycolipid was less active than lipid A but in turn induced greater enzyme synthesis than the intact LPS (Fig. 4). The lipid-free polysaccharide fraction did not stimulate the production of collagenase at the indicated concentration or at higher dosages.

DISCUSSION

Since other proteolytic enzymes do not attack the helical portion of the collagen molecule under physiological conditions, collagenase is believed to play an essential role in the degradation of this protein. Collagenase activity has been reported in extracts of macrophages obtained from human and dog lungs (2) and in media of cultured rabbit alveolar macrophages (3). In contrast, we found that neither cell extracts nor media from cultured peritoneal exudate macrophages contained measurable amounts of collagenase. The difference between the two macrophage populations may reflect the higher state of activation of alveolar macrophages (16) as a result of their continual contact with airborne stimulants. This metabolic difference suggested to us that peritoneal macrophages might produce a collagenolytic enzyme if adequately stimulated. Metabolic and synthetic activities of macrophages are stimulated after exposure to LPS, as evidence by increased respiration (4), elevated levels of hydrolyase enzymes (8), and enhanced phagocytic activity (17, 18). By exposing peritoneal macrophages to LPS we found that these cells can be stimulated *in vitro* to produce an enzyme capable of cleaving the collagen molecule.

The macrophage collagenase resembles other collagenases in some respects. It is inhibited by serum, ethylenediaminetetraacetate, and cysteine. Collagenases from several sources (19–21) cleave the collagen molecule into 75% and 25% fragments, while pieces 62–67% of the length of the collagen molecule were observed with the macrophage enzyme and have also been reported for purified collagenase from the uterus (22) and skin (23) of rats.

The data suggest that the collagenase was newly synthesized after activation of the macrophage cultures. First, a latent period was required prior to the appearance of the enzyme, but once synthesis was initiated it continued for more than 48 hr. Second, cycloheximide, an inhibitor of pro-

tein synthesis, prevented the appearance of collagenolytic activity. It is not clear whether the stimulated macrophages synthesize collagenase *de novo* or as a proenzyme. Recent studies indicate that tadpole tail collagenase is stored as a zymogen and converted to active enzyme after release (24). It should be possible to distinguish between *de novo* synthesis of collagenase and activation of a stored precursor by means of already established immunological procedures in the macrophage system.

The lipid moiety is the portion of the LPS that induces collagenase. Other studies have shown that the lipid component can duplicate the pyrogenicity, toxicity, complement consumption (25), and mitogenicity (26, 27) induced by the parent LPS. The mechanism by which the LPS activates the macrophage to produce collagenase is not clear. It is possible that the lipid moiety binds directly to the cell membrane, initiating macrophage activation. Alternatively, pinocytosis of the LPS (28) may stimulate the metabolic processes of the macrophage (4). Further, it is now well established that LPS is a potent mitogen for bone marrow-derived (B) lymphocytes (29, 30). LPS may not directly activate the macrophage, but may act as a mitogen for lymphocytes in the preparation which in turn may release a mediator(s) capable of altering macrophage functions. No collagenase production, however, has been observed in cultures of lymphocytes where macrophages were removed by adsorption (unpublished observation).

In aseptic inflammatory reactions, macrophages may be activated by other stimuli produced at the local site, such as products released from damaged tissue, immune complexes, and/or activating factors produced by other cells. In this regard, we have demonstrated that immune lymphocytes treated *in vitro* with specific antigen or with concanavalin A, a T cell mitogen, release a factor that can stimulate macrophages to produce collagenase (31). Thus, in the later phases of an immunologically induced inflammatory reaction, when lymphocytes and macrophages predominate, it may be the stimulated lymphocyte that in turn induces the macrophage to produce collagenase.

The *S. minnesota* LPS and the *S. minnesota* R595 glycolipid were kindly supplied by Dr. A. Nowotny. The authors thank Mr. Willard Lee for maintaining the cell cultures.

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