Bacterial Lipopolysaccharides Induce In Vitro Degradation of Cartilage Matrix through Chondrocyte Activation

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ABSTRACT The present studies demonstrate that bacterial lipopolysaccharides (LPS) induce cartilage matrix degradation in live explants in organ culture. Quintuplicate bovine nasal fibrocartilage explants cultured for 8 d with three different purified LPS preparations derived from *Escherichia coli* and *Salmonella typhosa* at concentrations ranging from 1.0 to 25.0 μg/ml resulted in matrix proteoglycan depletion of 33.3±5.8 to 92.5±2.0% (medium control depletion 17.7±0.7 to 32.4±1.4%). Matrix degradation depended on the presence of live chondrocytes because frozen-thawed explants incubated with LPS failed to show any proteoglycan release. Moreover, the addition of Polymyxin B (25 μg/ml) to live explants incubated with LPS abolished matrix release, whereas Polymyxin B had no effect on the matrix-degrading activity provided by blood mononuclear cell factors. A highly purified Lipid A preparation induced matrix degradation at a concentration of 0.01 μg/ml. Cartilage matrix collagen and proteoglycan depletion also occurred with porcine articular cartilage explants (collagen release: 18.3±3.5%, medium control: 2.1±0.5%; proteoglycan release: 79.0±5.9%, medium control: 28.8±4.8%). Histochemical analysis of the cultured explants confirmed the results described above. Gel chromatography of the proteoglycans released in culture indicated that LPS induced significant degradation of the high molecular weight chondroitin sulfate-containing aggregates.

These findings suggest that bacterial products may induce cartilage damage by direct stimulation of chondrocytes. This pathogenic mechanism may play a role in joint damage in septic arthritis and in arthropathies resulting from the presence of bacterial products derived from the gastrointestinal tract.

INTRODUCTION

Recent evidence suggests that in chronic inflammatory arthritis, articular cartilage damage may result from matrix degradation mediated by "activated" chondrocytes (for review see reference 1). A variety of factors secreted in vitro by traumatized connective tissue (2), fibroblasts (3), and mononuclear phagocytes (4, 5) has been shown to induce chondrocyte-dependent matrix degradation in living cartilage explants in organ culture. The present work demonstrates that purified bacterial lipopolysaccharide (LPS)[1] preparations can also mediate matrix degradation in cultured cartilage explants. The resorptive effects were shown to depend on the presence of live chondrocytes. They were not due to contaminating proteolytic enzyme activity in the bacterial products. These findings suggest that bacterial products may induce cartilage damage by direct stimulation of protease and collagenase secretion by chondrocytes. This pathogenic mechanism may play a role in joint damage in septic arthritis and in arthropathies resulting from the presence of bacterial products derived from the gastrointestinal tract.

METHODS

Materials. Bacterial lipopolysaccharides from *Salmonella typhosa* (trichloroacetic acid-extracted), *Escherichia coli* 0111:B4 (trichloroacetic acid-extracted), and 055:B5 (phenol-extracted) were obtained from Difco Laboratories Inc. (Detroit, MI). A purified *S. minnesota* R5 Lipid A preparation purified by method of Galanos et al. (6), containing <1%

Received for publication 22 February 1983 and in revised form 10 August 1983.

[1] *Abbreviations used in this paper:* LPS, lipopolysaccharide; MNC, mononuclear cell; PHA, phytohemagglutinin.

nucleic acid and <0.3% protein, was a gift from Dr. Robert Munford, University of Texas Health Science Center (7). This preparation was mitogenic for murine lymphocytes at a concentration of 0.01 μg/ml. Polymyxin B sulfate was obtained from Sigma Chemical Co. (St. Louis, MO).

Cartilage explant cultures. Cartilage breakdown assays involved the use of quintuplicate bovine nasal fibrocartilage disks and porcine articular cartilage explant cultures as described previously (5). Briefly, fibrocartilage disks measuring 4 mm diam × 1–2 mm thick were obtained from the nasal septum of 1-yr-old cows. Quintuplicate disks were cultured for 8 d at 37°C in 35-mm plastic petri dishes (Falcon Labware, Oxnard, CA) with 1 ml Dulbecco's modification of minimal essential medium (Grand Island Biological Co., Grand Island, NY) – 5% fetal calf serum containing the different LPS preparations or human mononuclear cells (MNC) supernatant (5) at 10% concentration as positive control. The culture media from each disk were changed once on day 4. Each experiment included base-line controls containing medium alone. To determine the role of chondrocyte activation, some experiments included explants killed before culture by freezing and thawing three times.

Articular cartilage explants were obtained from the metacarpophalangeal joints of 5–9 mo-old pigs. Paired explants measuring 2 mm wide × 5–8 mm long were cultured for 12–16 d over stainless steel grids in 35-mm plastic petri dishes containing 1.5 ml Dulbecco's modification of minimal essential medium – 10% fetal calf serum, 15 mg/dl ascorbic acid, and the LPS preparations or human MNC supernatants at 20% concentration. The culture media from each dish were changed three times per week and stored at −20°C until assayed for release of proteoglycan and hydroxyproline.

Histochemical assessment of proteoglycan and collagen depletion from the explants was carried out by comparing thickness, toluidine blue, and Van Gieson stains of histologic sections from explants divided in half before culture with control and LPS-containing media (2, 5).

MNC supernatants were obtained from cultures of 2×10^6 blood MNC/ml stimulated with 10 μl/ml phytohemagglutinin (PHA) (Wellcome Diagnostics, Dartford, England) for 48 h at 37°C (5).

Assays for cartilage matrix release. Determinations of matrix proteoglycan release into the culture supernatants were carried out on days 4 and 8 for bovine fibrocartilage cultures and on days 4, 9, 14, and 16 for the porcine articular explant cultures by a colorimetric method for chondroitin sulphate using metachromatic dye 1,9-dimethyl-methylene blue (Serva Feinbiochemica Gmbh & Co, Heidelberg, West Germany) in sodium formate buffer, pH 3.5 (8). Collagen breakdown of articular cartilage explants was measured by analysis of hydroxyproline in the culture media and explants previously hydrolyzed in 6 N HCl at 105°C for 20 h (9).

Characterization of proteoglycans released. Investigation of the molecular size profile of the proteoglycans released in culture was carried out by molecular-sieve chromatography, using Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ) columns measuring 1 × 20 cm equilibrated with phosphate buffered saline, pH 7.2 – 0.02% sodium azide. Prior to being applied to the column, the culture media from the bovine cartilage explant cultures were pooled, on days 4 and 8, freeze-dried, and reconstituted with one-tenth volume of distilled water. For comparison, intact proteoglycans were extracted from fresh cartilage disks with 4 M guanidine-HCl, pH 6.0, containing 0.1 M 6-aminohexanoic acid, 0.01 M EDTA, 0.001 M benzamidine HCl, 0.001 M phenylmethylsulfonyl fluoride, and 0.01 M *N*-ethylmaleimide as protease inhibitors (10). The column fractions were assayed for chondroitin sulfate content by the colorimetric method described above.

RESULTS

Proteoglycan release from bovine cartilage disks took place with the three different LPS preparations used in these experiments at a concentration of 10 μg/ml (Fig. 1). Matrix degradation was dependent on the presence of viable chondrocytes in the explants since proteoglycan release was completely abolished in frozen-thawed explants incubated with the LPS preparations. Frozen and thawed disks cultured in the absence of LPS showed identical release of proteoglycans (30.3±3.2% vs. 30.4±1.3% with LPS). Proteoglycan release was maximal or near maximal with LPS concentrations ranging from 25 to 10 μg/ml (25 μg/ml: 73.8±8.7%, 10 μg/ml: 80.1±5.3%). Significant release above background was consistently observed with concentrations as low as 1 μg/ml (33.3±5.8; control: 17.7±0.7%) (Fig. 2). Further evidence that LPS was directly responsible for chondrocyte-mediated matrix breakdown was obtained by the use of Polymyxin B, a peptide antibiotic capable of binding and neutralizing LPS (11, 12). As shown in Fig. 2, the addition of 25 μg/ml Polymyxin B to the culture media containing LPS resulted in almost complete inhibition of proteoglycan release from the cartilage explants. In contrast, Polymyxin B did not significantly affect matrix breakdown mediated by PHA-activated mononuclear cell supernatants.

Proteoglycan release was also shown to be mediated by low concentrations of a highly purified Lipid A preparation. In the experiment depicted in Fig. 3, Lipid A at a concentration of 1 μg/ml yielded maximal proteoglycan release. Significant matrix degradation (47.7±7.6%) above background levels (33.2±2.7%) was observed with as little as 0.01 μg/ml.

The experiments described above were performed

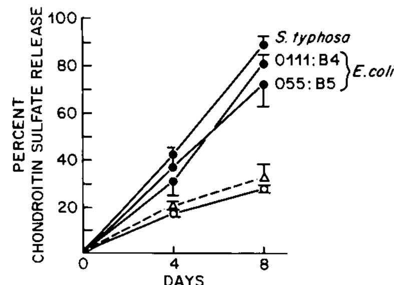

FIGURE 1 Effect of LPS on cartilage matrix proteoglycan release. ●, live cartilage explants cultured with three LPS preparations at a concentration of 10 μg/ml. Δ, dead cartilage explants cultured with *E. coli* 0111:B4 LPS, 10 μg/ml. O, medium control.

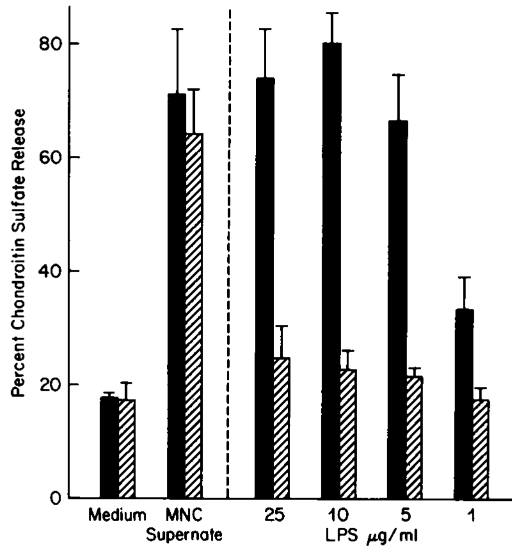

FIGURE 2 Effect of Polymyxin B on *E. coli* 0111:B4 LPS-mediated matrix proteoglycan cumulative release on day 8 of culture. Black columns, no Polymyxin B. Hatched columns, Polymyxin B, 25 μg/ml.

with fibrocartilage explants, a vascularized tissue containing fibroblasts, and endothelial cells as well as chondrocytes. To ascertain that the effect of LPS on cartilage matrix was mediated solely by chondrocytes, experiments using porcine articular cartilage explants were performed. In the experiment depicted in Fig. 4, triplicate articular cartilage explants were cultured for 16 d in the presence of *E. coli* 0111:B5 LPS, 10 μg/ml. Frozen-thawed explants cultured with LPS were included as controls. Both proteoglycan and collagen breakdown were measured in the supernates at days 4, 9, 12, and 16, and the remaining matrix macromolecules in the explants were quantitated at the end of the culture period. LPS-mediated proteoglycan release paralleled that induced by PHA-activated MNC supernatants used at 20% concentration. Cumulative proteoglycan release of 79.0±5.9% was measured on day 16 of culture with a control of 28.8±4.8%. The same supernatants were assayed for hydroxyproline as an index of collagen breakdown. Little hydroxyproline release was observed before day 8 of culture. Subsequently, the explants cultured with LPS showed evidence of significant collagen breakdown that was calculated to amount to >18% of the total collagen present in the explant. The MNC supernates induced collagen release of greater magnitude but with the same lag phase and time-course demonstrated for LPS. Frozen-thawed cartilage explants cultured with LPS for 12 d showed no evidence of matrix proteoglycan or collagen degradation.

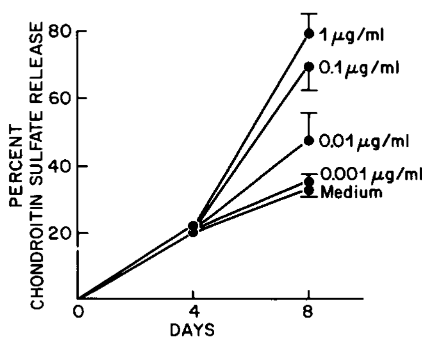

FIGURE 3 Effect of Lipid A on cartilage matrix degradation.

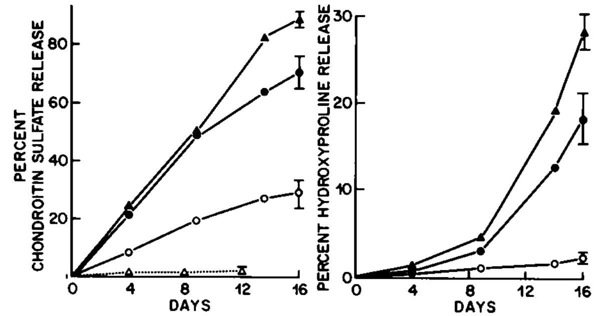

FIGURE 4 Articular cartilage matrix degradation by LPS. (*Left*) Proteoglycan release. (*Right*) Collagen release. ▲, mononuclear cell supernate; ●, LPS, 10 μg/ml; ○, culture medium alone; △, dead cartilage explants cultured with LPS.

Histochemical stains of the cultured paired explant halves confirmed the results obtained by biochemical analysis. Fig. 5 shows sections of explants cultured for 16 d with culture medium alone (left) and with LPS (right) stained for acidic proteoglycans with toluidine blue. There was almost complete disappearance of metachromatic material in the explants cultured with LPS. In addition, the experimental explants show a significant decrease in thickness commensurable with the loss of most of the proteoglycan and 20% of the collagen matrix.

Characterization of the proteoglycans released in culture in the presence or absence of LPS was carried out by gel chromatography on Sepharose CL-2B columns. Fig. 6 shows a comparison of the molecular size profiles of intact proteoglycans extracted from fresh bovine cartilage and proteoglycans released in culture in the presence or absence of LPS. It can be seen that the intact proteoglycans and the proteoglycans released in the presence of culture medium alone contain a sizeable proportion of high molecular weight chondroitin sulfate-containing aggregates. In contrast, the profile from explants incubated with 10 μg LPS shows a decrease of high molecular weight species and a relative increase in lower molecular weight chondroitin

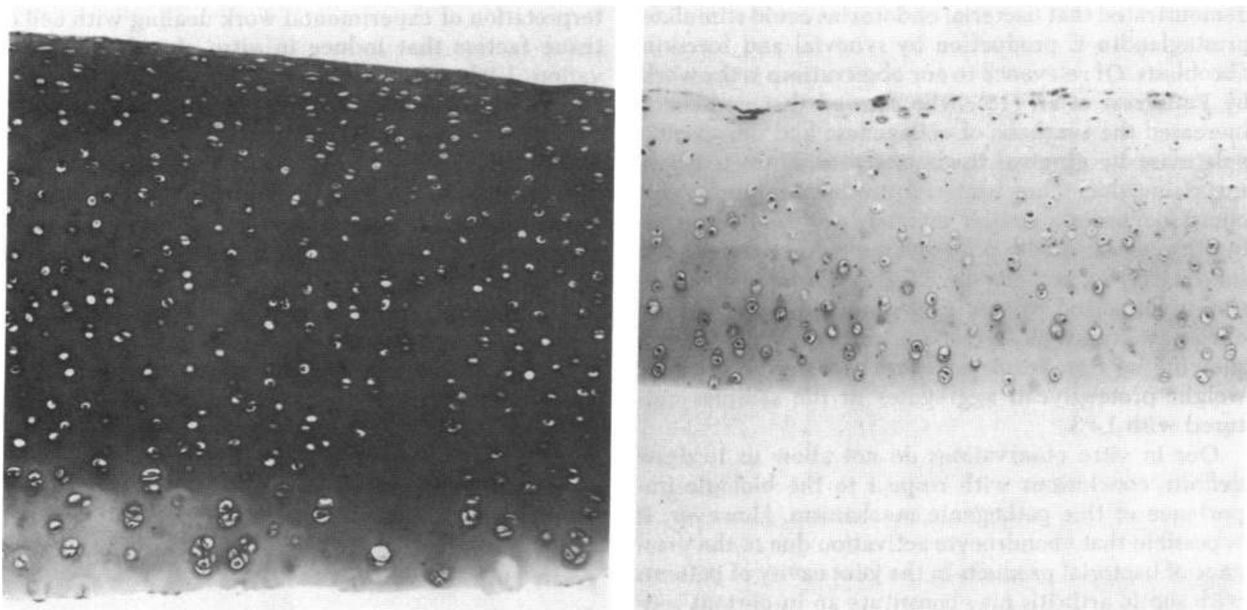

FIGURE 5 Matrix proteoglycan depletion of paired cartilage explant halves cultured for 16 d, assessed by toluidine blue stain. (*Left*) Cartilage explant incubated with culture medium alone. (*Right*) Cartilage explant cultured with LPS, 10 μg/ml. Notice the loss of metachromasia and the decrease in explant thickness.

sulfate-associated molecules. Chromatographic analysis of media obtained from explants cultured with MNC supernatants showed similar profiles (results not shown).

DISCUSSION

Pathologic articular cartilage resorption may be due to a decrease in matrix macromolecular synthesis by the chondrocytes, to an increase in the rate of degradation of proteoglycan and collagen, or to a combination of both processes. It is generally believed that the degradative mechanisms play a major role in cartilage damage in noninflammatory arthritides (13, 14). In chronic inflammatory arthritis, matrix degradation may be mediated by proteolytic enzymes in the synovial fluid, by the invasive pannus, or by the chondrocytes. There is a considerable body of evidence indicating that chondrocyte-mediated cartilage degradation may be of major importance in inflammatory arthritides (1). Chondrocyte activation resulting in in vitro cartilage matrix degradation may be induced by soluble factors secreted by connective tissue cells (2, 3) and mononuclear phagocytes (4, 5). The present work demonstrates that bacterial endotoxins also mediate matrix degradation in cartilage explants in organ culture. This effect was dependent on the presence of live chondrocytes because frozen-thawed explants cultured with LPS failed to show any evidence of proteoglycan or collagen release.

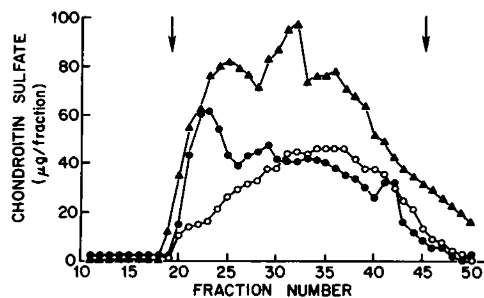

FIGURE 6 Molecular size profiles of proteoglycans released in culture in the presence or absence of LPS. Culture media from days 4 and 8 were pooled, concentrated 10-fold, and chromatographed on Sepharose CL-2B columns. ●, control; O, LPS, 10 μg/ml; ▲, intact proteoglycan. Each fraction was assayed for chondroitin sulfate content. The arrows indicate from left to right: column front (10–20 × 10^6 mol wt); IgG (1.5 × 10^5 mol wt).

Bacterial endotoxins are known to induce profound biologic effects on mononuclear phagocytes and lyphocytes (15). Fibroblasts also demonstrate metabolic changes when cultured in the presence of LPS. Buckingham et al. (16) showed that LPS stimulated synovial fibroblast hyaluronic acid production, glucose utilization, and lactate output. Recently, Yaron et al. (17)

demonstrated that bacterial endotoxins could stimulate prostaglandin E production by synovial and foreskin fibroblasts. Of relevance to our observations is the work by Pettigrew et al. (18), who showed that endotoxin increased the synthesis of collagenase and nonspecific gelatinase by gingival tissue explants. Thus, it is not surprising that these bacterial products interact with chondrocytes in a similar manner, probably stimulating the release of the proteolytic enzymes responsible for matrix macromolecule degradation. This conclusion is supported by the results obtained with the gel chromatography analysis of culture supernatants showing a significant decrease in high molecular weight proteoglycan aggregates in the samples cultured with LPS.

Our in vitro observations do not allow us to draw definite conclusions with respect to the biologic importance of this pathogenic mechanism. However, it is possible that chondrocyte activation due to the presence of bacterial products in the joint cavity of patients with septic arthritis may constitute an important contributing factor in cartilage damage. Smith et al. (19) have shown that co-cultures of live *E. coli* and *Staphylococcus aureus* with cartilage-induced proteoglycan degradation and chondrocyte death within 48 h. In these studies, matrix degradation was inhibited by small amounts of serum in the culture medium, suggesting that proteases produced by the bacteria may have been responsible for proteoglycan digestion. It is also possible that proteolytic enzymes released by the chondrocytes before their demise may have contributed to matrix degradation. These workers did not attempt to isolate the active bacterial products. In our hands, LPS was the only bacterial product tested that could activate chondrocytes. As previously shown for other biologic effects of LPS (15, 20), the ability to activate chondrocytes resided in the lipid portion of the molecule as established by the experiments using a highly purified Lipid A fraction of *S. minnesota* LPS. Incubation of cartilage explants with other bacterial products such as muramyl dipeptide (21) and streptolysin S (22) failed to induce matrix degradation (unpublished results).

The capacity of LPS to induce cartilage degradation suggests that this mechanism may also play a role in arthropathies where circulating bacterial products derived from the gastrointestinal tract localize to the joint cavity. In the arthritis associated with intestinal bypass syndrome (23), bacterial products originating in the gut have been identified in the synovial fluid obtained from affected joints (24). It is also possible that LPS-mediated chondrocyte activation may play a causative role in the group of reactive arthritides associated with infections (25) or those associated with idiopathic inflammatory bowel disease (26–28).

Our findings introduce a note of caution in the interpretation of experimental work dealing with cell or tissue factors that induce in vitro chondrocyte activation. LPS is commonly used to stimulate mononuclear phagocytes to secrete such factors (4). As a matter of fact, our early attempts to stimulate human monocytes with LPS led to the observations described here. Moreover, this ubiquitous bacterial product is frequently found in animal sera used to supplement the tissue culture media used in this type of experiment. The work described here suggests that the addition of Polymyxin B to active supernates may be necessary to distinguish between the chondrocyte-activating effects of cellular factors and those of LPS.

ACKNOWLEDGMENTS

I wish to thank Dr. Robert Munford for a generous gift of Lipid A, Mr. Ellis Lightfoot for expert technical assistance, and Ms. Debbie McInnis for secretarial help.

This work was supported by U. S. Public Health Service grants AM 16209 and AM 09989.

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