# The Effect of Bacterial Products on Synovial Fibroblast Function: Hypermetabolic Changes Induced by Endotoxin

ROBERT B. BUCKINGHAM and C. WILLIAM CASTOR with the technical assistance of PAUL F. HOAG

*From the Rackham Arthritis Research Unit, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48104*

ABSTRACT The effects of bacterial products on selected synovial fibroblast functions were studied. Extracts of commonly encountered microorganisms were prepared by sonic or mechanical disruption. "Purified" endotoxins were prepared from selected organisms, and in some cases were purchased commercially. Normal fibroblasts were derived from synovial connective tissue obtained from amputations or arthrotomy. The cells were grown as a monolayer on glass and were nourished by a semisynthetic nutrient medium.

Extracts of Gram-negative bacteria, applied to fibroblast cultures, markedly increased hyaluronic acid production, glucose utilization, and lactate output. Treatment of the extracts with heat at 100°C for ½ hr decreased their effectiveness by approximately 40%. Purified Gram-negative bacterial endotoxin stimulated synovial fibroblasts to an extent comparable to that caused by heat-treated whole extracts. The lipid moiety of the endotoxin molecule appeared to account for much of the stimulatory activity of the endotoxin. Extracts of commonly encountered Gram-positive cocci, yeast, and Mycoplasma had no stimulating capabilities. Corynebacterial extracts, however, had definite stimulating potential. Endotoxin-synovial cell interaction experiments demonstrated that endotoxin was bound to fibroblasts. Reassay of the endotoxin after extraction from the cells showed that it retained its stimulatory potential.

The metabolic phenomena stimulated by bacterial products duplicate the major known actions of connective tissue-activating peptide (CTAP). The observations made in this study suggest that bacterial products may participate in a fundamental way in the activation process, and indicate a possible role for bacterial products in synovial inflammation in humans.

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## INTRODUCTION

Connective tissue cells derived from diseased or normal synovial membrane can be propagated in a monolayer culture through 15 or more subcultures (1). The cells generated from rheumatoid synovium differ in several ways from "normal" cells, and continue to exhibit these differences during their life in cell culture (2, 3). This laboratory has demonstrated that rheumatoid cells are hypermetabolic, exhibit an unusually slow growth rate, and are relatively resistant to the suppressive effects of hydrocortisone on hyaluronic acid and collagen synthesis (2, 3).

The studies of others have suggested that rheumatoid cells exhibit decreased synthesis or release of a component immunologically like cartilage protein-polysaccharide (4). A recent study has cast doubt on the earlier finding that rheumatoid synovial fibroblasts, unlike normal fibroblasts, are resistant to infection with rubella virus (5). The "abnormalities" which characterize rheumatoid cells may provide insight into alterations occurring within chronically inflamed rheumatoid joints.

Identification of these differences stimulated a search for their pathogenetic basis. Recent work from this laboratory demonstrated a polypeptide in lymphocytes and other mammalian cells capable of inducing hypermetabolic behavior in normal cells and augmenting the existing hypermetabolism in rheumatoid cells (6–9). As a result of these studies, it has been suggested that the connective tissue-activating peptide (CTAP)[1] is a medi-

[1] *Abbreviations used in this paper:* CPC, cetylpyridinium chloride; CTAP, connective tissue-activating peptide; KDO, 2-keto-3-deoxyoctonate.

ator in the inflammatory reaction, acting to regulate the transition from the exudative to the reparative phase (8). More recently this laboratory demonstrated that rheumatoid synovial fibroblast strains have an activator polypeptide content approximately 4 times that of normal synovial cell strains, adding yet another abnormality to the list of altered characteristics of rheumatoid cells (9). It was postulated that excess endogenous activator peptide may explain many of the known propagable abnormalities of these cells (9).

No one, however, has discovered the inciting stimulus for these cellular alterations. Work attempting to demonstrate an infectious agent has failed to recover, in a reproducible way, infectious material from rheumatoid tissues. Recently, the focus of some investigators in this area has shifted toward the study of less typical microorganisms, such as slow viruses, as well as unusual bacteria, including diphtheroid organisms (10, 11). In addition, attention has turned toward a study of situations in which initially infectious materials within tissue may be inflammatory but not recoverable in a form which can be propagated.

This paper provides data which show that extracts of common as well as unusual Gram-negative bacteria stimulate the energy metabolism of synovial cells in monolayer culture and cause them to produce large amounts of mucopolysaccharide, thus duplicating the major known actions of CTAP. Specifically, we show that the cell wall lipopolysaccharide, or "bacterial endotoxin," is responsible for much of the activity of the bacterial extracts.

## METHODS

"Fibroblast cultures" were derived from synovial connective tissue obtained at amputations or arthrotomies. Explants from these sources were immobilized under cellophane, and after culture growth, the fibroblasts were dispersed with trypsin and transferred to larger flasks. Growing cells were nourished by semisynthetic medium containing 80% synthetic medium 1066, 10% fetal calf serum, and 10% pooled heat-inactivated human serum, supplemented with L-glutamine. Penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) were included in the culture medium. Sodium carbonate was used to adjust the pH to 7.4.

*Assay technique and biochemical measurements.* Assay of test materials was carried out by the procedure used to measure CTAP (8). $1 \times 10^6$ synovial fibroblasts were planted in replicate assay flasks with 2.0 ml serum-containing medium and allowed to adhere to the glass. Approximately 4 hr later, this medium was replaced with 2.0 ml of a nonserum-containing medium consisting of Eagle's synthetic medium supplemented with L-glutamine and containing HEPES[2] buffer, penicillin, and streptomycin sulfate. Experimental samples, including whole bacterial extracts, purified endotoxins, or modifications thereof, were then introduced into these flasks and allowed to interact with the cells for a 2 or 3 day period. Hyaluronic acid, released into the culture medium, was measured and expressed as micrograms mucopolysaccharide per milligram cell protein per 24 hr. Glucose uptake from media and lactate formation were also measured in selected cases. Protein was measured by the method of Oyama and Eagle (12). Mucopolysaccaride in the media was measured using a modified carbazole technique for uronic acids after isolation of the polymer (13). The Barker-Summerson procedure was used for lactate measurement and the glucostat method for glucose (14, 15). Cells used in assay were evaluated microscopically throughout the assay period.

*Propagation of bacteria and preparation of extracts.* Assorted known Gram-negative and Gram-positive bacterial strains were kept on nutrient agar at 4°C and periodically subcultured and reincubated overnight. For the preparation of bacterial extracts, a strain was inoculated in an appropriate nutrient medium, then harvested in late log phase and quantitated spectrophotometrically. Bacteria were then washed twice in distilled water. Extracts of the whole bacteria were prepared by sonication in buffer solution or water in a Raytheon sonicator (Raytheon Co., Lexington, Mass.) at full power for 5–20 min, or by mechanical disruption in a French press. Sonicates from the Gram-negative and Gram-positive bacteria were prepared from an initial concentration of 0.05 g wet organisms per ml of buffer solution. Endotoxin was prepared from the Gram-negative bacteria by methods described by Roberts (16), Boivin, Mesrobeanu, and Mesrobeanu (17), and Westphal and Lüderitz (18). In brief, the method described by Roberts involves extraction of whole bacterial cells by constant stirring in an 80°C water suspension for 30 min. The 6000 *g* supernate is then dialyzed and lyophilized. "Boivin antigen" is prepared by extraction with cold 5% trichloroacetic acid during homogenization in a high-speed homogenizer. Cell debris is centrifuged at 6000 *g* and the supernate is dialyzed, filtered, and lyophilized. The Westphal and Lüderitz method using phenol for extraction of endotoxin was applied by us as follows: bacteria were suspended in distilled water, and an equal portion of phenol: water (9:1, v:v) was added. This mixture was heated to 70°C in a water bath with constant stirring for 10 min or more. A 1500 *g* supernate was taken, dialyzed for 72 hr against distilled water, filtered, and concentrated. Methanol was added to precipitate the lipopolysaccharide. This precipitate was washed with cold methanol. The sediment was then dissolved in distilled water and the methanol removed by evaporation *in vacuuo*. The lipopolysaccharide could be lyophilized.

*Salmonella typhosa* 0901 endotoxin and *Escherichia coli* 0111: $B_4$ endotoxins were purchased from Difco Laboratories, Detroit, Mich. Endotoxins prepared from mutant *E. coli* strains J-5 and RC-59 were a gift of Dr. Edward C. Heath, Chairman, Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pa.

*"Lipid A" preparation.* "Lipid A" preparations were prepared by mild acid hydrolysis. Parent endotoxin was placed in 0.1 N hydrochloric acid and heated in a boiling water bath until a precipitate appeared (19). The precipitate, consisting of flocculated lipids, was sedimented by centrifugation, washed with distilled water, resedimented, and then suspended in distilled water with the aid of sonication. Sterilization was accomplished by repeated pasteurization (treatment at 66°C for 20 min followed by rapid cooling to 4°C) or by autoclaving at 100°C for 10 min.

*Enzyme and base hydrolysis studies.* Proteolytic enzyme treatment of whole extracts and endotoxin was carried out using pronase (Calbiochem, Los Angeles, Calif.) and trypsin (Worthington Biochemical Corp., Freehold, N. J.). 150 μg of either enzyme was added to 1 ml of the whole bac-

[2] *N*-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

terial preparation or to 100 μg of "purified" endotoxin. Samples were incubated for 4 hr in phosphate buffer, 0.1 M, pH 7.0. Alkaline hydrolysis of endotoxin was conducted in 0.1 N NaOH at room temperature for varying periods of time.

*Mucopolysaccharide isolation and characterization studies.* The mucopolysaccharide formed by synovial fibroblasts in response to "activation" by a whole extract of *E. coli* was isolated and purified for subsequent characterization studies. 95% ethanol was added to pooled specimens of culture medium in a ratio of 4 parts ethanol to 1 part medium. The resulting precipitate was extracted with acetone, the dry residue dissolved in 0.05 M tris buffer, pH 7.8, and treated with pronase, 0.4 mg/ml, for 6 hr. A second pronase digestion was carried out using identical conditions, and then the sample was dialyzed against distilled water. 1½ ml of 5% cetylpyridinium chloride (CPC) in 0.2 M $Na_2SO_4$ was added to the dialyzed specimen to form a CPC-mucopolysaccharide complex. This CPC-mucopolysaccharide precipitate was washed with water and dissolved in absolute methanol; then 1.5 cc of 10% sodium acetate in methanol was added to form the sodium salt of the mucopolysaccharide. This precipitate was further purified in the following way: the sodium salt was dissolved in 0.2 M $Na_2SO_4$; CPC was then added to isolate the mucopolysaccharide which would precipitate at this ionic strength. The remaining solution was diluted 6-fold with distilled water in order to isolate the mucopolysaccharide precipitable with CPC in dilute solution. This precipitate was washed with distilled water, dissolved in methanol, and the sodium salt was reformed by adding sodium acetate in methanol.

Characterization studies on the isolated mucopolysaccharide fraction included uronic acid measurement by the method of Dische (20), and by an orcinol method (21), to allow calculation of a carbazole: orcinol ratio. A sample was hydrolyzed with 4.0 N HCl at 100°C in a sealed glass tube for 20 hr and the hexosamine content measured (22) to allow calculation of the molar ratio of hexosamine: uronic acid in the hydrolyzed mucopolysaccharide. The hexosamine in the hydrolysate was identified by paper chromatography using authentic D-glucosamine and D-galactosamine as standards. Viscometry was carried out with several samples, using Ostwald viscometers with known buffer flow times. The relative viscosity, $\eta_r$, of a specimen is expressed as the ratio of its original efflux time to its efflux time after addition of 1.0 mg of testicular hyalunronidase in 0.05 ml of phosphate-buffered saline, pH 7.0. The intrinsic viscosity, useful as an index of molecular weight, can be calculated from the relative viscosity, knowing the concentration of mucopolysaccharide in the specimen (23, 24).

## RESULTS

*Effect of whole bacterial extracts on synovial fibroblasts.* Addition of whole bacterial extracts to replicate fibroblast cultures caused marked increases in the mucopolysaccharide synthesis rate (Table I). Whole extracts of two different pathogenic *E. coli* produced 7.5- and 18.0-fold increases over the untreated controls. Likewise, preparations from other Gram-negative organisms stimulated the synovial fibroblasts to produce large increases in mucopolysaccharide synthesis. Although the results with *Pseudomonas aeruginosa* extracts were not striking in this particular experiment, in most cases extracts of this organism have been associated with significant activation of fibroblasts. Extracts of *Neisseria gonorrhoeae* and *Corynebacterium acnes* produced modest though significant increases. On the other hand, whole extracts of Gram-positive bacteria, yeast, and Mycoplasma provided little stimulus. The data emphasize that the most active

TABLE I

*"Activation" by Microorganismal Extracts*

| Synovial cell line | Preparation assayed* | Hyaluronic acid synthesis rate‡ | Ratio, treated/control |
|---|---|---|---|
| | Control | 8.0 | 1.0 |
| | Control | 8.5 | 1.0 |
| | *E. coli* (biliary tract infection) | 60.0 | 7.5 |
| | *E. coli* (urinary tract infection) | 142.0 | 18.0 |
| | *Proteus mirabilis* | 55.0 | 7.0 |
| F. C. Fibroblasts | *Pseudomonas aeruginosa* type 38 | 11.5 | 1.5 |
| Type: Normal | *Klebsiella pneumoniae* | 31.0 | 3.8 |
| | *Corynebacterium acnes* | 18.0 | 2.2 |
| 1,000,000 cells/assay flask | *Neisseria gonorrhoeae* | 14.0 | 1.7 |
| | *Streptococcus*, β-hemolytic | 11.5 | 1.5 |
| | *Staphylococcus aureus* | 9.5 | 1.1 |
| | *Diplococcus pneumoniae* | 11.0 | 1.3 |
| | *Monilia albicans* | 9.5 | 1.3 |
| | *Mycoplasma hyorhinis* | 8.5 | 1.03 |
| | *Mycoplasma hominis* II | 8.5 | 1.03 |

* Extracts prepared by sonication of whole organisms in a Raytheon sonicator (Raytheon Co., Lexington, Mass.). The 600 *g* supernate taken as the whole bacterial extract. Sonicates were prepared from an initial concentration of 0.05 g wet organisms per ml of phosphate-buffered saline.

‡ Micrograms hyaluronic acid per milligram cell protein per 24 hr.

TABLE II

*Effect of Purified Endotoxin on Synovial Fibroblast Function*

| Endotoxin | Fibroblast* strain | Endotoxin concentration | Endotoxin preparative method‡ | Hyaluronic acid synthesis rates§ (ratio, treated/control) |
|---|---|---|---|---|
| | | *μg/ml* | | |
| *S. typhosa* 0901 | H. H. | 40 | TCA | 7.0 |
| *S. typhosa* 0901 | H. H. | 45 | PHW | 7.2 |
| *E. coli* 0111: $B_4$ | H. H. | 90 | TCA | 2.0 |
| *E. coli* 0111: $B_4$ | H. H. | 100 | PHW | 8.4 |
| *S. marcescens* | F. C. | 13 | TCA | 5.4 |
| *S. marcescens* | H. H. | 9 | TCA | 2.8 |

* Strains all derived from normal synovial tissue, initials refer to donor.

‡ Initials under preparative method indicate the following: TCA, trichloroacetic acid, PHW, phenol-water. *S. typhosa* and *E. coli* endotoxins purchased from Difco Laboratories. *S. marcescens* endotoxin was a gift of A. G. Johnson, Ph.D., Department of Microbiology, of Michigan Medical School.

§ Micrograms hyaluronic acid per milligram cell protein per 24 hr.

extracts were those derived from Gram-negative bacteria. This suggested that the active component might be associated with the endotoxins which these organisms are known to contain.

*Effect of purified endotoxin on fibroblast function.* Table II illustrates the effect of "purified" endotoxins on mucopolysaccharide synthesis. Purified endotoxins, prepared by the trichloroacetic acid or phenol-water method, produced maximum increases in mucopolysaccharide synthesis rates varying from 2.0- to 8.4-fold. The data show that activation phenomena occur with purified endotoxins. Furthermore, as demonstrated by Table III, the synovial cells are sensitive to remarkably low levels of *S. typhosa* endotoxin, with $2 \times 10^{-3}$ μg/ml being adequate to stimulate one million cells. The responses of the fibroblasts to endotoxin frequently fail to describe dose-response curves, although such curves have occasionally been obtained. Instead, relatively high doses have little more stimulatory effect than moderate doses; and very low endotoxin concentrations will on many occasions approximate the effects of moderate doses. The reasons for these unusual dose-response characteristics are not clear at present.

*Effect of endotoxin on lactate and glucose metabolism.* We also studied the effect of whole bacterial extracts and purified endotoxins on glucose uptake and lactate output.

TABLE III

*Dose-Response Characteristics of Endotoxin-Induced "Activation"*

| | Amount | Hyaluronic acid* synthesis rate |
|---|---|---|
| | *μg/ml* | |
| Control | | 6 |
| Control | | 4 |
| Endotoxin preparation‡ | | |
| *E. coli* 0111: $B_4$ | 260 | 25 |
| | 90 | 12 |
| | 0.09 | 12 |
| *S. typhosa* 0901 | 40 | 36 |
| | 13 | 42 |
| | 0.0009 | 10 |

* Micrograms hyaluronic acid per milligram cell protein per 24 hr.

‡ Endotoxins purchased from Difco Laboratories and prepared by them using modifications of Boivin (TCA) procedure (17).

TABLE IV

*Effect of Bacterial Products on Lactate and Glucose Metabolism*

| | Hyaluronic acid synthesis rate* | Residual glucose‡ | Lactate‡ |
|---|---|---|---|
| | | *mg/100 ml* | *μmoles/ml* |
| Control | 18.0 | 83.0 | 0.8 |
| Control | 17.5 | 80.0 | 0.5 |
| Whole bacterial preparation *E. coli* (wild type)§ | 117.3 | 12.2 | 8.5 |
| Whole bacterial preparation *E. coli* (wild type)§ | 123.3 | 20.8 | 7.5 |
| Purified endotoxin, 45 μg/ml *S. typhosa* 0901‖ | 93.7 | 35.3 | 5.5 |
| Purified endotoxin, 45 μg/ml *S. marcescens*¶ | 97.0 | 44.0 | 4.9 |

* Micrograms hyaluronic acid per milligram cell protein per 24 hr.

‡ Glucose and lactate were measured in the media after the assay period (14, 15).

§ Organisms obtained from Clinical Bacteriology Laboratory, University of Michigan Hospital; sonicates were prepared from an initial concentration of 0.05 g wet organisms per ml of phosphate-buffered saline.

‖ Purchased from Difco Laboratories.

¶ A gift of A. G. Johnson.

Representative results are shown in Table IV. Compared to the controls, there was marked stimulation of glucose uptake as well as lactate output by synovial fibroblasts in culture. The whole bacterial extracts were more active than the purified endotoxins in all parameters measured.

*Effect of enzyme, heat, and base treatment on endotoxin bioactivity.* Whole bacterial extracts and purified endotoxin were treated with proteolytic enzymes further to evaluate the active components (Table V). Neither pronase nor trypsin had any measurable effect on the ability of the whole extract of a pathogenic *E. coli* to "activate" fibroblasts. Likewise, no inactivation resulted from treatment of a purified *E. coli* 0111: $B_4$ endotoxin with either enzyme. On the other hand, heating whole bacterial preparations at 100°C for 10 min reduced the bioactivity of whole preparations. Activity reductions of 40% occurred consistently. Subsequent proteolytic enzyme treatment of this heat-treated endotoxin did not further reduce activity.

Table V also demonstrates that the "purified" *E. coli* endotoxin is less active than whole bacterial preparations. This endotoxin, obtained from Difco Laboratories, Detroit, Mich., had been prepared by a phenol-water

TABLE V

*Heat Treatment and Proteolytic Enzyme Exposure*

| Preparation assayed | Hyaluronic acid synthesis rate* |
|---|---|
| Control flask | 10.0 |
| Control flask | 10.5 |
| Whole bacterial preparation‡ | 77.0 |
| Pronase-treated | 79.0 |
| Trypsin-treated | 67.0 |
| Whole bacterial preparation‡ (100°C, 10 min) | 42.0 |
| Pronase treated§ | 87.0 |
| Trypsin treated‖ | 73.0 |
| *E. coli* 0111: $B_4$ endotoxin¶ (100 μg/ml) | 20.0 |
| Pronase-treated§ | 36.0 |
| Trypsin-treated‖ | 29.0 |

* Micrograms hyaluronic acid per milligram cell protein per 24 hr.

‡ *E. coli*, wild type, recovered from human urinary tract infection, extract prepared by sonication. Sonicates prepared from an initial concentration of 0.05 g wet organisms per ml of buffer.

‖ Trypsin (Worthington Biochemical Corp., Freehold, N. J.), 100 μg enzyme per ml extract or endotoxin (100 μg/ml), incubated 4 hr at 37°C.

§ Pronase (Calbiochem, Los Angeles, Calif.) was used in a concentration of 150 μg/ml bacterial extract or endotoxin (100 μg/ml).

¶ *E. coli* 0111: $B_4$ endotoxin, purchased from Difco Laboratories. Preparative method: phenol-water extraction.

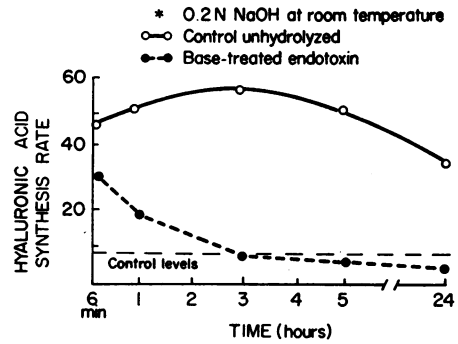


FIGURE 1 Alkaline hydrolysis of endotoxin. *S. typhosa* 0901 endotoxin (phenol-water preparation) was purchased from Difco Laboratories, Detroit, Mich.

purification procedure. In most cases, "purified" endotoxins, regardless of the preparative method used, stimulate mucopolysaccharide synthesis by synovial fibroblasts less than do whole bacterial extracts.

The active components of the whole bacterial extracts, therefore, were moderately heat-labile, but probably nonprotein. Endotoxin appears to be responsible for a major portion of this activity, but other materials may be important as well.

The effect of alkaline hydrolysis on endotoxin activity was examined. Fig. 1 demonstrates a time-dependent reduction in the activity of the hydrolyzed endotoxin. 30% of the activity had disappeared after 6 min and complete loss of activity occurred in 3 hr. Other investigators have studied the effect of alkaline hydrolysis on the activity of endotoxin in other assay systems. Pyrogenicity is not completely eliminated by such treatment. Lethality for mice, although not decreased in the first 3 hr, is virtually eliminated at 6–8 hr (25). Changes in mouse lethality associated with alkaline hydrolysis correlates with changes in molecular symmetry. Molecular dyssemmetry, postulated to include unfolding and swelling of the molecule, increases progressively between the 3rd and the 8th hr (25). Changes associated with alkaline hydrolysis which have been shown to occur in other assay systems are in the same direction as the time-dependent changes demonstrated here in a cell culture system.

*Preparation and assay of the lipid moiety of endotoxin: "Lipid A."* In 1933, Boivin et al. isolated a bacterial glycolipid (endotoxin) and investigated the effects of acid hydrolysis on the various activities of this preparation (17). Their studies and subsequent studies by others showed that mild acid hydrolysis will split the endotoxin molecule, releasing a lipid moiety which retains biological activity in many systems. After hydrolysis, the remaining endotoxin fractions consist of degraded polysaccharide as well as unknown amounts of degraded lipid. The lipid moiety, or "Lipid A," although

TABLE VI
*"Lipid A" Assay*

| Preparation assayed | Hyaluronic acid synthesis rate* |
|---|---|
| Control | 10.8 |
| Control | 11.0 |
| *S. typhosa* 0901 endotoxin, unhydrolyzed,‡ 45 μg/ml | 33.9 |
| Fraction A, "lipid moiety" extracted from 45 μg/ml *S. typhosa* 0901 endotoxin§ | 45.1 |
| Fraction B, "degraded polysaccharide" extracted from 45 μg/ml *S. typhosa* endotoxin‖ | 4.8 |

* Micrograms hyaluronic acid per milligram cell protein per 24 hr.

‡ Purchased from Difco Laboratories, prepared by a phenol-water method.

§ "Lipid A" prepared by placing parent endotoxin in 0.1 N HCl and heating at 100°C for 20 min, or until a precipitate appeared. The precipitate, consisting of flocculated lipids, was sedimented, washed, then suspended in distilled water with the aid of sonication.

‖ After precipitation of the lipid moiety, the supernatant fluid (degraded polysaccharide) was adjusted to pH 7.2 before assaying.

resembling the phospholipid portion of the endotoxin molecule as it occurs in the bacterial cell wall, is degraded to the extent that a significant amount of its total long-chain fatty acid content is removed (19). In addition, lipid preparations may not be entirely free from intact cell wall lipopolysaccharide, since the hydrolytic methods investigated leave carbohydrate attached to the endotoxin molecule (19).

Hydrolysis of *S. typhosa* 0901 endotoxin (Boivin-type) resulted in evidence suggesting that the "lipid moiety" has a stimulatory effect on synovial fibroblasts. As shown in Table VI, the unhydrolyzed endotoxin produced a 3-fold rise in mucopolysaccharide synthesis over control values. The lipid fraction was not diminished in biological activity, whereas the polysaccharide portion failed to show activity above the controls.

In view of our inability to evaluate the possibility that the activity of "Lipid A" was due to contaminating undegraded endotoxin, we studied, through the courtesy of Dr. Edward C. Heath of Pittsburgh, cell wall extracts of mutant *E. coli*. *E. coli* J-5, an epimerase-free mutant, lacks galactose and all sugars distal to galactose. Therefore, it consists of "Lipid A," 2-keto-3-deoxyoctonate (KDO), and a considerably shortened polysaccharide core (26). *E. coli* RC-59 contains only "Lipid A" and KDO as constituents of its cell wall glycolipid.[3] No polysaccharide core is present. Table VII shows the results of assay of the products of the mutant *E. coli*. Endotoxins from both the J-5 and RC-59 mutants retain the ability to activate synovial fibroblasts.

*Endotoxin-synovial fibroblast interaction.* Endotoxin-synovial fibroblast interaction experiments attempted to investigate endotoxin uptake by cells and to assess the effect of uptake on the endotoxin molecule. A normal synovial fibroblast cell line was treated with 30 μg/ml *E. coli* 0111 : $B_4$ endotoxin for 70 hr. The control for this experiment consisted of the same normal synovial cell line supported by nutrient medium for 70 hr without endotoxin. During the treatment period, both the control and endotoxin-treated cells were maintained in non-serum-containing medium. Both the control and treated cells were washed thoroughly with buffered saline, and cell extracts were prepared by a repeated freeze-thaw technique. Centrifuged extracts were then assayed for their ability to induce increases in mucopolysaccharide synthesis by fibroblasts, as in previous experiments. Table VIII shows that the saline extracts of the endotoxin-treated cells resulted in a 3.6-fold increase in mucopolysaccharide synthesis rate, whereas extracts of the controls produced a 2.6-fold rise. Treatment of the extracts with heat resulted in elimination of activity in the controls, but left residual activity in the extracts of the endotoxin-treated fibroblasts. Activity of the control cells was therefore presumed to be due entirely to heat-labile endogenous activator polypeptide (8), whereas the treated extracts must have contained another active principle.

A portion of the treated cell extracts was fractionated on Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and activity was demonstrated in the void volume eluate, suggesting a molecular weight of more than 150,000 for the active material.

Thus, extracts of the treated cells contained heat-stable activity with a molecular weight in the range of that

TABLE VII
*Assay of Endotoxins Prepared from Mutant E. coli*

| Preparation assayed | Hyaluronic acid synthesis rate* |
|---|---|
| Control | 8.4 |
| Control | 8.4 |
| *E. coli* 0111 : $B_4$ | 76.3 |
| *E. coli* 0111 : $B_4$ | 45.5 |
| *E. coli*: J5 | 19.9 |
| *E. coli*: J5 | 17.5 |
| *E. coli*: RC59 | 16.5 |
| *E. coli*: RC59 | 13.4 |

* Micrograms hyaluronic acid per milligram cell protein per 24 hr.

[3] Heath, E. C. 1971. Personal communication.

TABLE VIII
*Endotoxin-Synovial Fibroblast Interaction*

| Preparation assayed* | Hyaluronic acid synthesis rate, ratio, experimental/control‡ |
|---|---|
| Control (untreated) fibroblasts | 2.6 |
| Control (untreated) fibroblasts, 100°C 15 min | 1.0 |
| Endotoxin-treated fibroblasts | 3.6 |
| Endotoxin-treated fibroblasts, 100°C 15 min | 2.3 |

* A normal synovial fibroblast cell line treated with 30 μg *E. coli* 0111: $B_4$ endotoxin per ml nutrient medium. Endotoxin was purchased from Difco Laboratories (trichloroacetic acid preparation). Control untreated cells were derived from the same normal cell line. Extracts of the cells were assayed against normal synovial fibroblasts, before and after heat treatment. Activity of the extracts of untreated cells, removed by heat treatment, was due to endogenous CTAP.

‡ Synthesis rate expressed as micrograms hyaluronic acid per milligram cell protein per 24 hr. Numbers represent the ratio of experimental synthesis rate to control synthesis rate.

known for bacterial endotoxin. These findings suggest that endotoxin was taken up or adsorbed by the treated cells, and, further, that the endotoxin molecule did not lose its capacity to activate synovial fibroblasts during the interaction period.

*Characterization of the mucopolysaccharide.* 96% of the mucopolysaccharide recovered from the medium samples was precipitated with cetylpyridinium chloride from dilute solution rather than from 0.2 M $Na_2SO_4$. This suggested that the polymer formed by fibroblasts in response to stimulation by bacterial products was a nonsulfated one such as hyaluronic acid (27). The purified fraction was then subjected to further characterization studies. The Dische carbazole:orcinol ratio was 1.35, indicating that the uronic acid was largely glucuronic acid, and the molar ratio of hexosamine to uronic acid was 1.16:1. Chromatography of the purified sample showed that all of the hexosamine moved with D-glucosamine. No galactosamine was seen. The intrinsic viscosity $[\eta]$ of the analyzed mucopolysaccharide ranged from 29.6 to 54.0 dl/g, values characteristic of synovial fluid hyaluronate. These findings suggest that hyaluronic acid is the predominant mucopolysaccharide made by normal synovial fibroblasts in response to "activation" by a whole extract of *E. coli* D-10.

## DISCUSSION

These studies show that Gram-negative bacterial products have a marked effect on the behavior of synovial fibroblasts in monolayer culture. They stimulate these cells to produce large amounts of hyaluronic acid and to increase glucose uptake and lactate production. Specifically, cell wall lipopolysaccharide or endotoxin is responsible for much of this stimulation, although other constituents of the whole bacterial preparations may contribute by augmenting the endotoxin effect or by the additional stimulus of other unidentified bioactive materials. The lipid moiety of the endotoxin molecule, or "Lipid A," a specific submolecular fragment prepared by mild acid hydrolysis, retains activity comparable to that possessed by intact endotoxin. In addition, endotoxin-cell interaction experiments conducted over 3-day periods show that the extractable materials have not lost their activity. These experiments suggest that short-term metabolic handling of endotoxin by isolated fibroblasts results in no significant loss of activity in this system. Incomplete endotoxin breakdown or accumulation of active endotoxin metabolites such as "Lipid A" could account for such a phenomenon.

Changes induced in normal synovial fibroblasts by these preparations are significant in that they resemble the functional abnormalities exhibited by fibroblasts derived from rheumatoid synovial tissue (2). It is of interest that the microorganism need not be present in infectious form in these in vitro experiments in order for changes to occur. Rather, incomplete bacterial products, not infectious in the conventional sense, are capable of actively stimulating fibroblasts. In addition, the abnormal characteristics observed in vitro approximate the altered metabolic behavior known to occur in the inflamed rheumatoid joint (28, 29).

The way in which bacterial extracts participate in inducing these changes is not clear. The abnormalities produced, collectively referred to as "activation phenomena," duplicate the major known actions of connective tissue-activating peptide (CTAP). CTAP, postulated to be a mediator in the inflammatory reaction, is thought of as a regulator in the progression of inflammation from the acute to the chronic phase (8). In illnesses characterized by chronic synovitis, persistent high levels of activator materials might "freeze" inflammatory activity in the "reparative" phase. Materials from bacteria might participate with CTAP in producing hypermetabolic behavior by synovial fibroblasts, possibly by inducing formation of new CTAP, or by activating peptide present in inactive form. An alternative possibility is that bacterial extracts act independently of CTAP. Experiments designed to test the various possibilities are in progress.

These in vitro experiments are of interest in view of others' in vivo studies which show that Gram-negative bacterial endotoxin will produce synovial inflammation. Hollingsworth and Atkins reported that endotoxin injected into the rabbit knee joint induced a sustained inflammatory response (30). Their studies

showed that the rabbit synovium was sensitive to very small quantities ($5 \times 10^{-5}$ g) of endotoxin placed intra-articularly. Animals that were made "tolerant" to endotoxin had an undiminished inflammatory response after intra-articular injection (30). Furthermore, Aoki and Ikuta reported the experimental production of arthritis in rabbits during lethal *S. typhosa* 0-901 endotoxemia. Intravenous injection of 0.1 mg/kg caused marked synovial inflammation. The authors were able to demonstrate endotoxin within synovial lining cells of the treated rabbits by immunohistochemical techniques. Their studies demonstrated a decline in serum complement; this suggested to them that an immune mechanism might be partially responsible for the lesion (31).

In addition to the possible importance of endotoxin and other noninfectious bacterial extracts in producing rheumatoid inflammation, these materials might be instrumental in producing and sustaining inflammation in pyogenic arthritis, particularly in joints infected with Gram-negative bacteria. Braude, Jones, and Douglas demonstrated the persistence of endotoxin in rabbit synovial tissue long after an initial *E. coli* pyogenic arthritis had become sterile. Although the joints were sterile 2 wk after the onset of infection, joint swelling continued, and purulent exudate persisted up to 8 wk. *E. coli* somatic antigen could be recovered from the rabbit joints 2 wk after they had become sterile. The recovered endotoxin was shown to have retained certain of its toxic properties; that is, it produced a pyogenic response and a hemagglutinin antibody rise in recipient rabbits. The authors also showed that the intra-articular injection of purified endotoxin produced local inflammatory changes comparable to that caused by living organisms (32).

The concept of bacterial products as a cause for synovial inflammation is interesting with regard to other diseases in which bacteria are difficult to recover from joint fluid, such as gonococcal arthritis in the early migratory phase, as well as arthritis associated with subacute bacterial endocarditis. In ulcerative colitis and regional enteritis, chronic inflammatory diseases of the intestinal wall, bacterial products may play a role in the synovitis which sometimes occurs.

The present studies show that bacterial products exert multiple effects on isolated synovial cells. The data presented may contribute to understanding the mechanism of experimental endotoxin-induced arthritis in animals. In addition, these findings support the idea that endotoxin might be important in human diseases characterized by chronic synovitis. In this connection, the similarity of changes induced in normal cells by endotoxin to those which characterize rheumatoid cells is particularly interesting. It is possible that the persistence of endotoxin in articular tissues in undetoxified form might account for continued local inflammatory changes.

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