The role of lipopolysaccharides in endotoxin-induced thymocyte proliferation and chondrocyte collagenase synthesis

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Summary. Crude endotoxin preparations from Haemophilus actinomycetemcomitans and Bacteroides gingivalis showed activity in the two principal bio-assays for interleukin 1—the lymphocyte activating factor assay and stimulation of chondrocyte collagenase synthesis. Lipopolysaccharides purified from the crude endotoxins had reduced activity in the chondrocyte collagenase assay. The activity of the endotoxins may be due to synergic interaction between their lipopolysaccharides and other, as yet unidentified, bacterial components.

Introduction

The monokine interleukin 1 (IL 1) is a potent mediator of connective tissue destruction (Dinarello, 1984) and plays a key role in chronic inflammation, both of which are characteristic features of chronic inflammatory periodontal disease (CIPD). In CIPD it is not clear to what extent bacterial products from plaque cause tissue destruction directly, or indirectly via the release of inflammatory mediators such as IL 1. Furthermore, it has been demonstrated that at least one bacterial component, muramyl dipeptide, can mimic IL 1 (Chedid et al., 1984). We have, therefore, investigated the possibility that other bacterial cell-wall components may also possess IL 1-like activity. The standard method for bio-assay of IL 1 activity has been the lymphocyte activating factor (LAF) assay, i.e., the augmentation of murine thymocyte proliferation in the presence of sub-optimal concentrations of concanavalin A (Gery et al., 1972). An important drawback to this assay is its lack of specificity, because proliferation will also be stimulated by IL 2. An alternative assay, which is IL 2-independent, has recently been introduced—the stimulation of collagenase synthesis in cultured articular chondrocytes (Evequoz et al., 1984). An important drawback to this assay is its lack of specificity, because proliferation will also be stimulated by IL 2. An alternative assay, which is IL 2-independent, has recently been introduced—the stimulation of collagenase synthesis in cultured articular chondrocytes (Evequoz et al., 1984).

Because lipopolysaccharides (LPS) are considered to be important factors in the pathogenesis of CIPD (Daly et al., 1980) we decided to assay LPS from two periodontal pathogens, Bacteroides gingivalis and Haemophilus actinomycetemcomitans, for IL 1-like activity. As the biological activity of LPS preparations may depend on the method of extraction and purification employed (Bradley, 1979), we also investigated the effect of purification on the activity of the preparations in the IL 1 assay.

Materials and methods

Bacterial strains and cultivation

B. gingivalis (London Hospital strain W50) was grown in anaerobic conditions at 37°C in a medium consisting (/L) of yeast extract 5 g, peptone 20 g, NaCl 5 g, glucose 10 g, cysteine hydrochloride 0.55 g and horse serum 10 ml in distilled water. After 72 h the cultures were checked for contamination by staining and by subculture. The cells were then harvested by centrifugation, washed with saline and lyophilised. Lyophilised cells of H. actinomycetemcomitans (NCTC 9710) were obtained in the same way except that the medium consisted (/L) of tryptone 10 g, glucose 2 g, yeast extract 5 g, NaHCO3 5 g, dithiothreitol 50 mg, and biotin 1 mg in distilled water.

Preparation of bacterial components

LPS was obtained from the cells by the butanol extraction method of Morrison and Leive (1975). Briefly, the cells were suspended in 0.85% NaCl at 4°C and an equal volume of butanol was added. The suspension was mixed thoroughly at 4°C for 10 min and centrifuged at 35 000 g for 20 min. The aqueous phase was removed and the butanol, together with the insoluble residue, was re-extracted twice with approximately half the initial volume of 0.85% NaCl. The combined aqueous phases were centrifuged to remove particulate matter, dialysed against distilled water for 48 h and lyophilised. The crude preparation was resuspended in water, ultracentrifuged at 100 000 g for 1 h at 4°C and lyophilised; this was...
designated but-LPS. A portion of this was subjected to hot phenol-water (Westphal) extraction (Westphal and Jann, 1965), and purified by ultracentrifugation and enzymic digestion as described previously (Wilson et al., 1985), this was designated phen-LPS. The material separated from the LPS in the phenol phase was extensively dialysed and then lyophilised. A portion of the phen-LPS was extracted with phenol/chloroform/light petroleum ether (Galanos et al., 1969) and electrodialysed (Galanos and Luderitz, 1975). The resulting highly purified LPS was neutralised with NaOH; this was designated elec-LPS.

**Lymphocyte activating factor assay**

For the LAF assay (Gery et al., 1972), thymuses were obtained from 7-week-old BALB/c mice (King's College Hospital Medical School) and passed through a metal mesh to obtain single cell suspensions. The thymocytes were washed three times and suspended in RPMI 1640 (Gibco) supplemented with 10% complement-inactivated (56°C, 30 min) fetal calf serum (FCS), antibiotics, and 10 μM β-mercaptoethanol. Samples containing 1.5 × 10⁶ cells were pipetted into each well of a sterile 96-well flat-bottomed culture plate (Microtiter; Sterilin) and 20 μl of a concanavalin A (con A) solution containing 0.5 μg/ml was added to each well except for unstimulated control cultures; 100-μl volumes of the endotoxin preparations were then added to triplicate wells to give a final concentration of 50 μg/ml in a total volume of 200 μl/well. As an IL 1 control, supernate from con A-stimulated rat peritoneal macrophage cultures was used at a dilution of 1 in 10. The cultures were incubated for 48 h at 37°C in an atmosphere of CO₂ 5% in air and 0.5 μCi of ³H-thymidine (specific activity 24 Ci/mmol) was added to each well and the incubation continued for a further 18 h. Uptake of ³H-thymidine into the cells was measured by scintillation spectrometry (Rackbeta; LKB, Sweden) with external standardisation.

**Chondrocyte collagenase assay**

Articular cartilage was dissected from the knee and shoulder joints of 2-week-old New Zealand white rabbits, killed with chloroform. It was then sliced, washed in calcium- and magnesium-free phosphate-buffered saline, pH 7.0 (PBS), and incubated in hyaluronidase (Sigma; 5 mg/g of tissue) in PBS for 20 min at 37°C. The supernate was discarded and replaced with bacterial collagenase (Sigma; 3000–5000 U/g of tissue) and pronase (Sigma; 5 mg/g of tissue) in PBS for 1 h at 37°C. The enzyme solution was removed and the cartilage incubated overnight at 37°C in MEM with 10% FCS. The digest was filtered through a nylon mesh (75 μm pore size), the filtrate was discarded, and undigested tissue was treated further with collagenase for 2 h. Cells were dispersed by vigorous pipetting and were separated from the matrix by filtration, washed four times in PBS, and recovered by centrifugation. The final pellet was resuspended in MEM with 10% FCS and cultured in 25-cm² flasks. When confluent, the chondrocytes were resuspended after incubation with trypsin 0.25% and were seeded at a density of 5 × 10⁴ cells/well in triplicate 16-mm diameter wells each containing 1.0 ml of MEM with 10% FCS. When confluent, the cell layers were rinsed thrice in serum-free medium, and 1.0 ml of fresh serum-free medium containing the LPS preparations at concentrations of 50 μg/ml was added to each well (six wells per preparation). After incubation for 3 days the media were assayed for collagenase with reconstituted ³H-acetylated collagen fibrils (Gislow and McBride, 1972) after activation of latent collagenase by 4-aminophenyl mercuric acetate (APMA).

**Results**

The but-LPS preparations from both organisms stimulated thymocyte proliferation in the presence of con A (fig. 1). In the absence of con A, however, they did not have any stimulatory effect, indicating co-mitogenic activity characteristic of IL 1. These crude endotoxin preparations also stimulated the synthesis of collagenase in the articular chondrocytes (fig. 2) but did not have any intrinsic collagenolytic activity. Purification of these crude preparations by Westphal extraction, enzymic digestion, and ultracentrifugation to produce the phen-LPS resulted in a 78% reduction in activity for *B. gingivalis* preparations and a 26% reduction for *H. actinomyctemcomitans* preparations. Further purification by a hydrophobic extraction followed by electrodialysis (elec-LPS) reduced the activities to such an extent that in neither case were they significantly different from the controls. The material removed from the *B. gingivalis* but-LPS by phenol extraction contained no significant stimulatory activity, whereas that from *H. actinomyctemcomitans* did stimulate collagenase synthesis.

**Discussion**

These experiments demonstrate that endotoxins from *B. gingivalis* and *H. actinomyctemcomitans* give positive results in the two recognised bioassays for interleukin 1. Although the thymocyte proliferation assay lacks specificity for IL 1, these results demonstrate that the synthesis of chondrocyte collagenase is also stimulated by factors other than IL 1. In view of the ubiquity of endotoxins, care must be taken, therefore, to ensure that they are absent from material being assayed for IL 1 activity. These findings also have implications for the pathogenesis of periodontal diseases; should endotoxins demonstrate other IL 1-like activities they may play a role in perpetuating the inflamma-
Fig. 1. Incorporation of $^3$H-thymidine by mouse thymocytes in the presence (open bars) or absence (stippled bars) of sub-optimal concentrations of con A. Thymocytes were cultured in medium alone (C) or medium containing interleukin 1 (IL 1) from rat peritoneal macrophage cultures, or endotoxin preparations from *H. actinomycetemcomitans* (Ha) or *B. gingivalis* (Bg). Triplicate cultures were used for each preparation. Vertical lines represent one SEM; * indicates a significant difference from control (Student's $t$ test, $p < 0.05$).

The reason for loss of activity during purification is not known. The bone-resorbing activity of LPS resides in the lipid-A portion (Hausmann *et al.*, 1975). If the lipid-A region is also responsible for the activity of LPS on chondrocytes, the purification procedures used may have caused structural modifications resulting in the loss of activity. However, a more likely explanation is that the chondrocyte-stimulating activity resides in the material removed from the but-LPS by Westphal extraction. In the case of *H. actinomycetemcomitans*, stimulatory material appeared in the phenol phase, indicating that this material may be proteinaceous—perhaps an outer-membrane protein—or may represent a phenol-soluble fraction of LPS, e.g., one with a shorter polysaccharide chain or higher content of hydrophobic sugars (Knox and Parker, 1973). In contrast, no stimulatory material was recovered from the phenol phase of the *B. gingivalis* preparation. This material, although inactive on its own, may act synergically with LPS to produce the activity observed before separation. Synergic interactions of this type have been reported between LPS, muramyl dipeptide and prostaglandins, leading to increased bone-resorbing activity (Raisz *et al.*, 1981). Material removed from the but-LPS by phenol could include capsular material or other surface polymers, extracellular or surface-associated enzymes and outer-membrane proteins such as lipid A-associated protein.

The highly-purified elec-LPS had no collagenase-stimulating activity, again suggesting that the activity detected in the phen-LPS preparations may
also have been due to bacterial components co-purifying with the LPS. Likely contaminants would include outer-membrane proteins, as well as amines such as spermine, putrescine, and ethanolamine (Galanos and Luderitz, 1975), and these would have been eliminated by the hydrophobic extraction and electrodialysis. The collagenase-stimulating activity of the endotoxin preparations does not appear, therefore, to be attributable to LPS alone but depends on the presence of other bacterial components.

These results demonstrate firstly that endotoxins can give false-positive reactions in the LAF and chondrocyte collagenase assays for IL 1. Secondly, care must be taken in attributing a particular biological activity to LPS, because the activity may be due to other components which co-purify with it.

REFERENCES


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