Inhibition of fibroblast proliferation and collagen synthesis by capsular material from *Actinobacillus actinomycetemcomitans*

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Summary. Cytotoxic effects of bacteria found in dental plaque are usually attributed to lipopolysaccharides (LPS) or ill-defined toxins. Many bacteria implicated in periodontal disease produce surface exopolymers (capsules) recently shown to stimulate bone resorption. Capsular material and LPS extracted from *Actinobacillus (Haemophilus) actinomycetemcomitans* were purified and examined for their effects on cultures of human gingival fibroblasts. DNA and collagen synthesis were significantly inhibited by capsular material (0.1–50 µg/ml). LPS caused only modest inhibition of DNA synthesis at 10 and 50 µg/ml, and had no effect on collagen synthesis. Release of lactate dehydrogenase from fibroblasts was not increased by LPS nor by capsular material, showing that the inhibitory effects were not due to cell death. Capsular material, but not LPS, caused a pronounced increase in cell size; a doubling of the nuclear area occurred within 72 h exposure. These results indicate that the capsule of *A. actinomycetemcomitans* may play an active role in the tissue destruction characterising inflammatory periodontal disease.

Introduction

The clinical manifestations of periodontitis result from the effects of bacteria on host tissues. Bacterial products can exert a direct effect or can act indirectly by inducing inflammatory changes. In localised juvenile periodontitis (LJP) there is marked tissue destruction, especially bone resorption, but minimal inflammation (Baer, 1971; Hørmann and Frandsen, 1979), and the direct cytotoxic effects of bacterial products appear to play an important role. Such effects on the fibroblasts of the periodontium result in a decrease in cell numbers and a net loss of collagen (Page and Schroeder, 1976).

Actinobacillus (Haemophilus) actinomycetemcomitans has been implicated in LJP (Slots, 1979; Slots *et al.*, 1980; Zambon *et al.*, 1983) and attempts have been made recently to examine the mechanisms by which it damages periodontal tissues. *In vitro* soluble extracts of *A. actinomycetemcomitans* have been shown to inhibit the proliferation of several cell types including lymphocytes (Shenker *et al.*, 1982a), fibroblasts (Shenker *et al.*, 1982b; Stevens *et al.*, 1983), epithelial cells (Kamen, 1983), and endothelial cells (Taichman *et al.*, 1984). This inhibitory activity has been observed in both

leukotoxic and non-leukotoxic strains (Taichman *et al.*, 1982), and is therefore not attributable to a leukotoxin.

Lipopolysaccharide (LPS) is generally regarded as the component responsible for the cytotoxicity to fibroblasts demonstrated in plaque extracts from the teeth of patients with periodontal disease and in bacterial sonicates (Hatfield and Baumhams 1971; Aleo *et al.*, 1974). However, there have been no such studies with purified LPS preparations in which a contribution from other bacterial factors has been eliminated. This is important because components other than LPS, e.g., peptidoglycans, are known to affect connective tissue cells (Lengraf *et al.*, 1979) and recently we reported that capsular material (CM) purified from *A. actinomycetemcomitans* stimulates bone resorption *in vitro* at concentrations as low as 1 mg/ml (Wilson *et al.*, 1985). Therefore we decided to examine and compare the effects of LPS and CM from *A. actinomycetemcomitans* on human gingival fibroblasts *in vitro*.

Materials and methods

Bacterial cultivation

A. actinomycetemcomitans NCTC 9710 was kindly

supplied by Dr P. Marsh (CAMR, PHLS, Porton Down). The organism was grown in batch cultures at 37°C in anaerobic conditions in the following medium: Tryptone 10 g, glucose 2 g, yeast extract 5 g, NaHCO₃ 5 g, dithiothreitol 50 mg and biotin 1 mg in 1 L of distilled water. After incubation for 24 h a smear of the culture was stained by Gram's method to check for contamination and then centrifuged at 30 000 *g* for 30 min at 40°C. The harvested cells were washed with saline, recentrifuged and then lyophilised.

Preparation of capsular material

Capsular material was extracted from 1.1 g of lyophilised bacteria (Wilson *et al.*, 1985). Saline (100 ml) was added to the cells and the suspension was gently agitated for 1 h at 40°C, centrifuged at 30 000 *g* for 45 min and the supernate removed. This saline extraction was repeated twice and then acetone at -20°C was added to the supernate. The precipitate was centrifuged as above and the pellets redissolved, dialysed against distilled water and lyophilised. This crude CM was then purified by gel filtration and ion-exchange chromatography.

Preparation of LPS

LPS was extracted by the hot phenol-water method of Westphal and Jann (1965) after the removal of capsular material from the lyophilised cells. This crude LPS preparation was purified by treatment with DNAase, RNAase and Pronase and then ultracentrifugation (Kiley and Holt, 1980). The LPS and CM preparations were lyophilised and, for experimental use, dissolved directly in tissue culture medium.

Fibroblast cultures

Human gingival fibroblasts were grown from explants obtained during minor oral surgical procedures. The cells were grown in Eagles' MEM (Gibco-Europe), supplemented with fetal calf serum (FCS) 10%, 2 mM L-glutamine, NaHCO₃ 2.25 g/L, penicillin and streptomycin (each 100 U/ml) and incubated at 37°C in CO₂ 5% in air. Cells were subcultured at weekly intervals with trypsin 0.25% and used for experiments between passages 6 and 12.

Incorporation of ³H-thymidine

Fibroblast suspensions were seeded into 96-well culture plates (Microtitre, Linbro) at concentrations of 15 000 cells/well in 100 µl of MEM with FCS 10% and left overnight to attach. The following day culture medium was replaced by MEM with FCS 10% containing purified LPS or CM at concentrations of 0.1–50 µg/ml, in groups of six wells per concentration. Control cultures received only fresh medium. Cells were further incubated for 24 h and 0.5 µCi of 5-³H-thymidine (specific activity 20 Ci/mmol; Amersham International) was

introduced into each well 4 h before termination of the cultures. Trichloroacetic acid-insoluble material in each cell layer was dissolved in 0.4 M NaOH and radioactivity measured in 3.0 ml of scintillant (Unisolve I, Koch Light) by scintillation spectrometry (Rackbeta; LKB, Sweden) with external standardisation. Radioactivity was expressed as disintegrations/min (dpm)/well.

Collagen synthesis

The rate of collagen synthesis was estimated by the incorporation of ³H-proline into pepsin-resistant native collagen during a 24-h period (Webster and Harvey, 1979). 5-³H-proline (0.2 µCi; 2.3 Ci/mmol; Amersham International) was added to each well in 200 µl of MEM plus FCS 10% containing β-aminopropionitrile fumarate and L-ascorbic acid (50 µg/ml of each). On termination, the cell layer and culture medium together were extracted with pepsin 0.5 mg/ml in 0.5 M acetic acid for 16 h at 4°C, and the ³H-labelled collagen precipitated twice with 0.8 M NaCl; the carrier was 100 µg of acid-soluble rat skin collagen. The final collagen pellet was dissolved in 250 µl 0.5 M acetic acid and radioactivity was measured as described above.

Lactate dehydrogenase (LDH)

The release of LDH into culture medium was used as an index of cell lysis. Fibroblasts were seeded at a concentration of 15 000 cells/well and left overnight to attach. LPS or CM (50 µg/ml) was dissolved in MEM supplemented with 0.5% serum substitute Ultrosor G (LKB, Sweden) to eliminate the contribution of LDH from FCS. After incubation for 72 h, the culture medium was removed and 50-µl portions were retained for LDH measurement. Assay buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.5) 100 µl was added to each well and the cells extracted into this by freezing and thawing three times and 50 µl of the resulting extracts were then used to measure LDH activity in the cell layer. LDH was measured by reduction of pyruvate in the presence of NADH during a 5-h incubation period for culture supernates and 30 min for cell extracts. The absorbance of the hydrazone derivative of pyruvate was measured at 450 nm (Wroblewski and Gregory, 1961). Cell lysis was calculated as the ratio of medium LDH to total LDH, expressed as a percentage.

Nuclear area

Fibroblasts were seeded at a concentration of 15 000 cells/well and left overnight to attach. LPS or CM (50 µg/ml) was dissolved in MEM plus FCS 10% and 100 µl was added to each well (six wells/group). The plates were examined by phase contrast microscopy every 24 h, and after incubation for 72 h the cells were fixed with methanol and stained by Giemsa's method. The bottom of each well was removed and mounted on a glass slide. The nuclear area of 70 cells in each preparation was measured

Fig. 1. Effect of LPS and CM on DNA synthesis in human gingival fibroblasts; * $p < 0.05$.

by planimetry of the projected images with a computer image analysis system (Kontron 64, W. Germany).

Results

CM caused a concentration-dependent inhibition of ^3H -thymidine incorporation which was significant at a concentration of $0.1 \mu\text{g/ml}$ and above (fig. 1). LPS caused less inhibition, significant only at 10 and $50 \mu\text{g/ml}$. CM caused a profound inhibition of collagen synthesis; 75% reduction occurred at $50 \mu\text{g/ml}$ (fig 2). In contrast, LPS did not significantly alter the rate of collagen synthesis at any of the concentrations tested.

LDH release during 3 days was approximately

Fig. 2. Effect of LPS and CM on collagen synthesis by human gingival fibroblasts; * $p < 0.05$.

Table I. Effect of various concentrations of capsular material (CM) and lipopolysaccharide (LPS) on the release of lactate dehydrogenase (LDH) by human gingival fibroblasts

Component	Concentration ($\mu\text{g/ml}$)	LDH release (% $\pm$ SD)
Capsular material	0.1	5.28 $\pm$ 0.53
	1.0	5.16 $\pm$ 0.50
	10.0	5.08 $\pm$ 0.37
	50.0	5.45 $\pm$ 0.67
Lipopolysaccharide	0.1	5.75 $\pm$ 0.62
	1.0	5.30 $\pm$ 0.54
	10.0	4.97 $\pm$ 0.71
	50.0	5.60 $\pm$ 0.53
Control	...	5.04 $\pm$ 0.60

5% in control cultures and did not vary significantly in cultures containing CM or LPS at concentrations up to $50 \mu\text{g/ml}$ (table I). Microscopic examination of the fibroblasts confirmed that cell lysis had not occurred. However, CM-treated cells exhibited gross morphological changes; there was a progressive increase in cytoplasmic and nuclear area, detectable after 24 h and clearly visible after 72 h (fig. 3B). This was confirmed by measurement of the nuclear area which, in the CM-treated cells, was twice that of the control cells (table II). The nuclear area of LPS-treated cells was not significantly different from controls.

Discussion

These experiments showed that capsular material from *A. actinomycetemcomitans* is a potent inhibitor of gingival fibroblast proliferation, whereas LPS from the same organism had little effect. The lack of effect on LDH release, even at $50 \mu\text{g/ml}$, indicated that the reduction of ^3H -thymidine incorporation was due to inhibition of cell proliferation rather than to cell death. It is likely, therefore, that CM is responsible for the inhibitory activity found in crude extracts of *A. actinomycetemcomitans*. This is supported by the observations of Shenker *et al.* (1982b) and Stevens *et al.* (1983) who concluded that the fibroblast inhibitory factor was neither endotoxin nor a leukotoxin. Stevens *et al.* (1983) found that similar inhibitory activity present in a phosphate-buffer extract of *A. actinomycetemcomitans* was equal to that found in sonic extracts of the cultures, suggesting that loosely-bound surface components may have been responsible for the activity in the sonicates. Earlier demonstrations of cytotoxicity to fibroblasts by LPS

Fig. 3. Human gingival fibroblasts incubated for 72 h in: (a) MEM+FCS 10% (control); (b) CM 50 µg/ml; and (c) LPS 50 µg/ml. Giemsa stain; bar = 100 µm.

Table II. Nuclear area of human gingival fibroblasts after exposure to bacterial components (50 µg/ml) for 72 h

Cells	Mean nuclear area (µm ² ± SD)
Control	167.66 ± 31.27
CM-treated	355.17 ± 94.08*
LPS-treated	165.69 ± 31.08

* Significantly different from control (Student's *t* test, *p* < 0.01).

(Bergman and Nilsson, 1963; Alec *et al.*, 1974) used *Escherichia coli* as a source of LPS and the purification methods may not have excluded the presence of other bacterial components, including CM. In the present study, more rigorous purification procedures minimised possible contamination of the LPS. The inhibition of collagen synthesis by CM was in marked contrast to the lack of effect by LPS. Aleo (1980) found that LPS from *E. coli* stimulated collagen synthesis in a mouse fibroblast line, but the differences between the type of fibroblasts and the source and purity of the LPS preparations in this study and our own make direct comparison difficult. However, the lack of inhibition of collagen synthesis by LPS *in vitro* in both studies suggests that collagen loss in periodontal disease is not due to inhibition of its synthesis by LPS. The present studies have shown that CM, rather than LPS, may be the principal factor in bacterial sonicates and plaque extracts that inhibit fibroblast growth and collagen synthesis *in vitro*.

Histological examination of gingival tissue in the early stages of periodontitis has revealed a three-fold increase in fibroblast size, attributed to the action of lymphocyte products (Schroeder *et al.*, 1973). However, our finding that CM caused fibroblast enlargement *in vitro* suggests that bacterial components may contribute to this effect. Although the enlargement *in vivo* is thought to reflect cell morbidity (Page and Schroeder, 1977), CM produced this effect without cell lysis. This raises the possibility that CM may be responsible for some of the early pathological changes in periodontitis in addition to its potential for stimulating the bone resorption characteristic of the later stages of the disease.

REFERENCES

- Aleo J J, De Renzis F A, Farber P A, Varboncoeur A P 1974 The presence and biologic activity of cementum-bound endotoxin. *Journal of Periodontology* **45**:672–675.
- Aleo J J 1980. Stimulation of macromolecular synthesis by endotoxin-treated 3T6 fibroblasts. *Experientia* **36**:546–547.
- Baer P N 1971 The case for periodontitis as a clinical entity. *Journal of Periodontology* **42**:516–520.
- Bergman S, Nilsson S B 1963 Effect of endotoxin on embryonal

- chick fibroblasts cultured in monolayer. *Acta Pathologica et Microbiologica Scandinavica* **59**:161–164.
- Hatfield C G, Baumhammers A. 1971 Cytotoxic effects of periodontally involved surfaces of human teeth. *Archives of Oral Biology* **16**:465–468.
- Hørmann J, Frandsen A. 1979 Juvenile periodontitis. Localisation of bone loss in relation to age, sex and teeth. *Journal of Clinical Periodontology* **6**:407–416.
- Kamen P R. 1983 Inhibition of keratinocyte proliferation by extracts of *Actinobacillus actinomycetemcomitans*. *Infection and Immunity* **42**:1191–1194.
- Kiley P, Holt S C. 1980 Characterization of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. *Infection and Immunity* **30**:862–873.
- Lensgraf E J, Greenblatt J J, Bawden J W. 1979 Effect of group A streptococcal peptidoglycan and group A streptococcal cell wall on bone in tissue culture. *Archives of Oral Biology* **24**:495–498.
- Page R C, Schroeder H E. 1976 Pathogenesis of inflammatory periodontal disease. A summary of current work. *Laboratory Investigation* **34**:235–249.
- Page R C, Schroeder H E. 1977 Structure and pathogenesis. In: Schlager S *et al.* (eds) *Periodontal disease: basic phenomena, clinical management and occlusal and restorative interrelationships* Lea and Fabiger, Philadelphia, p 168.
- Schroeder H E, Munzel-Pedrazzoli S, Page R C. 1973 Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man. *Archives of Oral Biology* **18**:899–923.
- Shenker B J, McArthur W P, Tsai C C. 1982a Immune suppression induced by *Actinobacillus actinomycetemcomitans*. 1. Effects on human peripheral blood lymphocyte response to mitogens and antigens. *Journal of Immunology* **128**:148–154.
- Shenker B J, Kushner M E, Tsai C C. 1982b Inhibition of fibroblast proliferation by *Actinobacillus actinomycetemcomitans*. *Infection and Immunity* **38**:985–992.
- Slots J. 1979 Subgingival microflora and periodontal disease. *Journal of Clinical Periodontology* **6**:351–382.
- Slots J, Reynolds H S, Genco R J. 1980 *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infection and Immunity* **29**:1013–1020.
- Stevens R H, Gatewood C, Hammond B F. 1983 Cytotoxicity of the bacterium *Actinobacillus actinomycetemcomitans* extracts in human gingival fibroblasts. *Archives of Oral Biology* **28**:981–987.
- Taichman N S *et al.* 1982 Leukocidal mechanisms of *Actinobacillus actinomycetemcomitans*. In: Geneo R J, Mergenhagen S E (eds) *Host-parasite interactions in periodontal diseases*. American Society for Microbiology, Washington, D.C. pp 261–269.
- Taichman, N S, Klass J E, Shenker B J, Macarak E J, Boehringer H, Tsai C C. 1984 Suspected periodontopathic organisms alter in vitro proliferation of endothelial cells. *Journal of Periodontal Research* **19**:583–586.
- Webster D F, Harvey W. 1979 A quantitative assay for collagen synthesis in microwell fibroblast cultures. *Analytical Biochemistry* **96**:220–224.
- Westphal O, Jann K. 1965 Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. *Methods of Carbohydrate Chemistry* **5**:83–91.
- Wilson M, Kamin S and Harvey W. 1985 Bone-resorbing activity of purified capsular material from *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research* **20**:484–491.
- Wroblewski F, Gregory K F. 1961 Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. *Annals of the New York Academy of Sciences* **94**:912–932.
- Zambon J J, Christersson L A, Slots J. 1983 *Actinobacillus actinomycetemcomitans* in human periodontal disease: prevalence in patient groups and distribution of biotypes and serotypes within families. *Journal of Periodontology* **54**:707–711.