Stimulation of macromolecular synthesis by endotoxin-treated 3T6 fibroblasts

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Summary. The interaction of endotoxin with cultured fibroblasts resulted in a depression of cellular proliferation and an increased synthesis of macromolecules, namely collagenous and non-collagenous proteins. The collagen salt-soluble fraction was increased at the expense of the insoluble fraction, and both the salt-soluble fraction and collagen secreted into the medium was underhydroxylated.

It is fairly well established that the clinical expression of periodontal disease is accompanied by an increase of Gram-negative bacteria in the dental plaque surrounding the teeth[1,2]. Most of these Gram-negative bacteria contain a complex lipopolysaccharide (endotoxin), a potent inflammatory agent, as part of their cell walls. Endotoxin has been found to bind to the root surface (cementum) of teeth[3]. Since cementum is a relatively static tissue in comparison to the dynamic equilibrium and biologic turnover capabilities of the surrounding tissues, any change in its structure or chemical make-up may have long term effects. The supporting tissues of teeth in direct contact with cementum are primarily connective tissues. In order to determine whether endotoxin would cause any possible metabolic alterations in these tissues, cultured fibroblasts were used to study the effects of endotoxin on macromolecular synthesis, namely collagenous and non-collagenous protein synthesis.

Material and methods. 3T6 fibroblasts were grown in the Dulbecco-Voyt modification of Eagle's medium[4] containing 10% heat-inactivated calf serum and supplemented with ascorbic acid (50 µg/ml). 5-day old cultures were trypsinized and resuspended into a common suspension having approximately 100,000 cells per ml. The suspension was divided into 4 equal parts: 1 designated as the control, and the other 3 with 10, 20 and 30 µg/ml of commercial endotoxin added, respectively. Difco lipopolysaccharide B.E. *coli* 0111:B4 was used in these experiments. The dosages used in this study were based upon previous studies using the same model system[3,5]. Replicate cultures were prepared and grown for a 7-day period, the time required for the cultures to reach confluence. On day 7, L-[G-^{3}H] proline (2 mCi/ml of medium) was added and the cultures were harvested on day 9.

Total collagenous and non-collagenous protein in the cell layer and that secreted into the medium was measured. To measure the solubility of the cell layer collagen, the cells from 5 plates were pooled and sequentially extracted for 2 periods of 24 h each with 4–5 volumes of 1 M NaCl at 4 °C. The residue was re-extracted for 2 additional periods of 24 h each in 4–5 volumes of 0.1 M acetic acid at 4 °C, and the remaining residue was designated as insoluble collagen. For radiochemical analysis, each of the cell fractions and the medium after exhaustive analysis was treated with 20 µg purified bacterial collagenase (Advance Biofactors Corporation, Lynbrook, New York). After digestion, each sample was dialyzed against distilled water at 4 °C and samples of the diffusate and retentate were taken for counting; the remainder was hydrolyzed in 6 N HCl for 16 hours at 104 °C. The proline-hydroxyproline radioactivity ratios were measured by the method of Peterkofsky and Prockop[6]. All samples were counted by direct scintillation spectrometry in a Beckman LS-150 liquid scintillation spectrometer. Quantitation was based on both cell count and DNA analysis[7].

Results. Figure 1 shows that there is a depression of cell growth as the concentration of endotoxin is increased; these data support a previous study in which much larger concentrations of endotoxin exhibited cellular proliferation depression. It is obvious that the ability of depressing proliferation is not lost at lower concentrations.

Table 1. The effect of endotoxin concentration on cellular proliferation

Endotoxin*	Percent of control
10	92
20	61
30	54

* µg of endotoxin per ml of culture medium. Cell numbers were determined by DNA analysis. Each point represents the average of paired samples.

Table 2. Distribution of collagenous and non-collagenous protein

[^{3}H] Proline incorporation cpm/100 γ DNA		Collagenase digestible	Collagenase non-digestible
Cells			
Control		411	587
Endotoxin*	10	445	586
	20	967	1050
	30	1102	1375
Medium			
Control		256	638
Endotoxin*	10	258	1049
	20	480	1490
	30	532	1548

* µg of endotoxin per ml of culture medium. 7-day old cultures received labeled proline and were harvested on day 9. Each point represents the average of paired samples.

Table 3. Extractability of cell layer collagen

[^{3}H] Hydroxyproline as % of total		Neutral salt	Acid	Insoluble
Control		78.9	7.3	13.8
Endotoxin*	10	88.2	6.5	5.3
	20	90.6	6.2	3.1
	30	87.6	6.8	5.6

* µg of endotoxin per ml of endotoxin. Cells from 5 plates were pooled and sequentially extracted for 2 periods of 24 h each with 5 volumes of 1 M NaCl at 4 °C. The residue was re-extracted for 2 additional periods of 24 h each in 5 volumes of 0.1 M acetic acid at 4 °C, and the remaining residue was designated as insoluble collagen.

Table 4. Proline: hydroxyproline ratios in collagenase-digestible macromolecules

Cell fractions	Control	Endotoxin*		
		10	20	30
Salt soluble	1.90	4.46	3.96	4.93
Acid soluble	2.26	2.87	1.86	2.97
Insoluble	1.23	1.50	1.47	1.78
Medium	1.11	1.80	1.97	2.14

* µg of endotoxin per ml of culture medium.

The distribution of collagenous and non-collagenous protein in the cells and in the culture medium is shown in figure 2. Each point represents the average of paired samples. Endotoxin increased both collagenous and non-collagenous proteins both in the cell layer and in the culture medium. The increase was dose-dependent with a greater synthesis of collagen in the cell layer versus the culture medium, whereas the non-collagenous protein synthesis was greater in the culture medium versus the cell layer.

The effect of endotoxin on cell layer collagen extractability (figure 3) showed a slight but consistent increase in the saltsoluble fraction at the expense of the insoluble fraction; the acid-soluble fraction is relatively unchanged. The changes in the salt-soluble and insoluble fractions do not appear to be dose-related, at least at the concentrations used in these experiments.

In the endotoxin-treated cells, there is very little difference in the proline/hydroxyproline ratios in the acid-soluble and insoluble fractions, but the proline/hydroxyproline ratios of the salt-soluble fraction are definitely elevated signifying underhydroxylation. The macromolecules found in the medium are also underhydroxylated, but the degree of underhydroxylation in this fraction or in the salt-soluble fraction does not appear to be dose-related.

Discussion. These data show that endotoxin, in vitro, in the absence of the usual activation of an immune effector system found in vivo, can alter the synthetic capabilities and depress the proliferation of fibroblasts. This direct action of endotoxin may be due to its affinity for membranes. As a lipopolysaccharide, endotoxin is capable of interacting with phospholipids found in cell membranes[8] and thus, has a profound effect upon the physiological responses of the cell. Modified cell membranes may disrupt or alter membrane-bound enzymes, or contribute to a host of other metabolic alterations brought about by changes in membrane transport.

The hypermetabolism of fibroblasts induced by endotoxin in this study resembles the changes seen by Buckingham and Castor[9] who investigated the effects of extracts of Gram-negative bacteria on fibroblast cultures. They found a marked increase in hyaluronic acid production, glucose utilization and lactate output. Their studies demonstrated that endotoxin was bound to fibroblasts, but they had no conclusive evidence of how the metabolic changes occured. Whether membrane changes were solely responsible or whether the production of mediators resulted in hypermetabolism is not clear.

This study shows that endotoxin has a direct effect upon the metabolism and proliferation of a homogenous fibroblast population apart from the controlling and modifying influences of the host. The data presented may contribute to the understanding of the chronicity of periodontal disease. If cementum-bound endotoxin is allowed to remain in contact with the supporting tissues of teeth, the depression of cellular proliferation and the alterations in tissue metabolism make the prognosis of early eradication of the disease unlikely, and the development of the disease into chronic phases inevitable.

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The chromosome banding patterns of the aardvark *Orycteropus afer* (Tubulidentata, Orycteropidae)[1]

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Summary. C- and G-banding patterns of *Orycteropus afer* are described on the basis of fibroblast cultures obtained from a female individual.

Benirschke et al.[2], based on their conventional preparations, reported that the diploid chromosome number of the aardvark, *Orycteropus afer*, is 20. Its karyotype consists of 2 pairs of large subtelocentric plus 7 pairs of medium to small metacentric and submetacentric autosomes, and the sex pair. The X is a small metacentric and the Y is the smallest biarmed chromosome. We report here the C-band and G-band patterns of this species.

Skin biopsies of 2 sister aardvarks were obtained from Point Defiance Zoo in Tacoma, Washington. 1 sample was contaminated but the other gave rise to fibroblast cultures from which cytological preparations were made. C- and G-band procedures followed those described earlier[3].

From more than 100 metaphase plates in conventionally Giemsa-stained slides, we confirm the diploid number and the karyotype described by Benirschke et al. All chromosomes possess centromeric C-bands, but pairs 4 and 7 have an additional terminal C-band in the long arm, and pair 6 has an additional C-band in the short arm (figure 1). These additional C-bands are not obvious in the photograph, but they were consistently observed in our preparations. The amount and distribution of the C-band material in the aardvark karyotype is not unusual.

In the absence of male cells, the identification of the X chromosome in our karyotype follows that of Benirschke et al., hence the ? in figure 2, a G-band karyotype. Every chromosome pair can be unequivocally identified by G-banding.

Armadillos, anteaters, pangolins and the aardvark were originally classified into the single order Edentata. Subsequently, the pangolin was placed into a separate order Pholidota, and the aardvark, into another order Tubulidentata. The diploid number of the armadillos ranges from 58 to 64[4,5], and that of the anteaters ranges from 54 to 60[6]. A