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Inhibition of Endotoxin-Induced Depression of Cellular Proliferation by Ascorbic Acid (40856)

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Abstract. Previous studies have shown that cementum-bound endotoxin or endotoxin-like products from untreated periodontally involved teeth depress the proliferation of, and have direct toxic effects on, cultured fibroblasts. The purpose of this investigation was to study the effect(s) of ascorbate and several other compounds on the toxicity of commercially prepared endotoxin. The 3T6 fibroblasts were grown in Dulbecco–Vogt modification of Eagles' medium containing 10% heat-inactivated calf serum. Replicate cultures were prepared and grown for 5 days. The proliferation of endotoxin-challenged cells was monitored in the presence of either L-ascorbate, D-isoascorbate, dehydroascorbate, carbonylcyanide *m*-chlorophenylhydrazone, or cycloheximide. Under very specific conditions, ascorbate reversed the endotoxin-mediated depression of cellular proliferation. An uncoupler of oxidative phosphorylation, carbonylcyanide *m*-chlorophenylhydrazone also effectively reversed the effect of endotoxin while cycloheximide had no effect. Aside from the known involvement of ascorbate in connective tissue metabolism and repair, the findings in the study suggest that acute or prolonged ascorbate deficiency may potentially compromise host defenses to endotoxin. If endotoxin is involved as one of the factors in the initiation and progression of periodontal disease, as has been suggested, the probable connection between ascorbate deficiency and periodontal disease appears to be at least twofold: primarily, in etiology; and, second, in tissue repair and return to normal function.

The implication of bacterial endotoxins in the etiology and chronicity of inflammatory periodontal disease is well documented (1–8). Until the discovery by Aleo *et. al.* (9) that the cementum of periodontally-involved teeth exposed to the disease process harbors endotoxin, it was thought that the only source was that of bacterial plaque containing gram-negative organisms. Since cementum is a relatively static tissue when compared with the dynamic equilibrium and biologic turnover capabilities of surrounding tissues, any change in its structure or chemical makeup will have long-term effects.

In vitro studies (9) using cementum-bound or commercial endotoxin have shown that these products at low concentrations depress the proliferation of cultured fibroblasts without compromising cell viability; high concentrations, however, are toxic to cultured cells depressing both cellular proliferation and viability.

Recent *in vitro* studies in this laboratory (10) have shown that endotoxin-treated fibroblasts demonstrate an increase in macromolecular synthesis, namely collagenous and noncollagenous proteins. In analyzing the collagen, it was found that the 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. Because collagen synthesis was one of the macromolecules studied, ascorbic acid was added to the culture medium to insure maximum synthesis. During the course of these experiments, it was found that ascorbic acid had a consistent protective effect against endotoxin's depression of cellular proliferation.

The biochemical lesions of scurvy and the known actions of ascorbic acid in biologic systems are well known. Probably the best known and most intensively studied effect is the lesion in wound healing and connective tissue formation; wounds which are allowed to heal in a scorbutic animal lack tensile strength and reopen easily. The molecular lesion is related to a cofactor-like role of ascorbic acid in the action of prolyl and lysyl hydroxylases (11,

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12). What has received somewhat less emphasis is the interaction and pharmacologic action of ascorbic acid with drugs and a variety of chemicals. Many studies (13–15) have shown that vitamin C deficiency results in decreased metabolism of a variety of pharmacologic agents. Other studies (16) suggest a role for ascorbic acid in preventing the *in vivo* reaction of a variety of amines with sodium nitrite to form toxic nitrosamines, while others have shown ascorbic acid to be protective against the toxicity of heavy metals (17).

The purpose of this study was to show that ascorbate, under very specific conditions can protect against endotoxin-mediated depression of cellular proliferation. Several other compounds were also found to be protective; the relevance of these to the possible control of periodontal disease, *in vivo,* is discussed.

Material and methods. The 3T6 fibroblasts were grown in the Dulbecco–Vogt modification of Eagles' medium containing 10% heat-inactivated calf serum. Five-day-old cultures were trypsinized and resuspended into a common suspension having approximately 100,000 cells/ml. Replicate cultures were prepared from the common cell suspension and grown for a 4- to 5-day period, the time required for the cultures to reach late logarithmic phase. The concentrations of the test compounds introduced into the system were based upon previous studies (9, 18). Cell proliferation was measured by triplicate determinations of cell counts. Viability was determined by trypan-blue exclusion.

Results. The dose–response of cultured cells to L-*ascorbate.* Concentrations of L-ascorbate from 0 to 100 μg/ml of culture medium were used in this phase of the study. The cultures grew logarithmically for 4 to 8 days and then remained in a somewhat stationary phase for an additional 6 to 8 days before entering a declining period of growth.

Figure 1 shows the growth curves in the late logarithmic phase. There was a small stimulatory effect upon growth by L-ascorbate up to a concentration of 50 μg while higher concentrations definitely depressed cell proliferation; similar results

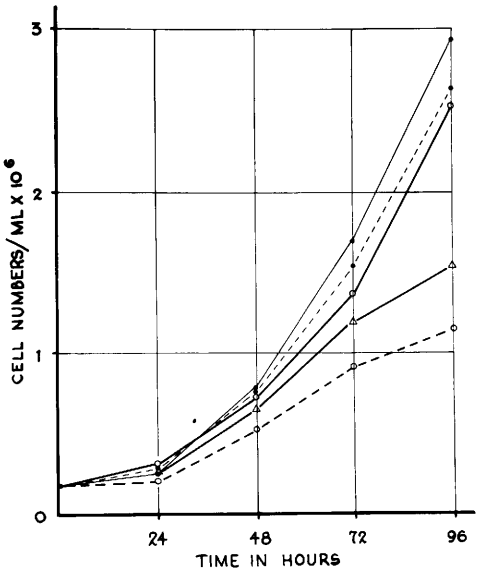

FIG. 1. Growth curves of 3T6 cultures in the presence of several concentrations of ascorbic acid. Cells were seeded at 100,000 cells/ml of culture medium and refed every 2 days. Minus ascorbate (control) ○——○; plus ascorbate in μg/ml: ●– –● 25; ●——● 50; △——△ 75; ○– –○ 100.

were obtained when substituting D-isoascorbate. Dehydroascorbate, on the other hand, had a slightly depressing effect upon cell growth, but it was decided to use 50 μg/ml of each in the study for uniformity.

The effect of L-*ascorbate on endotoxin-induced cell proliferation depression.* Cell proliferation depression by commercial endotoxin, Difco Lipopolysaccharide B.E. *coli* 0011:B4 has been described elsewhere (9); the concentration of 100 μg/ml of culture medium of endotoxin is the highest concentration of endotoxin which does not affect cell viability.

When L-ascorbate was added to endotoxin-challenged ascorbate-free cells at the initial seeding of the cultures, the ascorbate had no effect upon the endotoxin-induced depression of cellular proliferation. If the cells were pretreated with L-ascorbate for a period of 2 to 5 days before the endotoxin challenge, there was a gradual and definite protection against endotoxin toxicity; pretreatment beyond 5 days did not enhance the protective effect (Fig. 2).

In order to determine whether ascorbate

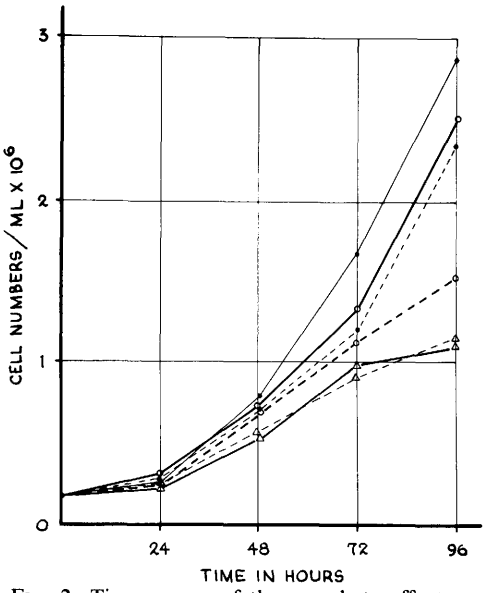

FIG. 2. Time course of the ascorbate effect upon endotoxin-challenged 3T6 cultures. Cultures were pretreated with ascorbate (50 μg/ml) for various time intervals before the endotoxin challenge (100 μg/ml). Minus ascorbate (control) ○——○; plus ascorbate (ascorbate control) ●——●; plus endotoxin (endotoxin control) △——△; ascorbate plus endotoxin (no pretreatment) △--△; ascorbate plus endotoxin (2-day pretreatment) ○--○; ascorbate plus endotoxin (5-day pretreatment) ●--●.

influenced the toxicity of endotoxin directly, endotoxin, at the concentration used in this study, was incubated with ascorbate for periods up to 5 days and then introduced into the culture system. This treatment did not alter or influence the toxicity of endotoxin suggesting that the observed protective effect of ascorbate is mediated by the cells.

The effect of other compounds on endotoxin-induced cell proliferation depression. Table I is a summary of all the agents used in the study. L-Ascorbate gave the maximum protection at 50 μg/ml while higher concentrations actually added to the cell proliferation depression. D-Isoascorbate gave an appreciable degree of protection and dehydroascorbate's protection was marginal. The uncoupling agent (carbonylcyanide *m*-chlorophenylhydrazone) (19), while depressing cell proliferation somewhat, gave an appreciable measure of protection against endotoxin.

Discussion. The present study demonstrates that ascorbate is effective against endotoxin-induced depression of cellular proliferation under very specific conditions; ascorbate-deficient cells must be pretreated with ascorbate for a definite period of time before the endotoxin challenge. Pretreatment of endotoxin with ascorbate had no effect upon endotoxin's toxicity suggesting that ascorbate's protective effect resides either at the cell surface or intracellularly. The lack of protection when both ascorbate and endotoxin are introduced simultaneously to ascorbate deficient cells indicates either a block in the trans-

TABLE I. EFFECTS OF ADDITIONS TO THE GROWTH MEDIUM ON CELLULAR PROLIFERATION IN ENDOTOXIN-TREATED CELLS IN LATE LOGARITHMIC PHASE

Addition or modification	Relative cell number as % of control cultures	
	Minus endotoxin	Plus endotoxin
None (control)	100	46
L-Ascorbate, 25 μg/ml	106	92
L-Ascorbate, 50 μg/ml	112	95
L-Ascorbate, 75 μg/ml	60	40
L-Ascorbate, 100 μg/ml	40	37
D-Isoascorbate, 50 μg/ml	104	74
Dehydroascorbate, 50 μg/ml	64	50
Uncoupling agent (carbonylcyanide *m*-chlorophenylhydrazone), 1.5×10^{-4} *M*	93	62
Cycloheximide, 1.0 μg/ml	60	54

[a] Cultures were grown as described under Methods and treated with each modification for a 5-day period before the endotoxin challenge (100 μg/ml of culture medium). Cultures were harvested in late logarithmic phase (4 days) and triplicate determinations of cell counts were performed.

port of ascorbate into the cells, or endotoxin-induced cellular damage not easily reversed by ascorbate.

The use of ascorbic acid in periodontal disease therapy has a long history with very little conclusive evidence that it is of value. Since endotoxin is intimately involved in periodontal disease, its presence in the cementum of teeth will serve as a constant detriment to any attempt to successfully treat the disease. The clinical application of these findings suggest that adequate tissue levels of ascorbate are a necessary prerequisite against endotoxin from bacterial plaque or cementum. Ascorbate under these conditions would appear to have at least a twofold purpose in the management of periodontal disease. First, cellular protection against the toxicity of endotoxin, and, second, in the recovery and regeneration of connective tissues subjected to the disease process.

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