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Inhibition of Superoxide Production in Human Polymorphonuclear Leukocytes by Oral Treponemal Factors

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The inhibition of superoxide (O_2^-) production by human peripheral blood polymorphonuclear leukocytes (PMNs) in the presence of oral treponemes, their cellular components, and their culture supernatants was investigated. Superoxide production was inhibited 56% by a 25-μg/ml phenol extract of a human clinical isolate. Inhibition by culture supernatants of both the clinical isolate and a reference strain was related to the bacterial phase of growth and viability, though inhibition also persisted in the decline phase. Inhibition of superoxide production was not evident when either opsonized or nonopsonized whole spirochetes were reacted with PMNs. The suppressive activity depended, therefore, on the treponemes either being disrupted or growing and releasing the inhibitory factor into the culture medium. These results suggest that oral treponemes possess factors which interfere with the activity of PMNs and thereby alter the inflammatory process in the diseased periodontal pocket.

Several studies have suggested that oral spirochetes have a contributory role in the pathogenesis of periodontal diseases. These studies include observations that spirochetes are found primarily on the apical surfaces of subgingival plaque, in direct contact with the pocket epithelium and migrating leukocytes (20), and that they invade the gingival connective tissue in acute necrotizing ulcerative gingivitis (19). The number of oral spirochetes increases dramatically in periodontally diseased, as opposed to nondiseased, sites (3, 21, 22), but it is markedly reduced after periodontal treatment (24). It was also suggested in a study on the microflora of chronic periodontitis that specific treponemes are among "the most suspect species" of the flora in this disease (27).

With the advent of new culture techniques that facilitate the isolation and growth of these fastidious microorganisms (2, 7, 18, 23, 36), a number of in vitro studies have been performed that show the effects of oral treponemes on a variety of host cells (4, 22, 34, 38). Moreover, proteolytic and fibrinolytic properties have been identified with oral treponemes (15, 28, 41). Since polymorphonuclear leukocytes (PMNs) are the predominant host defense cells that interact with the pocket microflora (35, 39, 43), their specific functions after challenge with the potential pathogens (i.e., phagocytosis, enzyme release, chemotaxis, etc.) are being investigated. It is now becoming evident that the production of free radicals by phagocytic cells plays an important role in the killing of ingested microorganisms (1, 16). When PMNs are stimulated by bacteria, they undergo a respiratory burst characterized by increased oxygen consumption (1, 16). More than 90% of the oxygen consumed goes into the generation of superoxide free radicals (O_2^-) (J. C. Fantone and P. A. Ward, Current concepts, The Upjohn Co., p. 12, 1985). Superoxide is the primary source of hydrogen peroxide (H_2O_2) through spontaneous dismutation and through an enzyme-catalyzed dismutation with superoxide dismutase (10). Whereas O_2^- alone is not a potent bactericidal agent, H_2O_2 and its subsequent metabolites are a primary means by which phagocytes kill bacteria (1, 6, 16). Therefore, any mechanism inhibiting O_2^- production will directly affect the ability of phagocytic cells to destroy bacteria.

The purpose of the present study was to investigate the interaction of PMNs with oral treponemes, their cellular components, and their extracellular products in the process of oxygen metabolite production.

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MATERIALS AND METHODS

Bacterial samples. Samples of human subgingival fluid were obtained from periodontal pockets of at least 6 mm. After the pocket was isolated and the gingiva was dried, sterile paper points were inserted for approximately 15 s, removed, and placed into sterile capped vials that contained reduced transport fluid (0.3 ml per vial) (37). Within 5 min of taking the samples, they were processed in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) (see below).

Isolation of spirochetes. A 10-μl portion of the bacterial sample was placed on a 0.2-μm-pore-size Millipore filter disk (Millipore Corp., Bedford, Mass.) positioned on the surface of prereduced GM-1 agar plates containing 2 μl of rifampin per ml (18) (see below). The plates were incubated for 7 days in an anaerobic chamber in an atmosphere of 5% H_2, 10% CO_2, and 85% N_2. The filters were then removed with sterile forceps, and the plates were allowed to incubate for an additional 5 days; a sample of the hazy, cloudy spreading growth was then removed from the periphery of the haze with a sterile loop and placed in 5 ml of GM-1 broth. This tube was vortexed for 45 s, and 100 μl of the resultant solution was spread with an L-shaped sterile glass rod over the surface of a fresh GM-1 plate. This plate was incubated for 7 days, after which isolated colonies of treponemes appeared. Colonies of similar and dissimilar morphology were isolated by conventional subculturing techniques. The

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colonies were subcultured, and isolated cells were cloned at least four times. Purity was confirmed by dark-field and transmission electron microscopy. The latter used a conventional negative-stain technique with 1% phosphotungstic acid (14).

Media. The isolation of treponemes was performed on both GM-1 (2) and NOS (18) media. The results reported in the present study relate to a clinical isolate grown in GM-1 medium. ATCC reference strain 35405 was grown in NOS medium. Growth curve profiles were established using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Long Island City, N.Y.). The cultures were harvested after 15 days of growth and processed to yield the different oral treponemal components.

Preparation of human leukocytes. Experiments were performed with preparations containing approximately 70% neutrophils obtained from freshly drawn normal adult whole blood in adenine, citrate, and dextrose, kindly provided by the Hadassah Hospital Blood Bank, Jerusalem, Israel. The leukocytes were separated by mixing 10 ml of blood with 1.5 ml of 6% dextran (Sigma Chemical Co., St. Louis, Mo.; molecular weight, 225,000) in saline. Erythrocytes were lysed by adding 3 ml of distilled water for 30 s and then washed with 0.8% saline. The washed leukocytes were suspended in Hanks balanced salt solution buffered with 3 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.33) or in Hanks balanced salt solution with HEPES to which 10 mM sodium azide (NaN_3) was added (40). The leukocyte suspensions were kept in polypropylene tubes in crushed ice. The viability of each leukocyte harvest was measured by the trypan blue exclusion technique and by the amount of lactate dehydrogenase released from leukocytes treated with and without the treponemal components.

Determination of superoxide (O_2^-). Superoxide was measured as the superoxide dismutase-inhibitable cytochrome *c* reduction by the method of Babior (1). Reaction mixtures contained 1.5×10^6 leukocytes in 0.8 ml of Hanks balanced salt solution with 10 mM NaN_3 and the different spirochetal components at various concentrations (see below) were incubated for 10 min at 37°C. Thereafter, 50 μl of group A streptococci (type 3, strain C203S) opsonized with histone (type II-A, 10,000 molecular weight; Sigma), cytochalasin B (2.5 μg/ml; Makor Chemicals, Jerusalem, Israel) (11), and 80 μM cytochrome *c* (type III; Sigma) were added and incubated for an additional 10 min at 37°C. Superoxide dismutase (30 μg/ml; Sigma) was added to the controls. Reaction mixtures also included preincubation of the various culture supernatants with the challenge bacteria before or simultaneously with the PMNs. The final volume for experimental and control vials was 1 ml. The reaction mixtures were centrifuged for 5 min at $1,000 \times g$, and the supernatant fluids were read at 575, 550, and 525 nm in a Kontron/Uvikon 810P double-beam spectrophotometer. The concentration of superoxide production was calculated from the formula $E_{550} = 2.1 \times 10^{-4}\ M^{-1}$ by the method of Babior (1) and expressed as nanomoles per 10^6 cells per 10 min. The inhibition of O_2^- production, observed after incubation of the different spirochetal components with the leukocytes, was expressed as the percentage of O_2^- produced when leukocytes were challenged with histone-opsonized streptococci alone.

Determination of hydrogen peroxide (H_2O_2). H_2O_2 was determined by the method of Thurman et al. (40). The reaction mixtures were the same as those described for superoxide determination, but without cytochrome *c* and superoxide dismutase. Briefly, after incubation of 1.5×10^6 leukocytes in 0.8 ml of Hanks balanced salt solution with 10 mM NaN_3 for 10 min at 37°C with the different spirochetal components, 50 μl of histone-opsonized streptococci and 2.5 μg of cytochalasin B per ml were added to a final volume of 1 ml and incubated for an additional 10 min at 37°C. Thereafter, 200 μl of 30% trichloroacetic acid was added to each test tube. The supernatants were transferred to clean test tubes, and 200 μl of $FeNH_4(SO_4)_2 \cdot 12H_2O$ (19 mg/ml of water) and 100 μl of 25% KSCN were then added. The contents of the tubes were thoroughly mixed, incubated for 5 min at room temperature, and centrifuged at $1,000 \times g$ for 5 min. The brown-red color which developed was read in the spectrophotometer at 480 nm. Results were expressed as nanomoles of H_2O_2 per 1.5×10^6 leukocytes per 10 min, according to a standard curve of H_2O_2. The inhibition of H_2O_2 production obtained by incubating the different spirochetal components with the leukocytes was expressed as a percentage of H_2O_2 produced when leukocytes were challenged with streptococci opsonized by histone alone.

Extraction of oral treponemal components. Growth medium supernatant (GMS) was separated from the bacterial pellet after various growth periods by centrifugation at $10,000 \times g$ for 30 min. Growth medium kept in the Coy anaerobic chamber for the same number of days underwent similar centrifugation. GMS and growth medium were heated at 56 and 98°C (boiling) for 30 min and treated with trypsin in an initial attempt to characterize the O_2^- inhibitory factor found in the treponemal supernatants.

The bacterial pellet was washed and suspended in 0.5 ml of normal saline at an optical density of 200 Klett units per ml, read at 540 nm. The suspension was then opsonized at 37°C for 30 min with fresh normal human serum at a final concentration of 10% (vol/vol).

Sonic extracts of the two oral treponemes and of *Actinobacillus actinomycetemcomitans* Y4 and *Capnocytophaga sputigena* 4 were obtained by disrupting lyophilized bacteria suspended in 4 ml of saline with a Sonicator cell disruptor (Heat Systems Ultrasonics, Inc., Plainview, N.Y.). In each case, samples were examined by dark-field microscopy to ensure homogeneous sonication. The sonicates were then centrifuged at $10,000 \times g$ for 30 min, and the supernatants were dialyzed against double-distilled water for 3 days with water changes twice daily. Lyophilization was performed, followed by protein content analysis with the Bio-Rad protein kit (Bio-Rad, Chemical Div., Richmond, Calif.).

Phenol extracts of the two oral treponemes and lipopolysaccharides (LPSs) of the two nontreponemal microorganisms were obtained by the method of Nowotny (29). Briefly, lyophilized treponemes were mixed with an equal volume of distilled water and 90% phenol in a 70°C water bath. After centrifugation at $3,000 \times g$ for 30 min, the upper phase was separated and the extraction procedure was repeated twice. The water phases were pooled and dialyzed against distilled water in the cold room for 3 days. After lyophilization, the crude extract was dissolved in cold methanol that contained 20% $MgCl_2$ in ethanol. The preparation was left standing in a cold room for 1 to 3 days for a precipitate to develop. After centrifugation at $3,000 \times g$ for 30 min, the sediment was dissolved in distilled water that contained cold methanol without $MgCl_2$. After standing again in the cold room for 1 to 3 days, the sediment was centrifuged, and dissolution was carried out for a third time as described above. The final sediment was dissolved in distilled water and lyophilized. This procedure was repeated to remove the methanol from the solution. For further purification, the extracts were centrifuged at 100,000 to 110,000 $\times g$ for 2 h. Protein content analysis was done as described above.

Statistical analysis. All the data were analyzed using the Student *t* test (25). A value of $P \leq 0.05$ was accepted as significant.

RESULTS

The results presented in the present study are from one of our clinical isolates, whose morphology is similar to that of *Treponema denticola*, with an axial fibril configuration of 2-4-2 and a protoplasmic cylinder width of 0.25 μm. The *T. denticola* reference strain described here has a similar morphology.

Cell viability in response to spirochete extracts and supernatants. PMNs incubated with either sonic extracts, phenol extracts, or supernatants of the spirochetes tested showed very little evidence of cell death. In all cases, no more than 6% of the PMNs counted showed uptake of trypan blue, and lactate dehydrogenase release was no more than 10% in any given assay (control was 8.9%).

Inhibition of superoxide production by human PMNs after interaction with extracts from oral treponemes. The inhibitory effects of oral treponemal components on the production of O_2^- free radicals when reacted with human peripheral blood PMNs are shown in Fig. 1. The phenol extracts of the two oral treponemes were very effective. At a final concentration of 25 μg/ml, the phenol extracts of the clinical isolate suppressed production of O_2^- by approximately 56%. In comparison, the reference strain treponeme caused a 52% inhibition. On the other hand, the same concentration of *A. actinomycetemcomitans* or *C. sputigena* LPS showed absolutely no inhibition. Moreover, *C. sputigena* LPS showed an 8% activation (data not shown). It took a fourfold increase in the concentration of *A. actinomycetemcomitans* LPS to approximate the effect of the two oral treponeme phenol extracts at 25 μg/ml. *C. sputigena* LPS at 100 μg/ml showed approximately 11% inhibition. At this concentration, the *T. denticola* phenol extracts registered an 80% inhibition of superoxide production. The sonic extracts were less inhibitory: at a final concentration of 100 μg/ml, the clinical isolate showed 28% inhibition, whereas the reference strain showed

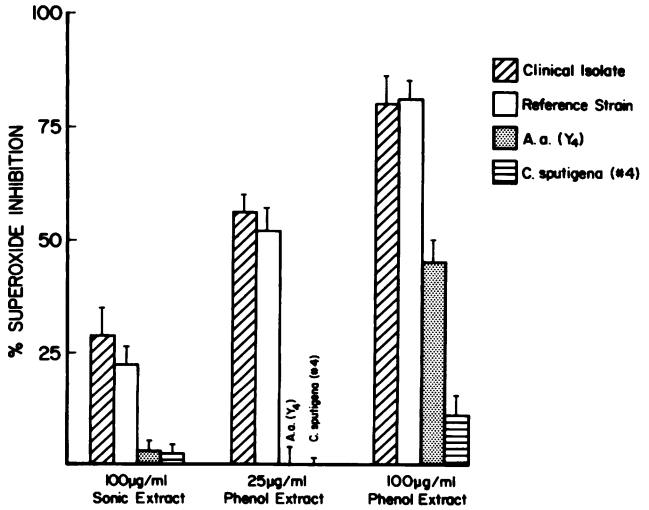

FIG. 1. Percent inhibition of superoxide production by extracts of oral treponemes and nontreponemal organisms. Each bar represents the mean of three experiments done in duplicate plus standard error. In all cases, $P \leq 0.001$ versus control. A.a., *A. actinomycetemcomitans*.

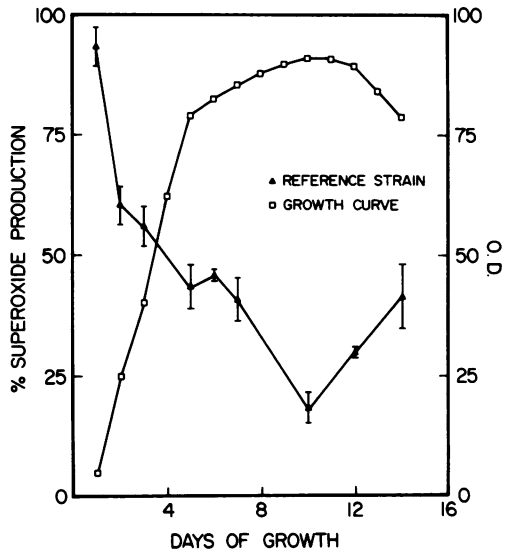

FIG. 2. Percent of superoxide production by 25 μl of reference strain culture supernatant. Each point represents the mean of four experiments done in duplicate plus standard error. In all cases, $P \leq 0.001$ versus control. The growth curve of the reference strain is also shown. O.D., optical density.

approximately 23% inhibition. In keeping with the negligible inhibition evinced by the two nontreponemal microorganisms, sonic extracts of *A. actinomycetemcomitans* caused only 3% inhibition and *C. sputigena* showed 2.5% inhibition. In contrast to the above results demonstrating the inhibition of superoxide production by extracts from oral treponemes, whole unopsonized spirochetes, both clinical isolate and reference strain, showed a negligible degree of inhibition (3 to 8%) and serum-opsonized spirochetes triggered a 25% activation in O_2^- production (data not shown). Serum alone enhances O_2^- production in PMNs (8).

Inhibition of superoxide production by PMNs after interaction with GMS. Culture supernatants (25 μl/ml of reaction mixture) of the two oral treponemes were potent inhibitors of O_2^- production (Fig. 2 and 3). A direct correlation between the increase in growth of the reference strain spirochete and the concomitant increase in inhibition of O_2^- production is shown in Fig. 2. A marked increase in inhibition was evinced from day 4 to day 10; the inhibition decreased thereafter. The most dramatic inhibition occurred during the exponential growth phase (days 1 to 5), showed a slight decrease during the retardation phase (days 5 to 6), increased during the onset of the stationary phase (days 7 to 10), and demonstrated a decrease in O_2^- inhibition toward the end of the stationary phase and the beginning of the decline phase (days 11 to 14). A similar activity profile for the clinical isolate is shown in Fig. 3. In addition, the relatively old culture supernatants of 22 and 34 days of growth maintained a superoxide inhibitory potential of approximately 48% long after the spirochetes ceased to proliferate. While less inhibitory than the 6- or 12-day growths, this inhibition was still significant.

When dose-response experiments were performed, the GMSs of both treponemes were inhibitory at concentrations as low as 1 μl/ml of reaction mixture. At this concentration, 7 to 10% O_2^- inhibition was evinced for both supernatants. Concentrations beyond 50 μl/ml of reaction mixture produced 100% inhibition in both cases. Preincubation of the various culture supernatants with the PMNs or simultaneous

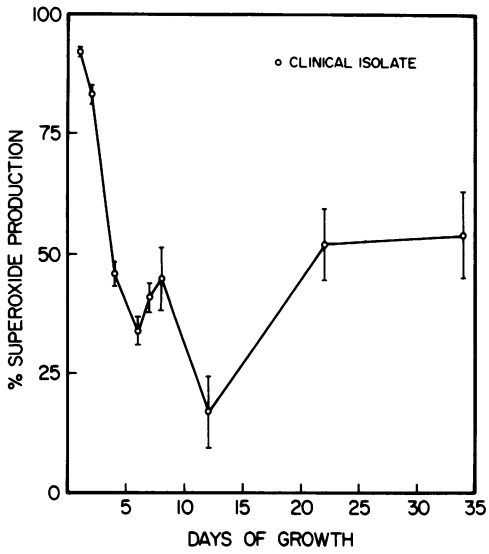

FIG. 3. Percent of superoxide production by 25 μl of clinical isolate culture supernatant. Each point represents the mean of four experiments done in duplicate plus standard error. In all cases, $P \leq 0.001$ versus control.

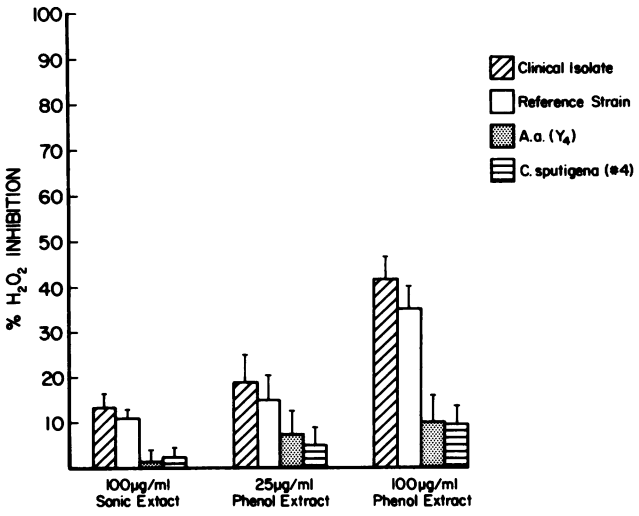

FIG. 4. Percent inhibition of H_2O_2 production by extracts of oral treponemes and nontreponemal organisms. Each bar represents the mean of three experiments done in duplicate plus standard error. In all cases, $P \leq 0.001$ versus control. A.a., *A. actinomycetemcomitans*.

incubation with the reaction mixture components gave similar results. However, when opsonized streptococci were preincubated with the various culture supernatants, washed, and reacted with PMNs, no inhibition of O_2^- production was observed.

H_2O_2 inhibition by oral treponemal extracts and GMS. Profiles of H_2O_2 production by peripheral blood PMNs treated with the spirochetal extracts and GMS indicated an inhibitory trend. Table 1 shows that on days 9, 14, and 16, the clinical isolate GMS was most inhibitory, approximating 50%. The reference strain was less inhibitory, with a mean inhibition of 36% for the same days of growth. However, when all data from days 3 through 28 were examined together, the mean inhibitions of H_2O_2 by both oral treponeme GMSs, 32% for the reference strain and 36% for the clinical isolate, was quite similar. Moreover, relatively old GMS (28 days) maintained H_2O_2 inhibitory potentials for the clinical isolate and the reference strain of 38% and 28%, respectively. The only effective inhibition of H_2O_2 production by the spirochetal extracts was evinced by their phenol extracts at a final concentration of 100 μg/ml (Fig. 4). At this concentration, the clinical isolate produced a 41.5% inhibition, whereas the reference strain showed a 35% inhibition. However, in relation to the two nontreponemal microorganisms, the two oral treponemes continued to demonstrate consistently more inhibition of free-radical production (Fig. 1 and 4).

Heating and trypsinization of the culture supernatants. Initial results, after the actively inhibitory culture supernatants of both *T. denticola* strains were heated at 56°C for 30 min and boiled for 30 min, showed that the factor probably responsible for the inhibition of superoxide production is thermostable. On the other hand, trypsinization of these culture supernatants reduced inhibitory activity by 60%.

DISCUSSION

The results of this study suggest the presence of an oral treponemal factor (OTF) which inhibits O_2^- production in human peripheral blood PMNs without any detrimental effect on PMN viability. Inhibition of superoxide production, while observed to some degree with the treponemal sonic extracts, was demonstrated mainly with the phenol extract and the culture supernatants. On the other hand, we found no inhibitory activity when PMNs were incubated with whole opsonized or nonopsonized spirochetes. The suppressive activity, therefore, depended on the spirochetes either being disrupted or growing and releasing the inhibitory factor into the medium. The increased suppressive effect on O_2^- production was initially directly related to oral treponemal growth and persisted without the need for treponemal viability (Fig. 2 and 3), even in the decline phase (22 and 35 days; Fig. 3). While lower than suppression during the early stages of growth, this suppression in O_2^- production plateaued to a significant 48%. These findings suggest (i) that the OTF found in the culture supernatant is released by the spirochetes during the exponential growth phase, (ii) that OTF activity is not lost from the medium even during the

TABLE 1. Percent inhibition of H_2O_2 by reference strain and clinical isolate treponeme GMSs[a]

Strain	% Inhibition[a] at day:									Mean cumulative % inhibition
	3	5	7	9	12	14	16	19	28	
Reference strain	29.8 ± 3.0	31.0 ± 2.0	20.0 ± 7.0	32.3 ± 4.0	31.0 ± 3.5	40.0 ± 2.0	36.0 ± 4.5	42.0 ± 4.5	28.0 ± 4.0	32.2 ± 3.8
Clinical isolate	26.5 ± 4.2	17.0 ± 7.0	21.3 ± 3.0	49.4 ± 5.0	34.0 ± 3.0	57.0 ± 5.2	48.4 ± 6.2	36.0 ± 3.5	37.9 ± 5.4	36.0 ± 4.7

[a] Mean ± standard error of four experiments done in duplicate. $P \leq 0.001$ versus control by the Student *t* test (25).

decline phase, and (iii) that the activity found during the decline phase could be due to components of the spirochetes, e.g., LPS-like macromolecules, which are liberated only during and after the disruption of the bacteria. This phenomenon is being further investigated.

Since in initial experiments each culture supernatant was preincubated with PMNs before challenge with opsonized streptococci, it was postulated that the site for OTF activity was the PMN itself. To exclude the possibility that the inhibition could have been due to attachment of OTF to the histone-opsonized streptococci, thereby affecting receptor recognition on the PMN surface membrane, the various culture supernatants were preincubated with the challenging bacteria (followed by washing) before reacting with the PMNs. The results showed that there was no interference in O_2^- production by the supernatants, indicating that the inhibitory effect was not a result of OTF affinity for the opsonized streptococci, but rather of affinity for the PMN itself. Furthermore, pretreatment of PMNs with GMSs was not a prerequisite for the inhibition of O_2^- production. Simultaneous incubation of PMNs, GMS, and opsonized streptococci caused an inhibition of O_2^- production of the same magnitude as when PMNs were pretreated with culture supernatant. This shows that OTF is capable of competitive inhibition with potential bacterial stimulants, a situation which could be of pathogenic importance in the periodontal pocket.

The current consensus is that O_2^- is formed by a one-electron reduction of molecular oxygen and is then dismutated to H_2O_2, with a theoretical quantitative ratio of two O_2^- to one H_2O_2 (1, 13, 17, 26, 30–32). Our results indicate that in some cases there was inhibition approximating this ratio (Fig. 1 and 4; sonic and phenol extracts of 100 μg/ml for the oral treponemes). However, in other cases this ratio was not upheld (Fig. 1 and 4 phenol extracts of 25 μg/ml for the oral treponemes and phenol extracts for *A. actinomycetemcomitans* and *C. sputigena*). Moreover, this ratio was not always maintained in the inhibition of H_2O_2 production by the two oral treponemal culture supernatants when compared with superoxide inhibition (Fig. 2 and 3, Table 1). These discrepancies are not fully understood. However, we did notice that variability in donor PMN responses to the same stimuli was most pronounced in those cases where inhibition of O_2^- was less than 20%. Moreover, not all stimuli of the respiratory burst induce the generation of equivalent amounts of superoxide and hydrogen peroxide (9, 13, 31, 32). Furthermore, it was recently demonstrated that much larger amounts of H_2O_2 than O_2^- were produced by histone-opsonized streptococci (12), suggesting that less inhibition of H_2O_2 in relation to O_2^- would ensue if an inhibitory agent were employed. It has been further suggested that, in histone-opsonized streptococci, peroxide might not only originate by dismutation from O_2^-, but might also be generated by a two-electron reduction of molecular oxygen by pathways analogous to those of glucose oxidase (12). These observations could explain in part the in vitro discrepancy in those cases where the ratio of inhibition did not approximate 2:1.

It has recently been suggested that succinic acid, a small-molecular-weight fatty acid and a major by-product of *Bacteroides* metabolism, is responsible for inhibiting superoxide production in PMNs (33). Our preliminary results of the characterization of the OTF seem to indicate a thermostable proteinlike substance responsible for the inhibition of superoxide production. Moreover, succinic acid was effective in inhibiting the neutrophil respiratory burst only at pH 5.5 (33), while the OTF is effective at neutral pH. Furthermore, succinic acid is not a major metabolic by-product of *T. denticola* (5). Further work on the isolation and characterization of the OTF is under way.

Disease-related properties of oral treponemes have been determined in vitro in recent years. *T. denticola* possesses proteolytic (15) and fibrinolytic properties (28), has a trypsinlike activity (22, 41), suppresses lymphocyte blastogenesis by mitogens (34), inhibits fibroblast proliferation (4), and, together with *T. vincentii*, is cytotoxic to epithelial cells (P. Baehni and G. Cimasoni, J. Dent. Res. **65**:767, abstr. no. 372, 1986). Recently, it was suggested that oral spirochetes "may limit fusion of lysosomes to phagosomes" by demonstrating a significant decrease in zymosan-induced degranulation when PMNs were pretreated with soluble extracts of spirochetes (3). Moreover, recent data on acute pericoronitis demonstrated that, although spirochetes are the predominant microorganisms in the exudates, they are not observed being phagocytosed by PMNs (42). In addition, whereas phagocytosis of other bacteria by PMNs was observed in all the exudates, lysis of the host PMNs was seen without eradication of the ingested bacteria (42). These in vivo observations, together with the in vitro results of Boehringer et al. (3), suggest that oral treponemes may interfere with ongoing neutralization of microorganisms by human PMNs. The results presented here further strengthen this contention. The significant inhibition of O_2^- production by the OTF and other oral treponemal components may alter the ability of PMNs to dispose of other noxious microorganisms, thereby enhancing the periodontal disease process. Therefore, it is important to determine the OTF mechanism of action and to learn whether it affects other cellular activities.

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