INFECTION AND IMMUNITY, Dec. 1984, p. 644–648
0019-9567/84/120644-05$02.00/0

Vol. 46, No. 3

Resistance of *Actinobacillus actinomycetemcomitans* and Differential Susceptibility of Oral *Haemophilus* Species to the Bactericidal Effects of Hydrogen Peroxide

K. T. MIYASAKI,* M. E. WILSON, H. S. REYNOLDS, AND R. J. GENCO

Department of Oral Biology, State University of New York at Buffalo, Buffalo, New York 14214

Received 28 March 1984/Accepted 22 August 1984

We compared the sensitivities of oral and nonoral isolates of *Actinobacillus actinomycetemcomitans*, *Haemophilus segnis*, *H. aphrophilus*, and *H. paraphrophilus* to the bactericidal action of reagent hydrogen peroxide (H_2O_2). Susceptibility to a range of H_2O_2 concentrations (10^{-6} to 10^{-3} M) was assessed by incubating bacterial suspensions for 1 h at 37°C in the presence of H_2O_2 and plating on chocolate agar to determine the concentration of H_2O_2 that would produce a 50% reduction in CFU (50% lethal dose). As a group, *A. actinomycetemcomitans* was more resistant to H_2O_2 than the oral haemophili, and *H. aphrophilus* was much more sensitive than all other organisms tested. The range of 50% lethal dose values for *A. actinomycetemcomitans* was between 8.5×10^{-5} and 10^{-3} M H_2O_2 or above. In contrast, *H. aphrophilus* exhibited 50% lethal dose values from below 1×10^{-6} to 3.4×10^{-4} M H_2O_2. The resistance of *A. actinomycetemcomitans* to H_2O_2 may be sufficient to protect these organisms from direct H_2O_2-mediated killing by host phagocytes.

The oral haemophili (*Haemophilus segnis*, *H. aphrophilus*, and *H. paraphrophilus*) and *Actinobacillus actinomycetemcomitans* are closely related, gram-negative, non-heme-requiring capnophilic bacteria that comprise part of the normal microflora of dental plaque (3, 11). The pathogenic potential of these organisms is regarded as low, although under certain circumstances they are capable of producing endocarditis, brain abscesses, and orofacial and bite wound infections (3). *A. actinomycetemcomitans* may also participate synergistically in actinomycotic lesions (3). Increases in the proportion of *A. actinomycetemcomitans* in plaque have been associated with localized juvenile periodontitis, a rapidly advancing form of periodontitis (5, 10). Such behavior in the oral environment has not been demonstrated in *H. aphrophilus* or *H. paraphrophilus*, indicating that there may be differences in periodontopathogenic potential among these organisms.

One explanation for this difference in pathogenicity may be a differential susceptibility of these bacteria to toxic oxygen metabolites, especially hydrogen peroxide (H_2O_2). Normal tissue levels of H_2O_2 are between 10^{-9} and 10^{-7} M (2). Both host leukocytes (principally polymorphonuclear and mononuclear phagocytes) and certain plaque bacteria, such as streptococci, generate much higher concentrations of H_2O_2, an oxygen metabolite that is known to be important to host oxidative bactericidal mechanisms (1, 6, 8, 12). Furthermore, H_2O_2 may be applied topically in high local concentrations in treating various forms of periodontal disease (9, 13, 14). Therefore, the ability of microbes to resist the killing or static effects of H_2O_2 may be an important determinant of pathogenicity and resistance to antimicrobial therapy.

The purpose of the present study was to determine the sensitivities of *A. actinomycetemcomitans* and related oral haemophili to the bactericidal effects of H_2O_2. We found that significant differences in sensitivity to H_2O_2 exist among the bacteria studied. In general, strains of *A. actinomycetemcomitans* were most resistant, whereas strains of *H. aphrophilus* were least resistant.

* Corresponding author.

MATERIALS AND METHODS

Bacteria. The bacteria used in this study are listed in Table 1. *A. actinomycetemcomitans* strains 650, 651, 652, and 653 were provided by Anne Tanner of the Forsyth Dental Center (11); strains 650, 651, and 653 are catalase deficient. *H. segnis* strains HK497, HK498, HK499, and HK500 were provided by Mogens Kilian of the Royal Dental College in Aarhus, Denmark (4). *H. segnis* strains 106, 336, 1144, and 1228 and *H. aphrophilus* strains PTB1, PTB30, PT22, PT74, PT90, and PT154 were isolated by Paulette Tempro in our laboratories, and the characterization of these strains will be published elsewhere (P. Tempro, J. Zambon, and J. Slots, manuscript in preparation). Cultures received from other laboratories were screened for biochemical characteristics by using the API 20E test strip (Analytab Products, Plainview, N.Y.). Fresh clinical strains were passed no more than three times after initial isolation, and samples were stored frozen at −70°C in 0.5% tryptone (Difco Laboratories, Detroit, Mich.) 0.14 M NaCl–0.5% bovine serum albumin (Pentex; Miles Laboratories, Inc., Elkhardt, Ind.)–10% glycerol (Fisher Chemical Co., Pittsburgh, Pa.) until assayed for sensitivity to H_2O_2. Bacteria were grown for 1 day at 37°C in 10% CO_2 on chocolate agar, which consisted of 5% hemolyzed defibrinated horse blood, 5 μg of equine hemin III (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.001% menadione (Sigma), 0.1% yeast extract (Difco), and 1% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) supplement in trypticase soy agar (BBL).

Bacterial suspensions. Initially, bacterial cell concentrations used in all assays were determined by counting particles in a Petroff-Hausser chamber (Petroff-Hausser, Blue Bell, Pa.). Subsequently, it was found that particle counts corresponded reproducibly to turbidimetric readings at 540 nm. In general, 10^9 bacterial particles corresponded to an optical density at 540 nm of 0.3.

Bactericidal activity assay. The bactericidal assay used in this study was adapted from a method described elsewhere

TABLE 1. Bacteria used in this study

Species and strain	Origin[a]	Comment
A. actinomycetemcomitans		
650	FDC, dental plaque	Localized juvenile periodontitis
651	FDC, dental plaque	Localized juvenile periodontitis
652	FDC, dental plaque	Papillon Lefevre
653	FDC, dental plaque	Papillon Lefevre
Y4	FDC, dental plaque	Localized juvenile periodontitis
67	SUNYAB, dental plaque	Localized juvenile periodontitis
Syl	SUNYAB, dental plaque	Localized juvenile periodontitis
Gar	SUNYAB, dental plaque	Localized juvenile periodontitis
Cra	SUNYAB, dental plaque	Healthy juvenile
9709	NCTC	Lumbar abscess
9710	NCTC	Lung abscess
29522	ATCC	Mandibular abscess
29523	ATCC	Septicemia
H. aphrophilus		
5906	NCTC	Endocarditis
5907	NCTC	Endocarditis
13252	ATCC	Unknown origin
19415	ATCC	Endocarditis
PTB1	SUNYAB, dental plaque	Localized juvenile periodontitis
PTB30	SUNYAB, dental plaque	Healthy gingiva
PT22	SUNYAB, dental plaque	Adult periodontitis
PT74	SUNYAB, dental plaque	Localized juvenile periodontitis
PT90	SUNYAB, dental plaque	Adult periodontitis
PT154	SUNYAB, dental plaque	Healthy gingiva
H. paraphrophilus		
29240	ATCC	Parietal abscess
29241	ATCC	Paronychia
29242	ATCC	Trachae
H. segnis		
10977	NCTC, dental plaque	Kilian HK 316
106	SUNYAB, dental plaque	Healthy gingiva
336	SUNYAB, dental plaque	Adult periodontitis
1144	SUNYAB, dental plaque	Localized juvenile periodontitis
1228	SUNYAB, dental plaque	Adult periodontitis
HK497	RDCA, saliva	
HK498	RDCA, saliva	
HK499	RDCA, saliva	
HK500	RDCA, saliva	

[a] FDC, Forsyth Dental Center; SUNYAB, State University of New York at Buffalo; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; RDCA, Royal Dental College, Aarhus, Denmark.

for enteric bacteria (7). Briefly, bacteria were diluted to a final concentration of 10^4 cells per ml in a diluent consisting of 0.5% tryptone, 0.14 M NaCl, and 0.5% bovine serum albumin (TSBSA). The concentration of a 30% solution of H_2O_2 (Fisher) was determined by absorbance at 230 nm and an extinction coefficient of 81 cm^{-1} M^{-1}. Serial dilutions of H_2O_2 were made in TSBSA. The bacterial suspension (0.1 ml) and the H_2O_2 solution (0.1 ml) were mixed in the wells of a microtiter plate in triplicate. The microtiter plate was then placed in a 37°C incubator for 1 h, after which 0.05 ml from each well was spread onto a chocolate agar plate containing 20 ml of solid medium. The number of CFU per plate was determined after incubating the plates for 2 days at 37°C in 5% CO_2. Fifty percent lethal dose (LD_{50}) values were determined graphically by plotting the percentage of control CFU as a function of log concentration of H_2O_2. The kinetics of peroxide-mediated bacterial death was examined by mixing 2.0 ml of H_2O_2 in TSBSA with 2.0 ml of bacterial cell suspension (10^4 cells/ml) in polystyrene tubes. In kinetic experiments, both the bacterial cell suspension and the H_2O_2 solution were prewarmed at 37°C, and the reactions were conducted in a 37°C water bath.

RESULTS

Dose response of bacterial survival with various H_2O_2 concentrations. Figure 1 illustrates the bactericidal effects of H_2O_2 at various concentrations. Three representative oranisms, *H. aphrophilus* strain 19415, *H. segnis* strain 1228, and *A. actinomycetemcomitans* strain 29523, are shown. The dose-response curves were generally sigmoidal as a function of the log H_2O_2 concentration, with 0 to 100% killing observed over a range of H_2O_2 concentrations of about 1 to 2 orders of magnitude. Often, the more resistant organisms exhibited broader ranges (compare strains 1228 and 29523). On the other hand, strains of *H. aphrophilus* exhibited a distinctly different level of susceptibility to H_2O_2 (compare strain 19415 with either strain 1228 or 29523).

Susceptibility of bacteria to the bactericidal effects of H_2O_2. The susceptibilities of 35 strains of bacteria to the lethal effects of H_2O_2 are compared in Table 2. Strains of *H. aphrophilus* were most sensitive to H_2O_2, with LD_{50} values usually below 1.2×10^{-5} M. Relatively greater resistance to H_2O_2 bactericidal effects was observed in the other bacteria studied. As a group, *A. actinomycetemcomitans* strains

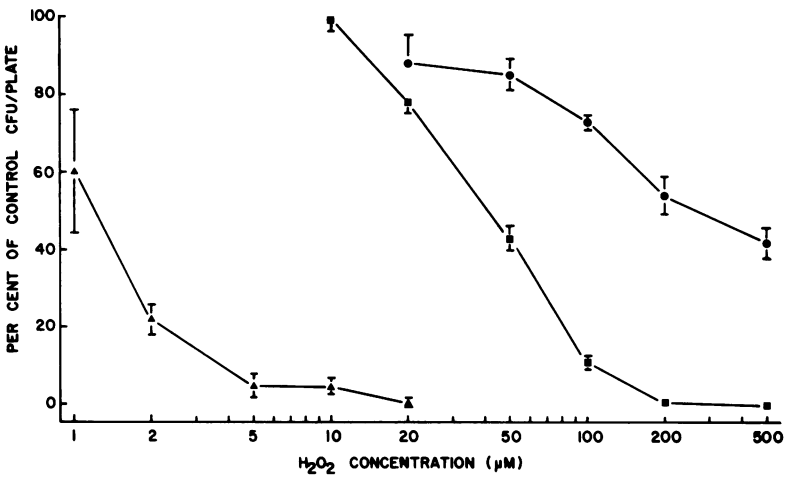

FIG. 1. Killing of three bacterial strains as a function of H_2O_2 concentration: (●) *A. actinomycetemcomitans* 29523, (■) *H. segnis* 1228, (▲) *H. aphrophilus* 14915. Bacterial cells at a concentration of 5×10^3 cells per ml were incubated at 37°C in TSBSA for 1 h in the presence of various concentrations of H_2O_2. Each point represents the mean of three trials, and each vertical bar represents one standard deviation.

were most resistant, often with LD_{50} values approaching 10^{-3} M. Recent clinical isolates of either *H. segnis* (strains 106, 336, 1144, and 1228) or *A. actinomycetemcomitans* (strains Syl, Gar, and Cra) exhibited no more resistance to H_2O_2 than did strains that had been maintained in laboratory culture for years. Two fresh clinical isolates of *H. aphrophilus*, designated PT154 and PT90, exhibited much higher resistance to H_2O_2 than most of the other strains of *H. aphrophilus*, including the four other recent isolates (PTB1, PTB30, PT22, and PT74).

Kinetics of H_2O_2-mediated bacterial killing. The kinetics of H_2O_2-mediated bacterial killing was examined using tubes rather than microtiter plates, since temperature could be controlled more readily in tubes maintained in a water bath. The time course of bactericidal action by different concentrations of H_2O_2 was followed for 90 min for four representative organisms (Fig. 2). In TSBSA in the absence of H_2O_2, no significant replication or loss of viability was observed in any of the four strains tested. Under the conditions of this assay, the kinetics of killing by H_2O_2 was usually not a simple logarithmic function of time. Higher concentrations of H_2O_2 resulted in more rapid killing, showing that LD_{50} values reflected differences in reaction rates as well as capacities.

DISCUSSION

Oral and nonoral strains of *A. actinomycetemcomitans*, *H. segnis*, *H. aphrophilus*, and *H. paraphrophilus* exhibit marked differences in sensitivity to the bactericidal effect of H_2O_2. In general, *A. actinomycetemcomitans* strains were most resistant, exhibiting LD_{50} values from 8.5×10^{-5} to approximately 1.0×10^{-3} M H_2O_2. In contrast, *H. aphrophilus* strains were least resistant, with LD_{50} values from less than 1.0×10^{-6} to 3.4×10^{-4} M H_2O_2. Fresh clinical isolates were no more resistant to H_2O_2 than were reference strains. For example, the reference strains of *A. actinomycetemcomitans*, ATCC 29522 and 29523, exhibited LD_{50} values comparable to those of the fresh clinical isolates Syl and Gar. The range of LD_{50} values exhibited by *A. actinomycetemcomitans* and the oral haemophili suggest that none would be killed by normal tissue levels of H_2O_2. However, most of the strains of *H. aphrophilus* would be sensitive to H_2O_2 levels generated within the phagolysosome of phagocytes, which probably approaches 10^{-4} M, based on the

TABLE 2. Susceptibility of *A. Actinomycetemcomitans* and the oral haemophili to the bactericidal effects of H_2O_2

Species and strain[a]	LD_{50}[b]
A. actinomycetemcomitans	
67	85
9710	85
9709	130
651	160
29522	160
Syl[c]	200
29523	280
Gar[c]	290
Y4	760
652	800
650	900
Cra[c]	1,000
653	>1,000
H. paraphrophilus	
29240	110
29242	130
29241	160
H. aphrophilus	
13252	<1.0
19415	1.3
PT22[c]	2.9
PT74[c]	3.0
PTB30[c]	3.4
5906	4.4
5907	7.5
PTB1[c]	12
PT154[c]	170
PT90[c]	340
H. segnis	
1228[c]	35
HK500	50
10977	80
106[c]	100
1144[c]	100
HK499	110
HK497	160
HK498	170
336[c]	800

[a] Strains are ranked according to susceptibilities.

[b] LD_{50} is the H_2O_2 concentration in molar concentration (μM) required to produce a 50% decrease in CFU after 1 h of incubation at 37°C in TSBSA, relative to controls incubated for 1 h at 37°C in TSBSA.

[c] Fresh clinical isolate.

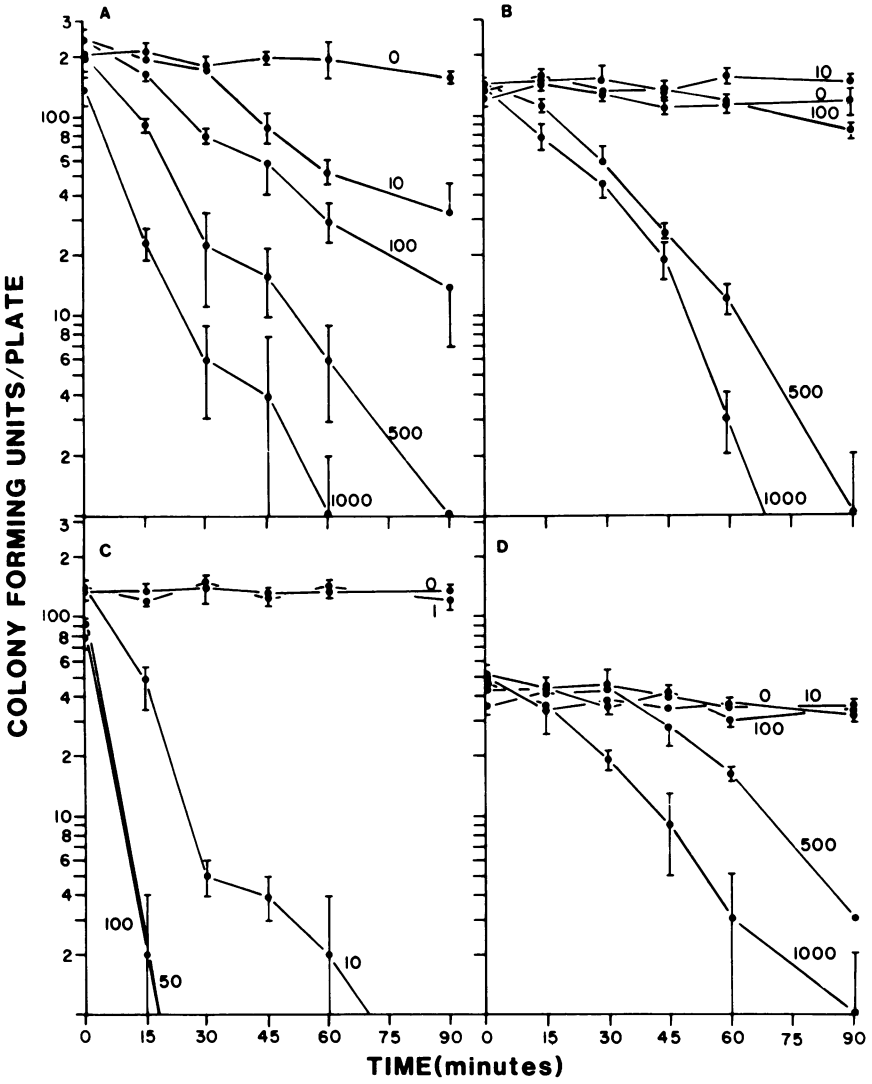

FIG. 2. Kinetics of H_2O_2-mediated bacterial killing in four bacterial strains: (A) *A. actinomycetemcomitans* 29523, (B) *H. paraphrophilus* 29241, (C) *H. aphrophilus* 13252, and (D) *H. segnis* 336. Bacterial cells at a concentration of 5×10^3 cells per ml were incubated at 37°C in TSBSA in the presence of various concentrations of H_2O_2. The concentrations of H_2O_2 in micromoles per liter are designated within each graph. Each point represents the mean of three trials, and each vertical bar represents one standard deviation.

concentrations of H_2O_2 that appear to be optimal for certain phagocyte enzymes (16).

Three serotypes (*a*, *b*, and *c*) of *A. actinomycetemcomitans* have been isolated from the dental plaque of patients with localized juvenile periodontitis (15). Serotype *c* organisms are infrequently isolated compared with serotype *b* organisms (and, to a lesser extent, serotype *a* organisms) (15). Although no differences in sensitivity to H_2O_2 were clearly evident among the three serotypes of *A. actinomycetemcomitans*, strains representing serotype *c* (strains 67, 9709, and 9710) exhibited slightly lower LD_{50} values than did strains representing serotype *b* (strains Y4 and 29522) or serotype *a* (strain 29523) or strains exhibiting 100% DNA homology with 29522 (strains 650 and 653) (11). It is intriguing to speculate that there is a relationship between susceptibility of serotype *c* organisms and their low frequency of isolation in patients with periodontal disease.

The mechanism(s) whereby *A. actinomycetemcomitans* resists H_2O_2-mediated injury is currently under investigation in this laboratory. We have found that the difference in resistance among these organisms to the bactericidal effects of H_2O_2 is not a function of catalase activity or phase of bacterial growth (K. T. Miyasaki, M. E. Wilson, J. J. Zambon, and R. J. Genco, submitted for publication). In related studies, this organism exhibited similar resistance to killing by a xanthine oxidase-mediated bactericidal system (unpublished observations), a known generator of superoxide anion. Additional study is required to assess the extent to which resistance of *A. actinomycetemcomitans* to H_2O_2-mediated killing is manifested in a similar resistance to oxidant injury induced by host phagocytic cells.

The data presented are consistent with hypothesis that the pathogenic potential of *A. actinomycetemcomitans* may be partly attributed to the resistance of this organism to bactericidal host defense mechanisms and, in particular, H_2O_2. These results also show that all of the oral haemophili and *A. actinomycetemcomitans* should be sensitive to the topical application of 3% H_2O_2 (approximately 0.5 M) in the treat-

ment of localized juvenile periodontitis and other periodontal diseases.

ACKNOWLEDGMENTS

We are grateful to Paulette Tempro, Pam Huber, J. J. Zambon, and J. Slots for providing carefully characterized bacterial strains and invaluable discussions.

This study was supported by Public Health Service grants DE07034 and DE04898 from the National Institute of Dental Research and by a grant from Kimberly-Clark Corp., Neenah, Wisc.

LITERATURE CITED

1. **Carlsson, J., Y. Iwami, and T. Yamada.** 1983. Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide. Infect. Immun. **40:**70–80.
2. **Chance, B., H. Sies, and A. Boveris.** 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59:**527–605.
3. **Frederiksen, W., and M. Kilian.** 1981. *Haemophilus-Pasteurella-Actinobacillus*: their significance in human medicine, p. 39–55. *In* M. Kilian, W. Frederiksen, and E. L. Biberstein (ed.), *Haemophilus*, *Pasteurella*, and *Actinobacillus*. Academic Press, Inc., New York.
4. **Kilian, M., and J. Theilade.** 1977. Amended description of *Haemophilus segnis* Kilian 1977. Int. J. Syst. Bacteriol. **28:**411–415.
5. **Mandell, R. L., and S. S. Socransky.** 1981. A selective medium for *Actinobacillus actinomycetemcomitans* and the incidence of the organism in juvenile periodontitis. J. Periodontol. **52:**593–598.
6. **Pick, E., and Y. Keisara.** 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods **38:**161–170.
7. **Rest, R. F., and J. K. Spitznagel.** 1978. Myeloperoxidase-Cl-H_2O_2 bactericidal system: effect of bacterial membrane structure and growth conditions. Infect. Immun. **19:**1110–1112.
8. **Root, R. K., and J. A. Metcalf.** 1977. H_2O_2 release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H_2O_2: studies with normal and cytochalasin B-treated cells. J. Clin. Invest. **60:**1266–1279.
9. **Rosling, B. G., J. Slots, R. L. Webber, L. A. Christersson, and R. J. Genco.** 1983. Microbiological and clinical effects of topical subgingival antimicrobial treatment on human periodontal disease. J. Clin. Periodontol. **10:**487–514.
10. **Slots, J., H. S. Reynolds, and R. J. Genco.** 1980. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. Infect. Immun. **29:**1013–1020.
11. **Tanner, A. C. R., R. A. Visconti, S. S. Socransky, and S. C. Holt.** 1982. Classification and identification of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by cluster analysis and deoxyribonucleic acid hybridizations. J. Periodontol. Res. **17:**585–596.
12. **Thomas, E. L., K. A. Pera, K. W. Smith, and A. K. Chwang.** 1983. Inhibition of *Streptococcus mutans* by the lactoperoxidase antimicrobial system. Infect. Immun. **39:**767–778.
13. **Wade, A. B., and R. B. Mirza.** 1964. The relative effectiveness of sodium peroxyborate and hydrogen peroxide in treating acute ulcerative gingivitis of the Vincent's type. Br. Dent. J. **111:**280–285.
14. **Wennstrom, J., and J. Lindhe.** 1979. Effective of hydrogen peroxide on developing plaque and gingivitis in man. J. Clin. Periodontol. **6:**115–130.
15. **Zambon, J. J., J. Slots, and R. J. Genco.** 1983. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. Infect. Immun. **41:**19–27.
16. **Zgliczynski, J. M.** 1980. Characteristics of myeloperoxidase from neutrophils and other peroxidases from different cell types, p. 255–278. *In* A. J. Sbarra and R. R. Strauss (ed.) The reticuloendothelial system: a comprehensive treatise. Plenum Publishing Corp., New York.