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Effects of hydrogen peroxide on growth and selected properties of *Porphyromonas gingivalis*

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Abstract

In this study we first evaluated the effects of hydrogen peroxide (H_2O_2) on growth and selected properties of *Porphyromonas gingivalis*, and compared them with those obtained by a reducing agent (cysteine). The growth of *P. gingivalis* was only moderately affected when H_2O_2 was added at concentrations up to 30 mM in a complex culture medium. However, when a defined basal medium was used, H_2O_2 at a concentration of 3 mM completely inhibited growth of *P. gingivalis*. Incorporation of cysteine at concentrations up to 30 mM in both media had no effect on growth. The effects of H_2O_2 and cysteine on cell-associated hemagglutinating and Arg-gingipain activities were evaluated using bacteria grown in the complex culture medium. Both activities were strongly decreased when H_2O_2 was added in the assay mixtures. This inhibitory effect of H_2O_2 was reversible. On the other hand, including cysteine in the assay mixtures increased both activities. H_2O_2 and cysteine had no effect on the expression of heat shock protein (HSP)-68 and HSP-75 by *P. gingivalis*, as determined by SDS-PAGE and Western immunoblotting analysis. In the second part of the study, we tested whether growth of selected oral bacterial species may modify the oxidation-reduction potential (Eh) of the environment. It was found that certain species were able to either decrease (*P. gingivalis*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Streptococcus mutans*) or increase (*Streptococcus sanguis*) the Eh of the medium. Our study provides evidence that an oxidizing agent such as H_2O_2 may affect the biology of *P. gingivalis*. Moreover, growth of some members of the oral microflora can generate oxidizing and reducing conditions, and thus potentially influence the ecology of subgingival sites by affecting strictly anaerobic bacteria such as *P. gingivalis*.

Keywords: *Porphyromonas gingivalis*; Growth; Protease activity; Hemagglutination; Heat shock protein; Hydrogen peroxide; Redox

1. Introduction

Under certain circumstances, specific bacteria found in subgingival plaque can increase in numbers and initiate periodontitis, a disease characterized by a significant breakdown of the tooth supporting tissues [1]. The microbial factors and ecological determinants that modulate the pathogenic and commensal bacteria in subgingival sites are under increasing scrutiny. A variety of environmental parameters, including oxidation-reduction potential (Eh), osmolarity, pH, temperature and nutrient restriction, can affect the microbial ecology of these sites [2,3]. Dur-

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ing the course of periodontitis, the formation of periodontal pockets is associated with a significant decrease in Eh thus resulting in a reduced environment favorable for the multiplication of strictly anaerobic periodontopathogens. Kenney and Ash [4] reported values in the range of +14 to −157 mV in these sites whereas the Eh of healthy gingival sulcus is approximately +74 mV.

Substances known to raise the Eh level act as an electron acceptor and thus cause a degree of oxidation of the environment. The beneficial clinical effect for periodontitis patients of such agents, including hydrogen peroxide (H_2O_2) and methylene blue, has been previously evaluated [5–7]. Few studies have evaluated the effect of oxidizing agents upon the physiology of periodontopathogens. *Actinobacillus actinomycetemcomitans* was reported to be relatively resistant to H_2O_2 [8] whereas methylene blue was found to kill *Porphyromonas gingivalis* [9]. In the present study, we evaluated the effects of H_2O_2 on growth, hemagglutination, Arg-gingipain activity and heat shock protein (HSP)-68 and HSP-75 response of *P. gingivalis*, and compared them with those obtained by a reducing agent (cysteine). In the second part of the study, we determined whether members of the oral microflora can modify the Eh level, thus conferring on them a potential to modulate the ecology of subgingival sites.

2. Materials and methods

2.1. Bacteria and growth conditions

P. gingivalis ATCC 33277 and ATCC 49417 were used in the study. Bacteria were grown in either a complex medium, Todd Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA), or a defined basal medium containing 1% bovine serum albumin (DBM-BSA) [10], both supplemented with hemin (10 μg ml^{-1}) and vitamin K (1 μg ml^{-1}). All cultures were incubated in an anaerobic chamber (N_2:H_2:CO_2/80:10:10) at 37°C.

2.2. Effects of hydrogen peroxide and cysteine on cell growth

H_2O_2 and cysteine were used as oxidizing and reducing agents, respectively. Various concentrations (0.3, 3 and 30 mM) of these agents were added to both the complex and the defined basal culture media to evaluate their effects on growth of *P. gingivalis* ATCC 49417. A combination redox cell with a platinum electrode and an Ag/AgCl reference electrode (Fisher Scientific, Montréal) was used for measuring the Eh level of all culture media prior to inoculation. A standard redox solution (Hanna Instruments, Laval) with a known Eh level was used to standardize the combination redox cell. A 5% (vol/vol) inoculum was used to inoculate the media and growth was monitored by measuring the final optical density at 660 nm after 48 h of incubation. Growth assays were performed in duplicate.

2.3. Effects of hydrogen peroxide and cysteine on hemagglutination and Arg-gingipain activity

The effects of H_2O_2 and cysteine on hemagglutination and Arg-gingipain activity of *P. gingivalis* (ATCC 49417 and ATCC 33277) cells were determined as previously reported [11]. Bacteria harvested in early stationary growth phase following culture in THB were suspended ($OD_{660nm} = 1$) in phosphate-buffered saline (PBS; 50 mM potassium phosphate, 0.15 M NaCl, pH 7.2) or in PBS containing either H_2O_2 (1.5, 7.5 and 15 mM) or cysteine (1.5, 15 and 37.5 mM). The combination redox cell was used to measure the Eh level of all buffers. In one assay, cells were treated with 15 mM H_2O_2 (1 h), washed twice in PBS and then assayed for hemagglutination and Arg-gingipain activity in the presence of 37.5 mM cysteine. All the above assays were performed in duplicate.

2.4. Effects of hydrogen peroxide and cysteine on the heat shock response

P. gingivalis ATCC 33277 was grown to mid-exponential growth phase ($OD_{660nm} = 0.6$), and 1-ml aliquots of the culture were prepared in sterile glass tubes. One tube was kept at 35°C and one was placed at 43°C. To the other tubes, H_2O_2 or cysteine was added at 1.5, 0.3 and 0.06 mM, and they were placed at 35°C. All tubes were incubated for 1 h. Cells were then harvested by centrifugation (10 000 × *g* for 2 min), the supernatant was dis-

carded, and 500 μl of 10% trichloroacetic acid (TCA) was added to the microtubes. After 1 h, cells were harvested, washed in PBS, and proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer. The HSP-68 (GroEL-like) and HSP-75 (DnaK-like) were detected by SDS-12.5% polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting using rabbit anti-HSP-68 and anti-HSP-75 antibodies (1:5000) as previously reported [12].

2.5. *Effect of growth of selected oral bacterial species on oxidation-reduction potential*

Selected oral bacterial species (*P. gingivalis* ATCC 49417 and ATCC 33277, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* NCTC 9336, *Fusobacterium nucleatum* ATCC 25586, *Peptostreptococcus micros* NY370, *Actinomyces naeslundii* 85.1, *Actinomyces viscosus* 54.2, *Streptococcus mutans* ATCC 10449, *Streptococcus mitis* ATCC 33399 and *Streptococcus sanguis* NY101) were inoculated in Todd Hewitt broth and cultivated for 48 h under either anaerobiosis (anaerobic chamber) or aerobiosis (orbital shaking outside the anaerobic chamber). The Eh was measured prior to inoculation and following bacterial growth, using the combination redox cell. In one experiment, values of Eh were recorded at various stages during the growth of *P. gingivalis* ATCC 49417. All assays were performed in duplicate.

3. Results

The effects of H_2O_2 and cysteine on growth of *P. gingivalis* ATCC 49417 in a complex medium (THB) and a defined basal medium (DBM-BSA) are presented in Fig. 1. At the highest concentration tested of the oxidizing agent (30 mM), the final OD obtained in the complex culture medium was only slightly decreased compared with the control medium without H_2O_2. At such a high concentration of H_2O_2, the Eh of the medium was +200 mV (prior to inoculation). On the other hand, when defined basal medium was used, H_2O_2 at a concentration of 3 mM (corresponding to an Eh of +200 mV) completely inhibited the growth of *P. gingivalis*. Addition into both media of the reducing agent cysteine at concentrations up to 30 mM did not appreciably modify the final OD reached by the culture. All the above observations were reproducible.

The hemagglutination and Arg-gingipain activity of cells of *P. gingivalis* ATCC 49417 and ATCC 33277 grown in THB were determined in the presence of various concentrations of either H_2O_2 or cysteine (Table 1). Both activities were strongly affected by the presence of the oxidizing agent, which increased the Eh level of the assay mixture. The hemagglutination was decreased but not entirely eliminated by the highest concentration of H_2O_2 tested (15 mM). The Arg-gingipain activity appeared to be much more sensitive to H_2O_2, since no residual ac-

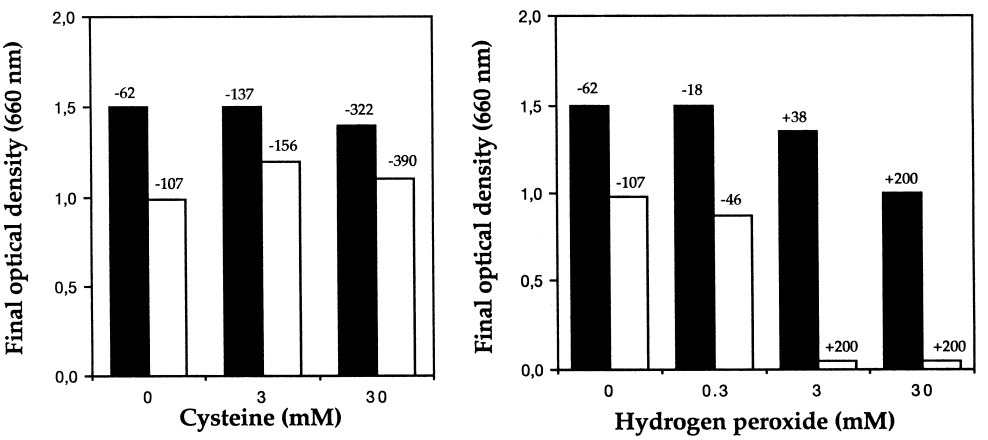

Fig. 1. Extent of growth of *P. gingivalis* ATCC 49417 for 48 h in a complex medium (THB; ■) and a defined basal medium (DBM-BSA; □) containing either cysteine or H_2O_2. Values of Eh (in mV) for the uninoculated media are indicated on the top of each column.

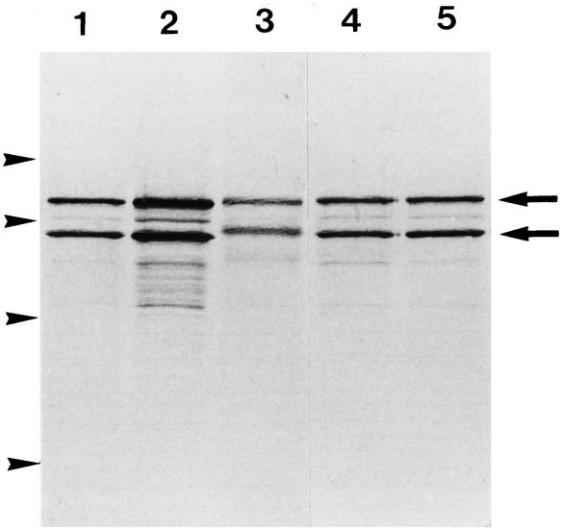

Fig. 2. Immunoblotting analysis of *P. gingivalis* ATCC 33277 for HSP68 (GroEL-like) and HSP75 (DnaK-like). Lane 1, unstressed control cells; lane 2, heat-stressed cells; lane 3, cells incubated with 1.5 mM H_2O_2; lane 4, cells incubated with 0.3 mM H_2O_2; lane 5, cells incubated with 0.06 mM H_2O_2. Each lane was loaded with 10 μg of protein. The arrows on the right side of the figure show the position of the HSP-68 and the HSP-75. The arrows on the left side of the figure indicate molecular mass markers which were from top to bottom: phosphorylase B (107 kDa), bovine serum albumin (69 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

tivity was detected even at the lowest concentration (1.5 mM). When cysteine was incorporated in the assay mixtures, higher hemagglutination and Arg-gingipain activity were detected as compared to the activities obtained in absence of the reducing agent. For both strains, the amount of the reducing agent needed for the increase in the protease activity was lower than that required for the hemagglutination. All these observations were found to be reproducible. The reversibility of the inhibitory effect of H_2O_2 on hemagglutination and Arg-gingipain activity of *P. gingivalis* was evaluated. Treating cells with 15 mM H_2O_2 for 1 h prior to measuring the activities in the presence of cysteine (37.5 mM) had no inhibitory effect as compared to untreated cells (data not shown). This indicates that the deleterious action of H_2O_2 could be reversed.

The effect of H_2O_2 on the expression of HSP-68 (GroEL-like) and HSP-75 (DnaK-like) by *P. gingivalis* ATCC 33277 is shown in Fig. 2. When cells were submitted to a heat stress at 43°C, protein bands corresponding to both HSPs increased in intensity when compared to unstressed cells. Incubating the cells in the presence of H_2O_2 had no such inducing effect on expression of both the HSP-68 and the HSP-75. Cysteine has also no effect on HSPs production (data not shown).

Since an oxidizing condition such as the one generated by H_2O_2 was found to affect growth and properties of *P. gingivalis* and thus represents an important environmental parameter, we tested whether growth of a number of oral bacterial species may modify the Eh of the environment. These experiments revealed that the Eh may be either decreased or increased by some species (Table 2).

Table 1
Effects of H_2O_2 on hemagglutination and Arg-gingipain activity of two strains of *P. gingivalis*

Redox agent	Eh (mV)[a]	Hemagglutinating activity		Arg-gingipain activity	
		Hemagglutination titer[b]		Hydrolysis of BA*p*NA (A_{405})	
		ATCC 49417	ATCC 33277	ATCC 49417	ATCC 33277
None	>+250	128/64	32/32	0.44/0.62	0.29/0.22
H_2O_2 (1.5 mM)	>+250	16/16	4/8	0/0	0/0
H_2O_2 (7.5 mM)	>+250	8/8	2/8	0/0	0/0
H_2O_2 (15 mM)	>+250	4/4	2/4	0/0	0/0
Cysteine (1.5 mM)	−98	128/128	32/32	1.11/1.27	1.47/1.48
Cysteine (15 mM)	−215	256/256	64/64	1.14/1.33	1.50/1.42
Cysteine (37.5 mM)	−242	256/256	128/64	1.12/1.38	1.41/1.48

[a]The Eh electrode could not measure values >+250 mV.
[b]Reciprocal of the highest dilution showing agglutination of sheep erythrocytes.

Table 2
Effect of growth of selected oral bacterial species on the final Eh of the medium

Bacteria	Eh values (mV)[a]	
	Aerobic culture	Anaerobic culture
Control (uninoculated)	+104	−75
P. gingivalis 49417	NG[b]	−360
P. gingivalis 33277	NG	−333
P. intermedia 25611	NG	−230
P. nigrescens 9336	NG	−213
F. nucleatum 25586	NG	−318
P. micros NY370	NG	−301
A. naeslundii 85.1	NG	−168
A. viscosus 54.2	+54	−153
S. mutans 10449	−75	−180
S. mitis 33399	+32	−211
S. sanguis NY101	+183	−173

[a]Measured after a 48-h incubation.
[b]NG, no growth.

Under aerobic conditions (orbital shaking outside the anaerobic chamber), the Eh of the uninoculated complex medium was +104 mV. Growth of *S. sanguis* increased the Eh to +183 mV whereas growth of *S. mutans* decreased it to −75 mV. Under anaerobic chamber conditions (N_2:H_2:CO_2/80:10:10), all bacterial species tested decreased the Eh to various extents, compared to the uninoculated medium (−75 mV). The greatest negative values of Eh were obtained following growth of *P. gingivalis* (−360 and −333 mV), *F. nucleatum* (−318 mV) and *P. micros* (−301 mV). Eh readings were determined at various stages during growth of *P. gingivalis* ATCC 49417 in THB (Fig. 3). The decrease of the Eh values occurred mostly during the first part of the exponential growth phase (from −125 to −250 mV). Thereafter, the Eh remained relatively stable prior to decreasing again later during the stationary growth phase.

4. Discussion

Strictly anaerobic bacteria such as those found in diseased periodontal sites may be described as bacteria that grow in reduced conditions (negative Eh values) and absence of significant amounts of free oxygen. A number of clinical studies have already proposed a therapeutic approach for periodontitis patients based on the application in periodontal pockets of an oxidizing agent that can increase the Eh [5–7]. Conclusions from these studies have been variable, most likely because the substances were eliminated from the diseased sites too rapidly to be effective. Incorporation of the oxidizing agents into a slow release carrier showed more convincing clinical improvements [13].

Few studies have evaluated the effects of oxidizing

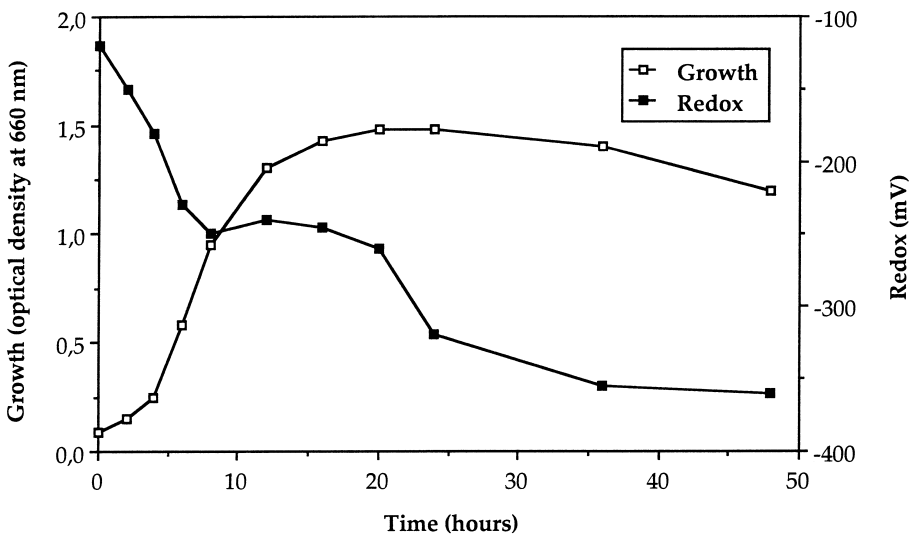

Fig. 3. Changes in Eh values observed during growth of *P. gingivalis* ATCC 49417 in THB.

agents and/or high Eh conditions on periodontopathogens. Socransky et al. [14] reported that a low Eh condition was required for growth of *Treponema microdentium* (reclassified as *Treponema denticola*) despite an oxygen-free environment. In our study, under conditions of positive Eh values obtained by adding 3 and 30 mM H_2O_2, the growth of *P. gingivalis* was only possible in the complex culture medium. No growth occurred in the defined basal medium. This observation suggests that bacteria grown under poor nutritional conditions, such as in DBM-BSA, are much more sensitive to the deleterious effect of an oxidizing agent. It is also possible that over-production of enzymes involved in elimination of toxic oxygen metabolites occurs when growth is carried out in a complex medium. It is presently unknown if the inability of *P. gingivalis* to grow in the defined basal medium containing H_2O_2 is related to the rise of Eh or the oxidation of electron donors (NADH and NADPH).

Our results indicated that H_2O_2 was deleterious for both hemagglutination and Arg-gingipain activity of *P. gingivalis*. On the other hand, reduced conditions obtained by adding cysteine increased both activities. The Arg-gingipain activity appeared to be more dependent on reducing conditions than the hemagglutinating activity. Previous studies have also reported that some properties of *P. gingivalis* were positively affected by reduced conditions [15,16]. More particularly, Smalley et al. [15] showed that the hemin-binding capacity of *P. gingivalis* was stronger under reduced conditions.

HSPs are highly conserved proteins that function to stabilize cellular components against stress conditions. A previous study [12] revealed that following a heat shock, *P. gingivalis* increased the synthesis of two important chaperones HSP-68 (GroEL-like) and HSP-75 (DnaK-like). In the present study, we noted that stressing the cells with H_2O_2 was not associated with an over-expression of these two HSPs. This result is in agreement with the recent study of Lu and McBride [17] who reported that GroEL- and DnaK-like proteins of *P. gingivalis* were not induced by an oxidative stress or a change in pH.

Since oxidizing conditions were found to affect the growth and some properties of *P. gingivalis*, the ability of oral bacteria to modify the Eh of the environment may represent the basis of positive and negative interactions occurring in subgingival sites between *P. gingivalis* and other bacteria. In our study, we found that growth of *S. mutans* was associated with a decrease of the Eh level in the culture medium. The presence of this bacterial species in subgingival sites may thus be potentially beneficial for *P. gingivalis*. It was previously reported that *S. mutans* can generate a reduced environment following growth in a complex medium [18]. This reduced environment was found to result from the accumulation of sulfhydryl compounds in the medium as well as the depletion of oxygen by a nicotinamide adenine dehydrogenase-dependent reduction. Ter Steeg et al. [19] showed that batch-wise enrichment of subgingival plaque samples with human serum resulted in the accumulation of black-pigmented anaerobic bacteria, *P. micros* and *F. nucleatum*. Accumulation of these bacteria coincided with a decline in Eh from −70 to −375 mV. This is in agreement with our study showing that *P. micros* and *F. nucleatum* could decrease the Eh of the environment to generate a reduced condition. Another interesting observation that we noted was the fact that cultivation of *S. sanguis* resulted in an increase of the Eh, which could be deleterious for *P. gingivalis*. The generation of this oxidizing environment is likely related to the ability of this bacterium to produce H_2O_2 [20]. In fact, a previous study demonstrated that inhibition of *A. actinomycetemcomitans* by *S. sanguis* was dependent on the production of H_2O_2 [21]. Interestingly, these in vitro data were supported by in vivo observations which indicated that the presence of *S. sanguis* correlated with the absence of *A. actinomycetemcomitans* in the sites [21].

In summary, this study provides evidence that the oxidizing agent H_2O_2 can negatively influence the growth of *P. gingivalis* as well as two of its biological properties that are thought to contribute to virulence by favoring colonization, neutralizing host defense mechanisms and causing tissue damage. It thus supports clinical reports showing a certain degree of beneficial effects of oxidizing agents in treatment of periodontitis [5–7,13]. Raising the Eh condition of the periodontal pocket by using an oxidizing agent may create an environment incompatible with growth and virulence of anaerobic periodontal pathogens.

Acknowledgments

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