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Effects of Subinhibitory Antibiotic Concentrations on *Porphyromonas gingivalis* Fibrinogen and Hemin Binding

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Porphyromonas gingivalis*, a gram-negative anaerobe, is one of the major causative agents in periodontal disease. In this study, the effect of sub-inhibitory concentrations of antibiotics on the binding characteristics of *P. gingivalis* was examined. *P. gingivalis* strains 381 and W50 were grown in broth containing one-half the minimum inhibitory concentration (MIC) of amoxicillin, doxycycline, metronidazole, penicillin, or tetracycline. Surface hydrophobicity, fibrinogen binding (soluble and immobilized) and hemin binding were evaluated. The surface hydrophobicity of strain 381 cultured in each antibiotic decreased, whereas strain W50 showed no statistically significant changes in surface hydrophobicity. All antibiotics used decreased soluble fibrinogen binding of strain 381. In contrast, all antibiotics, except penicillin, increased binding of soluble fibrinogen for strain W50. Binding of strain 381 to immobilized fibrinogen increased in the presence of amoxicillin and doxycycline; the binding of strain W50 to immobilized fibrinogen was not affected by any antibiotic. Hemin binding of strain 381 was unaltered by any antibiotic, whereas hemin binding by strain W50 was increased by amoxicillin, metronidazole and penicillin. These observations showed that sub-inhibitory concentrations of antibiotics could confer variable effects on the binding properties of *P. gingivalis*.

Key words : *Porphyromonas gingivalis*, antibiotic, sub-MIC, fibrinogen, hemin, binding

Introduction

Porphyromonas gingivalis is a Gram-negative black-pigmented anaerobe associated with several periodontal diseases (Mayrand and Holt, 1988). It has been suggested that the ability of pathogenic microorganisms to adhere to host tissues is a prerequisite for colonization, establishment, and initiation of disease (Finlay and Falkow, 1989). *P. gingivalis* has several virulence factors related to binding. Lantz *et al.* (1986) have shown that strains of *P. gingivalis* bind human fibrinogen with high affinity and specificity, and that these bacteria bind a specific region of the fibrinogen molecule (Lantz *et al.*, 1990; Lantz *et al.*, 1991). As with other pathogens, a requirement for the *in vivo* growth of *P. gingivalis* is its ability to obtain iron from the host. Although *P. gingivalis* is not able to synthesize heme, an important growth factor for this bacterium (Gibbons and MacDonald, 1960; Shah *et al.*, 1979), it is capable of both utilizing a broad range of hemin-containing compounds (Barua *et al.*, 1990; Bramanti and Holt, 1991) and binding hemin (Genco *et al.*, 1994; Tompkins *et al.*, 1997) *in vitro*. In a complex microbial community, such as exists in the gingival crevice, heme-requiring bacteria must also compete with one another for available heme.

Growth in a sub-minimal inhibitory concentration (sub-MIC) of an antibiotic produces numerous effects on bacterial cells, including morphologic alterations (Baker *et al.*, 1995; Choe *et al.*, 1998), changes in cell hydrophobicity (Peros and Gibbons, 1982; Wu *et al.*, 1995), and modulated bacterial adhesion, including altered ability to adhere to host cells (Shibl, 1985; Schifferli and Beachey, 1988a; Schifferli and Beachey, 1988b). Sub-MICs of clindamycin, erythromycin, or chloramphenicol decrease fibronectin binding to *Staphylococcus aureus*, whereas β-lactam antibiotics enhance this interaction (Proctor *et al.*, 1983). Cell surface properties, including glucan binding activity and hydrophobicity of *Streptococcus sobrinus* (Wu *et al.*, 1995), are affected by sub-MICs of antibiotics. Growth in sub-MICs of tetracycline decreases adherence of *Escherichia coli* to intestinal mucosal cells (Deneke *et al.*, 1985). *Neisseria meningitidis* and *Neisseria gonorrhoeae* show

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decreased expression of pili, decreased synthesis of pilin subunits, and decreased attachment to host cells when grown in sub-MICs of tetracycline (Stephens *et al.*, 1984).

Antibiotics are frequently used as an adjunct to conventional therapy in the treatment of some forms of periodontal disease (van Winkelhoff *et al.*, 1996). It is assumed that the ability of sub-MIC of antibiotics to affect the adherence properties of bacteria may be an important criterion in selecting a drug for therapy. In this study, we determined the influence of sub-inhibitory concentration of antibiotics that are generally used in periodontal treatment on the surface hydrophobicity of *P. gingivalis* and the binding of fibrinogen and hemin to *P. gingivalis*.

Materials and Methods

Bacteria and growth conditions

P. gingivalis laboratory strains 381 and W50 were obtained from stock cultures stored in the Department of Oral Microbiology, Seoul National University, Korea. Cells were cultured in prereduced trypticase soy broth (Difco, Detroit, MI, USA) containing 1 mg/ml of yeast extract (Difco), 5 μg/ml of hemin (Sigma Chemical Co., St. Louis, MO, USA) and 1 μg/ml of menadione (Sigma Chemicals Co.) under anaerobic conditions (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc., Cornelius, Oregon, USA) with an atmosphere of 90% N_2, 5% CO_2 and 5% H_2. Cultures were incubated for 72 h at 37°C. The turbidity of the bacterial suspension was measured by spectrophotometry. A standard curve was established for adjusting bacterial numbers.

Minimal inhibitory concentration (MIC)

To determine the MIC of the antibiotics commonly used for the treatment of certain kinds of periodontal diseases, stock antibiotic solutions of amoxicillin, doxycycline, metronidazole, penicillin and tetracycline (Sigma Chemical Co.) were prepared and filter sterilized. MICs were determined by two-fold serial macro-dilution of antibiotics in prereduced trypticase soy broth containing 1 mg/ml of yeast extract, 5 μg/ml of hemin and 1 μg/ml of menadione with an inoculum of approximately 10^5 cells/ml. Range of concentrations tested for each antibiotic was from 0.5 μg/ml to 1 mg/ml. The MIC was defined as the concentration of the highest dilution of antibiotic which inhibited the growth of bacteria. One-half the MIC was used as a sub-inhibitory concentration of the antibiotics.

Bacterial surface hydrophobicity

The relative surface hydrophobicity of *P. gingivalis* was determined by measuring their ability to absorb n-hexadecane (Hamada *et al.*, 1994). Briefly, bacterial cells cultured in sub-MIC of each antibiotic were washed twice in PUM buffer (0.115 M $K_2HPO_4 \cdot H_2O$, 0.05 M KH_2PO_4, 0.03 M urea, 0.8 mM $MgSO_4 \cdot 7H_2O$; pH 7.1) by centrifugation at 10,000 ×*g* for 5 min and suspended in the same buffer. Bacterial suspensions were adjusted to an optical density of 1.0 (1×10^9 cells/ml) at OD_{550}. Two milliliters of this suspension were placed in glass tubes (13×100 mm), and 400 μl of n-hexadecane (Sigma Chemicals Co.) was added. This mixture was vigorously mixed on a vortex mixer for 60 sec, then incubated for 15 min at room temperature. The OD_{550} of the aqueous phase was measured, and the hydrophobic activity (HP) was calculated using the formula, %HP=[OD (initial)-OD (expt)]×100/OD (initial); where OD (expt) is the OD_{550} measured after the 15 min incubation.

Soluble fibrinogen binding

Radiolabeled fibrinogen was prepared by the reductive alkylation technique (Rice and Means, 1971; Grinnell, 1980). Eighty-nine microliters of [^{3}H] formaldehyde (51.2 mCi/mmol, New England Nuclear, Boston, MA, USA) was added to a 5.0 ml solution of fibrinogen (5 mg/ml) in 0.05 M $Na_2B_4O_7 \cdot 10H_2O$, The reaction was carried out for 30 min at 4°C and stopped by three successive additions of 0.1 ml of 0.338 mM $NaBH_4$. Subsequently, the reaction mixtures were diluted to 15 ml with 0.01 M Tris buffer (pH 7.2) in 0.15 M NaCl, and dialyzed against 2 L of 20 mM phosphate buffered saline (PBS). The fibrinogen concentration of the preparation was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA). The radiolabeled fibrinogen stock preparation had a specific activity of 2,090 cpm/mg of fibrinogen. The stock [^{3}H] fibrinogen was diluted in PBS containing 0.1% BSA to yield a concentration of 100 μg/ml. Cultures of bacteria were washed twice in PBS containing 0.1% BSA and adjusted to a cell density of 1×10^9 cells/ml. The cell suspension (0.5 ml) was incubated with 100 μl of the diluted [^{3}H] fibrinogen for 30 min at room temperature on a rotator. The incubation mixture was washed twice with PBS containing 0.1% BSA to remove unbound fibrinogen, and the radioactivity in the cell pellet was measured.

Immobilized fibrinogen binding

Bacterial cultures were grown in 10 ml of media containing 10 μCi of [methyl-^{3}H] thymidine (83 curies/mmol, Amersham, Arlington Height, IL, USA) and sub-MIC of antibiotics, then washed three times with Hanks Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY, USA) containing 4 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 0.5% BSA (HBSS-BSA). The cells were resuspended in HBSS-BSA and adjusted to 5×10^8 cells/ml. Cell culture plates (24-well; Corning Inc., Corning, NY, USA) were coated overnight at 37°C with plasminogen-free human fibrinogen (75 μg/ml) (Sigma Chemical Co.) or BSA (75 μg/ml) in 0.05 M carbonate buffer (pH 9.5) containing 0.02% NaN_3. Protein-coated wells were washed twice with HBSS-BSA, then 0.5 ml of radiolabeled bacterial suspension was added. After a 1 h incubation on a rocking platform (18 cycles/min) at 37°C, unbound bacterial suspensions were aspirated, and wells were washed three times with HBSS-BSA. Bound bacteria were solubilized in 0.5 ml solution containing 1% sodium dodecyl sulfate, 8 M urea, and 1 M NaCl for 30 min at 37°C on rocker platform. This 0.5 ml solution and the subsequent 0.5 ml wash of the same solution were added to 10 ml of Scint-A XF (Packard, Meriden, CT, USA) and the radioactivity was counted in a scintillation counter. The cpm of the bacteria bound to BSA was subtracted from the cpm of bacteria bound to fibrinogen. The number of bound bacteria was calculated with the labelling efficiency (number of bacteria/cpm) of bacteria.

Hemin binding assay

The binding of hemin to *P. gingivalis* whole cells was measured by a modification of the procedure described by Deneer and Potter (1989). *P. gingivalis* cultures were grown, harvested by centrifugation at 10,000×*g* for 10 min, washed in PBS, and resuspended in PBS to 1×10^{10} cells/ml. Hemin was added to a final concentration of 30 μg/ml. After incubation for 30 min at 37°C, a 1.0 ml sample was removed and centrifuged for 1 min at 10,000×*g* in an Eppendorf centrifuge to pellet the cells. The OD_{400} of supernatant was measured by spectrophotometry. Cultures without added hemin, or hemin without added cells, served as controls. The concentration of hemin present in the supernatant fraction was calculated from a hemin standard curve. Binding of hemin to whole cells was calculated as the difference between the total amount of hemin added versus the amount remaining in the supernatant.

Statistical analysis of the data

The data were analyzed by Student's *t* test.

Results

The MIC of each antibiotic used in this study is shown in Table 1. The surface hydrophobicity of strain 381 was decreased by ~40% (metronidazole) to 90% (tetracycline) in the presence of the antibiotics used in the study (Fig. 1). For strain W50, however, there was no statistically significant changes in hydrophobicity with any antibiotic. Binding of soluble fibrinogen to *P. gingivalis* strain 381 decreased by up to 60% (amoxicillin) for all

Table 1. Minimal inhibitory concentration (MIC) of *P. gingivalis* 381 and W50

Antibiotics	MIC (μg/ml)	
	P. gingivalis 381	*P. gingivalis* W50
Amoxicillin	0.016	0.016
Doxycycline	0.016	0.016
Metronidazole	0.016	0.063
Penicillin	0.016	0.016
Tetracycline	0.063	0.031

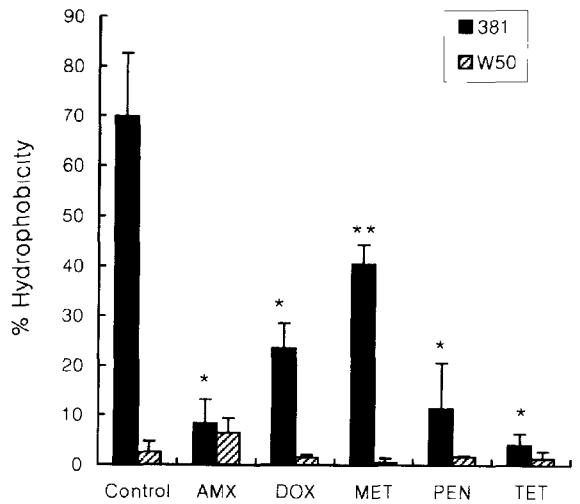

Fig. 1. The relative surface hydrophobicity of *P. gingivalis* 381 and W50. Bacterial suspensions cultured at sub-inhibitory concentrations of various antibiotics were adjusted to an OD_{550} of 1.0 (OD (initial)). After the addition of n-hexadecane, the OD (exp) of the aqueous phase was measured. The hydrophobic activity (HP) was calculated from the formula, %HP=[OD (initial)-OD (exp)]×100/OD (initial). Values are the means of triplicates; error bars represent standard deviations. *P<0.001; **P<0.05. Control, no antibiotic added; AMX, amoxicillin; DOX, doxycycline; MET, metronidazole; PEN, penicillin; TET, tetracycline.

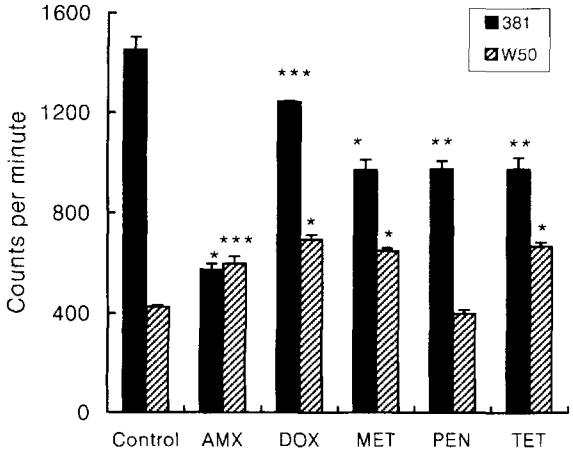

Fig. 2. Relative binding of soluble fibrinogen to *P. gingivalis* 381 and W50. The cell suspension (1×10^9 cells/ml) was incubated with [^{3}H] fibrinogen. After washing to remove the unbound fibrinogen, the radioactivity (cpm) in the cell pellet was measured. Values are the means of triplicates; error bars represent standard deviations. *P<0.005; **P<0.01; ***P<0.05. Control, no antibiotic added; AMX, amoxicillin; DOX, doxycycline; MET, metronidazole; PEN, penicillin; TET, tetracycline.

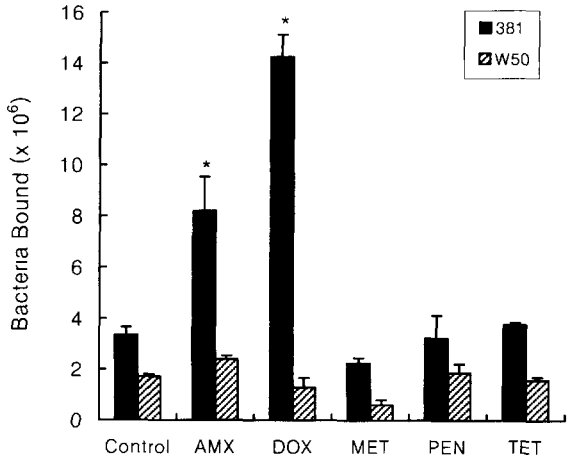

Fig. 3. Binding of *P. gingivalis* 381 and W50 to immobilized fibrinogen. [Methyl-^{3}H] thymidine-labeled bacteria were added into 24-well cell culture plates coated with fibrinogen or BSA. The bound cells were solubilized and the radioactivity (cpm) of the solubilized cells was counted. The cpm of the bacteria bound to BSA was subtracted from the cpm of bacteria bound to fibrinogen. The number of bound bacteria was calculated with the labelling efficiency (number of bacteria/cpm) of bacteria. Values are the means of triplicates; error bars represent standard deviations. *P<0.05. Control, no antibiotic added; AMX, amoxicillin; DOX, doxycycline; MET, metronidazole; PEN, penicillin; TET, tetracycline.

antibiotics tested (Fig. 2). In contrast, strain W50 showed increased soluble fibrinogen binding with all antibiotics except penicillin, which had no effect on soluble-fibrinogen binding of strain W50. The binding of strain 381 to immobilized fibrinogen (Fig. 3) was significantly increased by amoxicillin (~150%) and doxycycline (~325%), but showed no effect with metronidazole, penicillin or tetracycline. Strain W50 binding to immobilized fibrinogen was not affected by any antibiotic used. Strain 381 showed no significant changes in hemin binding with any antibiotic, whereas *P. gingivalis* W50 showed increased hemin binding with amoxicillin, metronidazole and penicillin. There were no changes with doxycycline and tetracycline for strain W50 (Fig. 4).

Discussion

The results of the present study suggest that fibrinogen and hemin binding of *P. gingivalis* can be affected when grown in the presence of sub-inhibitory concentrations of antibiotics. All of the antibiotics used in this study inhibited the binding of soluble fibrinogen to *P. gingivalis* 381 strain. In contrast, the binding of soluble fibrinogen to *P. gingivalis* W50 strain was increased with all of the antibiotics except penicillin. At this point, it is not clear why antibiotics affected the two strains

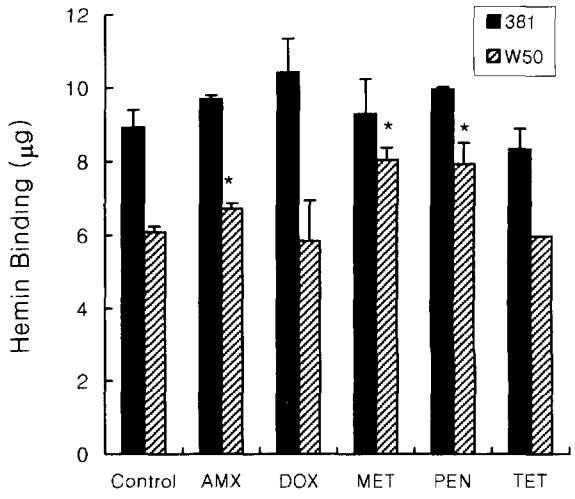

Fig. 4. Hemin binding by *P. gingivalis* 381 and W50. Hemin was added to a final concentration of 30 μg/ml to *P. gingivalis* suspensions (1×10^{10} cells/ml). After incubation, the OD_{400} of the supernatant was measured by spectrophotometry, and the concentration of hemin present in the supernatant fractions was calculated from a hemin standard curve. Binding of hemin to whole cells was calculated as the difference between the total amount of hemin added versus the amount remaining in the supernatant. Values are the means of triplicates and error bars represent standard deviations. *P<0.05. Control, no antibiotic added; AMX, amoxicillin; DOX, doxycycline; MET, metronidazole; PEN, penicillin; TET, tetracycline.

differently. Although the specific cell components of *P. gingivalis* that mediate binding of soluble fibrinogen have been described (Lantz *et al.*, 1991), it is not obvious from this study whether or not the effects of sub-inhibitory concentrations of these antibiotics on the binding of fibrinogen to *P. gingivalis* is due to direct effects on those cell surface components.

It is also possible that the antibiotics could affect non-specific binding properties of *P. gingivalis*. We observed that the surface hydrophobicity of *P. gingivalis* W50 was much lower than that of strain 381. Furthermore, all of the antibiotics used in this study inhibited the surface hydrophobicity of *P. gingivalis* 381, but had no effect on the hydrophobicity of *P. gingivalis* W50. In fact, the hydrophobicity of strain 381 remained statistically greater than that of strain W50 even in the presence of antibiotic, with the exception of amoxicillin and tetracycline. Thus, it may be assumed that the effects of the antibiotics on soluble-fibrinogen binding of *P. gingivalis* 381 are due in part to the differences in surface hydrophobicity caused by antibiotics.

The effects of sub-MIC of antibiotics on the binding of *P. gingivalis* to immobilized fibrinogen were different from that on the bacterial binding to soluble fibrinogen, i.e., only amoxicillin and doxycycline enhanced the binding of *P. gingivalis* 381 to immobilized fibrinogen. It has been suggested that the binding characteristics of bacteria to the immobilized proteins are different from those to the soluble form of proteins (Lowrance *et al.*, 1988; Gibbons *et al.*, 1991). Lowrance *et al.* (1988) reported that *Streptococcus sanguis* binds to a conformationally specific domain on the immobilized fibronectin molecule that is not exposed in soluble fibronectin. A similar phenomenon has been observed in the binding of oral streptococci to fibrinogen (Lee *et al.*, 1996). Our previous study showed that oral streptococci bind to immobilized fibrinogen, but not to the soluble form of fibrinogen (Lee *et al.*, 1996). It is tempting to assume that the binding of *P. gingivalis* to immobilized fibrinogen may be different from that to the soluble fibrinogen, and that the effects of antibiotics on the binding of *P. gingivalis* to the two forms of fibrinogen could be different. If fibrinogen binding is an adherence mechanism that mediates colonization, establishment or emergence of *P. gingivalis* in disease-associated periodontal microbiota, then antibiotics that inhibit fibrinogen binding may be effective in suppressing or eliminating this organism at concentrations well below those required to achieve a bacteriostatic effect.

In the hemin binding assay, three antibiotics were effective in increasing hemin binding by *P. gingivalis* W50, whereas none of the antibiotics affected the hemin binding by strain 381. Although the hemin binding of *P. gingivalis* has been characterized, the cell surface components involved in hemin binding has not yet been fully elucidated (Genco *et al.*, 1994; Tompkins *et al.*, 1997). Whether or not these antibiotics have any effect on hemin binding components is also not known.

Among the antibiotics used in periodontal treatments, the effects of sub-inhibitory concentrations of tetracycline have been studied extensively. Lantz *et al.* (1987) examined the effect of growth in sub-inhibitory concentrations of tetracycline on soluble-fibrinogen binding by *Prevotella intermedia* and found concentration-dependent inhibition of fibrinogen binding by the bacteria over the range of tetracycline concentrations from 0.0078 μg/ml to 0.0625 μg/ml. In the present study, sub-inhibitory concentrations of tetracycline inhibited the binding of soluble fibrinogen to *P. gingivalis* 381 and increased the binding to *P. gingivalis* W50, suggesting that there might be strain-to-strain variation in the effects of tetracycline. Oral bacteria, including *Actinomyces viscosus* and *P. gingivalis*, are also affected by growth in sub-inhibitory concentrations of tetracycline, exhibiting decreased adherence to saliva-treated hydroxyapatite (Peros and Gibbons, 1982), decreased fimbriation, and decreased hydrophobicity (Peros *et al.*, 1985). Our previous study on the effects of sub-inhibitory concentrations of antibiotics on cell surface properties of *Streptococcus gordonii* showed that the binding of *S. gordonii* to immobilized fibrinogen increased with β-lactam drugs, but was not affected by antibiotics that perturb protein synthesis (Choe *et al.*, 1998). This pattern of inhibition, however, was not observed for fibrinogen or hemin binding of *P. gingivalis* in this study.

The present study has demonstrated that sub-inhibitory concentrations of antibiotics can confer variable effects on binding properties of *P. gingivalis*. If the findings of this *in vitro* investigation are applicable *in vivo*, it will be worthwhile to consider using an antibiotic that decreases bacterial adherence.

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