J. Korean Soc. Microbiol., Vol. 33, No. 6, 1998

Effect of Subinhibitory Concentrations of Antibiotics on Cell Surface Properties of *Streptococcus gordonii* and *Staphylococcus aureus*

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Antibiotics were reported to be able to alter bacterial surface properties in subinhibitory concentrations (sub-MICs). The effects of sub-MICs of certain antibiotics on a bacterial surface property such as hemagglutination, as well as on the cell morphology were studied using *Streptococcus gordonii* and *Staphylococcus aureus*. The effect of sub-MICs of antibiotics on the binding of these bacteria to immobilized fibrinogen were also investigated. The MICs of antibiotics were determined by culturing *S. gordonii* and *S. aureus* in media supplemented with serially diluted drug solutions, and one-half the MIC was used as the sub-MIC of the drugs, unless stated otherwise. Sub-MICs of antibiotics did not affect bacterial agglutination of erythrocytes. Microscopic observation of *S. gordonii* grown at sub-MIC concentration of 0.02 μg/ml of amoxicillin revealed cell enlargement of 1.6 times those grown without the drug. When grown in the sub-MIC amount of 0.08 μg/ml of cefazolin, most *S. gordonii* cells were enlarged and elongated into rod-shape, resulting in 3 times the size of the cells grown without the antibiotic. The data from the fibrinogen-binding experiments showed that the binding of *S. gordonii* to immobilized fibrinogen was increased with all the β-lactam drugs tested; the binding of *S. aureus* to immobilized fibrinogen, on the other hand, was decreased with the same drugs. The results show that low concentrations of certain β-lactam antibiotics are able to cause alterations in cellular morphology of *S. gordonii* and affect the binding of *S. gordonii* and *S. aureus* to immobilized fibrinogen.

Key Words: *Streptococcus gordonii*, Sub-MIC of antibiotics, Fibrinogen binding

INTRODUCTION

The adherence of bacteria to the surfaces of host is the initial step for the subsequent colonization or invasion to the host (12). Several aspects which are associated with binding ability of bacteria are suggested as important virulence factors for inducing infective endocarditis (3). Studies on the binding of sanguis group streptococci (*Streptococcus sanguis, Streptococcus gordonii, Streptococcus oralis* and *Streptococcus parasanguis*) to attachment proteins such as fibronectin or laminin, and the aggregation

Received for publication : September 10, 1998, Accepted for publication : January 1, 1999

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of platelet by bacteria are the examples of recent research efforts (13, 14, 17, 29).

Recent studies indicated that the effect of prophylactic antibiotics on reducing the incidence of infective endocarditis is expected through the decrease of binding of causative bacteria such as *S. sanguis* to the damaged valve area (26).

Antibiotics were reported to be able to alter the bacterial surface properties at subinhibitory concentrations (sub-MICs). Especially, many investigators focused on the effects of antibiotics on bacterial adhesion (27). For example, sub-MICs of clindamycin, erythromycin, or chloramphenicol were shown to decrease the binding of *Staphylococcus aureus* to fibronectin, whereas β-lactam antibiotics enhanced this interaction (23). Also, subinhibitory concentrations of tetracycline were reported to decrease fibrinogen binding by *Bacteroides intermedius* (16). Furthermore, growth of *Actinobacillus actinomycetemcomitans* in sub-MIC of cefpodoxime induced morphological changes in the bacteria, causing the organisms to grow as filaments rather than coccobacilli (4). In addition, there are reports that bacteria including staphylococci and streptococci undergo changes in their morphology when grown at low concentrations of certain antibiotics (2). A recent work from our laboratory showed that fibrinogen was able to bind to oral streptococci (19).

In this study, the effect of various antibiotics of sub-MIC on a bacterial surface property such as hemagglutination, as well as on the cell morphology of two major causative agents of infective endocarditis, *S. gordonii* and *S. aureus* were examined. In addition, the effects of sub-MIC of antibiotics on the binding of these bacteria to immobilized fibrinogen were evaluated.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. gordonii* DL1 and *S. aureus* Cowan 1 were obtained from Dr. A. L. Sandberg (NIH, Bathesda, MD, USA) and from Dr. J. W. Kim (School of Dentistry, Kyungbook National University, Taegu, Korea), respectively. The bacteria were grown overnight in brain heart infusion (Difco, Detroit, MICH, USA) (BHI) broth. *S. gordonii* was cultured in the air supplemented with 5% CO_2, and *S. aureus* aerobically. The turbidity of bacterial suspensions was measured by spectrophotometer. A standard curve relating the culture turbidity and bacterial cell numbers was established and utilized.

Determination of minimal inhibitory concentrations (MIC) of antibiotics. To determine the MICs of the antibiotics commonly used for the prevention or treatment of infective endocarditis, the stock antibiotic solutions of genta-micin, streptomycin, erythromycin, penicillin, na-fcillin, ampicillin, oxacillin, amoxicillin, rifampin, vancomycin, cefazolin, cefalothin, and clindamycin (Sigma Chemical Co. St. Louis, MO, USA) were prepared and sterilized by filtering. Two fold serial dilutions of antibiotics were prepared and added to the culture media. Overnight cultures of *S. gordonii* and *S. aureus* were inoculated into BHI media supplemented with each antibiotics and cultured for 18~20 h at 37℃. MIC was the concentration of the highest dilution of antibiotics which inhibited the growth of bacteria (Table 1), and one-half the MIC was used as a subinhibitory concentration of the antibiotic.

Hemagglutination assay. Bacterial cells grown overnight at sub-MICs of each antibiotics were collected by centrifugation, washed twice with 0.01 M TBS (0.15 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.02% NaN_3; pH 7.2). The bacterial suspension was adjusted to an OD_{660} of 0.7, equating 2×10^9 cells per ml of suspension. Red blood cells of sheep and human (blood type A and B) were collected by spinning down (2,000 x g, 10 min), washed three times with PBS containing NaN_3 (0.02%) and BSA (2 mg/ml), and suspended to 1% (v/

Table 1. Minimal inhibitory concentrations of antibiotics against *S. gordonii* and *S. aureus*. Overnight cultures of both bacteria were inoculated into BHI media supplemented with two-fold serially diluted antibiotics and incubated for 18~20 h at 37℃. The MIC was the highest-diluted concentration of antibiotics which inhibited the growth of bacteria

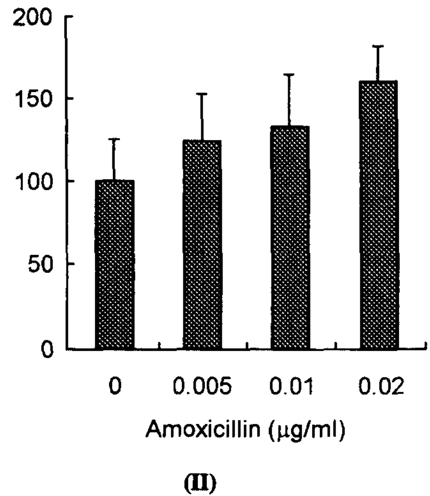
Antibiotics	MICs (μg/ml)	
	S. gordonii DL 1	*S. aureus* Cowan 1
Penicillin	0.005	0.01
Nafcillin	0.005	0.15
Oxacillin	0.04	0.08
Ampicillin	0.6	1.25
Amoxicillin	0.04	0.15
Cephalothin	0.08	0.15
Cefazolin	0.16	0.15
Vancomycin	0.6	1.25
Streptomycin	80	10
Gentamicin	5	1.25
Erythromycin	0.02	0.04
Clindamycin	0.08	0.04
Rifampin	0.08	0.005

v) in the same solution. Bacterial suspension was diluted in a twofold series with PBS in round-bottom microtiter plate (polyvinylchloride 96-well plate). A 25-μl aliquot of the diluted bacterial suspension and an equal volume of 1% erythrocyte suspension of sheep or human were mixed, vortexed for 30 sec and incubated overnight at 4℃. The hemagglutination titer was expressed as the last dilution showing complete agglutination of the erythrocytes.

Light microscopy. Bacteria cultured in BHI broth containing sub-MICs of antibiotics were Gram-stained and observed with light microscope.

Scanning electron microscopy. After growth with or without sub-MICs of antibiotics, the bacteria were washed with phosphate-buffered saline (PBS, 20 mM, pH 7.2), and fixed in suspension in 2.5% glutaraldehyde in PBS for 30 min on ice. The fixed bacteria were then allowed to settle onto glass coverslips previously coated with 1% gelatin. Bacteria were rinsed with PBS and postfixed in 2% osmium tetraoxide in PBS for 30 min. Bacteria were dehydrated through a graded ethanol series, subjected to critical point drying in CO_2 and sputter coated with gold-palladium. The bacteria were viewed in scanning electron microscope JEOL 840A at an accelerating voltage of 5 kV. In each group, sizes of not less than 30 bacterial cells were measured by the image analyzer.

Fibrinogen binding assay. The assay was performed as previously described (19). Cell culture plates (24-well; Corning, New York, NY, USA) were coated overnight with plasminogen-free human fibrinogen (Sigma Chemical Co. St. Louis, MO, USA) or BSA (0.5%) in 0.05 M carbonate buffer (pH 9.5) containing 0.02% NaN_3, at 37℃. Bacterial cultures were grown in 5 ml of BHI both containing 10 μCi of [methyl-^{3}H]thymidine (83 curies/mmol, Amersham, Arlington Height, IL, USA) and sub-MIC of antibiotics, washed 3 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) and adjusted to 5×10^8 cells/ml. Protein-coated wells were washed twice with HBSS-BSA and 0.5 ml of radiolabeled bacterial suspension was added. After 1 h incubation on rocker platform (18 cycles/min) at 37℃, unbound bacterial suspensions were aspirated, and wells were washed 3 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℃ on rocker platform. This 0.5 ml solution and the subsequent 0.5 ml wash of the same solution were added in 10 ml of Scint-A XF (Packard, Meriden, CT, USA) and the radioactivity was counted with scintillation counter. The number of bacteria bound to BSA was subtracted from the number of bacteria bound



(II)

Figure 1. Effect of various concentrations of amoxicillin on the morphology of *S. gordonii* cells. (I) Scanning electron microscopy of the cells grown in the presence of the drug of A) None, B) 0.005 μg/ml, C) 0.01 μg/ml, and D) 0.02 μg/ml. Bar, 1 μm. (II) Relative size of the cells. The changes of bacterial cell size were analysed by scanning electron microscopy. Values are the means of the cell size and error bars represent standard deviation.

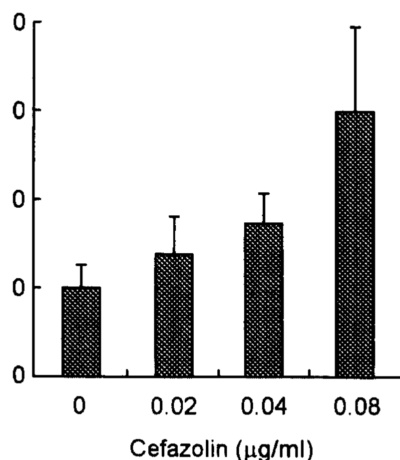
to fibrinogen.

RESULTS

Bacterial hemagglutination. *S. gordonii* grown in control BHI medium showed hemagglutination titer of 1/8 to 1/16, when tested with either sheep or human erythrocytes. Cultures of the same organism grown in antibiotic-supplemented media showed no difference from the normal hemagglutination capacity (data not shown). With *S. gordonii*, no differences in hemagglutination titer were observed between human and sheep blood or between human blood type A and B. *S. aureus* grown in media with or without antibiotics did not agglutinate erythrocytes at all (data not shown).

Microscopic observation of bacteria. Microscopic observation of *S. gordonii* grown at sub-MICs of certain antibiotics showed changes in cell size and shape. *S. gordonii* cells grown at sub-MICs of amoxicillin maintained coccus-shape, but the cells were much enlarged (Figure 1 (I)); the enlargement was proportional to the sub-MIC concentrations of amoxicillin. As shown in Figure 1 (II), at varying sub-MIC concentrations of amoxicillin, i.e., 0.005, 0.01, and 0.02 μg/ml, the average cell size was 1.2, 1.3, and 1.6 times the cells of the control culture, respectively. When cultured at sub-MICs of cefazolin or nafcillin, most *S. gordonii* cells were enlarged and elongated into rod-shape, and the typical changes in cefazolin are shown in Figure 2 (I). Enlargement of the organism was also proportional to the sub-MIC concentrations, resulting in 1.4, 1.7, and 3.0 times the cell size of the untreated culture, at sub-MIC cefazolin concentrations of 0.02, 0.04, and 0.08 μg/ml, respectively (Figure 2 (II)). Other antibiotics were not observed to affect cell size nor shape of *S. gordonii*. Most antibiotics used in this study did not affect the cell morphology of *S. aureus*, except that about 1% of cell populations grown at sub-MICs of ampicillin or cefazolin showed a tendency of becoming rod-shaped (data not shown).

Effects of sub-MIC of antibiotics on binding of bacteria to fibrinogen. Binding of *S. gordonii* to immobilized fibrinogen was considerably increased when grown at sub-MIC



(II)

Figure 2. Effect of various concentrations of cefazolin on the morphology of *S. gordonii* cells. (I) Scanning electron microscopy of the cells grown in the presence of the drug of A) None, B) 0.02 μg/ml, C) 0.04 μg/ml, and D) 0.08 μg/ml. Bar, 1 μm. (II) Relative size of the cells. The changes of bacterial cell size were analyzed by scanning electron microscopy. Values are the means of the cell size and error bars represent standard deviation.

Figure 3. The effect of sub-MIC on the binding of *S. gordonii* and *S. aureus* Cowan 1 to immobilized fibrinogen. Radiolabeled bacterial cultures (0.5 ml of 5×10^8 cells/ml) prepared in the presence of sub-MICs of antibiotics as described in Materials and Methods were added into 24-well cell culture clusters coated with 75 μg/ml of fibrinogen. After 1 h incubation at 37℃, bound bacterial cells were solubilized and their radioactivity was counted. Values are the means of duplicates and error bars represent standard deviations. The data are a representative of two experiments. [a]Not determined.

of most β-lactam antibiotics, such as penicillin, nafcillin, oxacillin, ampicillin, and amoxicillin. On the other hand, the binding of *S. aureus* to fibrinogen was significantly decreased with all β-lactam antibiotics including cephalothin and cefazolin (Figure 3). However, protein synthesis inhibitors like streptomycin, gentamicin, vanco-mycin, and nucleic acid synthesis inhibitor, rifampin, did not affect the binding of both *S. gordonii* and *S. aureus* to fibinogen.

DISCUSSION

Fibrinogen plays a role as a part of the fibrin-platelet matrix in infective endocarditis. Though the binding of *S. aureus* to fibrinogen has been extensively studied, the binding of oral streptococci, particularly *S. gordonii* to fibrinogen was reported by us only recently (19).

In the present study, it was found that sub-MICs of β-lactam antibiotics such as amoxicillin and nafcillin caused significant alterations in the morphology of *S. gordonii* and increased its binding to immobilized fibrinogen. One possible way by which these drugs may increase the binding of *S. gordonii* to fibrinogen is through the alterations in the cell wall. The adhesive molecules of *S. gordonii* responsible for the binding to laminin and fibronectin were found to be present on its cell wall (20, 28). Our previous study showed that the fibrinogen-binding protein of *S. gordonii* was apparently present on its cell surface, since intact bacterial cells bound fibrinogen, and the protein suspected for the binding could be released by briefly suspending the bacterial cells in a buffer solution (19). Fibrinogen-binding proteins distinct from the proteins for fibronectin-binding have been reported in *S. aureus* and *S. epidermidis* (9, 22). These proteins were found to be associated with bacterial cell surface. Since the mechanism by which *S. gordonii* attaches to fibrinogen is incompletely defined (19), it is difficult to predict what kind of alterations are brought about on the cell surface by exposure to the drugs.

Employing fibronectin as an attachment molecule, Proctor *et al.* (23) reported that sub-MICs of antibiotics which inhibit protein synthesis, such as lincosamines, erythromycin, and chloramphenicol decreased fibronectin binding to *S. aureus*, whereas β-lactam antibiotics enhanced this interaction. In our study in which fibrinogen was used as a substrate for bacterial attachment, β-lactam antibiotics enhanced the binding of *S. gordonii* to fibrinogen, whereas the same antibiotics reduced the binding of *S. aureus* to fibrinogen. The protein synthesis-inhibiting antibiotics, streptomycin and gentamicin, used in our studies, neither showed a significant decrease of the binding of *S. gordonii* to fibrinogen nor affect the binding of *S. aureus* to fibrinogen.

The effects of low concentrations of antibiotics as manifested above on the fibrinogen-binding of *S. gordonii* or *S. aureus* should affect the coagulation system whose activation ultimately leads to the formation of fibrin-platelet matrix on the heart-valve in infective endocarditis (11). A key protein in this process is the cell-associated tissue factor (TF). In an in vitro model of infective endocarditis, it was demonstrated that the expression of monocyte TF activity (TFA) depended not only on the adherence of these cells to a fibrin surface but also on an interaction with bacteria (5). Data from recent studies suggest that the main factor determining monocyte-dependent vegetational TFA is the number of vegetation-associated bacteria (6).

It is known that lipoteichoic acid (LTA) of gram-positive bacteria plays a significant role in promoting the binding of streptococci to epithelial cells and fibronectin (8, 10). Exposure of certain streptococci such as *S. sanguis*, and *S. aureus* to β-lactam antibiotics caused the release of LTA into the medium (15, 21). Moreover, the penicillin-induced LTA release from

stationary-phase cultures of *S. pyogenes* was paralleled with a reduction in the number of bacteria attaching to oral epithelial cells (1). These reports implyed that the β-lactam antibiotic-mediated changes in the binding of *S. gordonii* to fibrinogen might also arise through LTA. However, in *S. gordonii* cells, LTA does not appear to be an important receptor for fibrinogen, because fibrinogen-binding to the bacterial cells was not inhibited by LTA (19). Additionally, a fibronectin-binding activity of *S. pyogenes*, called ZOP, was recently identified, and the ZOP is known to be inhibited by LTA (18). Therefore, it may be that the binding of fibronectin by the ZOP is the activity previously attributed to LTA.

The observation in the present study that the hemagglutination ability of *S. gordonii* was not affected by the exposure to antibiotics indicates that fibrinogen-adhesion and hemagglutination do not necessarily involve the same ligand, as was exemplified by a study with *Neisseria meningitidis*; in this case tetracycline affected adhesion without altering hemagglutination (24). As in other reports (4, 25), our study also showed that antibiotics caused alterations in cellular morphology; enlargement and elongation of *S. gordonii* cells due to the exposure to amoxicillin, nafcillin, or cefazolin were consistently observed.

In conclusion, low concentrations of amoxicillin, nafcillin, or cefazolin were able to cause alterations in the morphology of *S. gordonii* cells, resulting in their enlargement and elongation. In addition, β-lactam antibiotics were found to cause enhancement of the binding of *S. gordonii* to immobilized fibrinogen and to reduce the binding of *S. aureus* to the same protein.

Acknowledgements

This work was supported by the Academic Research Fund (Basic Medical Sciences) from the Ministry of Education (1996), Republic of Korea, and by grant 01-96-091 from the Seoul National University Hospital Research Fund.

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