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Interaction between Clarithromycin and Biofilms Formed by *Staphylococcus epidermidis*

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Interactions between clarithromycin and biofilms formed by *Staphylococcus epidermidis* were investigated by using a clarithromycin-resistant strain. Treatment of the colonization with a relatively low concentration of clarithromycin resulted in the eradication of slime-like structure and a decrease in the quantity of hexose. Another result was increased penetration of antibiotics through the biofilm of *S. epidermidis*.

Biofilm bacteria are a major concern for clinicians in the treatment of infections because of their resistance to a wide range of antibiotics (17, 18, 24). Biofilms have in fact been found on the surfaces of biomaterials (5, 8, 25) and tissues (14–16) in chronic bacterial diseases that are characterized by resistance to chemotherapy (4, 9, 10, 12) and resistance to clearance by humoral or cellular host defense mechanisms (11, 13, 23). Some efforts have been made to eradicate biofilm bacteria efficiently. The combination of tobramycin and piperacillin (1) and the combination of antibiotics and dextranase (6) have been reported to be effective for eradicating biofilm bacteria. However, the clinical usefulness of these experimentally effective strategies is obscure. Recently, Takeda et al. (22) demonstrated that long-term low-dose administration of clarithromycin was very effective against diffuse panbronchiolitis and that clarithromycin was effective for the eradication of bacterial biofilms formed in vitro. In our previous report (26), we investigated interaction between clarithromycin and biofilms formed by *Pseudomonas aeruginosa* and presented the new finding that treatment of the biofilms with clarithromycin resulted in the eradication of slime-like structure by means of (i) inhibition of production of some polysaccharides of *P. aeruginosa* or (ii) destruction of exopolysaccharides. In this report, we investigated interaction between bacterial biofilms and clarithromycin using *Staphylococcus epidermidis*, which is well known to be the most common cause of prosthetic-device-related infection (2).

S. epidermidis 7646, a clinical isolate maintained in our laboratory, was used. This strain was selected from among more than 10 strains because it produces abundant slime and has poor susceptibility to clarithromycin (MIC: >200 μg/ml). The slime production was qualitatively measured by the methods of Christensen et al. (3).

The antimicrobial agents used were clarithromycin (Dainabot Co., Ltd., Tokyo, Japan), ofloxacin (Daiichi-Seiyaku Co., Ltd., Tokyo, Japan), and cefotiam (Takeda-Yakuhin Kogyo Co., Ltd., Osaka, Japan).

In vitro formation of bacterial biofilms on membrane filters and interaction with clarithromycin were carried at according to the methods by Yasuda et al. (26), with some modifications. A suspension of *S. epidermidis* was precultured for 20 h in 10 ml of Trypticase soy broth (Eikenkagaku Co., Ltd., Tokyo, Japan), and then it was washed with biological saline by centrifugation. Bacteria were resuspended in 5 ml of minimum medium (MM) [10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.47 g of Na citrate, and 4.0 g of glucose, dissolved in 500 ml of water) to 10^7 CFU/ml and were put into a plastic chamber with a 3-cm diameter in which a cellulose membrane filter (filter type, GS; pore size, 0.22 μm; Nihon Millipore Kogyo K.K., Tokyo, Japan), or a glass microfiber filter (filter type, GF/F; Whatman International Ltd., Maidstone, England), was set. In this medium, the number of viable bacteria gradually decreased. Each chamber was kept at 37°C for 10 days, then the medium in the chamber was discarded, and 5 ml of new medium containing 0 (for the control), 1, 5, 10, or 20 μg of clarithromycin per ml was added. The chambers were kept at 37°C for 5 more days. The filters, taken out from the chambers, were rinsed once with 1/15 M phosphate-buffered saline (PBS), pH 7.0, and were submitted for quantitative analysis of hexose (glass microfiber filter), for the measurement of penetration of antibacterial agents, and for electron microscopy investigation (cellulose membrane filter).

Quantitative analysis of hexose in or on bacteria colonizing the glass microfiber filters, and in the MM in which the biofilm mode of colonization of bacteria had occurred, was performed by the method of Roe (19). To obtain bacteria colonizing the filters, two membrane filters that had bacterial colonization on their surfaces were put into 4 ml of biological saline and were homogenized by a BT-10S homogenizer (Biotron). Homogenized samples were centrifuged at 1,100 × *g* for 15 min to remove fragments of the filters. Two milliliters of the bacterial suspension or MM was treated with 1 ml of 20% perchloric acid. The reaction mixture was kept in an ice bath for 40 min and then was centrifuged for 10 min at 750 × *g*. The supernatant was neutralized by 2 N KOH. Each sample (0.3 ml) was treated with 3 ml of anthrone reagent (a solution containing 0.05% anthrone, 1% thiourea, and 66% H_2SO_4, by volume) in an ice bath. The reaction mixture was boiled at 100°C for 15 min. After the mixture was cooled, its optical density at 620 nm was measured with a spectrophotometer (U-1000; Hitachi-Seisakusho, Tokyo, Japan). Quantitative analysis of bacterial protein was performed by the methods of Stickland (20). As the control for colonizing bacteria and MM, respectively, fresh biological saline and MM were used. A series of experiments was repeated three times.

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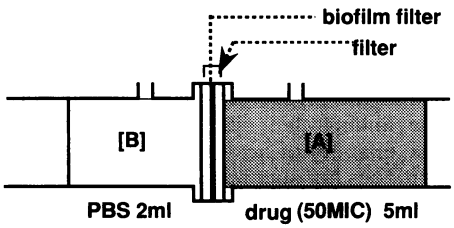

FIG. 1. Assay system for the measurement of penetration of antibiotics through bacterial biofilms.

For the measurement of penetration of antibiotics through bacterial biofilm, the membrane filters that had bacterial biofilms on their surfaces were set in the plastic test systems (26) illustrated in Fig. 1. For the control, fresh membrane filters without bacterial colonies were used in place of those with colonies. Ofloxacin or cefotiam was put into chamber A at concentrations 50 times the MICs (10 and 40 μg of ofloxacin and cefotiam, respectively, per ml). The systems were kept at 37°C. After 2 and 4 h, the concentrations of antibiotics in chamber B were measured by bioassay using *Bacillus subtilis* as the indicator. The experiment was repeated three times.

Membrane filters were processed for scanning microscopy by fixation for 90 min at 4°C in 2% glutaraldehyde in 0.1 M PBS (pH 7.4) plus 8% sucrose. This was followed by refixation for 90 min at 4°C in 2% osmic acid in the same buffer, dehydration in a series of aqueous ethanol solutions (50 to 100%), and drying with a VFD-20 drying apparatus (Hitachi-Seisakusho). Samples were then coated with platinum-palladium by using an E-102 ion coater (Hitachi-Seisakusho) and were examined with an S-400 scanning electron microscope (Hitachi-Seisakusho).

Figure 2 shows scanning electron micrographs of biofilms of *S. epidermidis* on the surface of membrane filters treated with 0 (control) (panel a), 5 (panel b), and 10 (panel c) μg of clarithromycin per ml for 5 days after incubation of the filters and bacteria for 10 days in MM. Abundant exopolysaccharides around bacterial colonies are shown in Fig. 2a, and dose-dependent decreases in the amount of exopolysaccharides are confirmed in Fig. 2b and c.

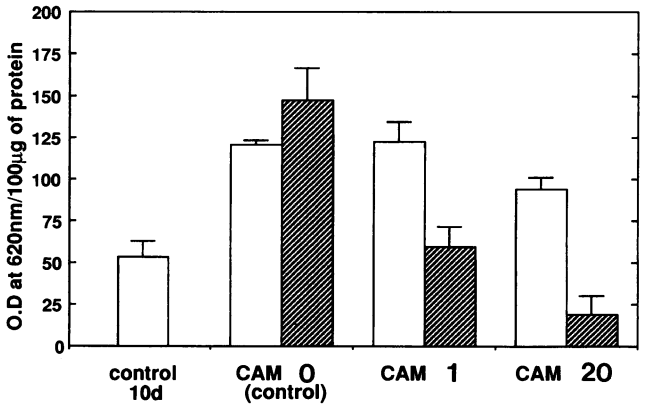

FIG. 3. Quantities of hexose on or in bacterial colonies (▭) and in the environment (▨). control 10d indicates the value obtained by using bacterial biofilms immediately before the addition of clarithromycin. The quantity of hexose in the environment was negligible because it was measured just after the change of the medium. CAM 0, 1, and 20, clarithromycin at 0, 1, and 20 μg/ml; O.D, optical density.

Figure 3 shows the optical densities at 620 nm per 100 μg of protein in samples. These values reflect the quantities of hexose in samples. The quantities of hexose not only in or on the bacterial colonization but also in MM in which bacterial biofilms had been formed clearly decreased dose dependently by treatments with 1 and 20 μg of clarithromycin per ml.

Figure 4 shows the permeabilities of ofloxacin and cefotiam through biofilms of *S. epidermidis*. The vertical axis indicates the penetration rates, with the value for the control assumed to be 100%. Permeabilities of ofloxacin and cefo-

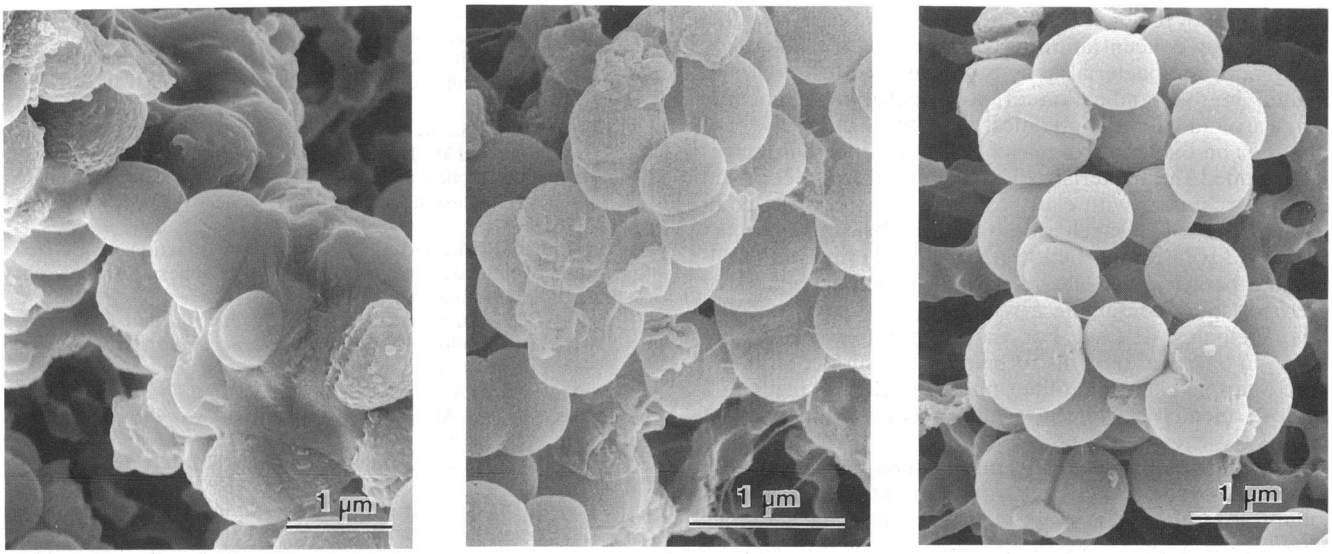

FIG. 2. Changes in structure of bacterial biofilms after treatment with clarithromycin. (a) Control (without clarithromycin); (b) 5 μg of clarithromycin per ml; (c) 10 μg of clarithromycin per ml.

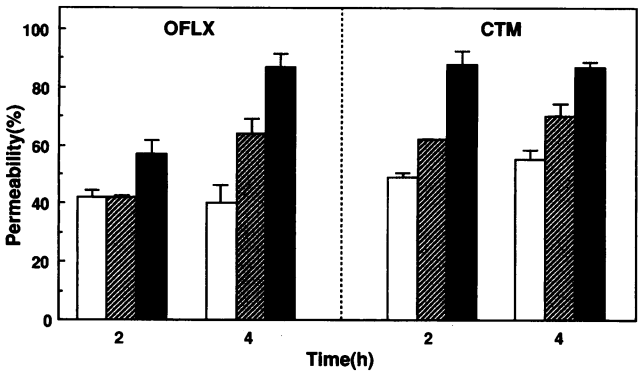

FIG. 4. Permeability of antibiotics through bacterial biofilms. □, control (without clarithromycin); ▨, 1 μg of clarithromycin per ml; ■, 10 μg of clarithromycin per ml. The vertical axis indicates the penetration rates, with the value for the control assumed to be 100%. OFLX, ofloxacin; CTM, cefotiam.

tiam through membrane filters were reduced by formation of *S. epidermidis* biofilm on their surfaces. On the other hand, the treatments of biofilm-bearing filters with 1- and 10-μg/ml concentrations of clarithromycin, a macrolide having poor antibacterial activity against the tested strain of *S. epidermidis*, increased the rates of penetration of antibiotics through the filters in a dose-dependent manner.

Foreign-body infections due to coagulase-negative staphylococci, including *S. epidermidis*, frequently do not respond to antimicrobial agents, and removal of prosthetic devices is often required (7). The biofilm mode of growth of bacteria on the surfaces of biomaterials has been well documented as one of the main causes of chronic prosthetic-device-related infections (5, 8, 25). Exopolysaccharides, also called glycocalyx, acting as a barrier against the penetration of antibacterial agents, are generally emphasized as one cause of the decrease in susceptibility of biofilm bacteria to the agents (9, 10). Therefore, if the polysaccharide glycocalyx matrix in the biofilm structure could be removed, eradication of biofilm bacteria should be possible.

Treatment of the biofilms formed by *S. epidermidis* with a relatively low concentration of clarithromycin, a macrolide having poor antibacterial activity against the tested strain, resulted in the eradication of polysaccharide glycocalyx matrix around bacterial colonies. Quantities of hexose, not only in or on bacterial colonies but also in the environment that included the bacterial colonies, remarkably decreased after the treatment with clarithromycin. Although the compositions of exopolysaccharides produced by *S. epidermidis* are not yet well established, Sutherland (21) reviewed findings that bacterial capsules consisted of homopolymers or heteropolymers containing a wide variety of monosaccharides, including hexose. For the quantitative analysis of hexose in bacterial biofilms formed on the surface of membrane filters, a glass microfiber filter was used in place of a cellulose membrane filter, because cellulose consists of hexoses.

Eradication of the glycocalyx matrix of biofilms resulted in an increase of the rate of penetration of antibiotics through biofilms. Therefore, it is supposed that bactericidal activities of antibiotics against bacteria in a biofilm may be enhanced by clarithromycin. In fact, our previous report (26) demonstrated that in vivo therapeutic effects of ofloxacin in the rat infection model, in which the biofilm mode of growth of *P. aeruginosa* is characteristic, were enhanced by oral coadministration of clarithromycin, a macrolide having no anti-*P. aeruginosa* activity.

The mechanisms of the activities through which clarithromycin eradicates the glycocalyx matrix are as yet unclear. However, the finding that clarithromycin suppresses the increase in the quantity of hexoses (including free hexoses) suggests that it does not suppress the step of polymerization of hexoses but rather suppresses a step or steps of the synthesis of monosaccharides. Moreover, the finding also suggests that the eradication of glycocalyx matrix by clarithromycin is not due to only a destruction of hexose-containing polysaccharides once produced, although the possibility of acceleration of the destruction by clarithromycin cannot be excluded.

The finding that clarithromycin has an activity to eradicate the glycocalyx matrix produced by not only *P. aeruginosa* but also *S. epidermidis*, both of which are highly resistant to clarithromycin, suggests that the activity is independent of the general modes of antibacterial activities of clarithromycin; it also suggests that there may be some macrolides, and/or some other related compounds, that have even stronger antibiofilm activity despite having no antibacterial activity.

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