ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1993, p. 1749–1755
0066-4804/93/091749-07$02.00/0

Vol. 37, No. 9

Interaction between Biofilms Formed by *Pseudomonas aeruginosa* and Clarithromycin

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Received 1 March 1993/Returned for modification 4 May 1993/Accepted 9 June 1993

Interactions between bacterial biofilms formed by *Pseudomonas aeruginosa* and clarithromycin, a macrolide having no anti-*P. aeruginosa* activity, were investigated. *P. aeruginosa* incubated for 10 days on membrane filters formed biofilms on the surfaces of the filters. The biofilms were characterized by dense colonizations of bacteria and thick membranous structures that covered the colonies. Treatment of the biofilms with a relatively low concentration of clarithromycin for 5 days resulted in an eradication of the membranous structures. Quantitative analysis of alginate and hexose was done to evaluate the quantity of polysaccharides in or on the biofilms. Treatment of the biofilms with clarithromycin decreased the quantity of alginate and hexose and therefore perhaps the quantity of polysaccharides as well. Eradication of the membranous structures of biofilms, or the decrease in the quantity of polysaccharides, resulted in an increase in the rate of penetration of antibiotics through bacterial biofilms. 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. It is suggested that clarithromycin eradicated glycocalyx produced by *P. aeruginosa*, or suppressed the production of glycocalyx, by unknown mechanisms and thereby enhanced the therapeutic efficacies of other antimicrobial agents against infections caused by *P. aeruginosa*.

Biofilm bacteria are a major concern for clinicians in the treatment of infections because of their resistance to a wide range of antibiotics (28, 29, 36). Biofilms have in fact been found on the surfaces of biomaterials (6, 8, 37) and tissues (20, 22, 23) in chronic bacterial diseases that are characterized by resistance to chemotherapy (5, 9, 12, 16) and resistance to clearance by humoral or cellular host defense mechanisms (15, 19, 35). Some efforts have been made to eradicate biofilm bacteria efficiently. The combination of tobramycin and piperacillin (2) and the combination of antibiotics and dextranase (7) have been reported to be effective for eradication of biofilm bacteria. However, the clinical usefulness of these experimentally effective strategies is not yet established.

Recently, Nagai et al. (24) showed that long-term low-dose administration of erythromycin was very effective against diffuse panbronchiolitis, although the maximum serum and sputum levels of erythromycin were below the MICs for clinically pathogenic *Haemophilus influenzae* and *Pseudomonas aeruginosa* strains, which were often isolated from the sputa of the patients. Macrolides have been known to have some activities in addition to antibacterial activities. Ichikawa et al. (18) demonstrated that erythromycin reduced neutrophils and neutrophil-derived elastolytic-like activity in the lower respiratory tracts of bronchiolitis patients. Goswami et al. (14) showed that erythromycin inhibited respiratory glycoconjugate secretion from human airways, and Fujimaki et al. (13) showed that the combination of tosufloxacin and erythromycin or clarithromycin (CAM) was remarkably effective for eradicating sessile biofilm bacteria.

In this study, we investigated an interaction between *P. aeruginosa* biofilms and CAM and also investigated the effectiveness of combination therapy with an antibacterial agent and CAM by using experimental infections in rats in which biofilms formed by *P. aeruginosa* are present.

MATERIALS AND METHODS

Animals. Male Wister-Imamichi rats weighing 140 to 150 g, purchased from the Imamichi Institute for Animal Reproduction (Saitama, Japan), were used. Rats were acclimatized for 5 to 7 days prior to use in a room in which an environment of controlled temperature, relative humidity, and a 12-h light-dark cycle was maintained.

Bacteria. *P. aeruginosa* 1008, a nonmucoid clinical isolate maintained in our laboratories, was precultured in Trypticase soy broth (Eikenkagaku Co., Ltd., Tokyo, Japan) at 37°C for 20 h before use.

Agents. Ofloxacin (OFLX) (Daiichi-Seiyaku Co., Ltd.,

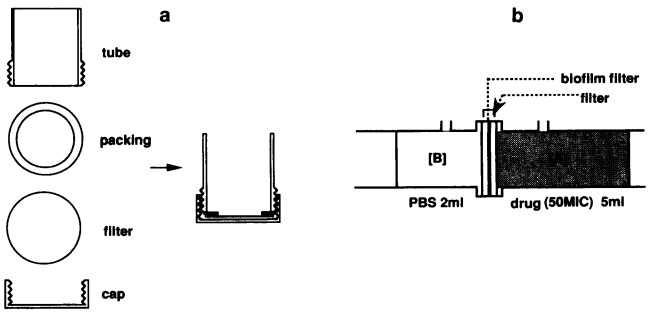

FIG. 1. (a) Plastic chamber for the formation of bacterial biofilms on membrane filters. (b) Assay system for the measurement of penetration of antibiotics through bacterial biofilms.

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Tokyo, Japan), CAM (Dainabot Co., Ltd., Tokyo, Japan), and gentamicin (GM) (Schering-Plough, Osaka, Japan) were used.

Determination of MICs. The MICs were measured on Mueller-Hinton II agar (Becton Dickinson) by the twofold dilution method. The inoculum size of the bacteria was 10^4 CFU per spot.

In vitro formation of bacterial biofilms on membrane filters and interaction with CAM. The suspension of *P. aeruginosa* precultured for 20 h in 10 ml of Trypticase soy broth was washed with biological saline (saline) by centrifugation. Bacteria were resuspended in 5 ml of saline to 10^7 CFU/ml and put into a plastic chamber of 3-cm diameter in which a membrane filter (filter type GS; pore size, 0.22 μm; Nihon Millipore Kogyo K.K., Tokyo, Japan) was set (Fig. 1a). Each chamber was kept at 37°C for 10 days, and then the saline in the chamber was discarded and 5 ml of new saline containing 0 (for the control), 0.1, 1, 5, 10, or 20 μg of CAM per ml was added. The chambers were kept at 37°C for 5 more days. The filters, taken from the chambers, were rinsed once with 0.067 M phosphate-buffered saline (PBS), pH 7.0, and submitted for quantitative analysis of alginate and hexose, for measurement of penetration of antibacterial agents, and for electron microscopy.

Quantitative analysis of alginate, hexose, and protein. Quantities of alginate and hexose in or on bacteria colonized on the membrane filters and in the saline in which bacterial biofilms had been formed were measured. To obtain bacteria colonized on the filters, two membrane filters that had bacterial colonization on their surfaces were put into 4 ml of saline and homogenized by a BT-10S homogenizer (Biotron). Homogenized samples were centrifuged at 1,100 × *g* for 15 min to remove fragments of the filters.

Quantitative analysis of alginate was performed by the method of Toyoda et al. (34) with some modifications. One milliliter of the bacterial suspension or saline was mixed with 3 ml of a 10% solution of copper sulfate. The reaction mixture was adjusted to pH 4.0 by 1 N HCl, kept at room temperature for 1 h, and centrifuged at 1,050 × *g* for 10 min. The precipitate was redissolved in 0.1 ml of 1 N NH_4OH and diluted with 0.9 ml of water. The sample (1 ml) was treated with 2 ml of copper-HCl reagent (40 ml of concentrated HCl plus 1 ml of 2.5% copper sulfate solution plus 9 ml of water) and 1 ml of naphthoresorcinol reagent (100 mg of 1,3-dihydroxynaphthalene was dissolved in 25 ml of water) and was kept in a boiling water bath for 40 min. After being chilled, the mixture was mixed with 4 ml of butyl acetate, shaken well, and centrifuged to separate the butyl acetate layer. After one wash with 20% NaCl solution, the optical density at 565 nm was measured. The quantity of alginate was calculated on the basis of a standard curve made with alginate solution.

The quantity of hexose was measured by the method of Roe (30). Two milliliters of the bacterial suspension or saline was treated with 1 ml of 20% perchloric acid. The reaction mixture was kept in an ice bath for 40 min and then centrifuged for 10 min at 750 × *g*. The supernatant was neutralized by 2 N KOH. The 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).

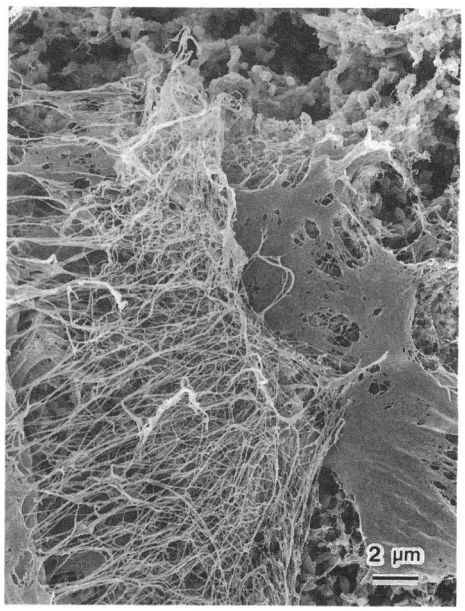

FIG. 2. Bacterial biofilm of *P. aeruginosa* formed on a membrane filter.

Quantitative analysis of bacterial protein was performed by the methods of Stickland (31). A series of experiments was repeated three times.

Measurement of penetration of antibiotics through bacterial biofilms. The membrane filters that had bacterial biofilms on their surfaces were set in the plastic test systems illustrated in Fig. 1b. For the control, fresh membrane filters without bacterial biofilms on their surfaces were used in place of those with biofilms. OFLX or GM was put into chamber A at concentrations 50 times the MICs 80 and 20 μg/ml for OFLX and GM, respectively. 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.

Induction of infection in pouches on the backs of rats and therapy by antibiotics. The methods used were described previously by Ajiki et al. (1). An air pouch on the back of a rat was formed by injecting 10 ml of air subcutaneously with a 21-gauge needle after trimming off the hair with a hair clipper. Just after removal of the needle, the needle hole in the skin was sealed with an adhesive agent. The next day, under anesthesia, the air in the pouch was aspirated and a carboxymethyl cellulose (CMC) pouch was formed by injecting 10 ml of sterilized 1.5% CMC (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) in saline. An infection was induced by inoculating 10^6 CFU of *P. aeruginosa* 1008 per pouch along with the injection of CMC.

The therapy was begun 4 days after the induction of infection, with oral administration of 200 mg of CAM or 100 mg of OFLX per kp per dose and coadministration of 200 mg of CAM plus 100 mg of OFLX per kg per dose. Drug administrations were continued twice a day for 5 days. Pouch exudates were sampled by syringe once a day for 6 days after the beginning of therapy. The numbers of viable bacteria in the pouch exudates were counted on Mueller-Hinton agar. The experimental schedule is illustrated in Fig. 7. Three or four rats were used for each group.

Scanning electron microscopy. Pieces of subcutaneous

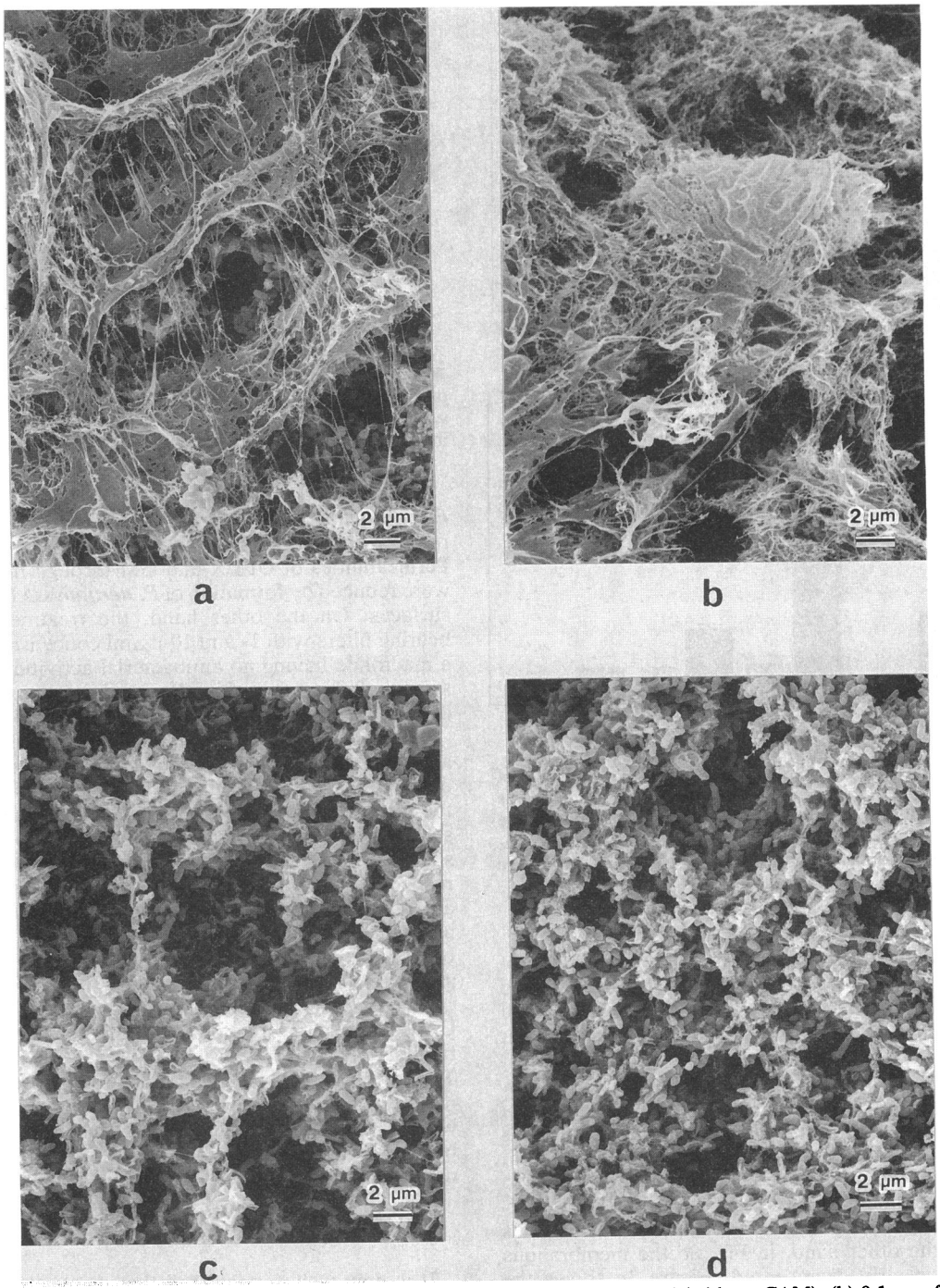

FIG. 3. Changes in structure of bacterial biofilms after treatment with CAM. (a) Control (without CAM); (b) 0.1 μg of CAM per ml; (c) 5 μg of CAM per ml; (d) 10 μg of CAM per ml.

tissue in the infected CMC pouches and 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 Ion-coater E-102 (Hitachi-Seisakusho) and examined by using an S-400 scanning electron microscope (Hitachi-Seisakusho).

RESULTS

MICs of antibiotics for bacteria. The MICs of CAM, OFLX, and GM for *P. aeruginosa* 1008 were >100, 1.56, and 0.39 μg/ml, respectively.

In vitro biofilm mode of growth of bacteria on the mem-

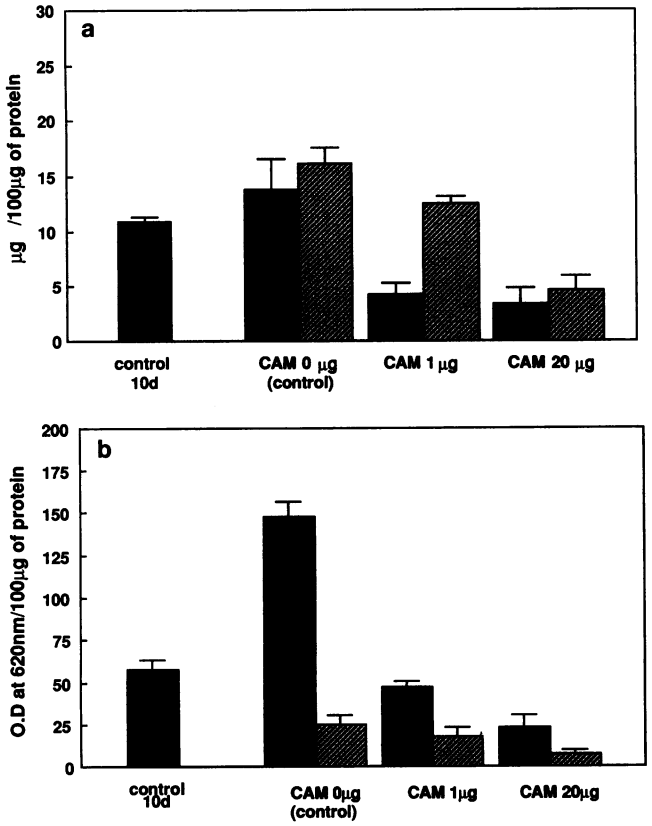

FIG. 4. Quantities of alginate (a) and hexose (b) on or in bacterial colonies (■) and in the environment (▨). Control 10d indicates the value obtained by using bacterial biofilm immediately before the addition of CAM. The quantity of alginate or hexose in the environment was negligibly small because it was measured just after the change of the medium. O.D., optical density.

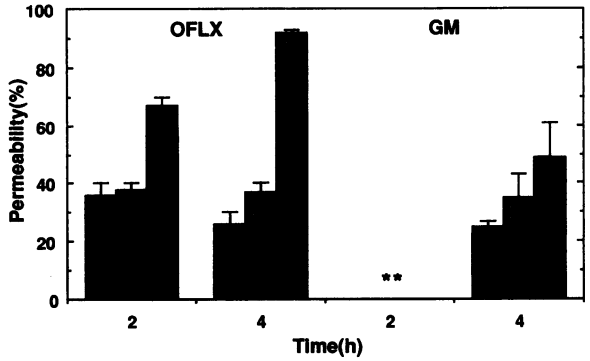

FIG. 5. Permeability of antibiotics through bacterial biofilms. ■, control (without CAM); ▨, 1 μg of CAM per ml; ▦, 10 μg of CAM per ml. The vertical axis indicates the penetration rates, with the value for the control assumed to be 100%. Asterisks indicate that the value was at the limit of determination.

brane surface. Figure 2 shows a scanning electron micrograph of the surface of a membrane filter that was incubated for 10 days with *P. aeruginosa*. Dense colonizations of bacteria and a thick membranous, or fibrous, structure that covers the colonies are observed.

Effects of CAM on the structures of bacterial biofilms. Figure 3a, b, c, and d show scanning electron micrographs of the biofilm mode of growth of *P. aeruginosa* on the surfaces of membrane filters after 5 days of treatment with 0, 0.1, 5, and 10 μg of CAM per ml, respectively. Figure 3a, similar to Fig. 2, shows the biofilm mode of growth of bacteria characterized by dense colonizations and thick membranous structures. On the other hand, in Fig. 3d the membranous structures have clearly disappeared. Figure 3c shows the middle condition between the quantities of membranous structure seen in Fig. 3a and d.

Changes in the quantities of alginate and hexose. Figure 4a shows the quantity of alginate per 100 μg of protein in samples, and Fig. 4b shows the optical density at 620 nm per 100 μg of protein. The values in Fig. 4b reflect the quantities of hexose in samples. The quantities of alginate and hexose not only in or on the bacterial colonization but also in the saline in which bacterial biofilms had been formed clearly decreased in a dose-dependent manner after treatment with 1 and 20 μg of CAM per ml.

Penetration of antibiotics through bacterial biofilms. Figure 5 show the permeabilities of antibiotics through biofilms of *P. aeruginosa*. The vertical axis indicates the penetration rates, with the value for the control assumed to be 100%. Permeabilities of OFLX and GM through membrane filters were reduced by formation of *P. aeruginosa* biofilms on their surfaces. On the other hand, the treatments of biofilm-bearing filters with 1- and 10-μg/ml concentrations of CAM, a macrolide having no antibacterial activities against tested strains of *P. aeruginosa*, increased the rates of penetration of antibiotics through the filters in a dose-dependent manner.

Therapeutic effect of combined use of OFLX and CAM against experimental *P. aeruginosa* infections in rats. The development of the biofilm mode of growth over time after the infection of *P. aeruginosa* in CMC pouches of rats is shown in Fig. 6a and b, which show electron micrographs of subcutaneous tissue at 1 and 2 days after the infection, respectively. Figures 6a′ and b′ are magnifications of Fig. 6a and b, respectively. It can be seen that many erythrocytes and some other cells, complicated fibrous structures, and bacteria, in one body, make the colonies of bacteria progressively larger and thicker.

The therapeutic effect of combined use of OFLX and CAM against the experimental infections in rats in which biofilms of *P. aeruginosa* were present is shown in Fig. 7. Treatment with CAM alone did not result in a decrease in the number of viable bacteria in a pouch, whereas treatment with OFLX alone resulted in a gradual decrease. The combined use of both drugs, however, resulted in a remarkable decrease in the number of viable bacteria.

DISCUSSION

It is now well documented that many chronic infections involve colonization of bacteria growing as an adherent biofilm within an extended polysaccharide glycocalyx (4, 16, 27, 33). Despite some efforts (2, 7), eradication of biofilm bacteria has been found to be difficult (3, 26). At present, two main causes are generally emphasized for the decrease in susceptibility of biofilm bacteria to antibacterial agents: the slow growth rate of biofilm bacteria (5, 10, 37) and exopolysaccharide, or glycocalyx, acting as a barrier against the penetration of antibacterial agents (9, 12). Therefore, if the polysaccharide glycocalyx matrix in the biofilm structure could be removed, eradication of biofilm bacteria should be possible. For the purpose of removing glycocalyx matrix, in this study we investigated the interaction between bacterial

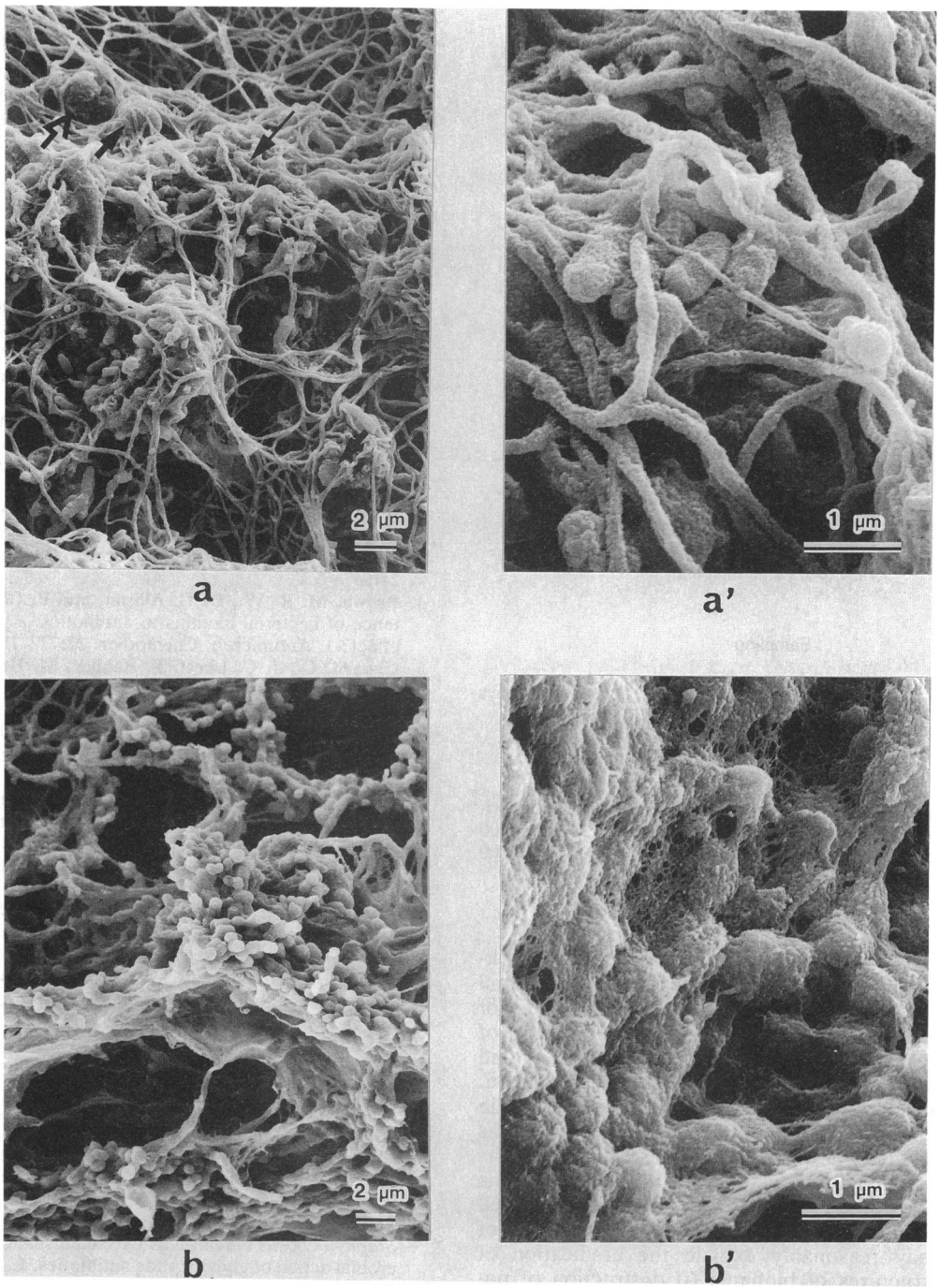

FIG. 6. Biofilm mode of growth of *P. aeruginosa* on subcutaneous tissues of rats 1 day (a and a') and 2 days (b and b') after infection. ➡, erythrocyte; ➡, another type of cell; ➡, fibrous structure.

biofilms that were formed in vitro by *P. aeruginosa* and CAM, because *P. aeruginosa* has been known as one of the major participants in biofilm-participating chronic infections (3, 9, 17, 20).

P. aeruginosa incubated for 10 days on membrane filters formed biofilms having very complex structures on the surfaces of the filters. The biofilms were characterized by dense colonizations of bacteria and thick membranous structures. So far, exact biochemical characterization of the membranous structures has not been done, although the structures have been ascertained to interact with ruthenium red, a dye that interacts with acidic polysaccharides (data not shown). Treatment of the biofilms with a relatively low concentration of CAM, a macrolide having no antibacterial activities against *P. aeruginosa*, for 5 days resulted in an eradication of the membranous structures that covered the bacterial colonizations. Moreover, quantities of alginate and hexose, not only in or on bacterial colonies but also in the

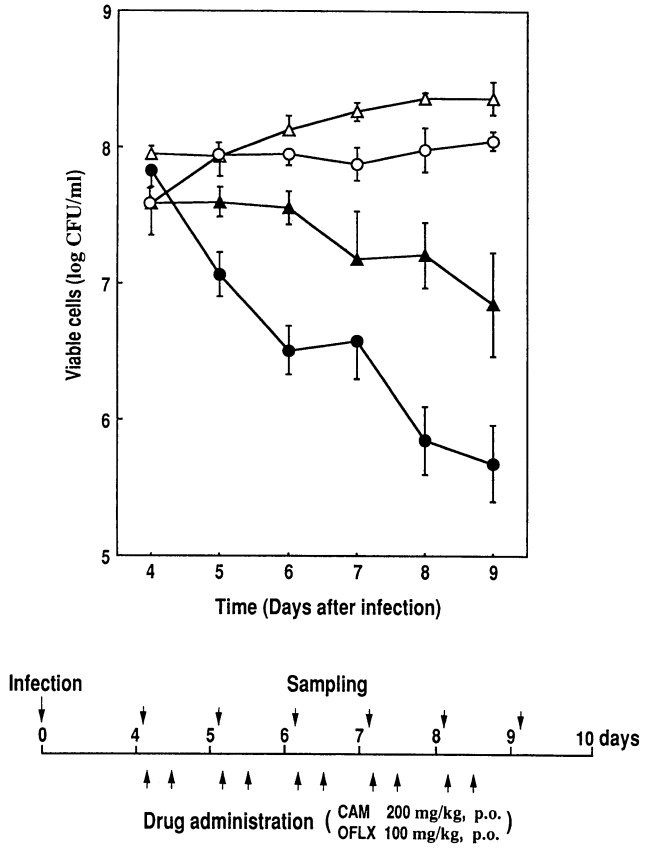

FIG. 7. Effects of combined use of OFLX and CAM on experimental infection in rats. ○, control (without therapy); △, CAM (200 mg/kg per dose); ▲, OFLX (100 mg/kg per dose); ●, CAM (200 mg/kg per dose) plus OFLX (100 mg/kg per dose).

environment that included the bacterial colonies, remarkably decreased after the treatment with CAM. Sutherland (32) reviewed findings that bacterial capsules consisted of homo- or heteropolymers containing a wide variety of monosaccharides, including hexose. However, Evans and Linker (11) reported that *P. aeruginosa* produced alginate. Alginate is reported to contain no hexose (21). Therefore, in this study we measured the quantities of alginate as well as hexose, because the *P. aeruginosa* strain used in this experiment was a nonmucoid type and because the compositions of polysaccharides produced by *P. aeruginosa* are not well established so far. From our experimental results, either of two reasons may reasonably explain the eradication of membranous structures of biofilms: (i) destruction of polysaccharide glycocalyx by CAM and (ii) inhibition of new synthesis of polysaccharides.

Eradication of the membranous structures, or eradication of the glycocalyx matrix, of biofilms resulted in an increase of the rate of penetration of antibiotics through biofilms. Therefore, it was supposed that bactericidal activities of antibiotics against bacteria in a biofilm might be enhanced by CAM. On the basis these experimental results and suppositions, we investigated the therapeutic effects of cotherapy with OFLX and CAM in an animal model of infection in which the biofilm mode of growth of *P. aeruginosa* is characteristic. The combination of OFLX and CAM resulted in an enhanced therapeutic efficacy of OFLX. Taking into consideration that CAM has no antibacterial activity against *P. aeruginosa* and that macrolides are generally known to decrease the bactericidal activities of quinolones in vitro (25), the synergy of OFLX and CAM in the therapeutic effect in the animal model of infection is very interesting. It may be reasonable to assume that the synergy originates from the activity of CAM to remove polysaccharide glycocalyx in or on bacterial biofilms. It is as yet uncertain if the activity of CAM to remove glycocalyx is independent of the general modes of antibacterial activities of CAM.

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