ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1992, p. 1208–1214
0066-4804/92/061208-07$02.00/0

Vol. 36, No. 6

Dynamic Interactions of Biofilms of Mucoid *Pseudomonas aeruginosa* with Tobramycin and Piperacillin

HOSMIN ANWAR,[1]* JANICE L. STRAP,[1] KAREN CHEN,[1] AND J. WILLIAM COSTERTON[2]

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9,[1] and Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4[2]

Received 15 July 1991/Accepted 13 March 1992

The dynamic interaction of planktonic and biofilm cells of mucoid *Pseudomonas aeruginosa* with tobramycin and piperacillin was investigated in a chemostat system. The results indicated that planktonic and young biofilm cells of the 2-day-old chemostat culture of *P. aeruginosa* were susceptible to killing by chemostat-controlled doses of either 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml or 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml. Complete eradication of the planktonic and young biofilm cells was observed after exposure of the cells to six chemostat-controlled doses of these antibiotics at 8-h intervals for 7 days. Regrowth of the organism was not observed after the termination of antibiotic therapy on day 7. A different picture was observed when antibiotic treatment was initiated on day 10 after inoculation. Viable old biofilm cells were reduced to approximately 20% after exposure to the chemostat-controlled doses of 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml. Complete eradication of old biofilm cells could not be achieved, and regrowth of the organism occurred after the termination of antibiotic therapy. These data suggest that young biofilm cells of mucoid *P. aeruginosa* can be effectively eradicated with the combination of piperacillin and tobramycin, while old biofilm cells are very resistant to these antibiotics and eradication of old biofilm cells is not achievable with the chemostat-controlled doses of piperacillin and tobramycin used in this study.

Pseudomonas aeruginosa is an opportunistic pathogen which rarely causes infection in the healthy host. However, the organism has emerged as a dominant pulmonary pathogen in cystic fibrosis (CF) patients (13, 25, 30). Severe lung damage caused by colonization of mucoid *P. aeruginosa* often results in the death of children with CF. The virulence determinants produced by this pathogen have been reviewed previously (10, 16, 21, 22).

Antibiotics with excellent activity against *P. aeruginosa* play an important role in reducing the mortality, increasing the life expectancy, and improving the quality of life of CF patients. Despite the use of aggressive antibiotic therapy in the treatment of *Pseudomonas* infections in CF patients (15), eradication of the organism from the lungs of CF patients is difficult to achieve at present (15, 21, 25, 30). It has been suggested that this is because of the biofilm mode of growth of the organism in the lungs of CF patients (8, 19, 24). The sticky alginate slime produced by mucoid *P. aeruginosa* is capable of binding antibiotic molecules, thereby significantly reducing the penetration of antibiotic through the glycocalyx matrix (7, 24, 28). There are those, however, who suggest that glycocalyx per se does not reduce the penetration of antibiotics but that it is actually the biofilm mode of growth that alters the physiology of embedded biofilm cells, resulting in changes of the permeability of antibiotics across the cell envelope of these cells (23). We believe that studies on the behavior of biofilms of mucoid *P. aeruginosa* may provide some insights into the formulation of effective strategies to combat biofilm-associated infections caused by *P. aeruginosa*.

An in vitro chemostat system has been developed to investigate the interaction of biofilm cells of *P. aeruginosa* with piperacillin and tobramycin (1, 3, 4). In this system, the organism was allowed to colonize inert solid surfaces and grow at a slow growth rate under conditions of iron restriction. These growth parameters have been suggested to play a profound role in affecting the physiology of *P. aeruginosa* in vivo (2, 5) and should be considered in the design of experiments to study the interaction of *P. aeruginosa* with antibiotics (2). Biofilm cells grown under these conditions were removed from the chemostat and exposed to fixed concentrations of antibiotics in test tubes. The studies revealed that young biofilm cells harvested on day 2 were still sensitive to piperacillin and tobramycin and could be effectively eradicated with relatively low concentrations of these antibiotics (1). However, old biofilm cells harvested on day 7 were very resistant to these antibiotics, and the eradication of the biofilm cells of mucoid *P. aeruginosa* was not achievable even when much higher concentrations of these antibiotics were used (1, 3, 4).

The results suggest that antibiotic therapy should be implemented as early as possible when dealing with biofilm-associated infections, because complete eradication of young biofilm cells colonizing the inert surfaces is still achievable (1, 3, 4). Delay in implementing antibiotic therapy is likely to result in the failure of the therapy, and recurrent infections may occur when antibiotic treatment is terminated. Although the studies have given some useful insights into the interaction of biofilm cells of mucoid *P. aeruginosa* with tobramycin and piperacillin (1, 3, 4), the dynamic interaction of these antibiotics with bacteria in biofilms was not considered in these studies. The biofilm cells were exposed to one dose of fixed concentrations of these antibiotics for a short time. Chemostats are open systems in which the trough and peak levels of antibiotics can be carefully controlled by monitoring the flow rates of antibiotic free medium added to the chemostats. In this report we discuss the dynamic interaction of planktonic and biofilm cells of mucoid *P. aeruginosa* with many doses of piperacillin and tobramycin for a period of a week. The usefulness of this

* Corresponding author.

system in the study of dynamic interaction of bacteria in biofilms with clinically important antibiotics is discussed.

MATERIALS AND METHODS

Bacteria. Mucoid *P. aeruginosa* M 579 was used throughout this study. The strain was maintained as freeze-dried cultures and recovered by growth on nutrient agar plates. After overnight incubation at 37°C, the growth from the plates was inoculated into 100 ml of iron-restricted tryptic soy broth (TSB-Fe) medium.

Preparation of growth medium. TSB-Fe was used for the cultivation of mucoid *P. aeruginosa* in this study. The procedure for removal of iron from complex laboratory medium has been described previously (17). It involved passing the tryptic soy broth (Difco Laboratories, Detroit, Mich.) through a column containing Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). Of the iron present in this complex laboratory medium, 85 to 95% was shown to be removed by this treatment (17). 2,2'-Dipyridyl (final concentration, 25 μg/ml) was added to the Chelex-treated medium to further restrict the availability of trace amounts of iron present in the medium (9). The method also removes other metal cations such as Mg^{2+} (17), and 0.4 mM $MgSO_4$, 0.5 mM NaCl, and 0.62 mM KCl (final concentrations) were added to the medium to avoid imposing additional nutrient depletion on the cells.

Growth of mucoid *P. aeruginosa* in the chemostat. An in vitro chemostat system described by Anwar et al. (1, 3, 4) was used throughout. Chemostats with 100 pieces of 1-cm-long (size 16) Masterflex silicone tubing (Cole-Parmer Instrument Ltd., Chicago, Ill.) were autoclaved at 121°C for 20 min. Five milliliters of *P. aeruginosa* grown to mid-exponential phase in TSB-Fe was inoculated into the chemostat. Fresh TSB-Fe medium was fed into the chemostat by using a peristaltic pump to give a dilution rate (D) of 0.125 h^{-1} (one volume change occurred every 8 h). Viable counts of planktonic cells of mucoid *P. aeruginosa* were determined on serially diluted samples incubated on nutrient agar (Difco). To determine the number of biofilm cells adhered to the silicone tubing, the tubing was removed, washed three times with 10 ml of phosphate-buffered saline (PBS) to remove nonadherent cells, and then placed in 1 ml of PBS. The contents were vortex mixed for 3 min, and the serially diluted samples were incubated on nutrient agar plates at 37°C. The efficiency of the removal of the biofilm cells of mucoid *P. aeruginosa* from the surface of the tubing was monitored by repeating on the vortex treated tubing the procedures described above. The results indicated that the first treatment removed over 99% of the adherent biofilm cells.

Dynamic interaction of planktonic and biofilms of mucoid *P. aeruginosa* with piperacillin and tobramycin. On day 2 (young biofilm) or day 10 (old biofilm), combinations of piperacillin and tobramycin in 5 ml of TSB-Fe medium (to give final concentrations of 250 μg of piperacillin per ml and 5 μg of tobramycin per ml or 500 μg of piperacillin and 5 μg of tobramycin per ml; the antibiotics were sterilized by filtration) were added every 8 h to the chemostat cultures containing the planktonic and biofilm cells of mucoid *P. aeruginosa*. The dilution rate of the chemostat was set to 0.125 h^{-1} so that one volume change of TSB-Fe medium in the chemostat occurred every 8 h. Samples were removed at hourly intervals, and viable counts were performed for both the planktonic and biofilm cells by plating onto nutrient agar plates and incubating at 37°C when the first doses of these antibiotics were added to the chemostat. At t = 8 h, the second doses of these antibiotics (same concentrations of piperacillin and tobramycin) were added to the chemostat, and the viable counts were determined before the addition of the next doses of the antibiotics. The addition of these concentrations of piperacillin and tobramycin to the chemostat cultures were repeated at 8-h intervals for 7 days. On day 7 (after initiation of antibiotic exposure), the antibiotic treatment was terminated and fresh TSB-Fe was continuously pumped into the chemostat at the same dilution rate for another 3 days to investigate the regrowth of the organism.

Antibiotic concentration analysis. Culture samples were collected from the effluent port of the chemostat before and after addition of antibiotics into the chemostat. The cells were separated by centrifugation at 5,000 × g for 15 min and the culture supernatants were sterilized by membrane filtration. The concentrations of piperacillin were analyzed by a spectrophotometric method (measurement of A_{254}), and the concentrations of tobramycin were determined by microbiological assay with the agar well method (26). *P. aeruginosa* DMX 568/1 (kindly provided by V. Deretic) cultivated in TSB-Fe plus 300 μg of carbenicillin per ml was used as an inoculum; this strain is resistant to piperacillin because β-lactamases are produced when the cells are cultivated in the presence of carbenicillin.

RESULTS

Kinetics of biofilm formation by mucoid *P. aeruginosa* M579. The population of the planktonic cells of mucoid *P. aeruginosa* reached approximately 4 × 10^9 cells per ml and remained constant until tobramycin and piperacillin were added to the chemostat. The kinetics of biofilm formation by this strain of *P. aeruginosa* is illustrated in Fig. 1. The number of biofilm cells colonizing the surface of the silicone tubings increased exponentially from day 1 to day 5. The number of sessile cells associated with the tubings was found to be approximately 1.5 × 10^9 cells per cm (length) on day 5, and this population continued to increase at a much slower rate to approximately 2.5 × 10^9 cells per cm on day 10. Since oxygen was supplied to the chemostat in the form of air bubbles which created turbulence, it was expected that some of the loosely bound sessile bacteria were released from the silicone tubings and this might contribute to the establishment of an equilibrium state from day 7 to day 10 (Fig. 1). It is important to note that the growth rate of biofilm cells is probably not strictly governed by the dilution rate, since the cells that are deeply embedded in the thick glycocalyx matrix may have very different access to the limiting nutrient in comparison with the planktonic cells. The same phenomenon may also be true when the organism forms biofilms in vivo.

Reaction of young biofilm cells of mucoid *P. aeruginosa* after exposure to the first chemostat-controlled dose of tobramycin and piperacillin. In this study, young biofilm cells were used to describe the properties of sessile cells cultivated to the second day in the chemostat and old biofilm cells were used to designate the properties of sessile cells cultivated to day 10 in the chemostat.

Two days after inoculation, 5 μg of tobramycin per ml plus 250 μg piperacillin per ml or 5 μg of tobramycin per ml plus 500 μg piperacillin per ml was added to the chemostat at 8-h intervals and the viability of the planktonic and young biofilm cells was monitored. The interaction of young biofilm cells (day 2 in the chemostat) of mucoid *P. aeruginosa* with

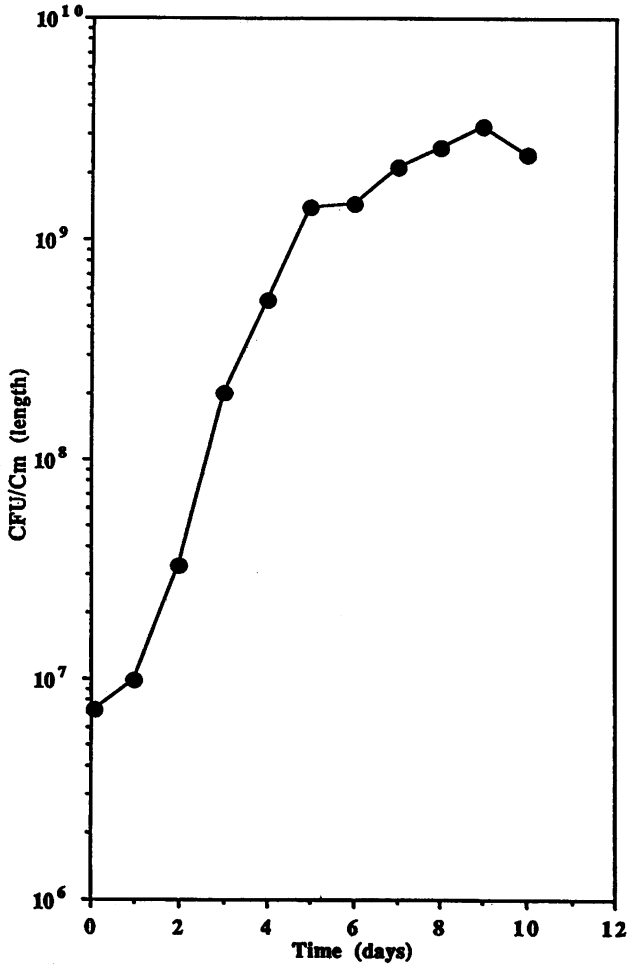

FIG. 1. Kinetics of biofilm formation of a mucoid strain of *P. aeruginosa* M 579 grown slowly under iron-restricted conditions in the chemostat.

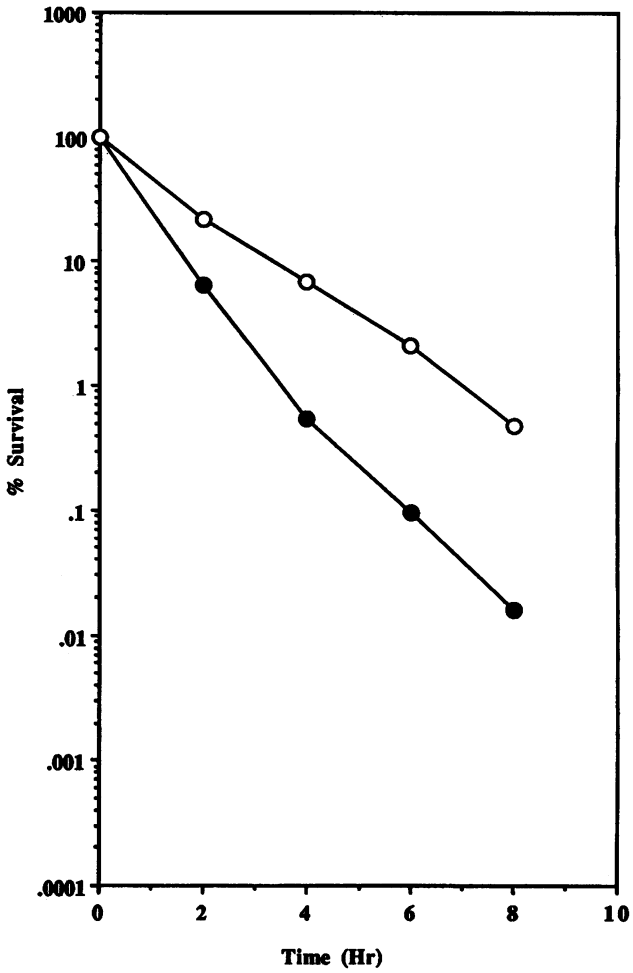

FIG. 2. Dynamic interaction of planktonic and young biofilm cells of the 2-day-old chemostat cultures of mucoid *P. aeruginosa* M 579 with the first dose of 500 μg of piperacillin plus 5 μg of tobramycin per ml. The dilution rate (0.125 h^{-1}) was chosen so that one volume change of the medium occurred every 8 h. Symbols: ●, planktonic cells; ○, biofilm cells.

500 μg of piperacillin per ml plus 5 μg of tobramycin per ml is illustrated in Fig. 2. The dilution rate of the chemostat was set to 0.125 h^{-1} so that one volume change of the medium in the chemostat occurred every 8 h. This corresponds to the frequency of the administration of clinical dosing of these antibiotics. The concentration of tobramycin was kept at 5 μg/ml because this concentration is believed to be achievable in the serum.

It can be seen that the planktonic and young biofilm cells of this strain of mucoid *P. aeruginosa* were sensitive to tobramycin and piperacillin (Fig. 2). The percentage of planktonic cells was reduced from 100 to approximately 0.05% after exposure to the first dose of 5 μg of tobramycin per ml plus 250 μg of piperacillin per ml for 8 h (data not shown). The percentage of young biofilm cells in the chemostat was reduced to approximately 10% when the cells were exposed to the same concentrations of antibiotics for the same period of time (data not shown). Increasing the concentration of piperacillin to 500 μg/ml resulted in a slight increase in the killing of both the planktonic and young biofilm cells (Fig. 2). The percentages of planktonic and young biofilm cells were found to be approximately 0.01 and 0.45%, respectively, after exposure to the chemostat-controlled first dose of these antibiotics (Fig. 2). At these concentrations of tobramycin and piperacillin, the planktonic and young biofilm cells were rapidly eradicated, indicating that this strain of mucoid *P. aeruginosa* was sensitive to the combination of these antibiotics.

Dynamic interaction of planktonic and young biofilm cells of *P. aeruginosa* with tobramycin and piperacillin for 7 days. The interaction of planktonic and young biofilm cells of *P. aeruginosa* with the chemostat-controlled doses of tobramycin plus piperacillin for a period of 7 days is illustrated in Fig. 3. It can be seen that both the planktonic and young biofilm cells of *P. aeruginosa* were sensitive to chemostat-controlled doses of 5 μg of tobramycin per ml plus 250 μg of piperacillin per ml. Eradication of planktonic and young biofilm cells was observed after exposure of young biofilm cells to six chemostat-controlled doses of tobramycin plus piperacillin (data not shown; the profile was similar to those observed in Fig. 3). Since the antibiotics were given at 8-h intervals, the results suggested that eradication of the planktonic and young biofilm cells was achievable after 2 days of antibiotic treatment. In this study, the antibiotic therapy was continued for an extra 5 days and no viable planktonic or

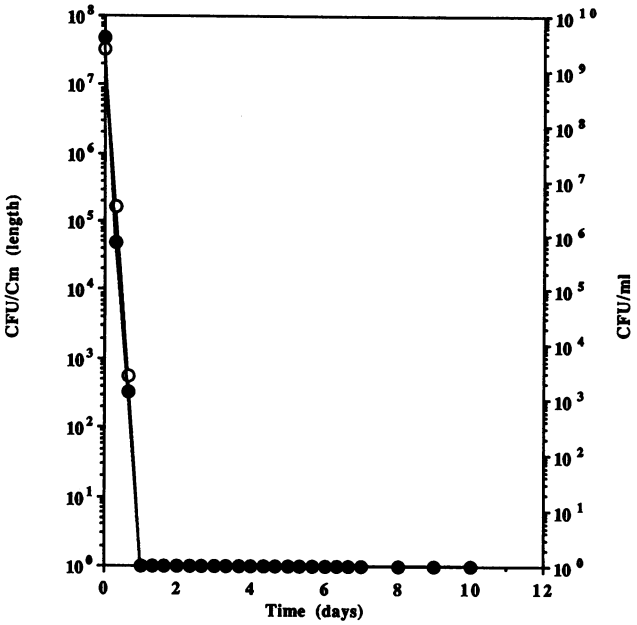

FIG. 3. Dynamic interaction of planktonic and young biofilm cells of the 2-day-old chemostat cultures of mucoid *P. aeruginosa* M 579 with doses of 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml at 8-h invervals for 7 days. Symbols: ●, planktonic cells; ○, biofilm cells.

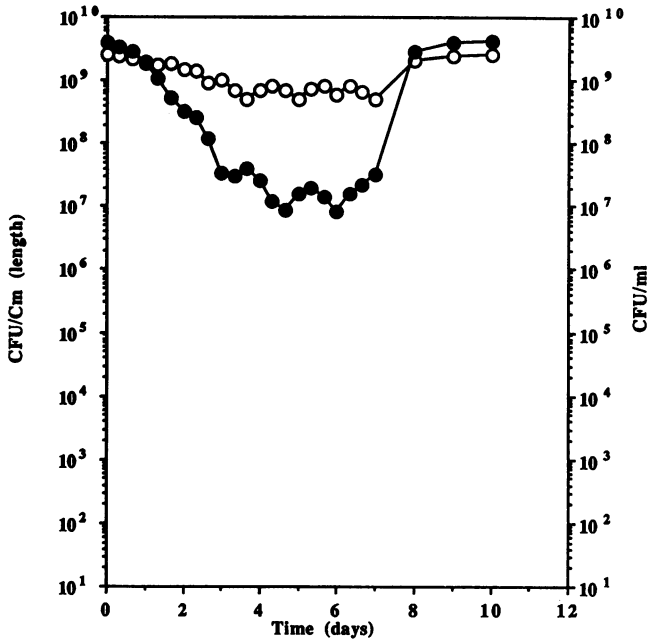

FIG. 4. Dynamic interaction of planktonic and old biofilm cells of the 10-day-old chemostat cultures of mucoid *P. aeruginosa* M 579 with doses of 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml at 8-h invervals for 7 days. Symbols: ●, planktonic cells; ○, biofilm cells.

young biofilm cells were found during this period of study (data not shown). Regrowth of the organism was not observed when the antibiotic treatment was terminated on day 7 (data not shown). Increasing the concentration of piperacillin to 500 μg/ml while keeping the concentration of tobramycin to 5 μg/ml showed a marginal increase in the activity of these antibiotics (Fig. 3). Four chemostat-controlled doses of these antibiotics at 8-h intervals (Fig. 3) were needed to eradicate the planktonic and young biofilm cells present in the chemostat. The eradication of young biofilm cells over several days might be partly due to slight accumulation of the antibiotics in the system with repeated doses and slow reduction in half-life. From this study, it is clear that the planktonic and young biofilm cells of mucoid *P. aeruginosa* are still sensitive to the combined bactericidal actions of piperacillin and tobramycin and eradication of this organism is achievable if antibiotic therapy is started on or before day 2 of colonization. Thus, 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml or 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml should be able to achieve eradication of the planktonic and young biofilm cells of mucoid *P. aeruginosa*.

Dynamic interaction of planktonic and old biofilm cells of mucoid *P. aeruginosa* with tobramycin and piperacillin for 7 days. Mucoid *P. aeruginosa* was allowed to colonize the surface of silicone tubings for 10 days before antibiotic intervention. It can be seen from Fig. 1 that heavy colonization of the surface of the tubings was observed at this time and the population of the old biofilm cells increased to approximately 2.5×10^9 cells per cm (length). Another observation was that the silicone tubing connecting the effluent port of the chemostat to the waste bottle was also heavily colonized by the organism, indicating that this organism is an excellent biofilm former.

Ten days after inoculation, 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml or 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml was added to the chemostat cultures and the viability of the planktonic and old biofilm cells was monitored. In both cases, the viability of the planktonic and old biofilm cells was not significantly reduced after exposure to the first chemostat-controlled dose of these antibiotics. Figure 4 illustrates the dynamic interaction of multiple doses of 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml with old biofilm of *P. aeruginosa* for a period of 7 days. Similar profiles with slightly less reduction in viable count were observed when the old biofilms of *P. aeruginosa* were exposed to the combination of 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml (data not shown). After the first chemostat-controlled dose of tobramycin and piperacillin, two different colonial types were detected on nutrient agar: one was opaque and the other was transparent. Each morphotype was streaked onto *Pseudomonas* isolation agar (PIA) to ensure that the chemostat culture was not contaminated. Both the opaque and transparent colonies grew on *Pseudomonas* isolation agar. Approximately 80% of the colonies observed on a given plate were opaque, while the remaining 20% were transparent. The opaque colony type was more resistant to both tobramycin and piperacillin than the transparent type. The MICs of tobramycin for opaque and transparent colonies were found to be 0.6 μg/ml and less than 0.15 μg/ml, respectively, and the MICs of piperacillin for opaque and transparent colonies were 26 and 6.5 μg/ml, respectively. Even after discontinuation of the chemostat-controlled doses of these antibiotics, these different colony types were observed in the same proportion. No such changes were noted with young biofilm cells or with the planktonic cells associated with them.

The chemostat cultures of planktonic and old biofilm cells

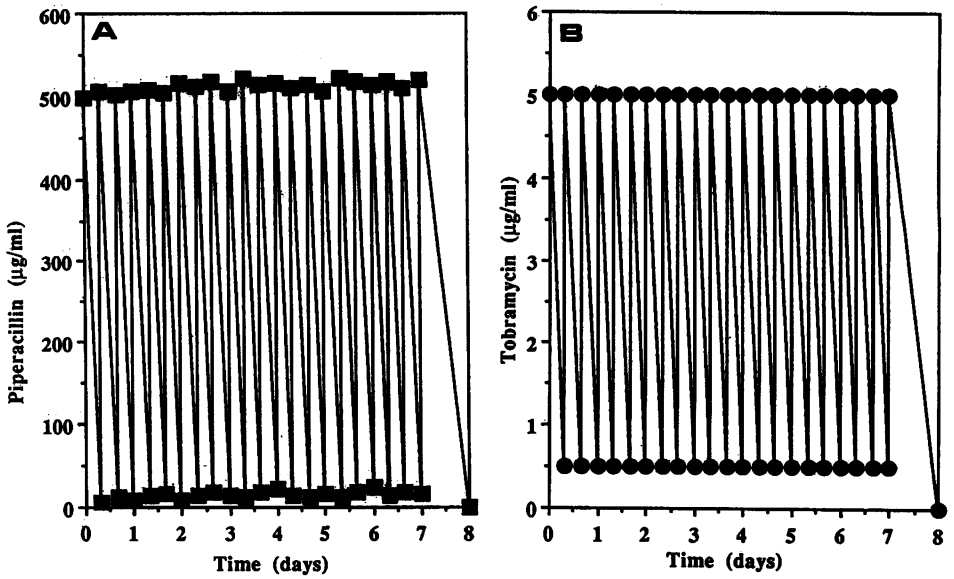

FIG. 5. Profiles of concentrations of piperacillin (A) and tobramycin (B) collected from the effluent port of the chemostat before and after addition of antibiotics to the chemostat.

of mucoid *P. aeruginosa* were very resistant to the combination of 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml (data not shown). Nine doses of these antibiotics given at 8-h intervals were needed to reduce the viability of the planktonic cells to approximately 10%. The population of the planktonic cells remained at the range of 5 to 10% during the course of antibiotic treatment. There was a significant reduction in the turbidity of the planktonic cell culture, but these concentrations of tobramycin plus piperacillin were not sufficient to eradicate the planktonic cells. Some of the planktonic cells observed in this experiment might have resulted from the release of old biofilm cells. The population of the planktonic cells increased to 100% after the termination of antibiotic treatment on day 7 (data not shown). The old biofilm cells were resistant to killing by the combination of 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml. The viability of the old biofilm cells adhering to the surface of the silicone tubings remained greater than 80% throughout this study and returned to 100% after the termination of the antibiotic treatment on day 7 (data not shown).

Figure 4 illustrates the dynamic interaction of the planktonic and old biofilm cells of mucoid *P. aeruginosa* with the combination of 500 μg of piperacillin plus 5 μg of tobramycin per ml. Increasing the concentration of piperacillin to 500 μg/ml significantly increased the killing of the planktonic and old biofilm cells of mucoid *P. aeruginosa*. However, these concentrations of piperacillin and tobramycin were not sufficient to achieve eradication of old biofilm cells. The population of the planktonic cells dropped to approximately 1% after exposure to the chemostat-controlled doses of these antibiotics for 3 days and it remained in the range of 0.2 to 1% for the course of antibiotic therapy. The population of the planktonic cells climbed back to 100% after the termination of antibiotic treatment on day 7.

The concentrations of piperacillin and tobramycin before and after the addition of antibiotics into the chemostat over the interval of the study are illustrated in Fig. 5A and B, respectively. It can be seen that the concentrations of piperacillin dropped to the level below 20 μg/ml before the addition of the next dose of antibiotics and increased to slightly over 500 μg/ml after the addition of the antibiotics to the chemostat (Fig. 5A). The concentrations of tobramycin decreased to approximately 0.5 μg/ml (minimum limit of detection, ~0.5 μg/ml) before the administration of the next dose of antibiotics and increased to 5 μg/ml after the addition of the antibiotics into the chemostat (Fig. 5B).

DISCUSSION

It is interesting that the chemostat-controlled doses of piperacillin and tobramycin used in this study can be used effectively to eradicate the planktonic and young biofilm cells of mucoid *P. aeruginosa* (Fig. 3). Combinations of either 250 μg of piperacillin per ml plus 5 μg of tobramycin per ml or 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml were sufficient to achieve eradication of 2-day-old young biofilm cells. Enhanced activity is often observed when β-lactam antibiotics are combined with aminoglycosides, and these compounds are commonly used in combination in the treatment of infections (1, 11, 18). We believe that these concentrations of antibiotics are achievable clinically, and if antibiotic therapy is initiated at the early stage of infection, one should observe eradication of young biofilm cells. Antibiotic molecules can still penetrate the newly formed biofilms to kill the young biofilm cells (1–4, 23). Note that air bubbles may be dislodging biofilm cells from the silicone tubings and also the wall of chemostat, and these cells are likely to be sensitive to antibiotics (14). It would be of interest to use the in vitro model developed by Haag et al. (14) to study the kinetic interaction of bacteria with antibiotics so that the results observed in this study can be compared with those obtained with other systems.

A very different picture is observed if the antibiotic treatment is delayed for an extra 8 days. The old biofilm cells of mucoid *P. aeruginosa* did not respond to the chemostat-controlled doses of piperacillin and tobramycin used in the study. Eradication of the infecting old biofilm cells was not achievable even when the concentration of piperacillin was

increased to 500 μg/ml. The population of the old biofilm cells of mucoid *P. aeruginosa* was reduced to approximately 20% after exposure to the chemostat-controlled doses of piperacillin plus tobramycin for 3 days. The percentage of survival remained in the range of 20 to 40% during the course of antibiotic therapy. The population of the old biofilm cells increased back to 100% after the termination of the antibiotic treatment on day 7. Eradication of planktonic and old biofilm cells was not observed after exposure of the chemostat culture to doses of 500 μg of piperacillin per ml plus 5 μg of tobramycin per ml for 7 days (Fig. 4). The data confirmed our previous observations that old biofilm cells of *P. aeruginosa* were very resistant to these antibiotics and the chemostat-controlled doses of these antibiotics were not sufficient to eradicate the biofilm cells colonizing the silicone tubings. From our studies, the results indicate that the best strategy to combat biofilm-associated infections is to initiate the antibiotic treatment as early as possible, because young biofilm cells of mucoid *P. aeruginosa* are still sensitive to the combination of piperacillin and tobramycin. Although these doses of antibiotics were not successful in eradicating the infecting pathogen, they may have reduced the population of the planktonic cells significantly so that the clinical symptoms were temporarily curtailed. This is obviously not the goal of antibiotic therapy, and hopefully the chemostat system described in this study can be used to optimize the performance of these antibiotics in the treatment of biofilm-associated infections.

The physiology of bacteria in biofilms is extremely complex and very different from those of planktonic cells. The physiology of bacteria in biofilms is likely to be influenced by the sites in which each individual biofilm cell is located in the multilayers of cells forming the biofilm (6, 7, 20, 29). Cells located on the surface of the biofilm may have easy access to nutrients and may also have fewer problems with the discharge of waste metabolic products. In contrast, cells that are embedded deep within the alginate glycocalyx matrix are likely to be less metabolically active because of poor access to essential nutrients and also because they have to deal with problems associated with the accumulation of waste metabolites in their milieu. The physiology of the bacterial cell can profoundly influence the sensitivity of the cell to antibiotics (2, 7). Under these circumstances, one would be able to suggest that the mechanisms of resistance of biofilm bacteria to antibiotics are extremely complex. This is partly due to the complex nature of the physiology of bacteria in biofilms. The alginate produced by old biofilm cells may bind the antibiotic molecules and therefore impede the penetration of the antibiotics (7, 24, 28). The ability of antibiotic molecules to cross the outer membrane of the old biofilm cells may be reduced significantly as a result of the alteration of the composition of the outer membrane (2, 7, 23). The reduction in the rate of the penetration of antibiotic molecules across the outer membrane of old biofilm cells embedded within the thick glycocalyx matrix may give the cells sufficient time to switch on the expression of antibiotic-degrading enzymes, enabling them to destroy the antibiotic molecules that have entered the cells. Induction of β-lactamases have been observed when sessile cells of *P. aeruginosa* are exposed to β-lactam antibiotics (12). It is therefore important to use biofilm-eradicating concentrations when dealing with bacteria in biofilms so that they can be killed instantaneously before the cells express antibiotic resistance factors to counteract the presence of antibiotics.

It is extremely frustrating to find that an antibiotic in which the efficacy against a particular group of pathogens has been extensively evaluated in the laboratory fails to eradicate that group of pathogens clinically. The problems of bacterial resistance to antibiotics have been the focus of recent discussions (27). The use of MIC in describing the susceptibility of bacterial pathogens to antibiotics has been scrutinized. MIC susceptibility tests can be deceiving since no one can guarantee that antibiotics with low MICs can be used successfully to control bacterial infections, especially biofilm-associated infections. MICs should only be used to identify resistant strains and not to indicate the usefulness of the antibiotic in the control of infections unless the concentrations and frequency of antibiotic administration required to eradicate the infecting pathogen are known. One problem of MIC testing is that planktonic cells are used in the determination of MICs, while in practice the antibiotics are used to eradicate biofilm cells. Clinicians fail to eradicate biofilm cells because concentrations of antibiotics much higher than the MICs are required to eradicate bacteria in biofilms (1, 3, 4). We think that the system described in this study will give more realistic information regarding the effectiveness of antibiotics in the eradication of bacteria in biofilms, because the concentrations and frequency of antibiotic administration that are required to eradicate the biofilm cells can be determined. Under these circumstances, it is logical for us to suggest that only antibiotics with known activity against biofilm cells of *P. aeruginosa* should be used to treat bronchiopulmonary infections of CF patients and that treatment should be initiated as early as possible to avoid the problems of dealing with old biofilms. Studies on the physiology of biofilm cells of this organism will assist in the design of effective therapy to combat *Pseudomonas* infections.

ACKNOWLEDGMENTS

This work was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada (H. Anwar and J. W. Costerton), which we gratefully acknowledge.

REFERENCES

1. **Anwar, H., and J. W. Costerton.** 1990. Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**:1666–1671.
2. **Anwar, H., M. K. Dasgupta, and J. W. Costerton.** 1990. Testing the susceptibility of bacteria in biofilms to antibacterials. Antimicrob. Agents Chemother. **34**:2043–2046.
3. **Anwar, H., M. Dasgupta, K. Lam, and J. W. Costerton.** 1989. Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. J. Antimicrob. Chemother. **24**:647–655.
4. **Anwar, H., T. van Biesen, M. Dasgupta, K. Lam, and J. W. Costerton.** 1989. Interaction of biofilm bacteria with antibiotics in a novel in vitro chemostat system. Antimicrob. Agents Chemother. **33**:1824–1826.
5. **Brown, M. R. W., H. Anwar, and P. A. Lambert.** 1984. Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiol. Lett. **21**:113–117.
6. **Caldwell, D. E., and J. R. Lawrence.** 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **12**:299–312.
7. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
8. **Costerton, J. W., J. Lam, K. Lam, and R. Chan.** 1983. The role of the microcolony in the pathogenesis of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5**:S867–S873.
9. **Deneer, H. G., and A. A. Potter.** 1989. Iron-repressible outer

membrane proteins of *Pasteurella haemolytica*. J. Gen. Microbiol. **135:**435–443.
10. **Deretic, V., J. F. Gill, and A. M. Charkrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. Bio/Technology **5:**469–477.
11. **Eliopoulous, H.** 1986. Aminoglycosides plus beta lactams against gram-negative bacteria. Am. J. Med. **80:**126–137.
12. **Giwercman, B., E. T. Jensen, S. S. Pedersen, and N. Hoiby.** 1990. Induction of β-lactamase production in *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. **35:**1008–1010.
13. **Govan, J. R. W.** 1988. Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis, p. 67–96. *In* E. Griffiths, W. Donachie, and J. Stephen (ed.), Bacterial infections of respiratory and gastrointestinal mucosae. IRL Press, Oxford.
14. **Haag, R., P. Lexa, and I. Werkhauser.** 1986. Artifacts in dilution pharmacokinetic models caused by adherent bacteria. **29:**765–768.
15. **Hoiby, N., B. Friss, K. Jensen, C. Koch, N. E. Moller, S. Stovring, and M. Szaff.** 1982. Antimicrobial chemotherapy in cystic fibrosis patients. Acta Paediatr. Scand. Suppl. **301:**75–100.
16. **Iglewski, B. H., L. Rust, and R. A. Beever.** 1990. Molecular analysis of *Pseudomonas aeruginosa* elastase, p. 36–43. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
17. **Kadurugamuwa, J. L., H. Anwar, M. R. W. Brown, G. H. Shand, and K. H. Ward.** 1987. Media for study of growth kinetics and envelope properties of iron-deprived bacteria. J. Clin. Microbiol. **25:**849–855.
18. **Klastersky, J., R. Cappel, and D. Danneau.** 1972. Clinical significance of in vitro synergism between antibiotics in gram-negative infections. Antimicrob. Agents Chemother. **2:**470–475.
19. **Lam, J., R. Chan, K. Lam, and J. W. Costerton.** 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. **28:**546–556.
20. **Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell.** 1987. *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface environments. Microb. Ecol. **14:**1–14.
21. **May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, N. A. Zielinski, A. Berry, R. K. Rothmel, T. K. Misra, and A. M. Chakrabarty.** 1991. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. **4:**191–206.
22. **Nicas, T. I., and B. H. Iglewski.** 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31:**387–392.
23. **Nichols, W. W., M. J. Evans, M. P. E. Slack, and H. L. Walmsley.** 1989. The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. J. Gen. Microbiol. **135:**1291–1303.
24. **Nickel, J. C., I. Ruseska, and J. W. Costerton.** 1985. Tobramycin resistance of cells of *Pseudomonas aeruginosa* growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27:**619–624.
25. **Pier, G. B.** 1986. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. **151:**575–580.
26. **Platt, T. B.** 1986. Microbiological assay of antibiotics in body fluids and tissues, p. 341–386. *In* A. Aszalos (ed.), Modern analysis of antibiotics, vol. 27, Marcel Dekker, Inc., New York.
27. **Sanders, C. C.** 1991. A problem with antimicrobial susceptibility tests. ASM News **57:**187–190.
28. **Slack, M. P. E., and W. W. Nichols.** 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. **10:**368–372.
29. **Van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder.** 1990. Influence of interfaces on microbial activity. Microbiol. Rev. **54:**75–87.
30. **Vasil, M. L.** 1986. *Pseudomonas aeruginosa*: biology, mechanisms of virulence, and epidemiology. J. Pediatr. Res. **108:**800–805.