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Enhanced Activity of Combination of Tobramycin and Piperacillin for Eradication of Sessile Biofilm Cells of *Pseudomonas aeruginosa*

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An in vitro chemostat system in which *Pseudomonas aeruginosa* can be cultivated at a slow growth rate and under iron limitation conditions was used to study the susceptibilities of sessile bacteria of mucoid and nonmucoid *P. aeruginosa* strains to tobramycin and piperacillin. Planktonic cells of both mucoid and nonmucoid *P. aeruginosa* strains were susceptible to tobramycin and piperacillin. None of the cells was found to be viable after 2 h of exposure to 200 μg of piperacillin plus 10 μg of tobramycin per ml. Young sessile bacteria were slightly more resistant to piperacillin or tobramycin than the planktonic cells were. However, eradication of young sessile bacteria could be achieved with a combination of piperacillin and tobramycin. None of these young biofilm bacteria were found to be viable after a 2-h exposure to 200 μg of piperacillin plus 10 μg of tobramycin per ml. Old sessile bacteria were very resistant to these antibiotics. Eradication of old sessile bacteria could not be achieved with either tobramycin (200 μg/ml) or piperacillin (200 μg/ml) alone. Combination of higher concentrations of tobramycin with piperacillin resulted in an enhancement of killing of the old sessile bacteria. Exposure of old sessile bacteria to 200 μg of piperacillin plus 100 μg of tobramycin per ml resulted in the reduction of the viable count to approximately 0.02%. The data suggest that the eradication of biofilm-associated infections is best carried out as early as possible. Enhanced activities against the sessile bacteria were achieved when higher concentrations of aminoglycosides were combined with β-lactam antibiotics.

Bacterial infections caused by colonization with sessile bacteria are a major cause of morbidity in patients who have received medical implants (11, 19, 24, 25). Because of their resistance to antibiotics, biofilm bacteria are a major concern to clinical microbiologists and clinicians engaged in the treatment of infectious diseases. Eradication of biofilm bacteria is often found to be difficult (3, 4, 8, 11, 19, 21, 23, 26). This may be achievable in the near future if some efforts are made to understand the interaction of sessile bacteria with antibiotics.

An in vitro chemostat system has recently been developed to study the interaction of biofilm bacteria with antimicrobial agents (3, 4). This system takes into account some growth parameters of bacteria in vivo. Slow growth rate, iron restriction, and the biofilm mode of growth were considered to be the key growth conditions of bacterial pathogens in vivo (1, 5, 14, 15, 20, 22, 29, 32). These parameters have been shown to be important environmental factors that govern the physiological state of the bacterial pathogens in vivo (5, 6, 14, 34, 35). The information on the interaction of antibiotics with bacteria cultivated under these conditions is important if we are to formulate strategies to eradicate biofilm bacteria in vivo.

The plasticity of the bacterial envelope plays an important role in the survival of bacteria in hostile environments (6, 7, 14). Alteration of envelope compositions, susceptibility to antibiotics, and resistance to host defenses in response to changes in the growth environment have been reported (2, 6, 13, 14). Any experimental protocol designed to test the biological activities of antibiotics should take into account the effects of growth environment on the susceptibility of the test organism to those antibiotics.

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β-Lactam antibiotics are commonly combined with aminoglycosides such as tobramycin in the treatment of *Pseudomonas* infections. Combination of these antibiotics may minimize the development of resistance to individual agents and, at the same time, achieve a synergistic effect (12, 18). However, planktonic cells cultivated in complex laboratory media were used in previous studies (18). It is therefore of interest to use the chemostat to study the synergistic effect of β-lactams and aminoglycosides on *Pseudomonas* biofilms. In this report we discuss the interaction of piperacillin and tobramycin with sessile bacteria of mucoid and nonmucoid strains of *Pseudomonas aeruginosa* cultivated under conditions mimicking those obtained in vivo.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* ATCC 27835 (nonmucoid strain), *P. aeruginosa* UAM 12 (an isogenic mucoid variant derived from *P. aeruginosa* ATCC 27835 by the method described by Ombaka et al. [27]), and *P. aeruginosa* 492 (a mucoid strain isolated from a patient with cystic fibrosis) were used throughout this study.

Media. Iron-depleted tryptic soy broth (TSB-Fe; Difco Laboratories, Detroit, Mich.) was used in this study. A total of 85 to 95% of the iron present in tryptic soy broth was shown to be removed following treatment with Chelex 100 (16). Mono- and divalent cations were added to TSB-Fe as described by Kadurugamuwa et al. (16).

Determination of MICs. TSB-Fe was used in the determination of the MICs of tobramycin (Sigma Chemical Co., St. Louis, Mo.) and piperacillin (Lederle Laboratories Division, Hampshire, United Kingdom) for these strains. Tobramycin and piperacillin were sterilized by membrane filtration. Fresh antibiotic solution was prepared daily and discarded after use. The MIC was determined by the tube dilution

method (16). The lowest concentration of antibiotic resulting in the complete inhibition of visible growth was taken as the MIC.

Tile construction. The tiles were made from a self-curing methyl methacrylate dental resin (product RR; De Trey Ltd., Weybridge, United Kingdom) as described by Keevil et al. (17). Instead of the stainless steel wire described by Keevil et al. (17), nylon thread was used to suspend the tiles (0.5 by 2 cm).

Continuous cultivation. The continuous culture system described by Ombaka et al. (27) was used. Chemostats with the acrylic tiles suspended inside were autoclaved at 121°C for 20 min. Fresh TSB-Fe was fed into the chemostat with a peristaltic pump. *P. aeruginosa* (5 ml; mucoid or nonmucoid) grown to the mid-exponential phase (optical density at 470 nm, 1) in TSB-Fe at 37°C was used as the inoculum. Fresh medium was added to the culture at a dilution rate of 0.05/h. For experiments with mucoid isolates, samples were removed daily and the serially diluted samples were spread onto nutrient agar and incubated at 37°C. This was to ensure that there were no nonmucoid *P. aeruginosa* revertants present in the chemostat. No nonmucoid revertants were isolated in the study.

Viable counts of bacteria in the suspension were determined on serially diluted samples incubated on nutrient agar (Difco) at 37°C. To quantitate biofilm bacteria on acrylic tiles, the tiles were first washed three times with phosphate-buffered saline (10 ml; pH 7.4) to remove nonadherent bacteria and were then placed in 1 ml of phosphate-buffered saline. The contents were vortex mixed for 3 min, and the serially diluted samples were incubated on nutrient agar (Difco) at 37°C. The efficiency of removal of biofilm bacteria from the tiles was checked by repeating the procedures described above on the treated tiles. The results indicated that the first treatment removed over 99% of the adherent bacteria.

Exposure of sessile and planktonic bacteria to tobramycin and piperacillin. The acrylic tiles were removed from the chemostat, washed three times with phosphate-buffered saline, and placed in 10 ml of TSB-Fe containing known concentrations of tobramycin, piperacillin, or both. For planktonic cells, 1 ml of the culture was diluted with TSB-Fe and transferred to TSB-Fe containing known concentrations of the antibiotics to give 10^8 cells per ml. The same number of sessile bacteria was exposed to the known concentration of the antibiotic. The samples were incubated at 37°C, and the viable counts of these samples were performed at the time intervals described above.

RESULTS

Growth of *P. aeruginosa* in the continuous culture system. The kinetics of growth and biofilm formation in the chemostats of the *P. aeruginosa* strains used in this study have been described previously (3, 4). The number of planktonic cells in the chemostat at the time of inoculation was 10^8 cells per ml. The value increased to 4×10^9 cells per ml by the next morning and remained constant throughout the experiments (7 days). The populations of the sessile bacteria increased exponentially from days 1 to 5. Biofilm bacteria of nonmucoid *P. aeruginosa* reached 2×10^8 cells per cm^2 on day 5 and remained constant until the experiment was terminated on day 7. The mucoid strains of *P. aeruginosa* were found to adhere to the acrylic tiles more efficiently than the nonmucoid strains. The sessile populations of mucoid isolates of *P. aeruginosa* were consistently higher than those of the nonmucoid strain. They reached 2×10^9 cells per cm^2 on day 5 and remained constant throughout the study. Air bubbles which create turbulence may result in the release of loosely bound biofilm bacteria from the tiles. It is important that the growth rate of the sessile bacteria is probably not strictly governed by the dilution rate, since the cells embedded in the glycocalyx matrix may have slightly different access to the limiting nutrient in comparison with the planktonic cells.

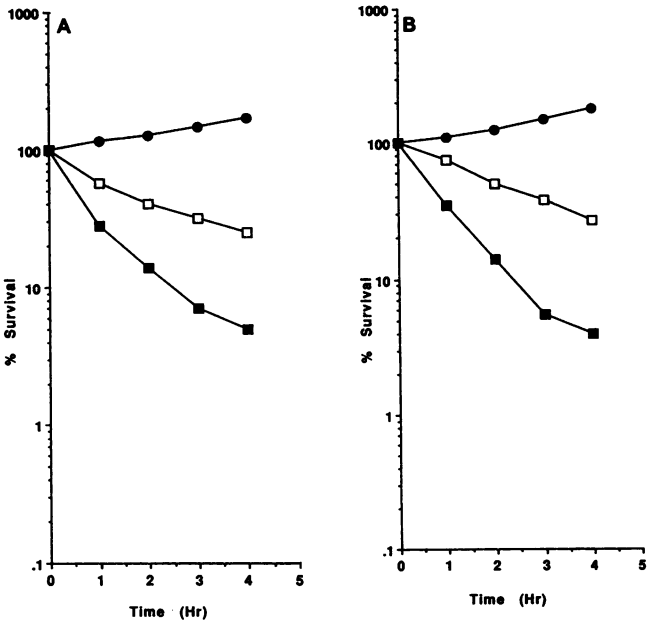

FIG. 1. Susceptibilities of planktonic cells of nonmucoid *P. aeruginosa* ATCC 27835 (A) and mucoid strains (UAM 12 or 492) of *P. aeruginosa* (B) to piperacillin. Symbols: ●, 0 μg of piperacillin per ml; □, 100 μg of piperacillin per ml; ■, 200 μg of piperacillin per ml.

Exposure of planktonic cells to tobramycin and piperacillin. The MICs of tobramycin for the planktonic cells of mucoid and nonmucoid strains were found to be 1 μg/ml. This indicated that both strains were extremely susceptible to this antibiotic. The kinetics of killing of planktonic cells following exposure to tobramycin have been reported previously (3, 4). Exposure of mucoid and nonmucoid strains to 10 μg of tobramycin per ml resulted in a prompt reduction in their viability. Less than 0.001% of the cells was found to be viable following treatment with this antibiotic for 5 h (3, 4). Similar results were obtained in this study (data not shown).

The MICs of piperacillin for the planktonic cells of mucoid and nonmucoid strains were found to be 16 μg/ml. Figures 1A and B show the kinetics of killing of planktonic cells of nonmucoid and mucoid strains of *P. aeruginosa*, respectively. A steady decrease in the viability of the cells was observed following exposure to 100 and 200 μg of piperacillin per ml. A total of 30% of the planktonic cells remained viable after exposure to 100 μg of piperacillin per ml for 4 h. An increase in the concentrations of piperacillin to 200 μg/ml resulted in an enhancement in the lethal effect of the antibiotic. A total of 4% of the planktonic cells were found to be viable after exposure to this concentration of piperacillin. In comparison with tobramycin, piperacillin did not seem to have a comparable activity against *P. aeruginosa* when it was tested on cells cultivated under the conditions described above.

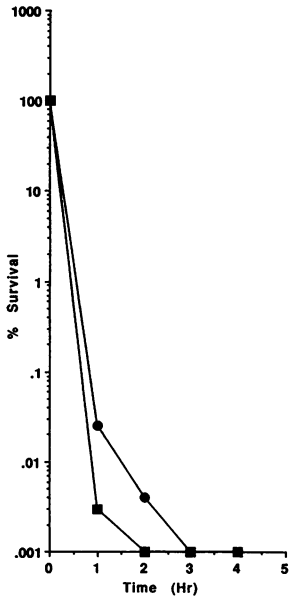

FIG. 2. Kinetics of killing of planktonic cells of mucoid (UAM 12 or 492) or nonmucoid (ATCC 27835) *P. aeruginosa* strains by a combination of piperacillin and tobramycin. Symbols: ●, 200 μg of piperacillin plus 5 μg of tobramycin per ml; ■, 200 μg of piperacillin plus 10 μg of tobramycin per ml.

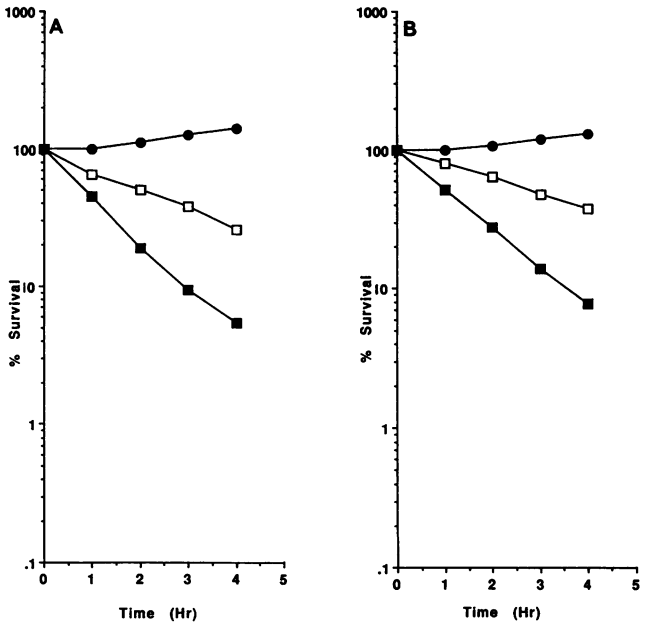

FIG. 3. Interaction of young sessile bacteria of nonmucoid *P. aeruginosa* ATCC 27835 (A) and mucoid *P. aeruginosa* (UAM 12 or 492) (B) with piperacillin. Symbols: ●, 0 μg of piperacillin per ml; □, 100 μg of piperacillin per ml; ■, 200 μg of piperacillin per ml.

Figure 2 shows the susceptibilities of planktonic cells of nonmucoid *P. aeruginosa* strains to the combination of piperacillin and tobramycin. The concentration of piperacillin was kept at 200 μg/ml. This was the highest concentration of piperacillin used in this study. Exposure of the cells to 200 μg of piperacillin per ml plus 5 μg of tobramycin per ml resulted in the prompt reduction of cell viability. Survival was reduced to less than 0.1% following exposure to these concentrations of antibiotics for 1 h. The viability continued to decline, and none of the cells was found to be viable after 3 h of exposure. A further increase in the concentration of tobramycin (10 μg/ml) resulted in an enhancement of killing of the planktonic cells. Similar susceptibility profiles were also observed for planktonic cells of mucoid *P. aeruginosa* strains (data not shown). The data suggest that planktonic cells of mucoid or nonmucoid isolates of *P. aeruginosa* can be eradicated easily with the combination of tobramycin with β-lactam antibiotics with proven activity against *P. aeruginosa*. Enhanced activity was clearly observed when these groups of antibiotics were combined.

Interaction of young sessile populations of mucoid and nonmucoid *P. aeruginosa* isolates with tobramycin and piperacillin. In previous studies (3, 4), we defined biofilm bacteria as young when they were harvested on day 2 and as old when they were harvested on day 7. Similar definitions were used in this study. The kinetics of the interaction of young biofilms of mucoid and nonmucoid strains with tobramycin have been described previously (3, 4). The young sessile populations were found to be more resistant to tobramycin than the planktonic cells were. Treatment of young sessile bacteria of nonmucoid strains with 5 μg of tobramycin per ml for 4 h resulted in a decrease in the viable counts by 97% (4). Eradication of the young biofilm could be achieved by exposure to 10 μg of tobramycin per ml (4). A young biofilm of mucoid strains of *P. aeruginosa* was found to be more resistant to the antibiotic than was a young biofilm of nonmucoid strains (3, 4). Approximately 98% of the mucoid sessile bacteria lost viability after exposure for 5 h to 10 μg of tobramycin per ml (3).

In this study, the investigation was extended to include the interaction of young sessile bacteria with piperacillin. Figures 3A and B show the susceptibility of young sessile populations of nonmucoid and mucoid *P. aeruginosa* to piperacillin, respectively. A steady decrease in the viability of the young sessile bacteria was observed following exposure of the cells to 100 μg of piperacillin per ml. An increase in the concentrations of the antibiotic to 200 μg/ml resulted in an enhancement of the killing of the young sessile bacteria. Exposure of the young biofilm of mucoid and nonmucoid *P. aeruginosa* isolates to 100 μg of piperacillin per ml for 4 h resulted in the reduction of cell viability to approximately 25%. Survival dropped to less than 10% when the concentration of piperacillin was increased to 200 μg/ml.

The susceptibilities of young sessile bacteria to the combination of piperacillin and tobramycin are presented in Fig. 4A and B. Enhanced activities were observed when young nonmucoid sessile bacteria (Fig. 4A) and young mucoid sessile bacteria (Fig. 4B) were treated with piperacillin-tobramycin. Combination of piperacillin (200 μg/ml) and tobramycin (5 μg/ml) was able to reduce the viable count of the young nonmucoid sessile population to 0.08% after a 4-h exposure (Fig. 4A). An increase in the concentration of tobramycin (10 μg/ml) while the concentration of piperacillin was kept at 200 μg/ml resulted in an enhancement of the killing effects, and survival of the nonmucoid biofilm bacteria dropped to less than 0.001% after a 4-h exposure (Fig. 4A). Young mucoid sessile bacteria were consistently found to be slightly more resistant than the nonmucoid isolates were (Fig. 4B). Treatment of young mucoid sessile bacteria to a combination of 200 μg of piperacillin per ml and 5 μg of tobramycin per ml for 4 h resulted in the reduction of cell viability to 2%. Enhancement of killing was observed when the concentration of tobramycin was increased to 10 μg/ml.

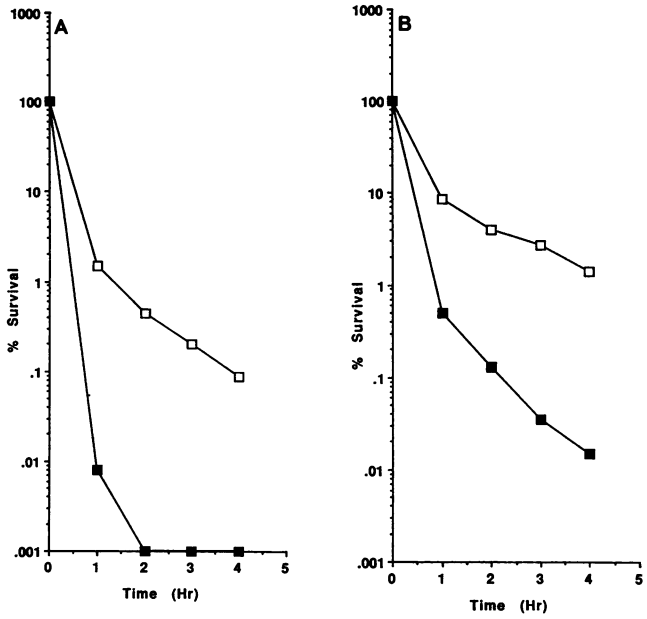

FIG. 4. Kinetics of killing of young sessile bacteria of nonmucoid *P. aeruginosa* ATCC 27835 (A) and mucoid *P. aeruginosa* (UAM 12 or 492) (B) by a combination of piperacillin and tobramycin. Symbols: □, 200 μg of piperacillin plus 5 μg of tobramycin per ml; ■, 200 μg of piperacillin plus 10 μg of tobramycin per ml.

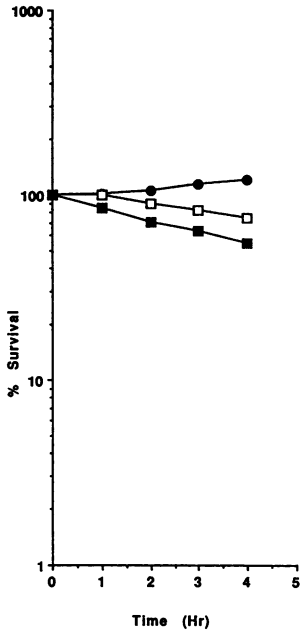

FIG. 5. Interaction of old sessile bacteria of mucoid (UAM 12 or 492) or nonmucoid *P. aeruginosa* ATCC 27835 to piperacillin. Symbols: ●, 0 μg of piperacillin per ml; □, 100 μg of piperacillin per ml; ■, 200 μg of piperacillin per ml. Closely similar kinetics were obtained for these strains.

Again, the results indicate that eradication of young sessile bacteria is achievable by a combination of piperacillin and tobramycin.

Resistance of old sessile bacteria of mucoid and nonmucoid *P. aeruginosa* strains to piperacillin and tobramycin. The activity of tobramycin against old sessile bacteria (harvested on day 7) of mucoid and nonmucoid *P. aeruginosa* strains has been reported previously (3, 4). This antibiotic alone has relatively poor activity against old biofilm. A 50% reduction of viability was observed when the old nonmucoid biofilm was exposed to 50 μg of tobramycin per ml for 5 h (1). Higher concentrations (up to 200 μg/ml) eliminated only 85% of the viable old mucoid sessile bacteria (3). Figure 5 shows the kinetics of killing of the old sessile populations of mucoid (UAM 12 and 492) and nonmucoid (ATCC 27835) *P. aeruginosa* strains by piperacillin. Treatment of the old sessile bacteria with 100 and 200 μg of piperacillin per ml for 4 h resulted in the reduction of cell viability to 87 and 69%, respectively. The data indicate that old sessile bacteria (Fig. 5) are more resistant than their young counterparts (Fig. 3A and B) are to piperacillin. Piperacillin alone does not seem to have the bactericidal activity needed for the eradication of old sessile bacteria. When old biofilm bacteria were released from the tiles by vortex mixing immediately before they were exposed to piperacillin, they showed the same susceptibilities seen in planktonic cells of the same strain. This observation implies that cells growing in the biofilm mode of growth are inherently resistant to this antibiotic.

The interaction of old sessile bacteria of nonmucoid and mucoid *P. aeruginosa* strains with piperacillin and tobramycin is shown in Fig. 6A and B, respectively. Combination of tobramycin and piperacillin resulted in an enhancement of the bactericidal activity of each antibiotic. A synergistic effect was observed when these antibiotics were combined. The concentration of piperacillin was kept at 200 μg/ml, while the concentration of tobramycin was increased from 25 to 100 μg/ml. The viability of old nonmucoid sessile bacteria was reduced from 100 to approximately 10% after exposure to 200 μg of piperacillin per ml plus 25 μg of tobramycin per ml for 4 h (Fig. 6A). An increase in the concentration of

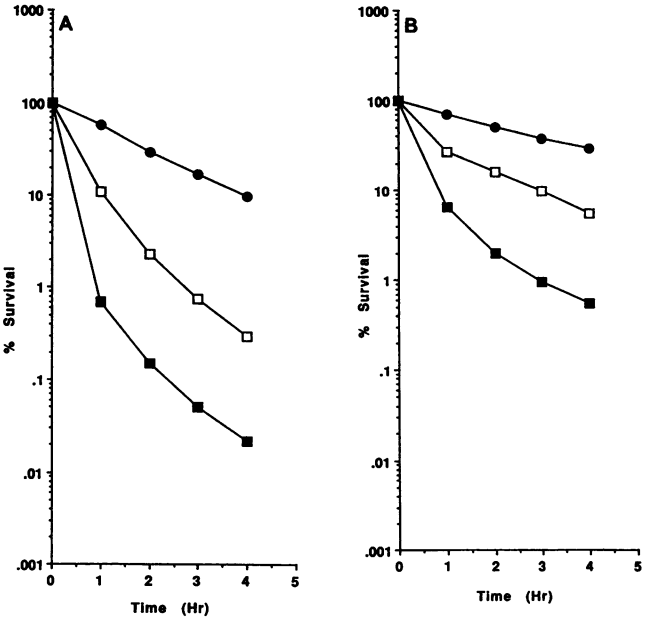

FIG. 6. Kinetics of killing of old sessile bacteria of nonmucoid *P. aeruginosa* ATCC 27835 (A) and mucoid *P. aeruginosa* (UAM 12 or 492) (B) by a combination of piperacillin and tobramycin. Symbols: ●, 200 μg of piperacillin plus 25 μg of tobramycin per ml; □, 200 μg of piperacillin plus 50 μg of tobramycin per ml; ■, 200 μg of piperacillin plus 100 μg of tobramycin per ml.

tobramycin resulted in an enhancement of killing of the sessile bacteria. Fewer than 0.02% of the cells remained viable after a 4-h exposure to 200 μg of piperacillin per ml plus 100 μg of tobramycin per ml (Fig. 6A). Similar profiles were observed for old mucoid sessile bacteria (Fig. 6B), except that the old mucoid sessile bacteria were consistently found to be more resistant than nonmucoid bacteria were (compare Fig. 6A and B). Alginate materials produced by mucoid *P. aeruginosa* strains may affect the achievement of bactericidal concentrations of these antibiotics at their targets (9, 10, 30, 32). The study indicates that a significant drop in the viability of old biofilm bacteria is achievable by the simultaneous use of piperacillin and tobramycin. It is important that higher concentrations of both antibiotics should be used to achieve the desired bactericidal activity and to prevent the emergence of resistant variants. Again, early diagnosis of the infection is of paramount importance since much lower concentrations of drug are needed to achieve eradication of the biofilm bacteria. Young biofilm bacteria can be eradicated easily and should not pose any threat to the life of the patient.

DISCUSSION

P. aeruginosa is a major pathogen that causes severe chronic pulmonary infection in patients with cystic fibrosis. Extensive lung damage caused by colonization by this organism often results in the death of children with cystic fibrosis. In this study, *P. aeruginosa* was found to be able to colonize acrylic tiles. The adherence of *P. aeruginosa* to the acrylic tiles is unlikely to be due to the interaction of specific surface components of *P. aeruginosa* with the surface of the acrylic tiles. No specific receptors are likely to be required for the initiation of this process. Adhesion of the organism to the surface of the acrylic tile resulted in the "gluing" of the pathogens to the tile, and the production of glycocalyx initiated their formation of microcolonies and biofilms on the tiles. This mechanism of bacterial adherence is extremely effective and has been shown to happen both in nature and in the human body (8–11).

The availability of broad-spectrum antibiotics with excellent anti-pseudomonal activity has significantly reduced the mortality, increased the life expectancy, and improved the quality of life of patients with cystic fibrosis. Antibiotics will continue to play a crucial role in the care of patients with cystic fibrosis. Eradication of the pathogen from the lungs is, however, not achievable at present. The presence of sticky slime and the microcolony mode of growth of the organism have been suggested to be the reasons for the failure of antibiotics in the clearance of the pathogen (8, 9, 26, 30, 31). Therefore, it is of paramount importance to conduct research in which these factors can be addressed.

Nutrient depletion and growth rate are known to affect the susceptiblity of microorganisms to antimicrobial agents (6, 7). It is therefore important to cultivate the test organism under conditions that mimic those observed in vivo. In this study, an in vitro chemostat system was selected because it enabled us to cultivate the cells under iron restriction conditions at a slow growth rate (3, 4). Biofilm was allowed to form on acrylic tiles, which could then be used in the study of susceptibility of sessile bacteria to antibiotics. This technique has been successfully used in the study of the interaction of sessile bacteria with tobramycin and piperacillin. It is hoped that the technique will be accepted as a standard method for obtaining biofilm bacteria in the study of the biological activity of antibiotics against sessile organisms.

Young sessile bacteria are more resistant than planktonic cells to tobramycin (3, 4). However, they can still be eradicated if higher concentrations of the antibiotics are used (3, 4). In this study, piperacillin was found to have some activity against young sessile bacteria. However, eradication of young sessile bacteria cannot be achievable with piperacillin alone. Combination of tobramycin and piperacillin resulted in an enhancement of killing of the sessile bacteria. This observation will be investigated further in experimentally induced biofilm infections in laboratory animals.

Old sessile bacteria formed by either mucoid or nonmucoid strains of *P. aeruginosa* are resistant to tobramycin (3, 4) or piperacillin. It is very difficult to eradicate this form of biofilm bacteria because the thick glycocalyx matrix prevents the accumulation of bactericidal concentrations of the antibiotics at the target (8–10, 26, 33). However, enhancement of bactericidal activity was observed when tobramycin was combined with piperacillin (Fig. 6A and B). Significantly higher concentrations of both antibiotics must be used to achieve killing of the old sessile bacteria. These concentrations of antibiotics may be too toxic to be used clinically. Improvement of the delivery of antibiotics to the sites of infection, such as the administration of sustained-released antibiotics directly to the peritoneal cavity in patients with Tenckhoff catheters, should increase the chances of eradicating the biofilm bacteria. This may be achieved because the exposure of sessile bacteria to higher concentrations of antibiotics can be extended. The concentrations of antibiotics required for eradication of biofilm bacteria can be determined by the approaches described in this report.

The data from this study indicate that the eradication of a biofilm-associated infection is best carried out as early as possible. It is still possible to eradicate the newly formed biofilm, provided that higher concentrations of antibiotics are used. A delay in implementing antibiotic therapy may result in failure of the therapy.

We believe that the ongoing exposure of sessile bacteria to the subinhibitory concentrations of antibiotics administered to patients with biofilm-associated infections may lead to a selective environment for antibiotic-resistant mutants of *P. aeruginosa* (28).

MICs have often been used to indicate the susceptibility of clinical isolates to a particular antibiotic. Antibiotic research has been directed toward finding those compounds with better MICs. This approach has been quite successful. A large number of broad-spectrum antibiotics with excellent activity against *P. aeruginosa* has been discovered. However, bacterial isolates which are found to be resistant to these antibiotics can be obtained as soon as these compounds are released for clinical use. This implies that the pathogens have been exposed to subinhibitory concentrations of antibiotics which allow them to develop resistance to that particular group of antibiotics. The strategies for the evaluation of antibiotics need to be reviewed if we are to prevent the appearance of resistant isolates. Dosages of antibiotics insufficient to eradicate old sessile bacteria have commonly been used to treat patients with biofilm-associated infections. This is mainly due to the lack of information available to the clinicians regarding the concentrations required for the eradication of old sessile bacteria. We feel that it is appropriate to introduce a new term in antibiotic research. Biofilm-eradicating concentration is the concentration of an antibiotic required to eradicate old sessile bacteria that are embedded in the thick glycocalyx matrix. It is important that any experimental design used to obtain biofilm-eradicating concentrations should include parameters

such as iron restriction and slow growth rate. These parameters are known to be important to the physiology of the bacterial cell in vivo (5, 6, 14, 15, 34, 35). The chemostat is a useful system for this type of study, in which the growth rate of the microorganisms can be effectively controlled. It is important that an in vivo model must be developed to verify the in vitro findings before these results are extrapolated to clinical situations. In the future, biofilm-eradicating concentrations may assist clinicians in prescribing more accurately the dosage of antibiotics required to resolve biofilm-associated chronic infections, whether these infections occur in tissues or in juxtaposition to medical devices.

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LITERATURE CITED

1. **Anwar, H., M. R. W. Brown, A. Day, and P. Weller.** 1984. Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiol. Lett. **24**:235–239.
2. **Anwar, H., M. R. W. Brown, and P. A. Lambert.** 1983. Effect of nutrient depletion on sensitivity of *Pseudomonas cepacia* to phagocytosis and serum bactericidal activity at different temperatures. J. Gen. Microbiol. **129**:2021–2027.
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-restriction conditions. FEMS Microbiol. Lett. **21**:113–117.
6. **Brown, M. R. W., and P. Williams.** 1985. The influence of environment on envelope properties affecting survival of bacteria in infections. Annu. Rev. Microbiol. **39**:527–556.
7. **Costerton, J. W.** 1988. Structure and plasticity at various organization levels in the bacterial cell. Can. J. Microbiol. **34**:513–521.
8. **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.
9. **Costerton, J. W., R. T. Irwin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35**:435–464.
10. **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.
11. **Dasgupta, M. K., K. B. Bettcher, R. A. Ulan, V. Burns, K. Lam, J. B. Dossetor, and J. W. Costerton.** 1987. Relationship of adherent bacterial biofilms to peritonitis in chronic ambulatory peritoneal dialysis. Peritoneal Dialysis Bull. **7**:168–173.
12. **Eliopoulous, H.** 1986. Aminoglycosides plus beta lactams against gram-negative organisms. Am. J. Med. **80**:126–137.
13. **Ellwood, D. C., and D. W. Tempest.** 1972. Effects of environment on bacterial cell wall content and composition. Adv. Microb. Physiol. **7**:83–117.
14. **Griffiths, E.** 1983. Availability of iron and survival of bacteria in infection. *In* C. S. F. Easmon, J. Jeljasewicz, M. R. W. Brown, and P. A. Lambert (ed.), Medical microbiology, vol. 3. Academic Press, Inc. (London), Ltd., London.
15. **Griffiths, E., P. Stevenson, and P. Joyce.** 1983. Pathogenic *Escherichia coli* express new outer membrane proteins when grown *in vivo*. FEMS Microbiol. Lett. **16**:95–99.
16. **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.
17. **Keevil, C. W., D. J. Bradshaw, A. B. Dowsett, and T. W. Feary.** 1987. Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques. J. Appl. Bacteriol. **62**:129–138.
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. **Kunin, C. M., and C. Steele.** 1985. Culture of the surfaces of urinary catheters to sample urethral flora and study the effect of antimicrobial therapy. J. Clin. Microbiol. **21**:902–908.
20. **Lam, C., F. Turnowski, E. Schwarzinger, and W. Neruda.** 1984. Bacteria recovered without subculture from infected human urines express iron-regulated membrane proteins. FEMS Microbiol. Lett. **24**:255–259.
21. **La Tourette Prosser, B., D. Taylor, B. A. Dix, and R. Cleeland.** 1987. Method of evaluating effects of antibiotics on bacterial biofilm. Antimicrob. Agents Chemother. **31**:1502–1506.
22. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Microbiol. **50**:285–309.
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., A. G. Gristina, and J. W. Costerton.** 1985. Electron microscopic study of an infected Foley catheter. Can. J. Surg. **28**:50–54.
25. **Nickel, J. C., J. Heaton, A. Morales, and J. W. Costerton.** 1986. Bacterial biofilm in persistent penile prosthesis-associated infection. J. Urol. **135**:586–588.
26. **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.
27. **Ombaka, E. A., R. M. Cozens, and M. R. W. Brown.** 1983. Influence of nutrient limitation of growth on stability and production of virulence factors of mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5**:S880–S888.
28. **Shand, G. H., H. Anwar, and M. R. W. Brown.** 1988. Outer membrane proteins of polymyxin resistant *Pseudomonas aeruginosa*: effect of magnesium depletion. J. Antimicrob. Chemother. **22**:811–821.
29. **Shand, G. H., H. Anwar, J. Kadurugamuwa, M. R. W. Brown, S. H. Silverman, and J. Melling.** 1985. In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infect. Immun. **48**:35–39.
30. **Slack, M. P. E., and W. W. Nichols.** 1981. The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*. Lancet **ii**:502–503.
31. **Slack, M. P. E., and W. W. Nichols.** 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. **10**:368–372.
32. **Ward, K. H., H. Anwar, M. R. W. Brown, J. Wale, and J. Gowar.** 1988. Antibody response to outer membrane antigens of *Pseudomonas aeruginosa* in human burn wound infection. J. Med. Microbiol. **27**:179–190.
33. **Warren, J. W., H. L. Muncie, Jr., E. J. Berquist, and J. M. Hoppes.** 1981. Sequelae and management of urinary infection in the patient requiring chronic catherization. J. Urol. **125**:1–8.
34. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
35. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64**:65–72.