

Kinetic Interaction of Biofilm Cells of *Staphylococcus aureus* with Cephalexin and Tobramycin in a Chemostat System

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Planktonic and young biofilm cells were completely eradicated after exposure of these cells to drug levels representing one loading and two maintenance doses of tobramycin and cephalexin. A very different picture was observed when antibiotic exposure was initiated on day 21. Complete eradication of the old biofilm cells was not observed even when the antibiotic exposure was continued for an extra 6 days. Regrowth of the organism was observed when the antibiotic exposure was terminated.

The implantation of a variety of medical devices, such as urinary catheters and cardiac pacemakers, has become an integral part of therapeutic strategies in modern medical care. The use of these devices has created problems related to clinical infections. The devices are frequently colonized by bacteria which form biofilms (7, 8, 10, 12-15, 20, 21). Skin floras such as *Staphylococcus aureus* and *Staphylococcus epidermidis* from patients and health care professionals have been shown to be implicated in these infections (7, 8, 10, 21). Millions of dollars are spent annually on antibiotics to deal with biofilm-associated infections, but the results are often disappointing (6, 10, 12, 16, 17). The antibiotics do not eradicate the bacteria colonizing the medical devices, which must therefore be removed. Quite often death occurs before this can be accomplished.

An in vitro chemostat system in which bacteria can be cultivated as biofilms at a low growth rate under conditions of iron restriction has been developed to investigate the interaction of bacteria in biofilms with antibiotics (1-5). By using this system, it was found that the planktonic cells of *Pseudomonas aeruginosa* were susceptible to piperacillin and tobramycin. Young biofilm cells (harvested on day 2) were still susceptible to these agents, whereas old biofilm cells (harvested on day 7) were very resistant to these antibiotics. Higher concentrations of piperacillin and tobramycin were required to eradicate the old biofilm cells of *P. aeruginosa* (1, 3).

In the studies mentioned above (1, 3-5), the experiments were carried out by removing the biofilms on inert surfaces from the chemostat and exposing them to fixed concentrations of antibiotics for 5 to 6 h (1, 3-5). The time-killing curves were then constructed to reveal the susceptibilities of bacteria in biofilms to antibiotics (1, 3-5). This exercise has provided us with information on the interaction of biofilm cells with the single dose of antibiotics required for their eradication. At this stage, we believe that it would be more useful to design an experiment in which the dynamic interaction of multiple doses of antibiotics with bacteria in biofilms for the duration of chemotherapy could be addressed. In this article, we report the kinetic interaction of biofilm cells of *S. aureus* with cephalexin and tobramycin. The usefulness of this system in the evaluation of the

effectiveness of antibiotics in the eradication of bacteria in biofilms is discussed.

S. aureus R-627 isolated from an infected Tenckhoff catheter was grown in iron-depleted brain heart infusion (BHI-Fe) broth containing added mono- and divalent cations (11) in chemostats as described previously (1, 3-5). One hundred pieces of 1-cm-long (size 16) Masterflex silicone tubing (Cole-Parmer Instrument Ltd., Chicago, Ill.) were incorporated into the chemostat culture for cell attachment and biofilm formation. A 5-ml sample of *S. aureus* grown to mid-exponential phase in BHI-Fe broth was inoculated into the chemostat. Fresh BHI-Fe medium was fed into the chemostat with a peristaltic pump to give a dilution rate of 0.17 h^{-1} (one volume change occurred every 6 h).

Viable counts of planktonic cells in the suspension were determined with serially diluted samples incubated on blood agar (Difco, Detroit, Mich.). To determine the number of biofilm cells that adhered on 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 blood agar (Difco) at 37°C .

On day 2 (young biofilm) or day 21 (old biofilm), tobramycin and cephalexin representing the loading dose (i.e., initial dose of antibiotics) were added to the chemostat cultures containing planktonic and biofilm cells so that the concentrations of these antibiotics were 70 and $500 \mu\text{g}$ per ml, respectively. These concentrations of antibiotics were chosen because high concentrations of antibiotics are often incorporated directly into the peritoneal fluid in the treatment of peritonitis in patients with peritoneal catheters (20, 21). The dilution rate of the chemostat was set to 0.17 h^{-1} so that one volume change of BHI-Fe medium in the chemostat occurred every 6 h. Samples were removed at hourly intervals for 6 h, and viable counts were obtained for both the planktonic and the biofilm cells by plating them onto blood agar and incubating them at 37°C . At 6 h, drug levels representing the maintenance doses of these agents ($62.5 \mu\text{g}$ of tobramycin per ml and $250 \mu\text{g}$ of cephalexin per ml) were added to the chemostat, and the viable counts were determined before the addition of the next dose. The addition of these antibiotics to the chemostat cultures was repeated at 6-h intervals for 7 days. On day 7 (after the initiation of antibiotic exposure), the antibiotic treatment was terminated, and fresh BHI-Fe medium was continuously pumped

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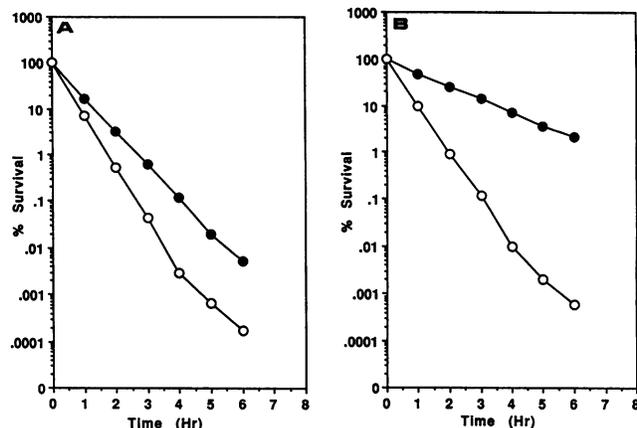


FIG. 1. Kinetic interaction of planktonic and biofilm cells of the 2-day-old (A) and 21-day-old (B) chemostat cultures of *S. aureus* R-627 with tobramycin and cephalixin at levels representing the loading doses (70 and 500 $\mu\text{g/ml}$, respectively). The dilution rate (0.17 h^{-1}) was chosen so that one volume change of the medium in the chemostat occurred in 6 h. Symbols: \circ , planktonic cells; \bullet , biofilm cells.

into the chemostat at the same dilution rate for another 3 days to investigate regrowth of the organism.

The kinetics of biofilm formation by *S. aureus* have been investigated recently (4). The population of the planktonic cells in the chemostat reached approximately 5×10^9 cells per ml 24 h after inoculation and remained fairly constant until tobramycin and cephalixin were added to the chemostat on either day 2 or day 21. The population of biofilm cells colonizing the surface of the silicone tubing increased exponentially from approximately 2×10^5 cells per cm (2 h after inoculation) to 8×10^7 cells per cm on day 6 (4). The population of biofilm cells remained fairly constant from day 10 to day 21. The population of the biofilm cells was 8×10^8 cells per cm before tobramycin and piperacillin were added to the chemostat on day 21.

The interaction of young and old biofilm cells of *S. aureus* with the kinetically controlled loading-dose combination of tobramycin (70 $\mu\text{g/ml}$) and cephalixin (500 $\mu\text{g/ml}$) is illustrated in Fig. 1A and B, respectively. The planktonic and young biofilm cells of the 2-day-old chemostat culture were found to be very susceptible to the drug levels representing the loading-dose combination of tobramycin and cephalixin (Fig. 1A). The percent viable planktonic cells was reduced from 100 to approximately 0.0002% following exposure to these antibiotics for 6 h (Fig. 1A). The young biofilm cells showed a very similar degree of susceptibility. The percent viable young biofilm cells was reduced from 100 to approximately 0.005% following exposure to these doses of antibiotics for 6 h (Fig. 1A).

From Fig. 1B, it can be seen that the planktonic cells of this 21-day-old chemostat culture were still susceptible to these doses of tobramycin and cephalixin. The percent viable planktonic cells was reduced from 100 to approximately 0.0005% following exposure to these antibiotics. However, the old biofilm cells of the 21-day-old chemostat culture were more resistant to these doses of antibiotics (Fig. 1B). The percent viable old biofilm cells was reduced from 100 to approximately 2.5% following exposure to kinetically controlled loading doses of these antibiotics for 6 h.

The interaction of planktonic and young biofilm cells with

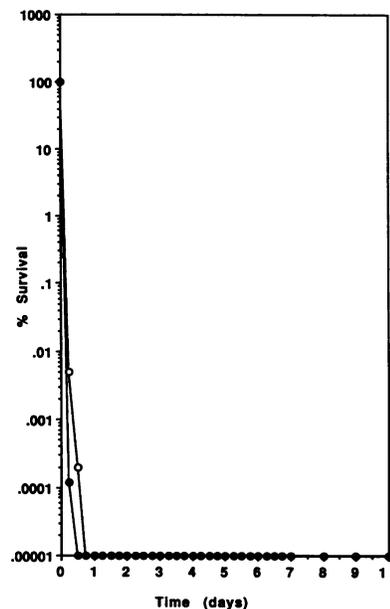


FIG. 2. Kinetic interaction of planktonic and young biofilm cells of the 2-day-old chemostat culture of *S. aureus* R-627 with tobramycin and cephalixin at levels representing the loading (70 μg of tobramycin and 500 μg of cephalixin per ml) and maintenance (62.5 μg of tobramycin and 250 μg of cephalixin per ml) doses of these antibiotics. The initial (first) dose was the loading dose, and those following were the maintenance doses. The dilution rate (0.17 h^{-1}) was chosen so that one volume change of the medium in the chemostat occurred in 6 h. Symbols: \bullet , planktonic cells; \circ , biofilm cells.

drug levels representing the loading and maintenance doses of tobramycin and cephalixin is illustrated in Fig. 2. The first dose of these antibiotics (70 μg of tobramycin and 500 μg of cephalixin per ml) was called the loading dose, and the second dose (62 μg of tobramycin and 250 μg of cephalixin per ml) was designated the maintenance dose. The viability of the planktonic cells dropped to approximately 0.0001% after exposure to the same concentrations of these agents for the same period. None of the young biofilm cells were found to be viable after exposure to the second maintenance doses of these antibiotics. However, the antibiotic administration was continued for 7 days, as this is routinely carried out in hospitals for the control of infection. No viable cells (either planktonic or young biofilm cells) could be detected in the chemostat for this period. The viability of the planktonic and young biofilm cells was monitored for 3 days after termination of antibiotic administration on day 7. Again, no viable cells could be detected, indicating complete eradication of planktonic and young biofilm cells.

The kinetic interaction of planktonic and old biofilm cells of the 21-day-old chemostat culture with tobramycin and cephalixin at levels representing the loading and maintenance doses is shown in Fig. 3. The percent viable old biofilm cells was reduced from 100 to approximately 2.5%. The viability of the planktonic cells dropped from 100 to approximately 0.005% after exposure to this dose of antibiotics under the same conditions for the same period. The percent viable old biofilm cells was decreased to approximately 0.08% after exposure to the first maintenance dose of these antibiotics. The population of the planktonic cells dropped to approximately 10 viable cells per ml after expo-

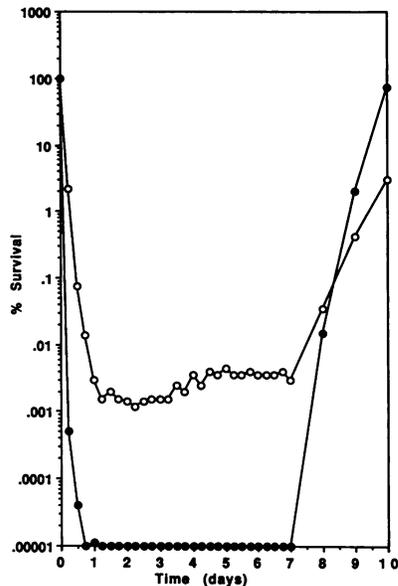


FIG. 3. Kinetic interaction of planktonic and old biofilm cells of the 21-day-old chemostat culture of *S. aureus* R-627 with tobramycin and cephalixin at levels representing the loading (70 μg of tobramycin and 500 μg of cephalixin per ml) and maintenance (62.5 μg of tobramycin and 250 μg of cephalixin per ml) doses of these antibiotics. The initial (first) dose was the loading dose, and those following were the maintenance doses. Symbols: ●, planktonic cells; ○, biofilm cells.

sure to drug levels representing one loading and two maintenance doses of these antibiotics. The viability of the old biofilm cells dropped to approximately 0.002% after exposure to one loading dose and four maintenance doses of these antibiotics. The population of old biofilm cells was found to remain constant at 0.002% (approximately 1,000 viable cells per cm) even when the addition of maintenance doses of these antibiotics was continued for 6 more days. The antibiotic treatment was discontinued on day 7, and fresh antibiotic-free BHI-Fe medium was pumped into the chemostat for 3 days. The viability of the planktonic cells increased to approximately 100% 3 days after termination of antibiotic treatment. The viability of the old biofilm cells increased to approximately 2% for the same period. The results revealed that the kinetically controlled loading and maintenance doses of tobramycin and cephalixin used in this study failed to eradicate the old biofilm cells of *S. aureus*.

The problems associated with in vitro antimicrobial susceptibility tests have recently been discussed (18). We believe that the interaction of bacterial pathogens with antibiotics in vivo is profoundly different from interactions observed in the in vitro susceptibility tests. In the in vitro susceptibility tests, the planktonic cells grown in complex laboratory medium are exposed to fixed concentrations of antibiotics. Planktonic cells are accessible to the antibiotics in the test tube, and the cells are killed instantaneously. A very different picture is observed in the interaction of pathogens with antibiotics in vivo. Antibiotics have access to planktonic cells and also biofilm cells that are located on the upper regions of biofilms. However, they do not have easy access to the biofilm cells that are deeply embedded in the glycocalyx matrix (1-6). Therefore, these biofilm cells may have sufficient time to turn on the expression of

antibiotic resistance factors such as β -lactamases (9) to counteract the presence of antibiotic molecules. Another factor that has not been taken into account in the experimental design of in vitro antimicrobial susceptibility tests is the kinetic interaction of antibiotics with bacterial cells. The concentrations of antibiotics in vivo are dependent on the rate of clearance of the antibiotics from the body, and this should be considered in the experimental design to evaluate the effectiveness of antibiotics in the eradication of bacterial pathogens. This factor is important because it determines the number of antibiotic molecules that are available to interact with bacteria in biofilms at any given moment. We believe that studies on the kinetic interaction of biofilm cells with antibiotics will give us some valuable insight into the dosage and frequency of antibiotic administration that should be used in the eradication of bacteria in biofilms. In this study, these antibiotics were added directly into the chemostat, as they are often added directly into the peritoneal fluid in clinical practice. Fresh antibiotic-free medium was then pumped into the chemostat at a rate determined by the pharmacokinetics of the antibiotics so that the peak and trough levels of the antibiotics could be simulated.

From this study, it can be seen that the planktonic and young biofilm cells of the 2-day-old chemostat culture can be effectively eradicated by the loading and maintenance doses of tobramycin and cephalixin (Fig. 2). From analysis of the data, we observe that the planktonic and young biofilm cells are completely eradicated after exposure of these cells to one loading and two maintenance doses of these antibiotics. Regrowth of the organism does not occur after the antibiotic treatment is terminated on day 7 (Fig. 2). Combinations of antibiotics with synergistic activities should be considered, and the concentrations of the antibiotics have to be sufficiently high to ensure complete eradication of the organism. Any delay in implementing antibiotic therapy may result in failure of the treatment.

A very different picture is observed when antibiotic treatment is initiated on day 21. We still observe a rapid decrease in the cell viability when the planktonic and old biofilm cells are exposed to the loading dose of tobramycin and cephalixin (Fig. 3). The cell viability is reduced to 0.002% when these cells are exposed to one loading and three maintenance doses of tobramycin and cephalixin (Fig. 3). Unfortunately, complete eradication of the infecting pathogen is not observed even when the antibiotic exposure is continued for an extra 6 days (Fig. 3). Regrowth of the organism is observed when the antibiotic exposure is terminated. It is clear that the loading and maintenance doses of tobramycin and cephalixin are not sufficient to eradicate the sessile bacteria completely. These doses of antibiotics are only sufficient to reduce the population of planktonic and biofilm cells to a level which curtails the clinical symptoms of infection. We believe that the in vitro chemostat system described in this study will enable health care professionals to determine the antibiotic dosing and also the frequency of antibiotic administration more accurately so that biofilm cells can be eradicated effectively. The system may also be used by pharmaceutical industries in the search for compounds with excellent activities against bacteria in biofilms. A chemostat system has recently been developed to investigate the interaction of planktonic cells of mixed populations of oral pathogens with antibiotics (19).

Antibiotic manufacturers invest millions of dollars annually to provide valuable information to health care professionals so that they can use antibiotics successfully in killing bacterial pathogens. We believe that the system described in

this study will assist them in the evaluation of the performance of antibiotics that are currently under development in the pharmaceutical industries. At this stage, our goal is to learn more about the behavior of old biofilms so that antibiotics with activities against old biofilm cells can be developed to combat their existence.

Regulatory authorities play a profound role in providing guidelines on the use of antibiotics in the treatment of infections. The incorporation of antibiotic susceptibility tests for bacteria in biofilms into the guidelines would stimulate the determination of concentrations of antibiotics required for the eradication of bacteria in biofilms. Clinical microbiologists and clinicians should be aware that much higher concentrations of antibiotics are required for the treatment of device-related infections. We believe that device-related infections can be successfully controlled if appropriate information regarding the dose and the frequency of antibiotic administration is available to clinicians.

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