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Interaction of Biofilm Bacteria with Antibiotics in a Novel In Vitro Chemostat System

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***Pseudomonas aeruginosa* was cultivated at low growth rates under iron-limiting conditions on acrylic tiles. Biofilm cells exhibited increased tobramycin resistance compared with that of planktonic cells, and cells in old biofilms were more resistant than were cells in young biofilms. However, on suspension of the biofilm bacteria, glycocalyx-mediated resistance was lost.**

The exposed outer surface of a bacterial cell plays an important role in the survival of the cell in nature and in the human body (4–6, 23). In addition to being able to adhere to mucosal surfaces (4–6, 23), microbes are also able to attach firmly to solid surfaces such as catheters (7, 8, 15, 19, 25). Cells bind to these surfaces by producing exopolysaccharide glycocalyx polymers, forming a matrix inside which microcolonies develop (7, 8). As the size and number of adherent microcolonies increase, the microcolonies coalesce to form biofilms (7, 8). The biofilm mode of growth confers on the pathogen both the ability to avoid attack by host defences and, as we show in this paper, increased resistance to antibiotics.

Because of their resistance to a wide range of antibiotics, biofilm bacteria are a major concern for clinicians in the treatment of infections (7, 15, 17, 20, 25). Infections resulting from colonization by biofilm bacteria are a serious problem for patients receiving implanted prosthetic devices. Both gram-positive and gram-negative bacteria have been implicated in these infections (7, 8, 15, 19, 25). Despite the large number of antibacterial agents available, none have been found to eradicate biofilm bacteria (15, 17, 20, 25).

Studies on bacteria isolated in vivo have indicated that these bacteria grow under iron restriction (1–3, 10, 11, 16, 22, 24). Because of high-affinity biological iron chelators such as transferrin and lactoferrin, iron, an essential nutrient, is not freely available to the invading pathogen (9, 18, 26, 27). In this study, a method for studying the interaction of biofilm with antibiotics which considers three important parameters—iron restriction, low growth rate, and the biofilm mode of growth—is presented. By using a novel in vitro chemostat system, the interaction of *Pseudomonas aeruginosa* with tobramycin, the antibiotic most commonly used to treat these infections, was investigated.

P. aeruginosa ATCC 27835 was grown in iron-depleted tryptic soy broth containing added mono- and divalent cations (12) in chemostats (as described by Ombaka et al. [21]). Tiles (0.5 by 2.0 cm) made from a self-curing methyl methacrylate dental resin (De Trey Ltd., Weybridge, United Kingdom) (14) were suspended in the culture medium. The dilution rate used in the continuous culture was 0.05/h (dilution time of cells in the culture, 13.86 h). MICs were determined by tube dilution (13). Viable bacterial counts were measured by using serially diluted samples incubated on nutrient agar (Difco Laboratories, Detroit, Mich.) at 37°C.

To measure the resistance of planktonic cells to tobramycin, 1 ml of culture was diluted to 10^8 cells per ml and exposed to a known concentration of the antibiotic. For biofilm bacteria, the acrylic tiles were washed three times with phosphate-buffered saline and the same number of cells was exposed to tobramycin in a final volume of 10 ml. The samples were incubated at 37°C, and viable counts were determined at various time intervals.

Figure 1 shows the kinetics of biofilm formation on acrylic tiles when *P. aeruginosa* was cultivated at a low growth rate in the chemostat. The number of viable bacteria colonizing each acrylic tile increased from day 1 to day 5 until a maximum of 2×10^8 cells per tile was reached. This figure remained constant throughout the remainder of the experiment (until day 7). The population of planktonic cells reached 3×10^9/ml on day 1 and remained constant throughout.

The MIC of tobramycin for the planktonic cells was found to be 1 μg/ml, indicating that this strain is relatively susceptible to this antibiotic. Figure 2 shows the susceptibility of day 7 planktonic cells to various concentrations of tobramycin. Similar results were obtained for day 2 and day 5 planktonic cells (data not shown). Tobramycin concentrations as low as 5 μg/ml reduced the percentage of viable cells to less than 1% after a 1-h exposure.

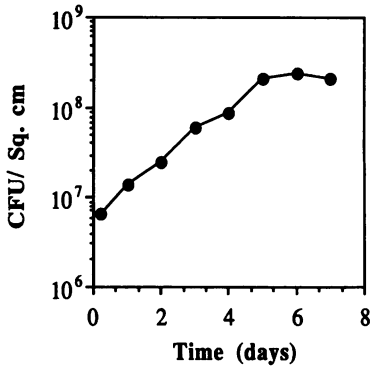

FIG. 1. Kinetics of biofilm formation of *P. aeruginosa* ATCC 27835 grown slowly under iron-restricted conditions (dilution rate, 0.05/h).

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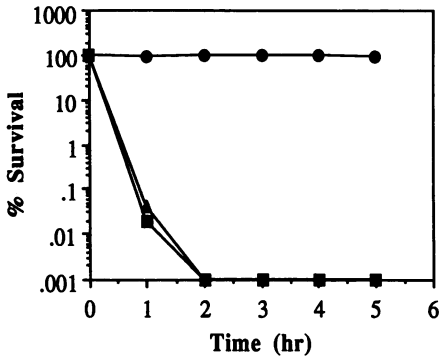

FIG. 2. Effects of tobramycin on the planktonic population harvested on day 7. Tobramycin was used at concentrations of 0 (●), 5 (▲), and 10 (■) μg/ml. Similar results were obtained for the planktonic populations harvested on days 2 and 5.

Our interest is in the susceptibility of biofilm bacteria to tobramycin. Tobramycin (5 μg/ml) was able to reduce the viable count of young (harvested on day 2) biofilm cells by 97% after a 4-h exposure (Fig. 3). Higher antibiotic concentrations were able to completely kill the biofilm bacteria. Old (harvested on day 7) biofilm cells, however, were unharmed after more than 5 h of exposure to 20 μg/ml (Fig. 4). Higher concentrations (up to 50 μg/ml) eliminated only 50% of the viable cells. In both cases, sessile bacteria suspended in phosphate-buffered saline showed the same susceptibility to tobramycin as did the planktonic cells described earlier.

The growth kinetics of the culture in a chemostat are such that fresh nutrients and oxygen must be added continually to maintain exponential growth. As a result, a small amount of turbulence is generated in the culture, releasing sessile bacteria from the outer surface of the biofilm. We believe that this turbulence is responsible for the decrease in biofilm development after day 5. The number of cells colonizing the acrylic tile at this stage may represent an early state of biofilm-associated infection in clinical situations.

Although young sessile bacteria are more resistant to tobramycin than planktonic cells are, they can still be eradicated provided high enough concentrations of antibiotic are used. Old biofilm bacteria, however, are affected only by lethal amounts of antibiotic. The presence of a thick glycocalyx matrix as proposed by Costerton et al. (7, 8) may be responsible for the tobramycin resistance exhibited by the sessile bacteria. It is therefore imperative that a biofilm-associated infection be treated at the earliest possible stage while it may still be susceptible to antibiotics.

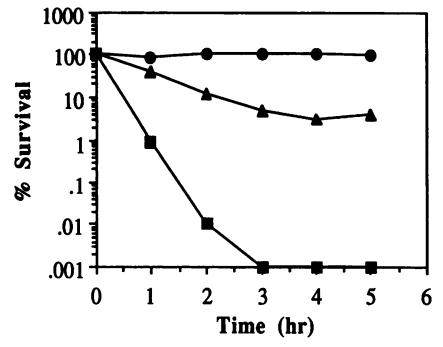

FIG. 3. Effects of tobramycin on the young sessile population harvested on day 2. Tobramycin was used at concentrations of 0 (●), 5 (▲), and 10 (■) μg/ml.

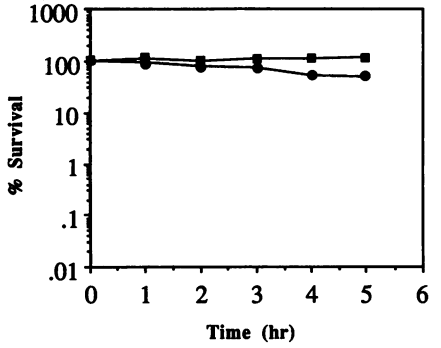

FIG. 4. Effects of tobramycin on the old sessile population harvested on day 7. Tobramycin was used at concentrations of 0, 5, 10, and 20 μg/ml (■) (averages of results obtained with all four concentrations) and 50 μg/ml (●).

This study provides the means to investigate the interaction of biofilm bacteria with antibiotics under conditions mimicking those that exist in vivo, a situation in which pathogens grow slowly, often as biofilms, under iron limitation. It is our belief that long-term exposure of bacteria to subinhibitory concentrations of antibiotic may create a selective environment for resistant variants. The system proposed here allows for the determination of antibiotic concentrations required to eradicate a biofilm-associated infection, thus decreasing the probability of creating such a selective environment.

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