ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1992, p. 2054–2056
0066-4804/92/092054-03$02.00/0

Vol. 36, No. 9

Pseudomonas aeruginosa Biofilm as a Diffusion Barrier to Piperacillin

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Received 11 March 1992/Accepted 22 June 1992

***Pseudomonas aeruginosa* 579 biofilms formed on dialysis membranes retarded piperacillin diffusion. Treatment of biofilms with 5.0 mM $CaCl_2 \cdot 2H_2O$ prevented diffusion. Biofilms permitted equilibration of [^{14}C]glucose. Thin-layer chromatography of fluids distal to untreated (viable and nonviable) and viable Ca^{2+}-treated *P. aeruginosa* 579 biofilms and fluids distal to a viable *P. aeruginosa* mutant noninducible for the expression of β-lactamase did not detect piperacillinoic acid.**

Pseudomonas aeruginosa can adopt a biofilm mode of growth (4, 5) with bacteria enmeshed in a mucoid exopolysaccharide (MEP) composed mainly of acetylated β-D-mannuronic acid and α-L-guluronic acid (18, 19). In a biofilm, *P. aeruginosa* cells are more resistant to aminoglycoside and β-lactam antibiotics than are planktonic cells (1, 9, 12, 16) and bacteria dispersed from the MEP (1, 4, 9, 16). Consequently, chronic infections ensue in the urinary tracts of catheterized patients and in the lungs of individuals afflicted with cystic fibrosis (4, 5, 11, 16). Infections in patients with cystic fibrosis are lethal (17).

Eradication of such *P. aeruginosa* populations requires an understanding of their antibiotic resistance mechanism(s). Bacterial physiology is undeniably important (2). Hyperproduction of MEP by adherent *P. aeruginosa* (5, 16) may influence physiology by decreasing nutrient availability (4, 10). MEP may also protect the bacteria by restricting the diffusion of aminoglycoside and β-lactam antibiotics into the biofilm (1, 9, 14, 16, 19). The latter role for soluble MEP has been questioned elsewhere (7, 14, 15, 21). However, MEP is a malleable polymer (18) whose role as a diffusion barrier may vary according to its soluble state (9, 10). Furthermore, since it is possible that the heterogeneity of *P. aeruginosa* biofilms (13) influences the localized structure of MEP (10), the relevance of the MEP as a diffusion barrier in intact biofilms may be more important than the role inferred from the studies conducted to date. We report here observations obtained by using a flowthrough dialysis apparatus in which the diffusion of a β-lactam antibiotic (piperacillin; Sigma Chemical Co., St. Louis, Mo.) across membranes colonized with *P. aeruginosa* 579 (*leu muc-23* FP^+ [8]) was assessed.

One liter of sterile 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical) (pH 7.0) or M-56 broth (3) cultures of *P. aeruginosa* 579 were connected via Tygon tubing (Cole-Parmer Instrument Co., Chicago, Ill.), routed through a Masterflex L/S peristaltic pump (1 to 100 rpm; Cole-Parmer), to a dialysis cell (Spectrum Medical Industries, Los Angeles, Calif.). The cell consisted of two plastic blocks (63 by 63 by 15 mm) each with a central well (4 by 20 mm) clamped around a 25-mm diameter cellulose dialysis membrane (molecular weight exclusion limit of 4,000; Spectrum Medical Industries). Two chambers were created, each having a fluid capacity of 1.0 ml. Membranes were pretreated with 0.1 M EDTA (Sigma Chemical) for 12 h at 25°C and rinsed 10 times with 100 ml of deionized distilled water each time prior to installation in the cell. The equipment was ethylene oxide sterilized before each experiment.

HEPES with 100 μg of piperacillin per ml was caused to flow (30 ml · h^{-1} in this and subsequent experiments) past one surface of three uncolonized membranes for 1.5 or 8 h. The distal chamber contained HEPES. The piperacillin concentrations in proximal and distal fluids were quantified in an agar diffusion bioassay (14) with *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 27217 as the indicator organism. The relationship between the 1 to 10 μg of piperacillin in the 100 μl of standard applied to the 5-mm-diameter wells in Direct Sensitivity Test agar (Oxoid, Basingstoke, United Kingdom) and the diameters of the resulting bacterial growth inhibition zones was linear and reproducible in 15 experiments. Zone diameters were sufficiently different to permit an accurate determination of the piperacillin concentrations in sample fluids (100 μl each). The concentrations of piperacillin in four proximal and distal chambers were 90 ± 2 (mean ± standard error, here and subsequently) and 75 ± 4 μg · ml^{-1}, respectively, after 1.5 h. After 8 h, both chambers displayed 90 ± 3 μg of piperacillin · ml^{-1} (n = 4). While binding of piperacillin to the membranes may have reduced the concentrations of unbound, biologically detectable antibiotic in the fluids on either side of the membranes, the equilibration of the remaining unbound antibiotic was evident.

In the colonization experiments, *P. aeruginosa* 579 cultures were grown at 25°C to an optical density (at 540 nm) of 0.80 (10^9 viable bacteria · ml^{-1}). A total of 20 ml of each culture was added to 980 ml of M-56, which was connected to the dialysis cell. The culture, aerated by the flow (200 ml · min^{-1}) of filter-sterilized air, was caused to flow for 30 h at 25°C through the proximal chamber of the cell. The distal chamber contained 1.0 ml of M-56. The medium and duration of flow were sufficient for the development of MEP by the adherent bacteria (9). Scanning electron microscopy examination (16) of the proximal surfaces of three membranes revealed confluent, multicell thick biofilms. Few bacteria were associated with the distal surfaces. The latter bacteria may have sloughed off from the proximal surface during removal of the membrane from the separation cell or during processing for electron microscopy. Whatever the

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origin of these bacteria, the dialysis membranes clearly restricted colonization solely to the proximal surface.

To assess whether colonization plugged the membranes, D-[U-^{14}C]glucose (Amersham, Burlington, Ontario, Canada) was added to the fluid distal to two untreated and two Ca^{2+}-treated (see below) *P. aeruginosa* 579 biofilms. A constant amount of radioactivity (80 nCi) was added to each distal fluid. Both chambers also contained HEPES supplemented with 2% (wt/vol) unlabelled glucose. The dialysis cell was statically incubated at 25°C for 8 h, and then 20 μl (each time) of each proximal and distal fluid was removed for four times. Each aliquot was added to 10 ml of Ecolite scintillation counting fluid (ICN Biomedicals Inc., Irvine, Calif.), and samples were counted for 10 min with an LS8000 scintillation counter (Beckman Instruments Inc., Irvine, Calif.). Proximal/distal ratios of disintegrations per minute approached equality for biofilms which were not treated (0.87 ± 0.18) or which were exposed to Ca^{2+} (0.88 ± 0.33). When two uncolonized membranes were tested, a similar result was obtained (0.89 ± 0.13).

Proximal surfaces of other colonized membranes received the flow of HEPES for 8 h (untreated [$n = 5$]) or for 60 min, after which time 500 ml of the buffer was supplemented with 1.0 M $CaCl_2 \cdot H_2O$ (final concentration, 5.0 mM; $n = 5$). The flow of the Ca^{2+}-supplemented HEPES was continued for 8 h. Both the untreated and the Ca^{2+}-treated biofilms were then exposed to the flow of HEPES containing 100 μg of piperacillin per ml. After 8 h, 100 μl (each time) of proximal and distal fluid was recovered three times for analysis by the agar diffusion bioassay. Proximal fluids displayed 90 ± 5 μg of piperacillin per ml. However, distal fluid from untreated and Ca^{2+}-treated biofilms displayed 15 ± 2 μg of piperacillin per ml and no piperacillin, respectively. In another experiment, HEPES plus 100 μg of piperacillin per ml was added to the distal chamber. Three biofilms were then exposed to the flow of HEPES ± Ca^{2+} through the proximal chamber for 8 h. When these distal fluids were recovered and assayed in triplicate in the agar diffusion bioassay, the piperacillin concentration was found to be 92 ± 5 μg · ml^{-1}.

Membranes were recovered for viable count determinations (16) prior to and following the application of piperacillin. Despite the 8-h exposure to 100 μg of piperacillin per ml, the biofilms contained many viable bacteria (an average of $4.7 \times 10^5 \cdot cm^{-2}$, in two untreated biofilms and $1.5 \times 10^6 \cdot cm^{-2}$ in two Ca^{2+}-treated biofilms). However, when the bacteria were ultrasonically dispersed (50 Hz for 5 min) from the MEP of untreated and Ca^{2+}-treated biofilms before ($n = 3$) and after ($n = 3$) exposure to piperacillin, the dispersed bacteria were susceptible to 7.81 μg of the β-lactam antibiotic per ml.

The agar diffusion bioassay would detect only the biologically active piperacillin molecule. Hydrolysis of piperacillin by *P. aeruginosa* β-lactamase would produce a molecule (piperacillinoic acid) which would not be detectable in the agar diffusion bioassay. To investigate this possibility, the piperacillin treatment experiment was repeated with two untreated, viable *P. aeruginosa* 579 biofilms, two 579 biofilms following ethylene oxide sterilization, two viable, Ca^{2+}-treated 579 biofilms, and three viable *P. aeruginosa* 4096 (*met pro blaI blaP*) biofilms. Preliminary experiments with three 579 biofilms found that no viable bacteria could be recovered after exposure to ethylene oxide. β-Lactamase production can be induced in *P. aeruginosa* biofilms following exposure to piperacillin (6). The nonviable bacteria in the ethylene oxide-treated biofilms would not produce β-lactamase. *P. aeruginosa* 4096, besides being noninducible for the expression of β-lactamase, is phenotypically nonmucoid (22). Thus, mucoid and nonmucoid, β-lactamase-deficient *P. aeruginosa* biofilms were examined. Distal fluids recovered following the 8-h exposure of the biofilms to 100 μg of piperacillin per ml were stored at −20°C until use. The fluids were qualitatively examined for piperacillin and piperacillinoic acid by thin-layer chromatography (TLC). Silica gel plates (60 A [20 by 20 cm; 250-μm layer]; Whatman International Ltd., Maidstone, United Kingdom) were used. The ascending solvent consisted of butyl acetate:butanol:acetic acid:methanol:phosphate buffer (pH 5.8) (80:15:40:5:24). Detection reagents were aqueous 0.5% (wt/vol) starch and 0.01 M iodine containing sodium azide (0.2 mg · ml^{-1}). Piperacillin (2 mg · ml^{-1} in deionized distilled water [$n = 6$]) was diluted (1:1) in deionized distilled water. Volumes (50 μl each) were applied to TLC plates (100 to 0.10 μg of piperacillin). Six other piperacillin solutions received *Bacillus cereus* penicillinase types I and II (Sigma Chemical; final concentrations, 50 μg · ml^{-1}). After incubation at 25°C (110 rpm for 15 to 18 h), a series of dilutions were prepared as described above and 50-μl volumes were applied to TLC plates. Chromatography of the standards resolved a minimum of 0.78 μg (15.6 μg · ml^{-1}) of undigested and β-lactamase-digested piperacillin. The undigested standards chromatographed as a prominent band having a relative mobility (R_f) of 0.23 and as two weakly evident bands ($R_f = 0.42$ and 0.50). Enzyme-digested samples chromatographed as two equally prominent bands ($R_f = 0.23$ and 0.42). TLC of distal fluids (50 μl each for five times) recovered from the viable and nonviable 579 biofilms and from the 4096 biofilms did not detect any bands.

Considered together, our observations are consistent with a role for *P. aeruginosa* 579 biofilms as a physical rather than an enzymatic diffusion barrier to piperacillin. That Ca^{2+} treatment of biofilms eliminated the diffusion of biologically active piperacillin across the *P. aeruginosa* biofilms is furthermore consistent with the behavior of the MEP as a molecular sieve. *P. aeruginosa* MEP is structurally altered by Ca^{2+} concentrations of 1.0 mM or more (9, 18). Permeation of piperacillin through condensed MEP may have been restricted in the Ca^{2+}-treated biofilms. At the pH of the HEPES buffer (7.0), piperacillin would be weakly anionic and so would not be expected to bind avidly to the polyanionic (20) MEP. Rather, a decreased porosity of the Ca^{2+}-condensed MEP may have been influential in the piperacillin resiliency of the *P. aeruginosa* biofilms. This hypothesis is testable (13). In environments such as the lungs of individuals afflicted with cystic fibrosis, the Ca^{2+} concentration employed in the present study may well be physiologically relevant (18).

We thank Don Woods, University of Calgary, for providing *P. aeruginosa* 4096 and the reviewers for their suggestions.

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