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Antibiotic Resistance of *Pseudomonas aeruginosa* Colonizing a Urinary Catheter in Vitro

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A modified Robbins Device was used to establish coherent biofilms of *Pseudomonas aeruginosa* on the surface of catheter material in an artificial urine milieu and the ability of an antibiotic to penetrate the biofilm and kill the enclosed bacteria was assessed. The *Pseudomonas aeruginosa* strain used had been isolated from a patient with urinary tract infection. Although planktonic (floating) cells of the *Pseudomonas aeruginosa* strain were inhibited by less than 1 mg/l of tobramycin and killed by 50 mg/l, contact with 1,000 mg/l of tobramycin for 12 h failed to kill all the sessile (adherent) bacteria in the biofilms on the surface of the catheter material. Surviving sessile bacteria recovered directly from the exposure to 1,000 mg/l of tobramycin were inhibited by 0.4 mg/l of this agent when tested as dispersed planktonic cells by standard MIC methods. It is suggested that growth within thick adherent biofilms confers upon cells of *Pseudomonas aeruginosa* a large measure of resistance to aminoglycosides and other antibiotics that may help to explain the frequent failure of antibiotic chemotherapy in catheter-associated urinary tract infections.

In their natural native aquatic habitats cells of *Pseudomonas aeruginosa* live predominantly in thick biofilms adherent to submerged surfaces (1). Within these biofilms, cells are surrounded by very large amounts of exopolysaccharide glycocalyx material (2) which forms a hydrated anionic matrix that concentrates nutrients near the cells but tends to exclude predatory bacteria, amoebae, bacteriophage and molecular antibacterial agents (3). Similarly, in aerobic industrial aquatic systems cells of *Pseudomonas aeruginosa* grow predominantly in glycocalyx-enclosed adherent biofilms on submerged surfaces (4). We used the original Robbins Device to study these adherent biofilms (4) and found the cells enclosed within these coherent anionic matrices were protected to a very great extent from the bactericidal activity of commercial chemical biocides (5). Bacterial biofilms have been found on critical surfaces within the hospital environment, and growth within these coherent adherent matrices has been shown to confer resistance to such bactericidal agents as chlorhexidine (6) because the agent in question must saturate a large number of ionic binding sites within the matrix before it reaches and kills the bacterial cells.

When cells of *Pseudomonas aeruginosa* enter the drainage bag systems of the urinary catheter they express their natural tendency to adhere to available surfaces (7) and to produce the alginate exopolymer (8) that mediates their irreversible adhesion to the colonized surface and their eventual formation of a coherent biofilm. When these organisms are recovered from the urine drainage systems and grown as individual unprotected cells in an in vitro culture, they appear to be sensitive to many antibiotics, but clinical experience suggests that the cells within established biofilms on catheter surfaces are not actually killed by treatment with clinical levels of these agents. To investigate this phenomenon we have modified the Robbins Device in order to establish coherent biofilms of *Pseudomonas aeruginosa* on the surfaces of catheter materials in a urine milieu, and we have used this system to assess the ability of antibiotics to penetrate the biofilm and kill the enclosed bacteria.

Materials and Methods

Modified Robbins Device. The original Robbins Device (4) was modified to develop an artificial multiport sampling catheter. The modified Robbins Device was constructed from an acrylic block 41.5 cm long with a 2 mm × 10 mm lumen. Twenty-five evenly spaced sampling ports were devised such that catheter material (0.5 cm^2) attached to sampling plugs would lie flush with the inner surface without disturbing flow characteristics. The sampling plugs could be removed and replaced aseptically. The modified device was connected by diameter type tubing to a 2 l reservoir held

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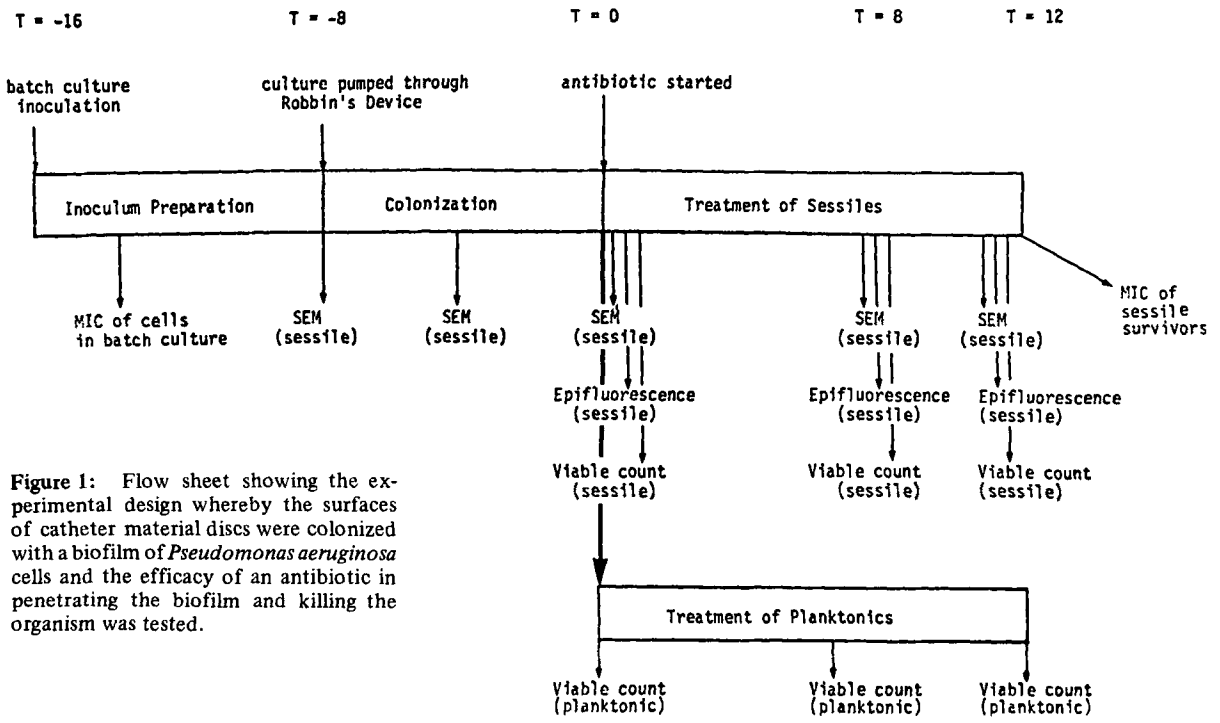

Figure 1: Flow sheet showing the experimental design whereby the surfaces of catheter material discs were colonized with a biofilm of *Pseudomonas aeruginosa* cells and the efficacy of an antibiotic in penetrating the biofilm and killing the organism was tested.

in a 37 °C water bath. Medium containing bacteria was pumped from this reservoir (artificial bladder) through the device (artificial catheter) by a peristaltic pump set to deliver 60 ml/h. The device exposed discs cut from a urinary catheter (Silkolatex, Rusch) to the bacteria-containing urine and these discs could be aseptically removed from the system for analysis. Before each experiment the entire experimental apparatus was sterilized with ethylene oxide.

Microbiology. The strain of *Pseudomonas aeruginosa* used in these experiments was freshly isolated from a patient with urinary tract infection and identified by routine microbiological methods. The medium used was artificial urine adapted from that of Minuth et al. (9) and contained 0.65 g/l $CaCl_2 \cdot 2H_20$, 0.65 g/l $MgCl_2 \cdot 6H_20$, 4.6 g/l NaCl, 2.3 g/l Na_2SO_4, 0.65 g/l sodium citrate, 0.020 sodium oxalate, 2.8 g/l KH_2PO_4, 1.6 g/l KCl, 2.0 g/l NH_4Cl, 12.0 g/l urea and 1.1 g/l creatinine, supplemented with 0.4 % nutrient broth (Difco) to enhance bacterial growth. The bacteria were stored on slants at −70 °C and serially cultured at 8 h intervals to provide an inoculum for the reservoir. On the basis of standard growth curve data developed in batch cultures of the organism in this medium, the reservoir was inoculated with a 2 % inoculum so that the reservoir delivered logarithmic phase cells to the modified Robbins Device throughout the 8 h colonization period (Figure 7). The MIC of logarithmic phase cells of the strain were determined using standard methods (10).

Experimental Design. Artificial urine containing logarithmic phase cells of *Pseudomonas aeruginosa* was passed through the modified Robbins Device for 8 h during the colonization period of the experiment (Figure 1) and the development of the bacterial biofilm was monitored by regular sampling of catheter material surfaces by scanning electron microscopy (SEM). At the end of the 8 h colonization period (t = 0 h), individual sample discs bearing sessile (adherent) bacteria were aseptically removed for examination by SEM and epifluorescence microscopy and performance of viable counts, and the artificial urine medium containing the logarithmic phase bacterial cells was transferred in equal amounts to 50 ml flasks in which these planktonic (floating) bacteria were exposed to various concentrations of tobramycin for either 8 or 12 h (Figure 1). At t = 0 h, flow was started from the antibiotic-medium reservoir so that artificial urine containing tobramycin flowed through the modified Robbins Device during the treatment period of the experiment (Figure 1). Discs of catheter material were removed from the treated device at 8 h and 12 h and the sessile bacteria in the biofilm were examined by SEM, epifluorescence, and viable counting. At 12 h a disc bearing sessile bacteria was removed from the system and rinsed in the artificial urine medium, and the adherent cells were aseptically scraped into fresh artificial urine medium in which their MIC of tobramycin was determined.

Aerobic Plate Counts. The sampling surface was washed with phosphate-buffered saline (PBS), scraped with a sterile blade and the scrapings and the catheter disc itself were placed in 5 cc of sterile PBS and ultra-sonicated at a very low output in order to disperse the cells. Dilution series were made up to 10^{-4} and plated on nutrient agar from which quantitative plate counts were obtained.

Epifluorescence. Quantitative sessile bacterial counts were obtained using a modified quantitative epifluorescence technique (1). Briefly, this involved fixing the aseptically removed catheter specimens in 0.5 % glutaraldehyde (in 0.1 M cacodylate buffer). The specimens were stained with 0.01 % acridine orange for 2 min, rinsed with filter-sterilized phosphate buffer (pH 7.5) and destained with isopropanol. Because of problems due to fluorescing catheter material, the technique was modified by counter-staining the specimens with a 1 % aqueous solution of malachite green for 10 min. The specimens were air dried, mounted on slides and examined with a Zeiss Standard 16 microscope fitted for epifluorescence microscopy. Cells were counted, and numbers of sessile cells/cm^2 calculated.

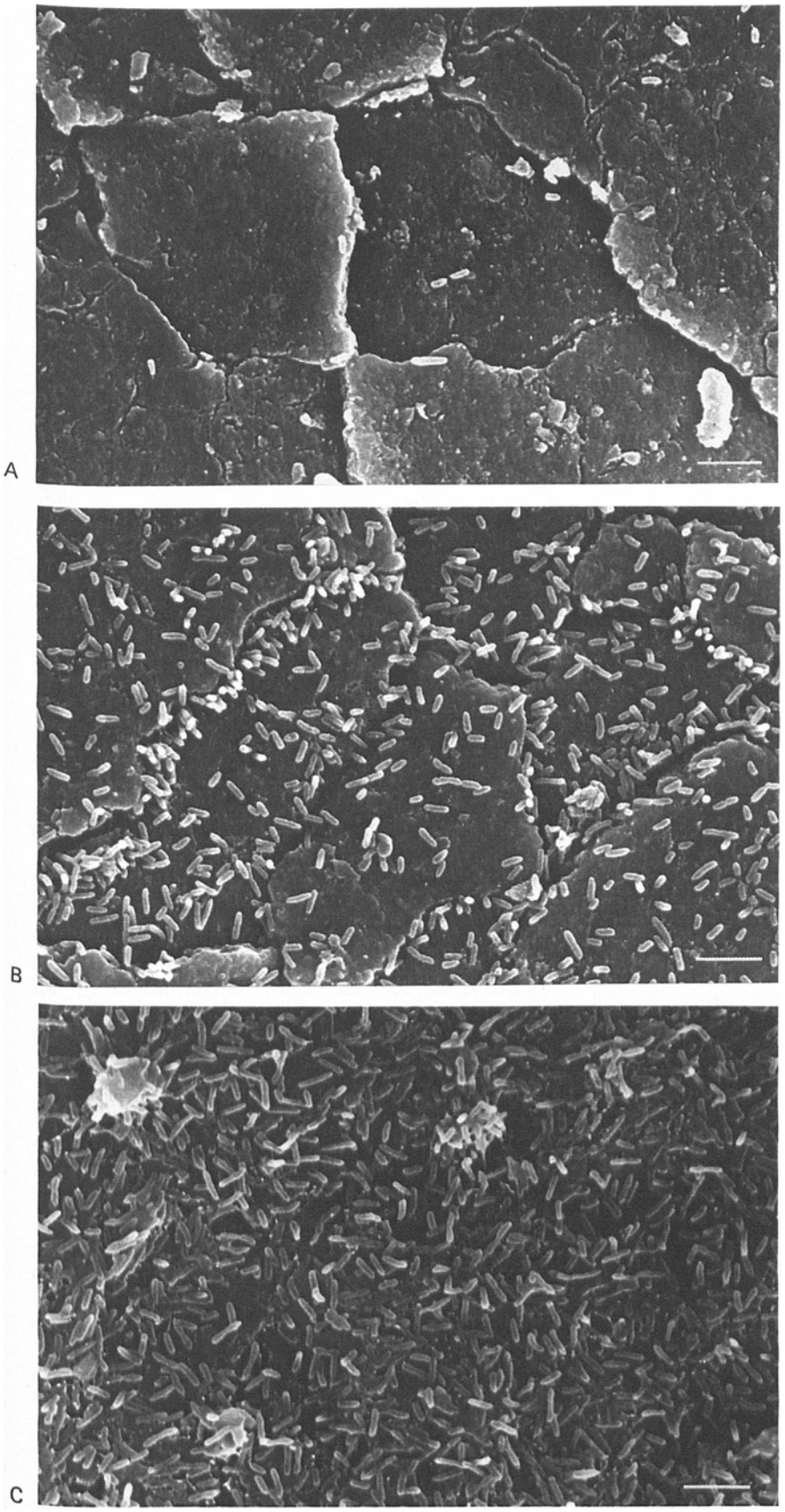

Figure 2: Scanning electron micrographs of catheter material surface after contact with a *Pseudomonas aeruginosa* strain in artifical urine. A: After 5 min the irregular plate-like surface of the latex material was sparsely colonized by rod-shaped bacteria. B: After 1 h large numbers of rod-shaped cells adhered to the surface but had not yet generated significant amounts of exopolysaccharide glycocalyx or formed a confluent biofilm. C: After 8 h a thick bacterial biofilm occluded the plate-like surface and rod-shaped bacteria were partly buried in the dehydration condensed residue of the exopolysaccharide matrix of their confluent biofilm. Bars, 5 μm.

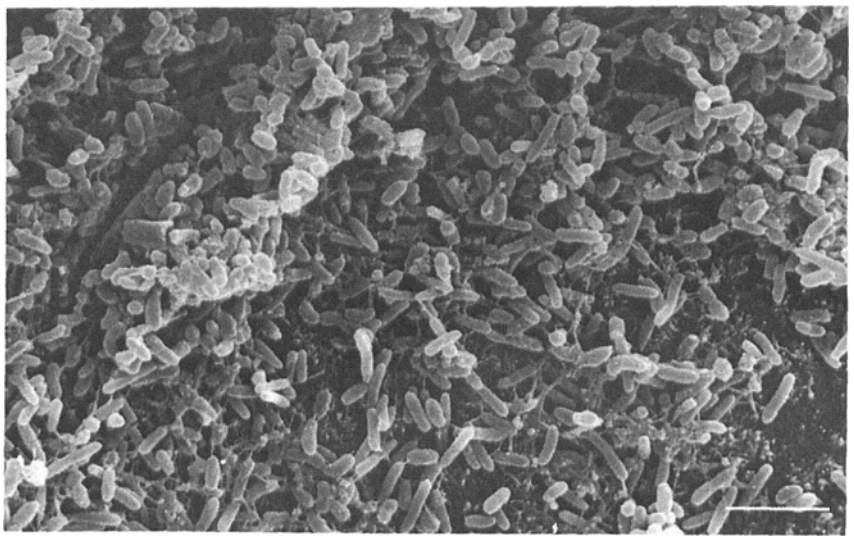

Figure 3: Scanning electron micrograph of the luminal surface of a urinary catheter recovered from a patient with catheter-associated *Pseudomonas aeruginosa* UTI. Note the confluent biofilm composed of rod-like bacterial cells and the amorphous dehydration-condensed residue of the biofilm matrix. Bar, 5 μm.

Scanning Electron Microscopy. Catheter specimens were placed in a fixative solution consisting of 5 % glutaraldehyde in cacodylate buffer (0.1 M pH 7.2) for 1 h at 22 °C, followed by dehydration in a series of aqueous ethanol solutions (20–100 %) and Freon 113-ethanol solution (30–100 %) and then air dried. Samples were coated with gold in a sputter coater and examined using a Hitachi 5450 scanning electron microscope.

Transmission Electron Microscopy. Material scraped from the catheter surface was fixed in 5 % glutaraldehyde in cacodylate buffer (0.M pH 7.2) with 0.15 % ruthenium red for 24 h at 20 °C. The material was then washed five times in the buffer, postfixed in 2 % OsO_4 in buffer, washed five more times in the buffer, and dehydrated through a series of acetone washes. All of the solutions used in processing the specimen, from the washes after glutaraldehyde fixation to dehydration with the 70 % acetone solution, contained 0.05 % ruthenium red. After further dehydration in propylene oxide, the specimens were embedded in Spurr (11) low viscosity embedding resin, sectioned, stained with uranyl acetate and lead citrate, reinforced with evaporated carbon, and examined with a Hitachi 600 transmission electron microscope at an acceleration voltage of 60 kV.

Results

Spectrophotometric monitoring of the growth of the *Pseudomonas aeruginosa* strain in artificial urine confirmed that the cells remained in the logarithmic phase of growth throughout the 8 h colonization period. As these cells flowed through the modified Robbins Device, they began to colonize the discs of catheter material at 5 min (Figure 2A) and had colonized a large proportion of the surfaces of these discs by 1 h (Figure 2B). At 8 h the plate-like surface structure of the catheter material was completely occluded by a confluent biofilm (Figure 2C) within which the bacterial cells were partially buried in the dehydration-condensed residue of their glycocalyx exopolysaccharides. Recovery of sessile biofilms and plating of their dispersed cells showed that catheter materials were colonized by $2.1–5.8 \times 10^8$ cells/cm^2 at the end of the 8 h colonization period (t = 0h). This adherent biofilm of cells of *Pseudomonas aeruginosa* developed on a catheter material surface in an artificial urine milieu closely resembles the biofilms seen on the luminal surfaces of urinary catheters removed from patients with catheter-associated *Pseudomonas aeruginosa* UTI (Figure 3).

Transmission electron micrographs of ruthenium red stained sections of biofilm material scraped from the catheter disc surface (Figure 4) demonstrate extracellular condensed electron dense fibrous strands between the bacterial cells. The distribution of this condensed exopolysaccharide glycocalyx material suggests that in its hydrated state it occupied all of the intercellular space within the thick bacterial biofilm and constituted its functional matrix.

The MIC of tobramycin for planktonic cells of the *Pseudomonas aeruginosa* strain taken from the batch cultures used to inoculate the experimental system was 0.6 mg/l. When the planktonic cells were removed from the experimental system at t = 0 h and transferred to flasks with various concentrations of tobramycin their MBC of tobramycin was found to be 50 mg/l. When sterile artificial urine containing 1,000 mg/l of tobramycin was flowed past the colonized discs of catheter material bearing 2.1×10^8 cells/cm^3, in the modified Robbins Device, a large number (1.1×10^7 cells/cm^3) of cells remained viable at 8 h and an almost equally large number (6.1×10^6 cells/cm^2) at 12 h. When these tobramycin-treated biofilms were examined by epifluorescence microscopy, which does not distinguish between live

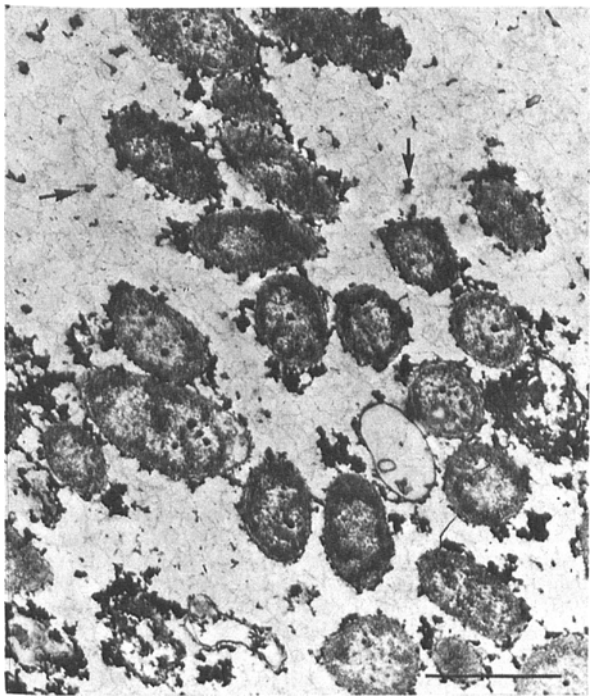

Figure 4: Transmission electron micrograph of a ruthenium red stained preparation of biofilm material scraped from a catheter disc. Note the formation of a very extensive reticulum of electron-dense condensed fibers (arrows) throughout the intercellular spaces in the biofilm. This is the residuum of the extensive glycocalyx of these cells following 99 % condensation by the dehydration of this structure in preparation for electron microscopy. Bar, 1 μm.

and dead cells, the adherent cell numbers were found to remain constant at 0 h, 8 h and 12 h. SEM, which also fails to distinguish live from dead bacteria, also showed very thick biofilms containing very large numbers of bacterial cells on the surfaces of catheter materials at 12 h, following tobramycin treatment. When the tobramycin-treated biofilms were rinsed in fresh artificial urine, and the component cells were recovered and dispersed, their MIC of tobramycin was found to be 0.4 mg/l.

Discussion

Proper use of sterile technique and the closed urinary system has decreased the incidence of bacteriuria in short term catheterization (12, 13); however, the risk of infection increases with each day of catheterization (14) and almost all chronically catheterized patients develop bacteriuria (15). In fact, catheterization of the urinary tract remains the most common cause of nosocomial infection in medical practice (16) resulting in significant morbidity (14) and even mortality (17).

Systemic antibiotic therapy usually fails to eradicate a catheter-associated bacteriuria or if the infection is presumably cured, the bacteriuria quickly recurs after cessation of therapy (13, 14, 18–20). Although this failure of chemotherapy in catheter-associated infections is generally accepted, the reason is unclear. A new concept developing in industrial and environmental microbial ecology is that the predominant bacteria in aqueous systems are sessile organisms attached to inert surfaces in biofilms, rather than the floating planktonic bacteria upon which standard microbiologic investigations are based (1, 3). This sessile population appears to be relatively resistant to biocides in industrial systems (5). We have demonstrated that this same phenomenon of bacterial growth and antibiotic resistance occured on a urinary catheter removed from a patient with a *Pseudomonas aeruginosa* urinary tract infection (unpublished observation).

The original Robbins device is used to study and quantitate bacterial biofilm plugging in oil and water pipes (4) and our modification has proved useful in demonstrating in vitro bacterial colonization of catheter surfaces and the susceptibility of these bacteria to antibiotics. The catheter materials were colonized by a bacterial biofilm covering the entire surface within 8 h of being exposed to the infected artificial urine (Figure 1C). Transmission electron microscopy demonstrated that the bacteria in the biofilm were enclosed in an extensive exopolysaccharide matrix or glycocalyx which condensed during the dehydration process to an electron-dense residue (Figure 3).

The MIC of tobramycin for the *Pseudomonas aeruginosa* strain was less than 1 mg/l, while the MBC for the bacteria in batch culture was 50 mg/l. However, when the sessile bacteria growing in biofilm adherent to the catheter surfaces were exposed to high concentrations of tobramycin (1,000 mg/l) they demonstrated relative resistance. Aerobic bacterial plate counts confirmed that the bacteria were still viable, even after having been exposed to levels of tobramycin exceeding one thousand times their planktonic MIC. However, when these same sessile bacteria were scraped from the catheter surfaces and subsequently tested in pure culture, their MIC value was comparable to that of the planktonic bacteria. Thus, the bacteria growing on the catheter surface did not develop permanent resistance to tobramycin. The inability of the antibiotic to kill the bacteria was only evident when tested against sessile bacteria growing in biofilms. Under usual clinical conditions, levels of plasma tobramycin rarely exceed 8 mg/l which usually results in urine concentrations of 50–200 mg/l (21). Therefore, the biofilm enclosed bacteria in the in vitro system cannot be killed with tobramycin levels obtainable in urine. These observations explain the futility of using systemic antibiotics in catheterized patients once significant bacteriuria has occurred.

Acknowledgements

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