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The effect of electrical currents and tobramycin on *Pseudomonas aeruginosa* biofilms

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The combined use of antibiotics with low levels of electrical current has been reported to be more effective in controlling biofilms (the bioelectric effect) than antibiotics alone. An electrical colonisation cell was designed to study the effect of antibiotics on biofilms formed on a dialysis membrane away from the electrode surface. To avoid the electrochemical generation of toxic products, *Pseudomonas aeruginosa* biofilms were formed in minimal salts medium that excluded chloride-containing compounds. Under these conditions, electrical currents of up to 20 mA cm^{-2} did not prevent biofilm formation or have any detrimental effect on an established biofilm. Tobramycin alone at concentrations of 10 μg ml^{-1} did not affect the biofilm, but were significantly enhanced by 9 mA cm^{-2}. The effect of tobramycin concentrations of 25 μg ml^{-1} were enhanced by a 15 mA cm^{-2} electrical current. In both cases higher levels of electrical current, up to 20 mA cm^{-2}, did not further enhance the effect of the antibiotic. The possible mechanisms of action of the bioelectric effect have been reported to involve electrophoresis, iontophoresis and electroporesis, thus overcoming the biofilm biomass and cell wall barriers. Our results suggest that other factors may also be important, such as the metabolic activity and growth rate of the bacteria. Such factors may be critical in maximising antibiotic efficacy.

Keywords: biofilms; *Pseudomonas aeruginosa*; antibiotics; tobramycin; electrical current

Introduction

Bacterial biofilms have added complications to current medical practices because of their resistance to a wide range of antibiotics, resulting in chronic infections [44]. In many instances, bacteria will preferentially grow as biofilms on any biomedical material and associated surface [26]. Medical prosthetic devices that are totally implanted such as orthopaedic prostheses [20], heart valves and cardiac pacemakers [2], internal catheters [28] and artificial vascular grafts [25] may become contaminated with thick bacterial films and will require surgical removal. Transdermal devices including urinary catheters [27,31], dialysis tubing [21,37], intravascular catheters for drug delivery [19] and contact lenses [30] provide ideal surfaces for biofilm formation. Associated medical equipment such as dental drills, endoscopes [42] and contact lens cases [30] are also prone to contamination by bacterial biofilms.

Once established on a surface, biofilms are often difficult to control and nearly impossible to eradicate by conventional antimicrobial therapy. Nickel *et al* [32] demonstrated that *Pseudomonas aeruginosa* within a biofilm survived antibiotic concentrations 20 times the minimal inhibitory concentration for the organism. Various researchers have reported similar observations with other species of bacteria such as *Escherichia coli, Staphylococcus aureus* and *S. epidermidis* [44]. The mechanisms of the antimicrobial resistance exhibited by the biofilm have been related to its 3-dimensional structure; the resistance is lost as soon as the structure is disrupted [23]. This suggests that the production of copious amounts of exopolysaccharide by the bacteria during biofilm formation and growth may protect the innermost cells either by binding the antibiotic or providing a niche which has a lower permeability to the antibiotic than its surrounding aqueous phase [14,22]. Furthermore, antimicrobial agents are more effective against actively growing cells, implying that bacteria within the biofilm may have an altered growth rate and physiology, resulting in increased resistance to these compounds [4,12]. The persistence and resistance of bacterial biofilms to antimicrobial treatment has led to numerous studies to develop different strategies in an attempt to control and possibly eradicate the contaminant and the resultant infections.

With the improved application and increased use of medical implants and associated devices, contact lens use and operative techniques, the eradication of biofilms has been a challenge for clinicians. This has prompted extensive studies into new ways of controlling these persistent infective contaminants. The efficacy of killing bacteria by physical methods such as super high magnetic fields [33,35], ultrasonic treatment [34], high pulsed electrical fields [6,29,38,39] and low electric fields, both on their own [9] and as enhancers of biocides [3] and antibiotics [7], are currently being investigated. Davis *et al* [8] observed that very low currents of 200 and 400 μA, using silver, carbon and platinum electrodes killed planktonic cells of Gram-positive and Gram-negative bacteria and *Candida albicans* in synthetic urine. The biocidal effect was primarily attributed to iontophoresis, the generation of ions from chlorine-containing components in a medium [8,11]. When a simple salts medium containing no chlorine was used, only

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a marginal reduction in the bacterial population was observed [10].

Most of these studies involved the eradication of planktonic bacteria. However, several addressed the possibility of applying these technologies to highly resistant biofilm bacteria. Previous work by Blenkinsopp *et al* [3] demonstrated a bioelectric effect, that low electrical currents enhanced the efficacy of glutaraldehyde, kathon and a quarternary ammonium compound on *P. aeruginosa* biofilms when the biofilm was formed on one of the electrodes. Recently, work undertaken in one of our laboratories showed that low electrical currents and tobramycin increased the killing efficiency against *P. aeruginosa* biofilms on both the electrode and on adjacent metallic surfaces [7]. The present study assesses whether a low electrical current can enhance the bactericidal effect of tobramycin when the biofilm is formed on a surface suspended between two electrodes, rather than on the electrodes themselves. To undertake this study a device was designed to colonise a semipermeable surface with the biofilm held away from the electrode surface to avoid mechanical and surface electrochemistry effects.

Materials and methods

Culture and chemostat growth conditions

A catheter-associated urinary tract isolate of *Pseudomonas aeruginosa* EX226 was obtained from the Royal Devon and Exeter Hospital, UK. The strain, stored on glass beads in 10% glycerol at −70° C with minimum subculturing to retain its phenotypic characteristics, was revived on nutrient agar plates and inoculated into a basic minimal salts medium (MS medium). The basic minimal salts medium adapted from Gilbert *et al* [18] excluded chloride-containing compounds and contained (g L^{-1}): $(NH_4)_2SO_4$, 0.793; $MgSO_4 \cdot 7H_2O$, 0.123; KH_2PO_4, 2.67; Na_2HPO_4, 11.4; buffered to pH 7.4 with 10% H_2SO_4. A 10-ml volume of 0.1 M glucose solution, sterilised separately, was added to 1.0 L of medium as the sole carbon source.

The organism was grown in continuous culture for the colonisation studies in a 2-L culture vessel connected to an Electrolab P300 agitation unit (Electrolab Ltd, Tewkesbury, UK) and an Anglicon computerised control panel (Anglicon Instruments Ltd, East Sussex, UK). A 10-L volume of MS medium with 10 mM glucose was connected to an assembled sterile chemostat via a Gilson peristaltic pump. A volume of 10 ml of an exponentially growing culture of *P. aeruginosa* was inoculated into the chemostat vessel containing 500 ml of medium and was grown in batch for 6 h. Fresh 10 mM glucose–MS medium was fed into the chemostat at 15 ml h^{-1} to give a working volume of 1150 ml and a dilution rate (D) of 0.013 h^{-1} (one volume change occurred every 3.2 days).

Antibiotic sensitivity testing planktonic bacteria

Tobramycin was purchased from Sigma Chemical Co (St Louis, MO, USA). Stock solutions of tobramycin for the antibiotic sensitivity assays were prepared in sterile distilled water and were filter-sterilised prior to use. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for *P. aeruginosa* EX226 were determined by the standard macrodilution method [24,40] in Mueller–Hinton (M–H) medium supplemented with 50 μg ml^{-1} Ca^{2+} and 25 μg ml^{-1} Mg^{2+}. Each tube, containing 2 ml of antibiotic solution in M–H medium, was inoculated with 100 μl of a logarithmic phase culture to a final cell concentration of 10^5 CFU ml^{-1}. The MIC was defined as the lowest concentration of antibiotic at which there was no visible bacterial growth. The minimal bactericidal concentrations for the antibiotics were determined by plating 100 μl from each test tube that had no visible bacterial growth and incubating the plate at 37° C overnight. The MBC was defined as the minimum concentration of antibiotic that inhibits growth of 99.9% of the bacteria [40].

The sensitivity of planktonic *P. aeruginosa* to tobramycin in MS medium was established by inoculating 10 mM glucose–MS medium containing 0, 5, 10, 25 and 50 μg ml^{-1} tobramycin. Each vial was inoculated with 1 ml of a 1/10 dilution of the chemostat culture to a concentration of 10^7 CFU ml^{-1} and incubated in a stationary incubator at 37° C. A 1-ml sample was removed at 12 h and another at 24 h. The cells were centrifuged and resuspended in sterile 1/4 Ringer's solution to remove the antibiotic. The suspension was serially diluted and plated onto nutrient agar plates for viable counts.

Electrical colonisation cell

An electrical colonisation cell (ECC) was designed so that a biofilm was formed on a surface away from the electrodes, avoiding electrochemical and mechanical disturbances, yet remaining in the path of the electric current. The design was based on the two parallel electrode plates with a biofilm formed on a membrane suspended in parallel, approximately equi-distant from each electrode (Figure 1). The ECC was divided into two chambers by a membrane clamped in place with an O-ring. The 5.31 cm^2 circular stainless steel electrodes (BSS–EN.58B; 0.08% C, 0.70% Si, 0.70% Mn, 0.03% S, 18.00% Cr, 8.00% Ni, 0.60% Ti and 71.89% Fe) were placed at either end of the cell to produce an electrical current through a biofilm colonised on one side of the dialysis membrane. Wires connecting the electrodes to a power generator were affixed to the centre of each electrode plate and sealed with silicone. An electrolyte solution was pumped through both chambers separately at 50 ml h^{-1} via silicone tubing and a peristaltic pump. A direct current was produced from a power generator that provided a constant current that changed polarity every 32 s in a square wave function. The ECC were ethylene oxide-sterilized prior to use.

Biofilm formation

Biofilms were formed on a treated dialysis membrane. To remove residual sulphides and surfactants from the dialysis membranes, they were treated with 0.3% w/v sodium sulphide at 80° C for 1 min, washed with hot distilled water for 2 min, acidified with 0.2% v/v sulphuric acid and again rinsed with hot distilled water (60° C). The membranes were rinsed with a solution of 0.1 M Tris buffer in 1/4-strength Ringer's (pH 7.2) overnight and rinsed further with distilled water and placed between glass separators in distilled water for autoclaving. Sterile membranes were asepti-

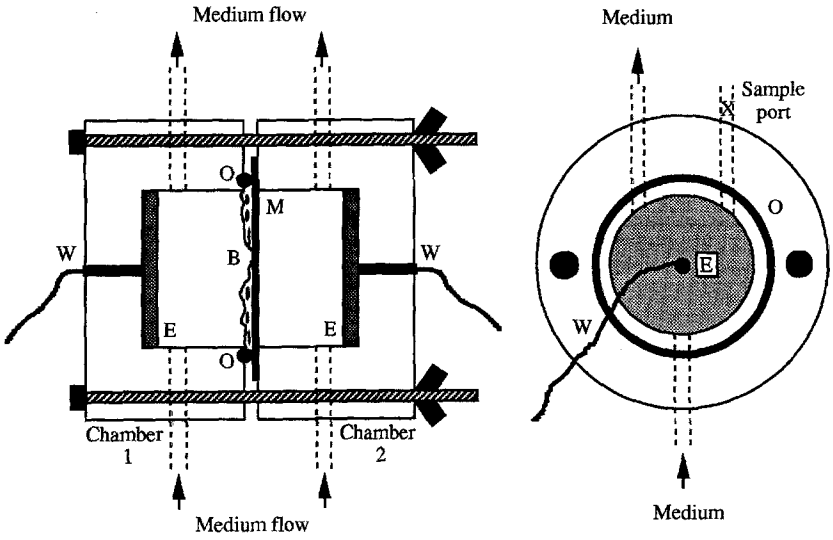

Figure 1 A schematic diagram of the electrical colonisation cell. Two circular stainless steel electrodes (E) at either end of the cell were connected to a power generator by wires (W) attached to the center of each electrode. Two chambers were formed by a dialysis membrane (M) clamped and sealed with an O-ring (O). The medium was passed through the ports at the bottom of the cell and out through the ports at the top avoiding the accumulation of gases generated by the current. Separately, a culture was pumped into chamber 1 to form a biofilm on the membrane and sterile medium was pumped into chamber 2 to complete the electrical curcuit. A sample port provided access to the chambers asceptically

cally placed and clamped into a sterile ECC just prior to colonisation.

P. aeruginosa was colonised onto one side of the dialysis membrane surface by connecting the ECC to the chemostat in a recirculating loop through one chamber. To complete the electrical circuit through the cell, the other chamber remained sterile and was connected to a reservoir of sterile MS medium without glucose (electrolyte medium). The chemostat culture was pumped through the ECC at 50–60 ml h^{-1} for 1–2 h and then connected to sterile 10 mM glucose–MS medium to allow the formation of 12-, 24- or 48-h biofilms. Each colonisation experiment required two ECCs, one connected to an electrical source and the other remained as a control. Bacterial growth was monitored by viable counts, total cell counts and SEM. All colonisation experiments were composed of a minimum of three replicates.

Antibiotic and electrical treatment of biofilms

Biofilms were formed on dialysis membranes over 12, 24 and 48 h and then treated for a further 12 h with either antibiotic, current or both. Once the biofilm was formed, the tubing was reconnected to a reservoir containing prewarmed (37° C) 1.0 mM glucose–MS medium and tobramycin for 12 h. To compare the effect of increasing concentrations of tobramycin in the presence and absence of electrical current, 48-h biofilms were exposed to 0, 10, 25 or 50 μg ml^{-1} tobramycin in the presence of either 0 or 9 mA cm^{-2} of electrical current. The dialysis membrane was removed aseptically from the ECC, placed into 1/4-strength Ringer's solution and the effect of the treatment was determined by total and viable cell counts. Biofilms that formed over 12, 24 and 48 h were treated with 0 and 10 μg ml^{-1} tobramycin in the absence and presence of 9 mA cm^{-2} current. The response was assessed by viable cell counts. The effect of increasing electrical current density (0, 9, 15 or 20 mA cm^{-2}) in the presence of 0, 10 or 25 μg ml^{-1} tobramycin was assessed by viable counts.

Bacterial enumeration

The biofilm bacteria were enumerated by washing the dialysis membrane in 1/4-strength Ringer's solution to remove any non-adherent cells and dividing the membrane into four sections. One section was placed into 1 ml 1/4-strength Ringer's solution with several sterile glass beads to optimise dispersal of the bacteria from the dialysis membrane during vortexing. The membranes with the adherent bacteria were vortexed, sonicated in an ultrasonic bath for 5 min, followed by further vortexing and an appropriate dilution was spread on to nutrient agar plates.

Total bacterial populations of both planktonic and sessile cells were enumerated by fluorescent staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences Inc, Warrington, PA, USA) and enumerated using a fluorescent microscope [43]. Bacteria were stained by fixing 0.5 ml of a cell suspension with 20 ml of 75% glutaraldehyde for 2 h at room temperature or overnight at 10° C. The cells were washed with 1/4-strength Ringer's solution by microcentrifugation (10 000 × *g* for 5 min) in a sterile 2-ml Eppendorf tube and resuspended in 0.9 ml 1/4-strength Ringer's and 0.1 ml of a 5.0 mM CTC solution. The cells were incubated at 60° C for 1 h and diluted to 10 ml with 1/4-strength Ringer's solution. The suspension was vortexed, sonicated in an ultrasonic bath for 5 min and vortexed again to disperse the cells. Between 3 to 6 ml of a stained cell suspension was filtered onto a 0.22-μm pore size polycarbonate filter (Poretics Corp, Livermore, CA, USA) and counted using an Olympus microscope equipped with a BH2-RFCA epi-fluorescence illumination system and a mercury lamp (Olympus Optical Co (UK) Ltd, London, UK). The dichroic block was fitted with a B excitation filter (400–500 nm) and

IB barrier filter (cut off at approximately 490 nm) with a supplementary filter, 20 0590 (cut off at 590 nm).

Scanning electron microscopy

Surfaces containing adherent bacteria were rinsed in 1/4-strength Ringer's solution and fixed with 3.0% v/v glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 2 h at room temperature or overnight at 10° C. The specimens were washed with cacodylate buffer and dehydrated in a series of 30%, 50%, 70% and 100% ethanol in water. Samples were air-dried, mounted and sputter-coated with gold. They were viewed on a Sterioscan 100 scanning electron microscope (Leica, Cambridge, UK) at 15 or 25 kV accelerating voltage.

Transmission electron microscopy

Surfaces containing biofilm bacteria were fixed in 3% (v/v) glutaraldehyde – 0.1% (w/v) ruthenium red in 0.1 M cacodylate buffer (pH 7.4) for 12 h at 4° C and washed twice with sterile 0.1 M cacodylate buffer. The biofilms were enrobed in 3% (w/v) agarose by placing a drop on the membrane and after solidifying, the agar with the biofilm was carefully removed from the dialysis membrane surface. To prevent damage to the biofilm, another drop of agarose was placed on the bottom and the agarose-embedded biofilms were trimmed into rectangular strips of approximately 1 mm × 1 mm × 1 cm. The cells were post-fixed with 1% (v/v) osmium tetroxide for 1 h at ambient temperature, washed three times with cacodylate buffer, prestained with uranyl acetate for 1 h in the dark and dehydrated with an acetone/water dehydration series. The agar and biofilm strips were oriented flat in an embedding mold and embedded in Spurr's low viscosity resin. The specimens were mounted for transverse sections of the biofilm. The ultrathin sections were stained with Reynold's lead citrate and viewed under a JEOL 100S electron microscope at 80 kV.

Statistics

The data were compared pairwise using the Student's *t*-test for independent samples. Differences were considered significant at $P \leq 0.05$, however particular comparisons where the P values were different are indicated in the text. The tests were done using the Excel statistics package for Macintosh computers.

Results

Biofilm formation

P. aeruginosa attached to the dialysis membrane and formed thick biofilms, increasing in bacterial numbers with increasing colonisation time (Figure 2). The electrical current did not prevent bacterial attachment and subsequent biofilm formation. The increase in the biofilm was attributed to the increase in cell growth and division rather than attachment, since no further bacteria were introduced into the system after the first 2 h. Comparisons of the viable counts of biofilms formed in the presence and absence of an electrical current demonstrated no significant difference for the three periods studied. The total cell numbers on the surface produced a trend where there was a slight increase

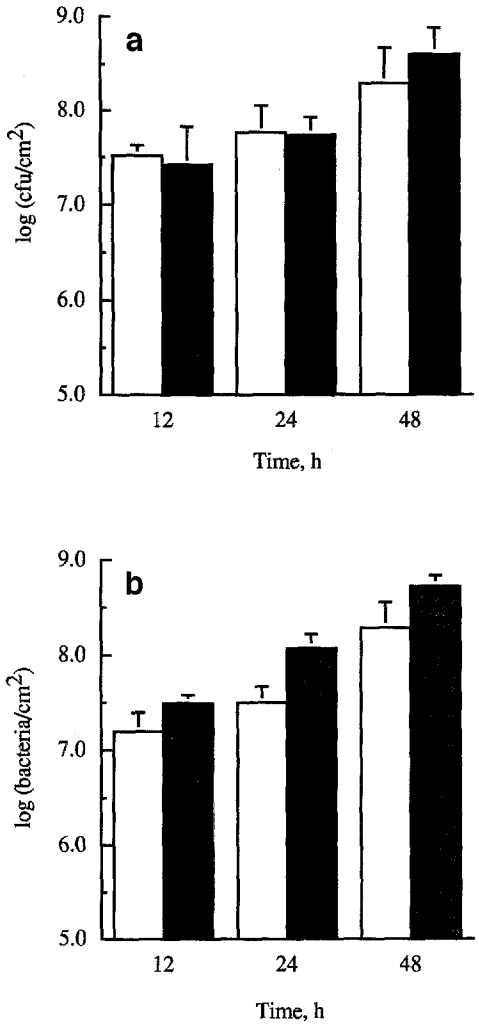

Figure 2 The effect of 0 and 9 mA cm^{-2} current density or *Pseudomonas aeruginosa* biofilm formation by (a) viable counts and (b) total bacterial numbers. The data are from a minimum of three experiments and S. E. bars are shown. □ 0 mA cm^{-2}; ■ 9 mA cm^{-2}

in cell numbers in the presence of an electrical current. This trend was more apparent in the 24- and 48-h biofilms. By 48 h, the cell numbers were *ca* 10^9 cells cm^{-2}, suggesting thick biofilm formation. This growth was a result of nutrient being supplied from one side of the biofilm only while the other chamber contained an electrolyte medium to complete the electrical circuit (Figure 1).

Scanning electron microscopy confirmed the formation of confluent biofilms covered in exopolymer for both the electrically-treated and untreated biofilm (Figure 3). Although thick biofilms were observed under both conditions, it is clearly visible from the transmission electron micrographs (Figure 4) of cross-sections that the biofilm formed in the presence of an electrical current (Figure 4b) was thicker than that formed in the absence of an electrical current (Figure 4a). From the transmission electron micrographs, the thickness of the 48-h biofilm formed in the absence of an electrical current was estimated at approximately 15–36% that of the biofilm formed in the presence of an electrical current. This was supported by the total cell numbers, which estimated that the biofilms formed in the

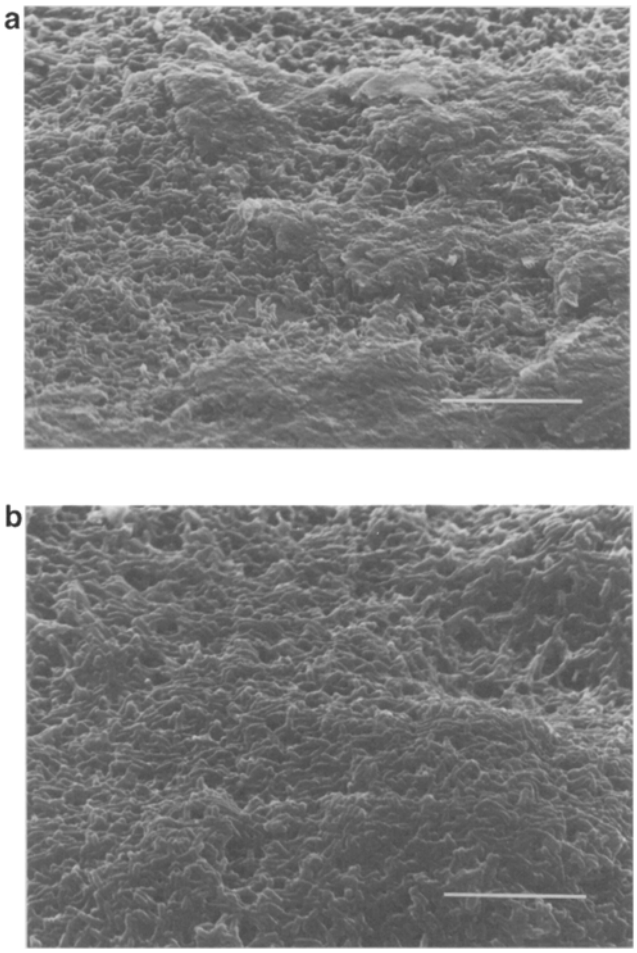

Figure 3 Scanning electron micrographs of a 48-h *P. aeruginosa* biofilm formed on a dialysis membrane in the (a) absence and (b) presence of 9 mA cm^{-2} current density. Bar = 10 μm

absence of current were 37% of the biofilm formed in the presence of electrical current. The bacterial cells appeared to be slightly larger when exposed to an electrical current during biofilm formation and growth but there was no other unusual cell morphology or membrane damage visible from the transmission electron micrographs.

Susceptibility tests on planktonic cells

The MIC and MBC for tobramycin were 1.0 μg ml^{-1}, although this does not fully reflect its activity against planktonic cells of *P. aeruginosa*. The starting bacterial population for standard MIC and MBC determinations, which were cultivated in M–H medium, were 10^5 CFU ml^{-1}, while the starting population for the planktonic assessment of tobramycin (Table 1) was 10^7 CFU ml^{-1}. When the concentration of tobramycin was increased, the bacteria were killed more rapidly. Treatment with the higher concentrations of antibiotic still resulted in a small number of colonies on the plates.

Effect of increasing antibiotic concentration

The bactericidal activity of increasing concentrations of tobramycin against thick *P. aeruginosa* biofilms formed over 48 h was monitored after a 12-h exposure (Figure 5). The viable cell numbers decreased with increasing concentrations of the antibiotic (Figure 5). The electrical current enhanced the antimicrobial effect by significantly killing more cells at 25 and 50 ($P = 0.002$) μg ml^{-1} tobramycin than the antibiotic alone. However, the presence of an electrical current and 10 μg ml^{-1} tobramycin did not significantly reduce the population from that treated with antibiotic alone. Some bacterial cells were removed from the membrane surface when treated with both the antibiotic and antibiotic with current (Figure 5). The total cell counts followed a similar trend for both treatments, although the viable cell population was reduced, indicating that the electrical current was bactericidal but did not remove the biofilm from the surface.

Effect of biofilm age

Biofilms formed over all three time periods were unaffected by 10 μg ml^{-1} tobramycin in the absence of an electrical current over the 12-h treatment (Figure 6a). With the application of 9 mA cm^{-2} electrical current in addition to 10 μg ml^{-1} tobramycin, the 12-h biofilm population was reduced by nearly 2 log values (Figure 6b). This combination of the electrical current and tobramycin did not affect the older biofilms formed over 24 and 48 h when compared to the starting population (Figure 6b).

Increasing current density

To determine if the bactericidal effect of tobramycin on a 24-h biofilm could be further enhanced by increasing field strengths, comparisons were made for 0, 10 and 25 μg ml^{-1} tobramycin at different levels of current (Figure 7). The 24-h biofilm population was reduced with the increased current and the increased tobramycin concentration (Figure 7). In the absence of antibiotic, there was no significant change in population with increasing current density once the biofilm was established. The population was not significantly decreased by the addition of 10 μg ml^{-1} tobramycin alone, but a combination of electrical current (9 mA cm^{-2}) and 10 μg ml^{-1} tobramycin produced a significant decrease in viable cells when compared to the untreated biofilm. This significant reduction in cells did not continue from 9 to 15 and 20 mA cm^{-2} suggesting that the bioelectric effect was optimised between 0 and 9 mA cm^{-2}. At 10 μg ml^{-1} tobramycin, an approximately linear relationship was observed between the viable counts over the range of currents studied. Although there are two values not lying close to the straight line included in the calculation, a reasonable fit of $r^2 = 0.762$ and a negative slope were obtained with a significant correlation ($P = 0.05$) between the viable cell counts and the electrical current. At 25 μg ml^{-1} tobramycin alone, there was an initial decrease in viable cell numbers from the starting biofilm population and this was further enhanced by application of 15 and 20 mA cm^{-2} but not with 9 mA cm^{-2}. Similarly, there is a significant correlation ($P = 0.05$) between viable counts and current densities for the 25 μg ml^{-1} tobramycin.

Discussion

P. aeruginosa infections associated with medical devices, although not as common as *E. coli* or infections caused by

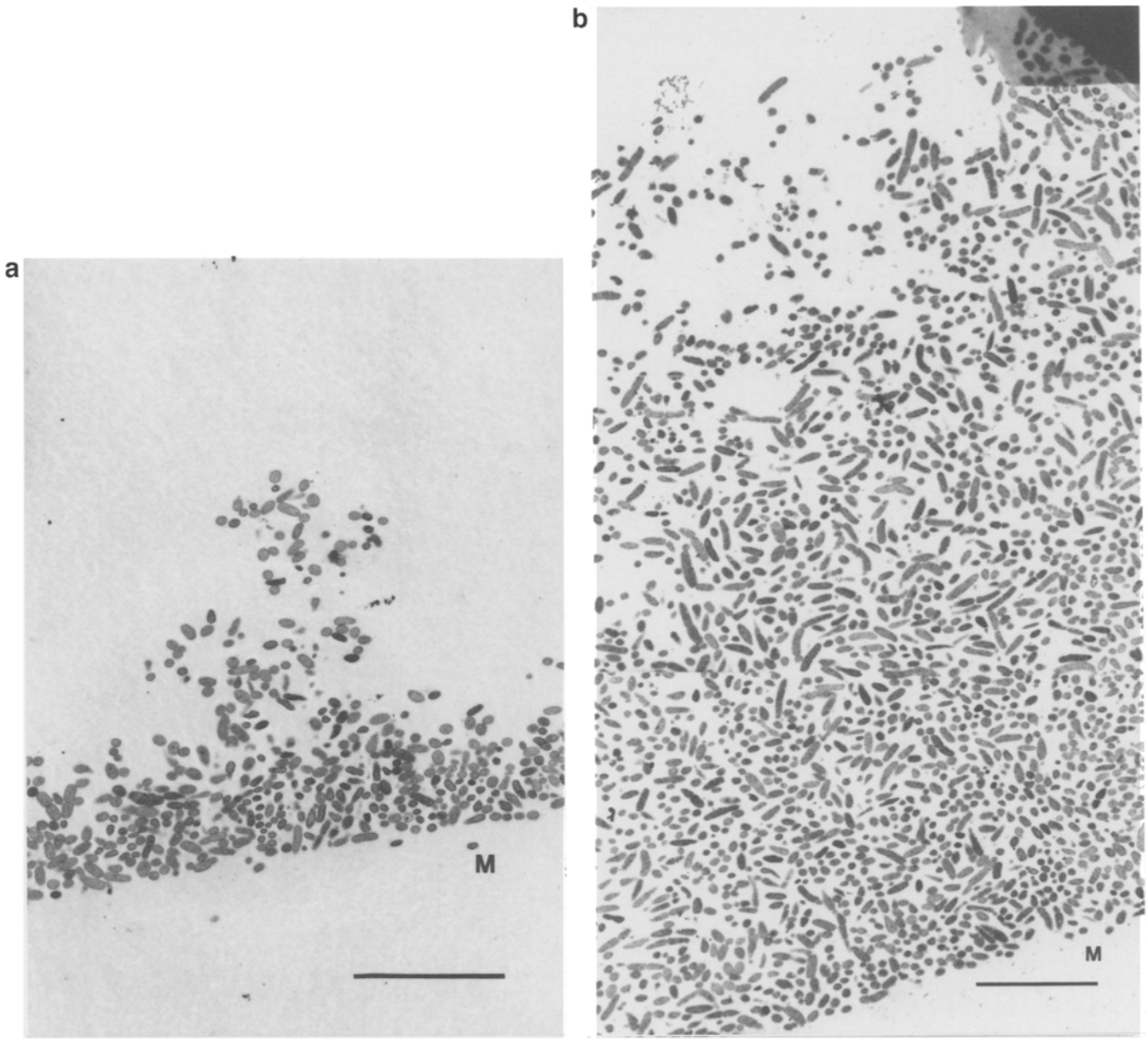

Figure 4 Transmission electron micrograph of a transection of a 48-h *P. aeruginosa* biofilm in the (a) absence and (b) presence of 9 mA cm^{-2} current density. The membrane (M) was present at the base of each micrograph. Bar = 10 μm

Table 1 The effect of tobramycin on planktonic *Pseudomonas aeruginosa*

Tobramycin ($\mu g\ ml^{-1}$)	Viable counts, log ($CFU\ ml^{-1}$)	
	12 h[a]	24 h[a]
0	7.00	7.36
5	4.77	3.48
10	3.90	2.85
25	<2.00[b]	<2.00[b]
50	<2.00[b]	<2.00[b]

[a]Viable counts after 12 or 24 h of exposure to the antibiotic
[b]The detection limit for viable cell counts is 10^2

Gram-positive bacteria, are far more serious when acquired. *P. aeruginosa* biofilms tend to be difficult to treat with conventional antibiotic therapy and therefore new strategies for their control are continually under investigation. Furthermore, *P. aeruginosa* biofilms have been models for biofilm research and antibiotic testing, hence are an ideal choice for our investigation.

The electrical colonisation cell was designed to develop biofilms away from electrodes. This allows separate investigations of the effect of the electrical current and the electrode chemistry (Figure 1). The electrodes are a source of both electrochemical and physical activities which could potentially affect the biofilms formed on their surface. Such activities would include the formation of gas bubbles (H_2 and O_2) at the electrode surface which could dislodge the biofilms from the substratum. Although this mechanical

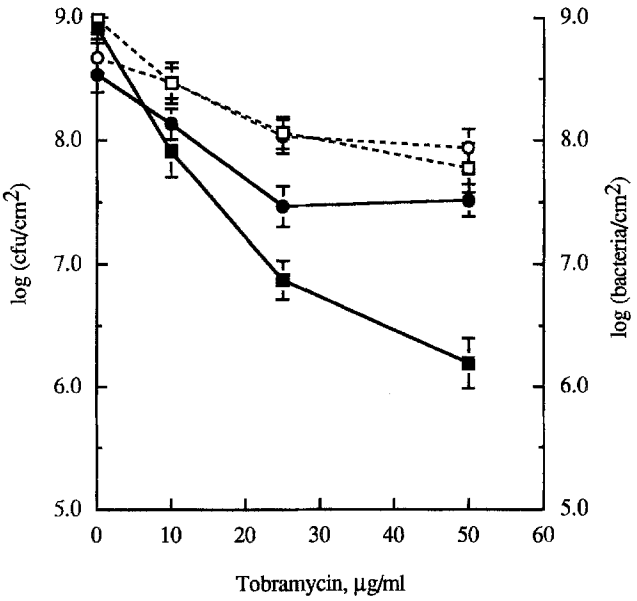

Figure 5 The effect of tobramycin on a 48-h *P. aeruginosa* biofilm in the absence (●, ○) and presence (■, □) of 9 mA cm^{-2} electrical current. The viable counts (●, ■) were compared with the total bacterial counts (○, □) remaining on the surface. The data are from a minimum of three experiments and S. E. bars are shown

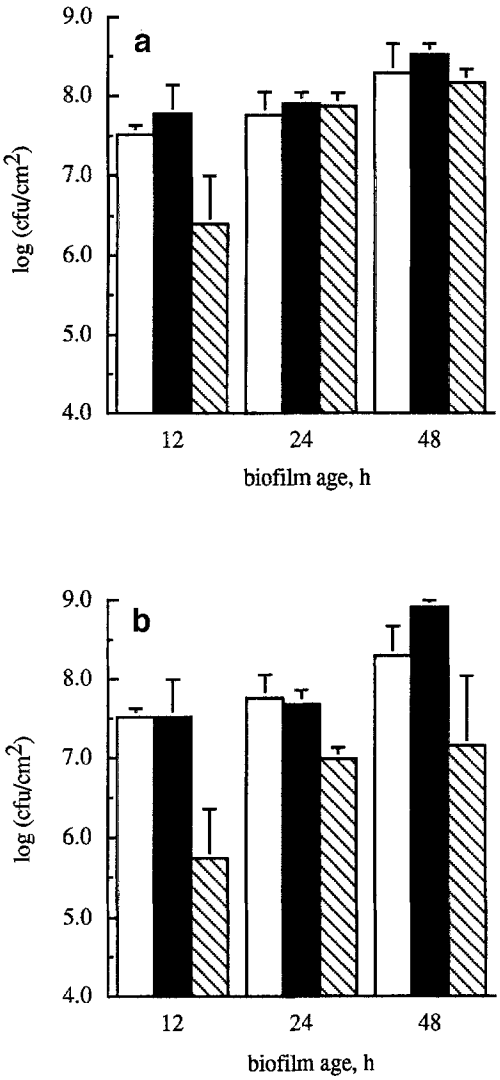

Figure 6 The effect of tobramycin on *P. aeruginosa* biofilms formed over different lengths of time in the (a) absence and (b) presence of 9 mA cm^{-2} current. The graph shows the initial viable biofilm population present at 12, 24 and 48 h followed by the viable population after a further 12-h treatment with 0 or 10 $\mu g\ ml^{-1}$ tobramycin. The data are from a minimum of three experiments and S. E. bars are shown. □ biofilm; ■ 0 $\mu g\ ml^{-1}$; ▨ 10 $\mu g\ ml^{-1}$

disruption is beneficial as it may help dislodge the biofilm, it does not aid our understanding of how the electrical current alone enhances the activity of antibiotics and biocides. The ECC allows us to study the contribution of the electric field alone on the enhancement of antimicrobial efficacy. It was designed to provide an electrical field through the vertical axes of a biofilm (from the top to the bottom of the biofilm) while Costerton *et al* [7] designed a device which provided a current along the horizontal axes of the biofilm (from one side of the biofilm to the other side). At low electric fields, this configuration may contribute to the differences between the results from the two designs.

To determine the effect of antibiotic and electric current on biofilms, factors that could produce lethal effects on bacteria in the absence of an antimicrobial agent were removed from the system. Davis *et al* [11] reported that chloride-containing biocides are generated by passing an electrical current through media which include chloride substances such as NaCl, $CaCl_2$ and NH_4Cl. In the absence of any antibiotics or biocides, the medium containing the chloride compounds had the highest antimicrobial effects in the presence of an electrical current [10]. To avoid the possibility of iontophoresis, chloride-containing compounds were eliminated from the medium in this study.

The MIC and MBC of tobramycin for the *P. aeruginosa* strain used in this study was sufficiently low to be regarded as indicating that the strain is susceptible to the antibiotic. When inoculum size was increased from 10^5 to 10^7 CFU ml^{-1} and a defined medium was used, the bactericidal effect of the antibiotic changed [17]. Firstly, increasing inoculation size increases the concentration of the antibiotic required for a bactericidal effect [5]. Although a higher concentration of tobramycin was required than indicated by the MBC to kill the planktonic bacteria, the concentration was still insufficient to kill the biofilm cells (Table 1). Secondly, by not shaking the culture during incubation it is likely to be oxygen-limited and slow growing, resulting in a reduced antimicrobial activity [5,13,17]. We have confirmed previous studies comparing planktonic and biofilm bacteria, which showed that the survival of viable planktonic cells was decreased below the detection level at 25 and 50 $\mu g\ ml^{-1}$ of antibiotic, while these concentrations were not sufficient to kill the bacteria within the biofilm (Table 1, Figure 5).

A suggested mechanism for the bioelectric effect is that the current drives charged molecules and ions into the biofilm matrix, thus increasing mass transfer [7,10,36]. Similarly, the electrical current may drive the antibiotic into the cell through the membrane [6]. Even though the antibiotic may be moved into the cell and to the target site much more quickly in potentially lethal concentrations, it is still dependent on the rate of growth and metabolism of

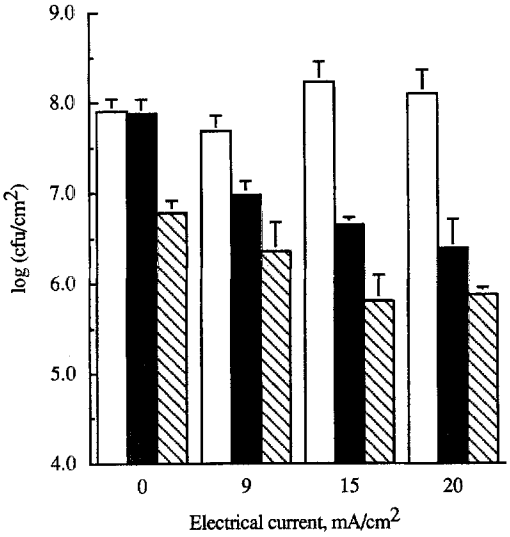

Figure 7 The effect of increasing electrical current on a 24-h *P. aeruginosa* biofilm at 0, 10 and 25 μg ml^{-1} tobramycin. The data are from a minimum of three experiments and S. E. bars are shown. □ 0 μg ml^{-1}; ■ 10 μg ml^{-1}; ▨ 25 μg ml^{-1}

microorganism for its antimicrobial activity [41,45]. Tobramycin is bactericidal by binding to ribosomes and affecting protein biosynthesis. By increasing the growth rate of the microorganism the bactericidal rate may be increased [16].

Previous work has shown greater killing of bacteria by the combination of an electrical current and antibiotic and biocides [3,7]. Biocides are not generally dependent on the growth rate and activity of the bacterium, but on contact time for the chemical to act. Certain systemically used antibiotics more specifically interact with the distinct physiology of prokaryotic cells making them dependent on the metabolic activity of the cell [15]. Consequently, bactericidal effects of these antibiotics are not only related to the concentration but to the time the bacteria are exposed to the antibiotic. Costerton *et al* [7] treated the biofilms for 24 h and longer to observe the bioelectric effect. In the present experiments, treating biofilms with tobramycin for 12 h may not allow sufficient time for the organism to respond to the same degree as Costerton *et al* [7] have shown.

Anwar and Costerton [1] demonstrated that the age of a biofilm or the length of time allowed for its formation influences its susceptibility to antibiotics. We observed that antibiotics alone did not significantly reduce biofilm population formed over 12, 24 and 48 h but with the application of an electrical current, the 12-h biofilms were significantly reduced with 10 μg ml^{-1} tobramycin (Figure 6). This suggests that the biofilm age and activity are again limiting factors in antibiotic effectiveness.

In theory, by increasing the electrical current, the charged molecules and antibiotics might be transported more quickly to the target site and thus increase cell death. Our results have shown that increasing the current from 9 to 15 or 20 mA cm^{-2} at 10 μg ml^{-1} of tobramycin does not significantly increase the bactericidal effect as might be expected. The viable counts were reduced linearly but not proportionately with increasing current in the presence of tobramycin. Rather, the electrical effect was optimised between 0 and 9 mA cm^{-2}. This leads us to conclude that there must be another limiting factor for the antimicrobial activity of tobramycin. The electrical current may transport nutrients and ions to the innermost cells in the same way it may electrophoretically transport antibiotics into the biofilm. This is supported by the comparison between biofilms formed in the presence and absence of an electrical current. Although population numbers are not significantly different (Figure 2), the electron micrographs show substantial differences (Figures 3 and 4). Gas production at the electrodes increases the dissolved oxygen in the aqueous phase with the increase in current over the range studied (data not shown). Whether this is able to reach the depths of the biofilm is not yet known, but the cells at the surface would have more oxygen available for growth. This in turn would increase metabolic activity and account for some of the bactericidal effects observed by the combined antibiotic and electrical current treatment. Both in the presence and absence of 9 mA cm^{-2} current, the transmission electron micrographs show apparently healthy cells with no indication of membrane damage. The electrical current therefore does not appear to damage the cells and leave them more susceptible to the antibiotic; indeed the electrically-treated cells appeared larger than the untreated and this may also indicate increased metabolic activity (Figure 3).

This paper reports that a bioelectric effect was observed when a biofilm formed between two electrodes was treated with a combination of tobramycin and an electrical current, although the effect of electrical current was lower than previously reported. The absence of chloride-containing compounds in the medium may have contributed to the reduced efficacy of the combination of current and antibiotic. In conclusion, we suggest that transport of the antibiotics to the bacteria is only part of the bactericidal process. The result of the bioelectric effect is also dependent on the metabolic activity of the cells within the biofilm.

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