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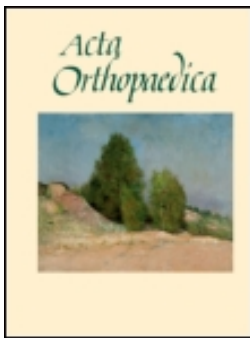
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Pseudomonas aeruginosa biofilm formation and slime excretion on antibiotic-loaded bone cement

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Background Infection is an infrequent but serious complication of prosthetic joint surgery. These infections will usually not clear until the implant is removed and re-implantation has a high failure rate, especially when *Pseudomonas aeruginosa* is involved.

Material and methods We examined *Pseudomonas aeruginosa* biofilm formation on plain and gentamicin-loaded bone cement with confocal scanning laser microscopy (CSLM). Two different stains were applied in order to visualize and quantify the distribution of bacterial cells and extracellular polymeric substances (slime) from the bone cement surface to the top of the biofilm. Staining with LIVE/DEAD viability stain differentiated between live and dead bacteria within the biofilm, and slime production was evaluated after staining with Calcofluor white.

Results CSLM showed that the biofilm was a nonuniform structure of variable thickness, with differences in local bacterial cell and slime densities. Incorporation of gentamicin in bone cement resulted in a 44% reduction in bacterial viability, while the slime density increased significantly. In addition, conventional plate counting showed the development of small-colony variants on gentamicin-loaded bone cement with a decreased sensitivity for gentamicin (MIC: 8 mg/L), as compared with normal-sized colonies taken from plain and gentamicin-loaded bone cement (MIC: 3 mg/L). The enhanced slime production on antibiotic-loaded bone cement, together with the formation of small-colony variants, resulted in decreased susceptibility to antibiotics—probably concomitant with the onset of persistent and relapsing infections.

Interpretation In the clinical situation, our findings help to explain the frequent re-implantation failure of joint replacements infected with *P. aeruginosa* when the procedure has been performed using antibiotic-loaded bone cement.

■

Several studies have shown that local administration of antibiotics through antibiotic-loaded bone cement results in successful and cost-effective prevention of infections in total joint arthroplasty (Josefsson and Kolmert 1993, Espehaug et al. 1997). However, several in vitro studies have shown that bacteria are able to adhere and grow on antibiotic-loaded cement surfaces, despite the high local concentrations of the antibacterial substance (Chang and Merritt 1992, Oga et al. 1992, Kendall et al. 1996, Van de Belt et al. 2001). After adherence, bacteria encase themselves in a hydrated matrix of polysaccharides and proteins to form a slimy layer known as a biofilm (Costerton et al. 1999). Slime production is usually triggered by adherence to surfaces but some bacterial strains, particularly *Pseudomonas aeruginosa,* are notorious producers of slime. Slime formation promotes intercellular adhesion, captures nutrients, and protects bacteria against the deleterious effects of antibiotics (Donlan and Costerton 2002). It has been reported that *P. aeruginosa* cells in biofilms are far less sensitive to antibiotic treatment than their non-attached individual planktonic counterparts (Nickel

et al. 1985). The protective mechanisms in biofilms turn out to be distinct from those that are responsible for conventional antibiotic resistance. In biofilms, poor antibiotic penetration, slow growth, and the formation of more persistent cells have been hypothesized to constitute a multilayered defense (Costerton et al. 1999). Reduced antibiotic susceptibility contributes to the persistence of biofilm infections, such as those associated with total joint replacements. In general, biofilm infections will not clear until the implant has been removed.

Re-implantation after removal of an infected joint replacement has a high failure rate, especially when Gram-negative organisms are involved (*P. aeruginosa* having the worst prognosis) (Cherney and Amstutz 1983). The aim of this study was to evaluate the in-vitro formation, viability and slime excretion of *P. aeruginosa* biofilms on antibiotic-loaded and plain bone cement. Biofilm formation and viability were assessed by conventional plate counting and confocal scanning laser microscopy (CSLM), while slime production was demonstrated using CSLM after staining of the biofilm with Calcofluor white.

Materials and methods

Cement disc preparation

Commercially available Palacos R bone cement (Schering-Plough, Utrecht, the Netherlands) was used with and without 1.25% (w/w) gentamicin base. Cements were prepared by mixing the powdered polymethylmethacrylate with the liquid monomer in a bowl with a spatula. Manual mixing was done according to the manufacturer's instructions, and resulted in doughy cement. This was spread over a polytetrafluoroethylene mold (200 × 40 × 3.2 mm), containing holes of 6 mm diameter. This filled mold was pressed manually between two glass plates and covered with copier overhead film (Océ, MC 110, 's-Hertogenbosch, the Netherlands) to facilitate removal after hardening, up to the time specified for final hardening. After 24 h, the cement discs were pulled out of the mold, and stored under dark conditions at room temperature until further use. The total surface area of each disc was 1.2 cm^2 and one disc weighed about 100 mg. All procedures were carried out under sterile conditions.

Bacterial strain, growth conditions and biofilm formation

A gentamicin-susceptible *P. aeruginosa* 5148 strain (MIC value of 2 mg/L according to E-testing) was used. *P. aeruginosa,* an opportunistic human pathogen and ubiquitous environmental bacterium, is capable of forming biofilms on surfaces. The bacterial strain was isolated from a hip prosthesis that had been retrieved after septic loosening. It was aerobically cultured from cryopreservative beads (Bio Trading, Benelux B.V., Mijdrecht, the Netherlands) onto blood agar plates at 37°C for 24 h. One colony from this plate was used to create a preculture in 10 mL Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) under the same incubating conditions. This procedure yielded a mean growth density of 7.8×10^8 CFU/mL culture medium, as determined by enumeration of colony-forming units (CFU) after growth of serial dilutions on TSB agar plates. The preculture (20 μL) was used to inoculate a second culture (10 mL) in test tubes together with cement samples of plain or gentamicin-loaded Palacos R. After 24 and 48 h, the cement discs were placed in fresh TSB (10 mL), to provide new nutrients.

Biofilm evaluation with conventional plate counting

The cement discs were removed from the test tubes after 72 h, put into 2 mL of 0.9% NaCl, vortexed for 10 sec and finally sonicated for 60 sec before microbiological evaluation. Serial dilutions were poured onto TSB agar plates. After incubation for 24 h, the number of CFU on each cement disc was determined and expressed relative to the surface area of the bone cement (CFU/cm^2). The experiments were carried out in triplicate.

MIC values of the colonies recovered with conventional plate counting were determined in duplicate using an E-test (AB Biodisk, Dälvagen, Sweden).

Biofilm evaluation with CSLM

Biofilm formation was visualized by CSLM after staining the discs for 30 min in the dark at room temperature with the LIVE/DEAD BacLight viability stain (Molecular Probes Europe BV, Leiden, the Netherlands) containing SYTO 9 dye (3.34 mM) and propidium iodide (20 mM). Besides the

Slime production and viability of the P. aeruginosa strain as estimated using CSLM, together with bacterial growth and MIC values resulting from conventional plate counting. The bacterial growth results are given as mean (standard deviation) from three separate experiments

Palacos G bone cement	Slime production	Viability bacteria (%)	Bacterial growth (CFU/cm^2)	MIC value (mg/L)
Plain	+	91	3.24×10^7 (0.93)	3
Gentamicin-loaded	++	47	2.06×10^7 (1.02)	8

intact biofilm, scrapings of the cement discs were also streaked over microscope slides and stained. Confocal images were collected using a Leica TCS-SP2 confocal scanning laser microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with beam path settings for FITC- and TRITC-like labels. Stacks of about 20 images were collected using a 40× objective lens. The stain differentiates live from dead bacteria by staining living bacteria fluorescent green and the dead ones fluorescent red, while the background remains virtually non-fluorescent. Subsequently, the ratio of green bacteria to the total (red and green) bacteria was calculated and expressed as a percentage of viable cells. In each sample, two representative sites were chosen for visualization. To compare the images, we kept the laser power and pinhole settings constant. The counting of stained scrapings from the cement discs was carried out in triplicate, for quantitative analysis of bacterial viability.

Evaluation of slime with CSLM

Calcofluor white, a polysaccharide-binding dye, has been used to stain the extracellular matrix of biofilms formed by bacteria (Stewart et al. 1995). Thus, to determine whether the adhered structures of *P. aeruginosa* were encased in a polysaccharide matrix (slime), the biofilm was stained with Calcofluor white (50 mM). Staining was done in duplicate for 15 min in the dark at room temperature, and the slime production was then observed by CSLM.

Results

The biofilm evaluation results obtained with conventional plate counting and with CLSM are sum-

marized in the Table. CSLM clearly indicated enhanced slime formation in biofilms formed on gentamicin-loaded bone cement as compared with biofilms on plain bone cement. Note that with CSLM, a reduced bacterial viability (%) was observed on gentamicin-loaded cement, which is in line with the reduced CFU/cm^2 counts by plating (although of course conventional plate counting fully disregards dead organisms, unlike CSLM). A noticeable increase in the MIC value of organisms after growth on gentamicin-loaded cement could be seen.

Discussion

Biofilm evaluation with conventional plate counting

Interestingly, standard microbiological plate counting yielded two macroscopically different colonies on the gentamicin-loaded bone cement discs. Some of the colonies were of normal size, while other colonies (approximately 60% of the total) were smaller with somewhat rough edges. Drenkard and Ausubel (2002) also found these small *P. aeruginosa* colonies with rough edges in cystic fibrosis patients and called them rough small-colony variants. The term "small-colony variant" (SCV) refers to a phenomenon whereby certain variants of bacteria grow slowly on routine media and yield unexpectedly small colonies in comparison to the normal-growing parental strains; it does not imply genetic reversion (Roggenkamp et al. 1998). Drenkard and Ausubel (2002) found that some variants of *P. aeruginosa* appear to be naturally resistant to antibiotic therapy and isolation of SCVs is possible in an in-vitro kinetic model after exposure to antibiotics (Langford et al. 1989).

The numbers of colony-forming units harvested from the plain and gentamicin-loaded cement discs

were 3.2×10^7 and 2.0×10^7 CFU/cm^2, respectively, indicating a reduction in bacterial growth of approximately 36% due to the incorporation of gentamicin in the cement. The normal-sized colonies found on plain and gentamicin-loaded bone cement had an MIC value of 3 mg/L, while the SCV(s) found on the gentamicin-loaded bone cement had an MIC value of 8 mg/L. The rather low reduction in bacterial growth reduction caused by gentamicin-loaded bone cement is possibly due to the formation of SCVs, as reduced sensitivity to antibiotics is a characteristic of SCVs. The MIC values of a broad range of antibiotics for SCVs of *P. aeruginosa* are 2-fold to 8-fold higher than values for normal parental strains (Haussler et al. 1999). Moreover, Chuard et al. found that SCVs show a dramatic reduction in susceptibility once they are attached to a surface and have reached the stationary phase, such that adherent SCV can be almost fully resistant to antibiotics (Chuard et al. 1997).

Biofilm evaluation with CSLM

CSLM has been introduced as a new analytical method that can provide a better understanding of biofilm structure, and has been used extensively in cell biology (Wright et al. 1993). Unlike scanning electron microscopy, sample preparation for CSLM is fast and does not require dehydration of samples. Furthermore, CSLM is non-destructive, enabling analysis of live biological samples even if the sample is thick. By moving the sample in the z-direction, stacks of optical sections can be generated which can be used to localize labeled structures in three dimensions. In addition, the LIVE/DEAD BacLight viability stain can differentiate live from dead bacteria, in terms of membrane integrity. A small number of studies have already demonstrated that CSLM can clearly differentiate live from dead bacteria in biofilms when this stain is used (Auty et al. 2001, Takenaka et al. 2001).

In this study, CSLM in combination with LIVE/DEAD stain showed that *P. aeruginosa* biofilms on plain and gentamicin-loaded bone cement had a non-uniform structure, characterized by variable thickness and different local bacterial densities (see Figure 1 A and B). On gentamicin-loaded bone cement, significant changes in these parameters occurred in the biofilm over distances of 10

µm or less. Though the biofilm was several tens of micrometers thick in places, bacteria-free areas were also observed in the same sample. The structure of the biofilm consisted mainly of a complex organisation involving mushroom-like aggregates, whereas the structure of biofilms on plain bone cement is of a relatively-featureless, flat type. Bacterial viabilities within biofilms on plain and gentamicin-loaded bone cement amounted to 91% and 47%, respectively, corresponding to a 44% reduction in viability due to gentamicin-loading. Consequently, it can be concluded that the reduction in viability observed by CSLM using the LIVE/DEAD stain is in good agreement with the 36% bacterial growth reduction on gentamicin-loaded versus plain bone cement found with conventional plate counting.

CSLM of *P. aeruginosa* biofilms stained with Calcofluor white revealed slime production, both on plain and gentamicin-loaded bone cement discs (see Figure 1 C and D). However, by comparison of Figures 1 C and D, it can be seen that the bacteria on gentamicin-loaded bone cement discs are more heavily encased in a denser slime matrix than bacteria on plain cement discs. Extra slime production by the bacterial strain on gentamicin-loaded bone cement may be a survival mechanism in order to overcome the high local antibiotic concentrations released. The enlarged slime-enclosed biofilm on gentamicin-loaded bone cement results in a reduced supply of oxygen, causing the bacteria in the lower layers of the biofilm to go into a 'dormant' state: the small-colony variants (Bayston 2000). Although on agar plates these SCVs can be distinguished from normal parental strains with larger surface colonies, microscopy has revealed no significant differences in the cell size or morphology of SCVs (Haussler et al. 1999, Looney 2000).

It is difficult to extrapolate our in-vitro results to the clinical situation, but the suggestion can be made that gentamicin-loaded bone cement stimulates slime production in *P. aeruginosa* infections to protect the organisms, thereby decreasing the efficacy of loading the bone cement with antibiotic. This may explain why only 32% of prosthetic joint infections caused by slime-positive organisms are successfully treated with antibiotics, while all infections caused by slime-negative organisms
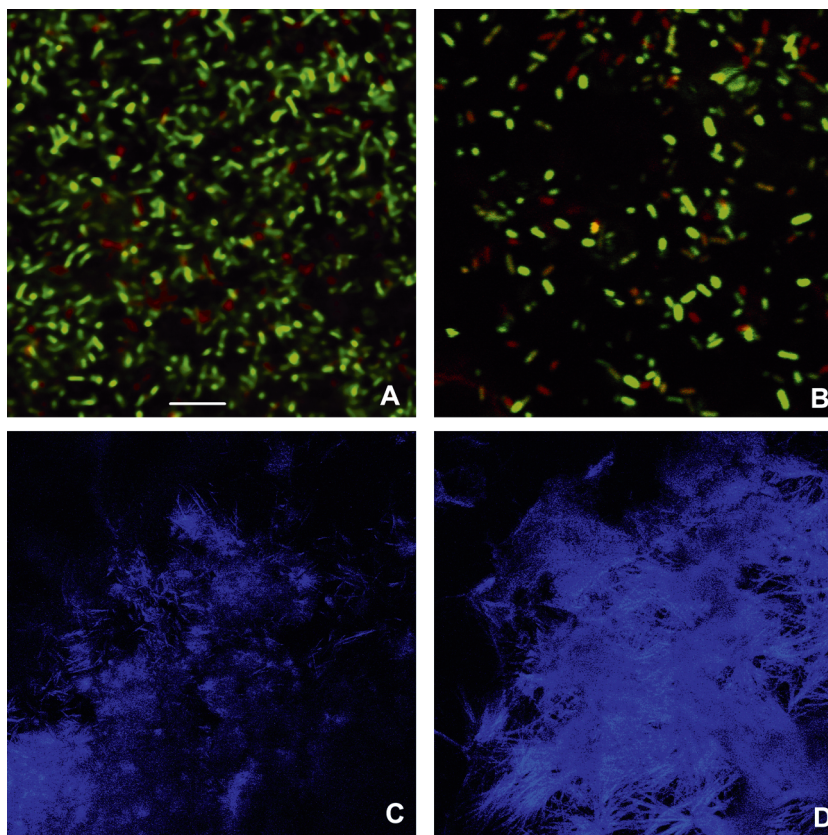
CLSM images of in-vitro grown *P. aeruginosa* biofilm on bone cement discs. Overlay projection (158 × 158 × 20 μm) of the biofilm on plain (A) and gentamicin-loaded bone cement (B), after LIVE/DEAD BacLight staining. The live bacteria are green and the dead bacteria are red. The projection image includes all the slices in an image stack. The bar equals 10 μm. The blue clouds represent concentrations of extracellular polysaccharide substances (slime) created by the bacteria on plain (C) and gentamicin-loaded bone cement (D). An analysis of the XZ-plane revealed that not only was the surface of the structures stained, but there was staining throughout the depth of the structure. The projection image is the middle slice in the image stack of the biofilm.

can be cured by antibiotic treatment (Davenport et al. 1986). Moreover, the antibiotic-loaded bone cement also stimulates the formation of SCVs, with decreased sensitivity to gentamicin, which is the onset to recurrent and antibiotic-resistant infections. The link between SCVs and persistent and relapsing infections has been strengthened over the past decade, especially in patients with chronic osteomyelitis (Von Eiff et al. 2000).

In conclusion, enhanced slime production by *P. aeruginosa* on antibiotic-loaded bone cement, together with the formation of small-colony variants, resulted in decreased susceptibility to antibiotics. In the clinical situation, these observations help to explain the frequent re-implantation failure of joint replacements infected with *P. aeruginosa* even when the procedure has been done with antibiotic-loaded bone cement.

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