Gentamicin release from polymethylmethacrylate bone cements and *Staphylococcus aureus* biofilm formation

Hilbrand van de Belt[1,2], Daniëlle Neut[1,2], Willem Schenk[1], Jim R van Horn[1], Henny C van der Mei[2], Henk J Busscher[2]

Departments of [1]Orthopedic Surgery, University Hospital Groningen, Hanzeplein 1, NL-9713 GZ Groningen, The Netherlands, [2]Biomedical Engineering, University of Groningen, Antonius Deusinglaan 1, NL-9713 AV Groningen, The Netherlands. Tel +31 50 36331–40. Fax –59. Correspondence: Dr. H.J. Busscher
Submitted 99-07-30. Accepted 00-04-26

ABSTRACT – We measured the formation of a *Staphylococcus aureus* biofilm in vitro on unloaded and gentamicin-loaded bone cements (CMW3 and Palacos R) and related the formation to antibiotic release rates. All experiments were done in triplicate. Microbial growth on gentamicin-loaded cements occurred despite the release of antibiotic. Biofilm formation on gentamicin loaded CMW3 bone cement was one fourth to one fifth less than on the unloaded bone cement, while biofilm formation on Palacos R bone cement was not significantly affected by antibiotic loading. More gentamicin was released from CMW3 (79 mg) than from Palacos R (70 mg), but the percentage gentamicin released after one week relative to the total amount incorporated was significantly lower for CMW3 (4.7%) than for Palacos R (8.4%). After one day, subinhibitory concentrations of antibiotics were eluted from the cements. We concluded that antibiotic-loaded bone cement does not necessarily inhibit the formation of an infectious biofilm in vitro.

■

Microorganisms causing infection of a prosthesis are difficult to eradicate due to the formation of a biofilm on the prosthetic surface; the adhering bacteria are embedded in a matrix of secreted, adhesive exopolymers, composed mainly of polysaccharides, "glycocalyx". The resistance of periprosthetic infections to host defense mechanisms and to antimicrobial therapy is largely related to the protective environment of the glycocalyx (Dougherty and Simmons 1982, Costerton et al. 1987, Gristina 1987, Dobbins et al. 1988). Heck et al. (1995) reported that 90% of orthopedic surgeons in the USA used antibiotic-loaded bone cement. However, since the first reports of antibiotic release from polymethylmethacrylate (PMMA) bone cement (Buchholz and Engelbrecht 1970, Wahlig et al. 1972), conflicting reports have been published concerning the elution rate, the amount of antibiotics released and the clinical efficacy.

None of the currently used antibiotic-loaded bone cements release more than 15% of the antibiotic incorporated, and it has been suggested that vacuum mixing even further reduces the release (Kuechle et al. 1991). Little is known about the actual antibiotic concentration around an implant. 88% of the patients with an infected total hip, in which gentamicin-loaded bone cement was used primarily, harbored at least one staphylococcal strain resistant against gentamicin (Hope et al. 1989). Thus, there is a risk that the long time low concentration of gentamicin around an implant may induce antibiotic-resistant strains (Van de Belt et al. 1999).

Our aim was to study *Staphylococcus aureus* biofilm formation on two types of unloaded and gentamicin-loaded bone cements (CMW3 and Palacos R) and the kinetics of gentamicin release.

Material and methods

Bone cement

Polymethylmethacrylate (PMMA) CMW3 bone cement (DePuy International Ltd., Cornford road, Blackpool, Lancashire FY4 4QQ, England) and Palacos R (Schering-Plough, Maarssen, The Netherlands) was used with or without a 2.5 and 1.25 w/w% loading of gentamicin (or 4.2 w/w% and 2.0 w/w% gentamicin sulfate), respectively. The cement was mixed in a bowl with a spatula during 60 s. The cement was then poured in a polytetrafluoroethylene mold (200 × 40 × 3.2 mm), containing 6 mm diameter holes. This mold was pressed between 2 glass plates for 25 min. After that, the samples were pulled out of the mold, and stored under dark, sterile conditions at room temperature. The surface area of each disc was 1.17 cm^2 and one disc weighed 100 mg.

Bacterial strains, growth condition and biofilm formation

Staphylococcus aureus ATCC 12600 was grown for 24 h at 37 °C in ambient air in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, Great Britain, pH 7.3). This preculture was used to inoculate a second culture (400 mL) which was grown overnight and used to inoculate the modified Robbins device (MRD, McCoy et al. 1981). This device is a hollow rectangular cube (620 × 20 × 20 mm) with 10 holes into which cement discs can be plugged. These discs were glued onto the sample holders with silicone paste under aseptic conditions.

The MRD was inoculated with the overnight culture of *S. aureus* and left for 5 h. Thereafter, fluid flowed through the device for 72 h with TSB growth medium at a rate of 63mL/h. 3 runs were performed and in each, newly prepared cement discs were placed in the device. Plain cement discs were placed in positions 1–5 and gentamicin-loaded cement discs were placed in positions 6–10. The temperature of the MRD was kept at about 37 °C during the experiment.

Biofilm evaluation

The cement discs were removed from the MRD, put in 2 mL of Reduced Transport Fluid, RTF (NaCl 0.9 g/L, $(NH_4)_2\ SO_4$ 0.9 g/L, KH_2PO_4 0.45 g/L, Mg_2SO_4 0.19g/L, K_2HPO_4 0.45 g/L, Na_2EDTA 0.37 g/L, L-Cysteine HCl 0.2 g/L, pH 6.8), vortexed for 10 s and finally sonicated for 60 s for microbiological evaluation. Serial dilutions were streaked onto TSB agar plates. After incubation overnight, the number of Colony-Forming Units (CFU) on each cement disc were counted and expressed relative to the surface area of the bone cement (CFU/cm^2). Percentage biofilm formation on antibiotic-loaded bone cement was calculated relative to biofilm formation on the unloaded cements to eliminate biological variations between runs.

For scanning electron microscopic (SEM) evaluation, discs with or without gentamicin were glued on a cryoholder and flushed with distilled water. Cryofixation was performed at –210 °C with nitrogen slush after which the samples were put in the cryo transfer system (CT 1500). Freeze drying was performed at –120 °C for 5 min. The samples were then sputter-coated with gold and palladium (3 nm) and examined at 5.0 kV in a JEOL field emission scanning electron microscope type 630 1F.

Gentamicin release rates

A gentamicin-loaded cement disc was immersed in 10 mL phosphate buffer saline (PBS) (NaCl 8.76 g/L, K_2HPO_4 0.87 g/L, KH_2PO_4 0.68 g/L, pH 7.4) and stirred at 37 °C. At designated sampling intervals (6, 24, 30, 48, 72, 168 h), the disc was removed, a 1 mL sample of the PBS solution was taken, and the disc was placed in fresh 10 mL PBS. In these samples, gentamicin levels were determined using fluorescence polarization immunoassay (Abbott AxSym; Abbott Laboratories, Abbott Park, Illinois), based on competitive-binding (Price and Newman 1991).

Statistics

Data for gentamicin release were evaluated for statistical significance, as described by Matthews et al. (1990) for serial measurements in medical research, by calculating the area under the gentamicin release curves versus time and expressing this area as a summary variable for further statistical analysis, using the Student t-test. Percentage biofilm formation was examined for statistical significance at each point in time and for peak re-

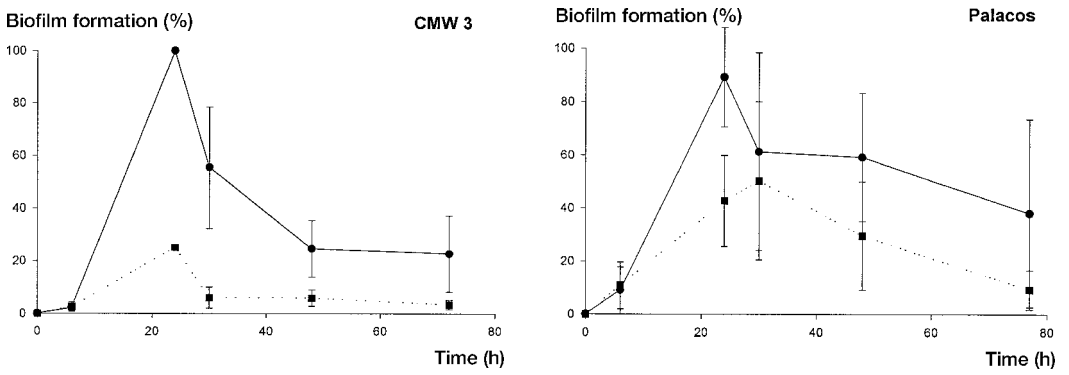

Figure 1. The numbers of infectious *S. aureus* (CFU/cm^2) isolated from unloaded (●) and gentamicin-loaded CMW3 (left graph) and Palacos R (right graph) bone cement (■) as a function of time, expressed relative to the maximal number of CFU/cm^2 isolated from an unloaded cement disc per run. Results presented are averages of 3 different experimental runs with bars indicating the SD.

sponse (Matthews et al. 1990), also using the Student t-test. Three study units were used for all experiments and a 95% ($p < 0.05$) confidence level was adopted for statistical significance.

Results

Biofilm evaluation

Figure 1 summarizes the number of CFU/cm^2 on CMW3 and Palacos R bone cement discs as a function of time as expressed relative to the maximum number of CFU/cm^2 recovered from an unloaded cement disc during a run. Biofilm formation was always maximal on unloaded bone cements after 24 h and did not differ significantly between unloaded CMW3 and Palacos R bone cements, with an average maximum over all experiments carried out of 3.4 (SD 2.6) × 10^8 CFU/cm^2 due to biological variations. By expressing all results relative to this maximum number, however, the biological variations between runs were eliminated.

Fewer ($p < 0.05$) infectious organisms were isolated from biofilms on the antibiotic-loaded CMW3 bone cement than from biofilms on the unloaded bone cements at all times during the study except after 6 h (i.e., 24, 30, 48, and 72 h), including the peak summary measure after 24 h. Only immediately after the start of an experiment (i.e., after 6 h), was there no significant difference in biofilm formation on unloaded and gentamicin-loaded CMW3 bone cements. Gentamicin-loaded Palacos R bone cement, on the other hand, did not acquire a significantly lower number of infectious microorganisms than unloaded Palacos bone cement during the study, except after 24 h.

Figure 2 shows scanning electron microscopic features of the biofilms on both types of CMW3 bone cements and confirms the quantitative biofilm evaluation described above. Fewer *S. aureus* are found adhering to the gentamicin-loaded bone cement than on the unloaded bone cement. Furthermore, on the unloaded bone cement, the adhering organisms appear to be covered with a slimy film, that also forms patches on the cement, while on the gentamicin-loaded bone cement such a slimy film is absent and the organisms seemed to be connected by thin slimy threads.

Gentamicin release rates

Figure 3 shows the gentamicin release as a function of time. During the first hours, a high amount of gentamicin was released, which subsequently decreased rapidly. From Figure 3 it can be calculated that only 4.7 (SD 0.2)%, equivalent to 79 (SD 3.6) μg, of the total amount of gentamicin incorporated in the CMW3 and 8.4 (SD 0.4)% (70, SD 3.2) μg of the total amount in Palacos R, bone cement disc was released, while most of this gentamicin (for CMW3 79, SD 2.1% and for Palacos R 66, SD 2.8%) was actually released during the first 6 h after exposure to an aqueous fluid. The total amounts of gentamicin released, measured according to Matthews et al. (1990), were significantly higher for CMW3 than for Palacos R.

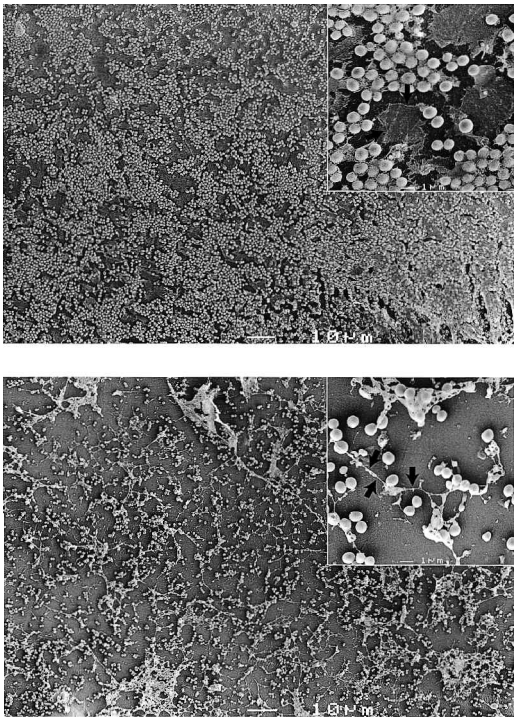

Figure 2. Cryo scanning electron micrographs of *S. aureus* on unloaded (top) and gentamicin-loaded (bottom) CMW3 bone cement showing the difference in adherence on both type of discs. Slimy films and threads on the antibiotic-loaded cement are indicated by arrows. The bar equals 10 μm for low magnification micrograph, and 1 μm for the insert.

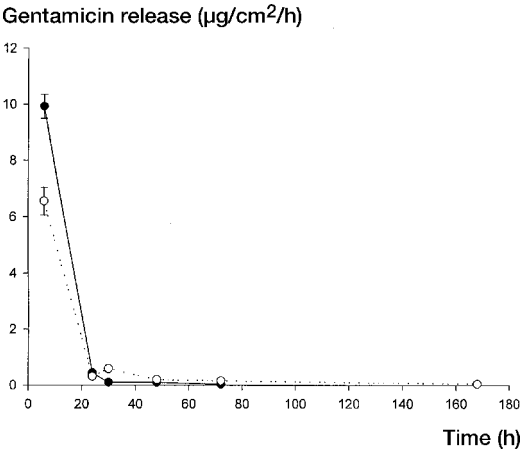

Figure 3. Gentamicin release rates of CMW3 (●) and Palacos R (●) bone cement as a function of time during exposure to phosphate-buffered saline. Results are averages of three experimental runs with bars indicating SD.

Discussion

CMW3 bone cement used in this study is a low-viscosity cement. The release of gentamicin from low-viscosity cements is only half of that from high-viscosity bone cements in vitro, but in vivo studies have shown the opposite (Lindberg et al. 1991). Palacos R was reported to elute between 3% and 7% of the total amount of gentamicin incorporated (Picknell et al. 1979, Chohfi et al. 1998), which is similar to our results, while CMW3 released 5%. In a clinical study, Palacos R was found to release 13% of the gentamicin after 16 days (Törholm et al. 1983). The viscosity influences the porosity and therefore the antibiotic release, and more porous cements may have an increased area for elution. Other variables influencing the release of antibiotics from bone cements are the type and concentration of the antibiotic as well as possible combinations (Baker and Greenham 1988).

Ideally, an antibiotic-loaded bone cement should release antibiotic during a short period, after which the release should stop, to prevent subinhibitory concentrations thereafter so as not to induce bacterial resistance (Hope et al 1989). Since the pharmacokinetics of our cements did not satisfy these requirements, bone cements which more efficiently release antibiotics for a limited time only should be developed (Van de Belt et al. 1999).

Bacterial adhesion, the primary step in the formation of an infectious biofilm, to PMMA was similar after 30 min on unloaded and gentamicin-loaded PMMA, but as soon as the adhering bacteria were allowed to grow for 24 h, fewer *S. aureus* and *Proteus mirabilis* were found on the antibiotic-loaded PMMA (Chang and Merritt 1992). Viable bacteria were also isolated from tobramycin- and vancomycin-impregnated cement discs after 96 h immersion in a bacterial suspension in broth (Kendall et al. 1996). In a "flow colonization chamber", bacterial adhesion was less on tobramycin-loaded cement discs than on unloaded discs, but tobramycin release did not kill the adhering bacteria. (Oga et al. 1992). Arizona et al. (1992) reported increased gentamicin resistance of bacteria after adhesion to plain polymethylmethacrylate, but their results should probably not

be considered as gentamicin resistance, but rather as confirming the generally greater antibiotic resistance of biofilm bacteria, as compared to planktonic ones.

Our study shows that the formation of an infectious biofilm on bone cements follows a similar temporal pattern on unloaded and gentamicin-loaded bone cement, with the maximum number of viable bacteria in the biofilm after 24 h. Once the biofilm has reached a certain thickness, the continuous flow of medium over the cement surface causes detachment of parts of the biofilm, finally resulting in a steady state biofilm which can be quantified after several days. To what extent this process occurs in the interface between bone and cement is uncertain. Bacterial growth along this interface in vivo is slower due to less nutrition and there is no detachment due to flow. Furthermore, even though the amount of gentamicin released is small, the local concentration in the narrow interface might be higher than in the Robbins device. Nevertheless, we observed gentamicin release rates similar to those found in vivo, which allowed adhesion and growth of a staphylococcal strain on the gentamicin-loaded bone cement.

Arizona T, Oga M, Sugioka Y. Increased resistance of bacteria after adherence to polymethylmethacrylate. An in vitro study. Acta Orthop Scand 1992; 63: 661-4.

Baker A S, Greenham LW. Release of gentamicin from acrylic bone cement. J Bone and Joint Surg (Am) 1988; 70: 1551-7.

Buchholz H W, Engelbrecht H. Uber die depotwirkung einiger antibiotica bei vermischung mit dem kunstharz Palacos. Chirurg 1970; 41: 511-5.

Chang C C, Merritt K. Microbial adherence on polymethyl-methacrylate (PMMA) surfaces. J Biomed Mat Res 1992; 26: 197-207.

Chohfi M, Langlais F, Fouraster J, Minet J, Thomazeau H, Cormier M. Pharmacokinetics, uses, and limitations of vancomycin-loaded bone cement. Inter Orthop 1998; 22: 171-7.

Costerton J W, Cheng K J, Geesey G G, Ladd T I, Nickel J C, Dasgupta M, Marrie T J. Bacterial biofilms in nature and disease. Ann Rev Microbiol 1987; 41: 435-64.

Dobbins J J, Seligson D, Raff M J. Bacteria colonization of orthopedic fixation devices in the absence of clinical infection. J Infect Dis 1988; 158: 203-5.

Dougherty S H, Simmons R L. Infections in bionic man: The pathobiology of infections in prosthetic devices-Part I. Curr Prob Surg 1982; 19: 221-64.

Gristina A G. Biomaterial-centered infection; microbial adhesion versus tissue integration. Science 1987; 237: 1588-95.

Heck D, Rosenberg A, Schink-Ascani M, Garbus S, Kiewitt T. Use of antibiotic-impregnated cement during hip and knee arthroplasty in the United States. J Arthrop 1995; 10: 470-5.

Hope P G, Kristinsson K G, Norman P, Elson R A. Deep infection of cemented total hip arthroplasties caused by coagulase negative staphylococci. J Bone Joint Surg (Br) 1989; 71: 851-5.

Kendall R W, Duncan C P, Smith J A, Ngui-Yen J H. Persistence of bacteria on antibiotic-loaded acrylic depots. Clin Orthop 1996; 329: 273-80.

Kuechle D K, Landon G C, Musher D M, Noble P C. Elution of vancomycin, daptomycin and amikacin from acrylic bone cement. Clin Orthop 1991; 264: 302-8.

Lindberg L, Önnerfält R, Dingeldein E, Wahlig H. The release of gentamicin after total hip replacement using low- or high-viscosity bone cement. Inter Orthop 1991; 15: 305-9.

Matthews JNS, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medical research. Br Med J 1990; 300: 230-235.

McCoy W F, Bryers J D, Robbins J, Costerton J W. Observations of fouling biofilm formation. Can J Microbiol 1981; 27: 910-7.

Oga M, Arizono T, Sugioka Y. Inhibition of bacterial adherence to tobramycin-impregnated PMMA bone cement. Acta Orthop Scand 1992; 63: 301-4.

Picknell B, Mizen L, Sutherland R. Antibacterial activity of antibiotics in acrylic bone cement. J Bone Joint Surg (Br) 1979; 59: 302-7.

Price C P, Newman D J. Heterogeneous fluoroimmunoassay. In: Principles and practice of immunoassay (Eds. Price C P, Newman D J). Stockton Press, New York 1991; 365-92.

Törholm C, Lidgren L, Kahlmoter G. Total hip joint arthroplasty with gentamicin-impregnated cement. A clinical study of gentamicin excretion kinetics. Clin Orthop 1983; 181: 99-106.

Van de Belt H, Neut D, Van Horn J R, Van der Mei H C, Schenk W, Busscher H J. Antibiotic resistance–to treat or not to treat? Nature Medicine 1999; 5: 358-9.

Wahlig H, Schliep H J, Bergmann R, Hameister W, Grieben A. Über die Freisentzung von Gentamicin aus Polymethylmethacrylat. II. Expirimentelle Untersuchungen in vitro. Chir 1972; 331: 193-212.