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Release of antimicrobial peptide Dhvar-5 from polymethylmethacrylate beads

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Osteomyelitis is still a major cause of morbidity and remains a difficult complication to treat in orthopaedic surgery. The treatment of choice is a combination of systemic and local antibiotics. The insertion of gentamicin-loaded polymethylmethacrylate (PMMA) beads into the bone results in high local concentrations of gentamicin and low systemic concentrations. However, the effectiveness of this treatment is being hampered by the emergence of antimicrobial resistance. New antimicrobial agents are therefore needed. One new class of promising antibiotics is antimicrobial peptides (AMP). Derived from natural human peptides, these have a low tendency to induce antimicrobial resistance. Dhvar-5 is an antimicrobial peptide based on histatin-5, which is found in human saliva and consists of 14 amino acids. It has demonstrated bactericidal activity *in vitro*. In order to develop a new local treatment using Dhvar-5 for osteomyelitis, we investigated its release from PMMA beads and its antimicrobial activity against a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) before and after release from PMMA beads. Specific amounts of Dhvar-5 were incorporated into PMMA mini beads, containing 120, 600 and 1200 μg of Dhvar-5, respectively. Dhvar-5 was released from the beads in all three groups. Total release from the 120 μg beads was 9 μg per bead after 7 days. However, the release per bead in the 600 and 1200 μg beads was far more, respectively, 416 and 1091 μg over a 28 day period. After release, the Dhvar-5 also retained its antimicrobial activity against MRSA. On the basis of these data we conclude that the amount of Dhvar-5 release from PMMA beads is not proportionate to the amount incorporated; instead, it demonstrated an exponential relationship to the amount of total peptide released. Furthermore, the released peptide remained biologically active against a clinical isolate of MRSA.

Keywords: antimicrobial peptides, Dhvar-5, PMMA beads, release kinetics

Introduction

Mixtures of antibiotics with the artificial resin polymethylmethacrylate (PMMA) and antibiotics have been used for several decades to prevent or to treat orthopaedic infections.[1] These mixtures are used as bone cements in primary total joint arthroplasty to reduce the initial incidence of infections.[2–5] In addition, antibiotic-containing PMMA beads in combination with systemic antibiotics have been used extensively to treat osteomyelitis.[6–10]

PMMA beads containing gentamicin have long been the primary choice for treating osteomyelitis.[10–12] Gentamicin is a broad spectrum antibiotic, which is active against both Gram-positive and Gram-negative bacteria.[13] The exothermic polymerization process of the PMMA does not influence the bioactivity of gentamicin,[8] and the long-term release profiles of gentamicin from PMMA beads have been demonstrated in kinetic release studies.[8] Overall, the use of gentamicin-loaded PMMA beads results in high local bone and soft tissue concentrations and low systemic concen-

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trations, reducing systemic effects.[8] On the other hand, the high systemic dosage of gentamicin needed to reach sufficient tissue penetration would create serious toxic side effects.

However, recent studies have shown an increase in antibiotic-resistant bacteria such as gentamicin-resistant *Staphylococcus aureus* (GRSA) and methicillin-resistant *S. aureus* (MRSA).[14] Prevalence of MRSA in nosocomial infections has exceeded 30% in for example Southern Europe and the USA.[14,15] In addition, amongst a broad variety of pathogens, there is a continuing shift towards antibiotic resistance.[14] Neut *et al.* demonstrated staphylococci on retrieved gentamicin-loaded PMMA beads, raising concern about the efficacy of this treatment option.[16] As the current antibiotic therapy for osteomyelitis is becoming a serious medical problem, there is an urgent need for better antimicrobial agents.

Antimicrobial peptides are a new and promising class of antibiotics, derived from naturally occurring peptides.[17] As part of the innate immune system, these are found on epithelial surfaces, in secretion fluids and in neutrophils, and thus form a first line of host defence.[18] Studies have shown that they have a low tendency to induce resistance because of the evolutionary difficulty in changing bacterial membrane structure.[17,18]

Dhvar-5 is an experimental antimicrobial peptide based on the amino acid composition of histatin-5, which is an antifungal peptide found in human saliva.[19] It has been constructed synthetically of 14 amino acids to create a net positive charge at the C terminus and a hydrophobic N terminus.[20,21] This positive charge is presumed to play a crucial part in its antimicrobial activity;[20] allowing it to disrupt and to penetrate the negatively charged bacterial cell wall; and after cellular entrance it is targeted to intracellular organelles.[22] The Dhvar-5 molecule has both fungicidal and bactericidal activity *in vitro* against among others MRSA.[21,23]

As the use of PMMA beads containing antibiotics is a successful approach for the treatment of osteomyelitis, the present investigation was carried out in order to analyse the release characteristics of the Dhvar-5 antimicrobial peptide from pre-hardened PMMA beads *in vitro*. Local administration of the antimicrobial peptide Dhvar-5 could create an alternative for the treatment of osteomyelitis. However, since the release patterns of antimicrobial peptides from bone cements are not yet known, we investigated the release of the antimicrobial peptide Dhvar-5 from PMMA beads.

Materials and methods

PMMA beads

The Dhvar-5 antimicrobial peptide was commercially synthesized (UCB-Bioproducts, Braine-l'Alleud, Belgium) and was available as a white freeze-dried amorphous powder. Exact amounts of Dhvar-5 were thoroughly mixed with 1 g of cement powder (Osteopal, Biomet Merck, Darmstadt, Germany). Then, 0.5 g of liquid monomer component was added to the cement powder and mixed to a paste. Individual components were cooled to 4°C until the mixing procedure, which was done under sterile conditions. The obtained paste was subjected to a slight vacuum in a syringe for 5 s, and subsequently injected into a custom-made mould. After polymerization around a stainless steel wire (Ø 0.3 mm) for 20 min, a chain of 10 beads could be retrieved (Figure 1), all with the same configuration and weight. The beads were stored at –20°C until the release experiment. Three different amounts of Dhvar-5 were added to the co-polymer cement powder, with the following weight/weight percentages (w/w%) of the dry cement powder: 0.5%, 2.5% and 5.0%. After correction for evaporated monomer, the beads contained 120, 600 or 1200 μg Dhvar-5 antimicrobial peptide, respectively. In addition, a control group of PMMA beads was made without Dhvar-5.

Release experiment and analysis

Individual beads (n = 10 per group) were placed in a 96-well polystyrene microtitre plate. Then 150 μL of distilled H_2O (release medium) was added and the bead fully submerged. The release medium was exchanged at preset times of 30 min, 1, 2 and 4 h, and thereafter every 24 h continuing for 28 days. During the interim periods, the 96-well plate was sealed to prevent evaporation of release medium.

The frequent exchange of the release medium in the initial stages of the experiment prevents the concentration of peptide rising to a high level, which could reduce the concentration gradient between the bead and the elution fluid. In the later stages of the release experiment, the peptide release decreases, which allows for exchange of the release medium every 24 h.

The experiment was carried out at room temperature and under shaking conditions. Then the medium was stored in Eppendorf containers at –20°C until analysis. Also a release experiment was conducted in which the pH was monitored; there was no change in the levels throughout the experiment.

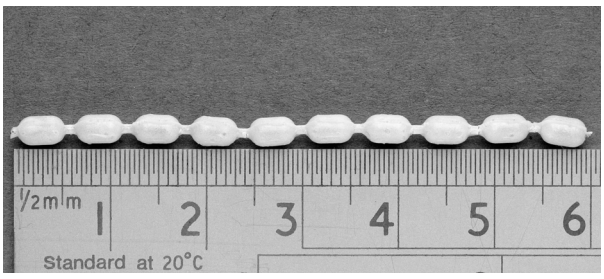

Figure 1. A chain of 10 custom-made PMMA-mini beads as used in this study.

As the Dhvar-5 peptide is a very small peptide of only 14 amino acids, there is no suitable antibody available for its detection. Therefore, we used a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA) to measure the Dhvar-5 concentration in the release medium. This method detects the peptide present in the release medium. Serial dilutions of Dhvar-5 were used as a reference on every extinction measurement. Extinction was measured at 540 nm with a benchmark microplate reader and analysed with Microplate Manager software, version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA). To correct for background extinction, the control group measurements were subtracted from the experimental measurements. The detection limit of the BCA-assay is 5 μg/mL; in preceding experiments, this was verified for the Dhvar-5 peptide. The volume of release medium, 150 μL, enabled a detection limit of 0.75 μg per bead. The accuracy of the assay ranges from 0.4% to 6.7% as calculated from the mean error of the true value, and the precision ranges from 3.8% to 7.0% as calculated from the coefficient of variance.

In a preceding experiment, we investigated the adhesion of the Dhvar-5 peptide to glass, polypropylene and polystyrene. A solution of Dhvar-5 was stored for 24 h in each container and the concentration analysed by capillary zone electrophoresis (CZE). Furthermore, another experiment was carried out in which the released peptide was analysed by means of CZE for control of the integrity of the peptide. Also, the stability of the Dhvar-5 peptide was analysed by heating it to 120°C for 20 min and by storing a stock solution for 26 weeks at 37°C, subsequently an antimicrobial assay (described below) was carried out to evaluate its antimicrobial activity.

Antimicrobial assay

Antimicrobial activity was analysed by a modified killing assay as described by Helmerhorst *et al.*[24] In short, an MRSA clinical isolate was used as the test organism, an overnight culture was washed three times in PBS and diluted to contain ~2×10^8 colony forming units (cfu)/mL. Of this suspension, 50 μL was added to the serial dilution series of Dhvar-5 and release media of day one in a 96-well culture plate to yield a final volume of 200 μL. After 1 h of incubation under agitation at 37°C, 25 μL was diluted 200-fold in PBS and the surviving bacteria determined by plating 25 μL on blood-agar plates. After 24 h of incubation at 37°C, the percentage of killing was determined by the following equation: [1 – (number of viable counts of sample/average number of counts in control samples)] × 100%, and the IC_{50} values were determined as the concentration at which 50% of the MRSA inoculum survived. The IC_{50} values were compared to evaluate the antimicrobial activity of Dhvar-5 after release from the PMMA beads.

Statistical methods

Variables were compared using a *t*-test for independent samples assuming unequal variances and one-way analysis of variance. Differences were considered significant at $P < 0.05$.

Results

Individual beads of the same size and configuration 3 × 5 mm (Figure 1) and weight 33.8 ± 0.2 mg were retrieved. The CZE data demonstrated not only a single peak throughout the release experiment but also no shift in peak position during the experiment (data not shown). Furthermore, the CZE analysis demonstrated no adhesion of Dhvar-5 to glass, polypropylene and polystyrene. The Dhvar-5 peptide demonstrated identical antimicrobial activity after being heated to 120°C for 20 min, and also after storage for 26 weeks at 37°C (data not shown).

The Dhvar-5 release patterns per group of beads are shown in Figures 2 and 3(a). The 120 μg Dhvar-5 bead (n = 5) released 9 ± 4.7 μg per bead, corresponding to 7.5% of the incorporated peptide over a 7 day period. The 120 μg Dhvar-5 beads showed a significantly lower release ($P < 0.00001$) at every measurement compared with both the 600 and 1200 μg Dhvar-5 beads.

The 600 μg Dhvar-5 beads (n = 10) released 416 ± 56 μg over a 28 day period (Figure 3b) resulting in 70% of the incorporated antimicrobial peptide being released. Furthermore, at day 9, 80% of the total release was reached. The 1200 μg Dhvar-5 per bead group (n = 10) released 1091 ± 80 μg Dhvar-5 over a 28 day period (Figure 3b), 80% of the total release was reached at day 8. Furthermore, the 1200 μg Dhvar-5 per bead group released 91% of the added antimicrobial peptide over the same 28 day period. The 1200 μg

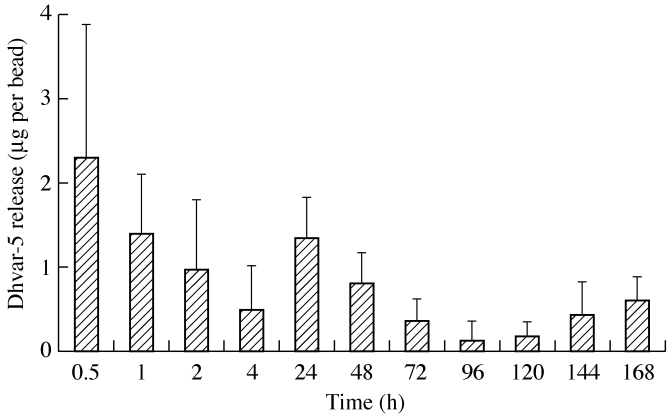

Figure 2. Dhvar-5 release pattern for the 120 μg/bead group (values are a means ± S.D. of five beads).

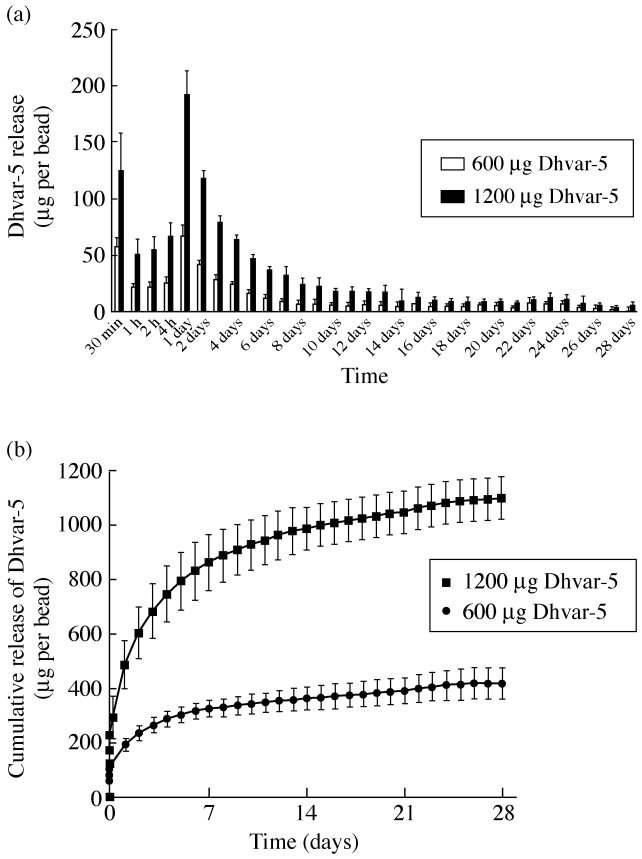

Figure 3. (a) Absolute Dhvar-5 release from the 600 and 1200 µg/bead groups; (b) cumulative release profile computed from the data in (a). The cumulative values of the 1200 µg/bead group were significantly higher than those of the 600 µg/bead group throughout the experiment (values are mean of 10 determinations ± S.D. from two different experiments).

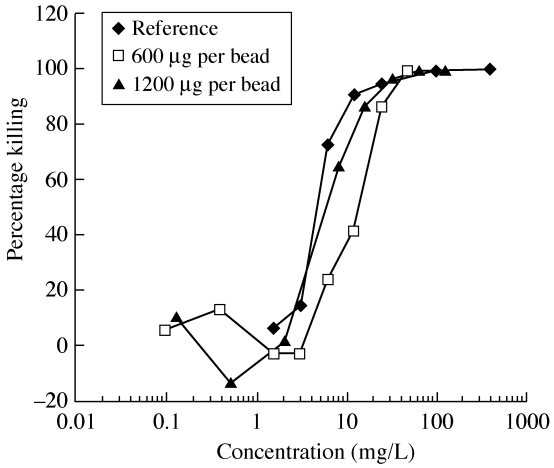

Figure 4. Comparison of the MRSA killing activity between a stock solution of Dhvar-5 peptide (reference peptide), and released peptide from the 600 and 1200 µg/bead groups. For these experiments, the peptide released on the first day was used. The antimicrobial active range of Dhvar-5 against MRSA is 4.9 ± 0.5 µg/mL (IC_{50}) *in vitro*. Each measurement point is an average of four determinations.

Dhvar-5 beads released significantly more peptide over a 28 day period ($P < 0.00005$). The highest release concentrations from the 1200 µg bead were measured on the first day at 1276 (± 146) µg/mL.

The 600 and 1200 µg Dhvar-5 beads displayed similar release patterns, with an initial high release period during the first week, followed by a lower level of continued release. The release remains statistically higher in the 1200 µg Dhvar-5 beads than the 600 µg beads until day 22, indicating the 1200 µg beads have reached the completion of release at this day (Figure 3a). The same release profile was found in the 120 µg bead group. However, the experiment was terminated because the absolute release was below the detection of our protein assay (Figure 2).

The results of the killing assay are shown in Figure 4. The released Dhvar-5 peptide from the 600 and 1200 µg Dhvar-5 beads showed IC_{50} values of 14.6 ± 6.8 and 7.0 ± 3.0 µg/mL against MRSA, respectively, as compared with the reference dilution of Dhvar-5: 4.9 ± 0.5 µg/mL against MRSA. Furthermore, there was no significant difference between the IC_{50} values of the released and reference solutions of Dhvar-5.

Discussion

Currently, the surgical treatment of osteomyelitis consists of a thorough debridement, usually followed by a temporary placement of gentamicin-containing PMMA beads.[6,9] However, the emergence of resistant bacteria is starting to undermine the efficacy of this treatment option.[14,16,25] To overcome these problems, clinicians have started to use alternative antibiotics, such as vancomycin mixed in manually made PMMA beads.[26] But intermediate vancomycin resistance is now emerging, prompting fears of losing the last refuge for treating antibiotic-resistant bacteria.[25,27]

Antimicrobial peptides are a new class of promising antimicrobial agents with a low tendency to induce resistance *in vitro*.[17,18] Also, clinical applications for antimicrobial peptides are currently under investigation, such as the saliva substitute xanthan loaded with antimicrobial peptides for treating oral candidosis.[28] Furthermore, Phase II and III clinical trials have been conducted with the antimicrobial peptide $rBPI_{21}$ to improve the clinical outcome of severe meningococcal sepsis.[29,30] Van't Hof *et al.* have reviewed the current antimicrobial peptides under clinical investigation.[20]

The study presented here demonstrates that the antimicrobial peptide Dhvar-5 is released from PMMA beads for a sustained period of time and Dhvar-5 retains its antimicrobial activity after release (Figure 4). Also, the quantity of release was dependent on the amount of Dhvar-5 added to the cement. The release pattern of Dhvar-5 from the beads resembled the release pattern of gentamicin only in the 120 µg (0.5% w/w) Dhvar-5 beads.[8,31] This resulted in 8% release of the incorporated Dhvar-5 (Figure 3b). However, when the amount of peptide added was increased to 600 µg (2.5% w/w)

per bead, the amount of peptide released increased to 70% (Figure 3b). Furthermore, if the quantity was increased to 1200 μg (5.0% w/w), the released amount increased to 91% of incorporated Dhvar-5 (Figure 3b). The percentages of released Dhvar-5 are far more than previously described in the literature for other antibiotics.[1,8,31]

Known factors that influence antibiotic release from PMMA are surface roughness and porosity.[32] Van de Belt *et al*. demonstrated that porosity corresponded best with the eventual amount of release.[32] The high amount of Dhvar-5 release that has been observed in this study may be explained by the structure of the peptide, a freeze-dried amorphous powder, which has a relatively high volume compared to gentamicin sulphate. It may be hypothesized that at higher concentrations of Dhvar-5, the peptide creates a porous network throughout the bead, allowing diffusion from the core of the bead. To confirm this hypothesis, ultrastructural studies have commenced and will provide additional information.

The limitations of this study are the release conditions which could have simulated the *in vivo* conditions more accurately. However, the release medium dH_2O was chosen because of its lack of protein, which enabled us to measure the release of Dhvar-5 with a BCA protein assay. Furthermore, from the literature it is known that physiological ionic conditions can influence the activity of antimicrobial peptides,[19] so for reasons of comparison with other experiments using different peptides (unpublished data), the medium dH_2O was used. Also the temperature was not physiological, though constant. We therefore conclude that the experiment was conducted under controlled conditions, although not physiological conditions.

The presented data show that the antimicrobial peptide (Dhvar-5) release kinetics from PMMA bone cement is dependent upon the amount of peptide added: a higher percentage of incorporation resulted in a higher percentage of peptide released. Furthermore, Dhvar-5 retains its antimicrobial activity after release from the PMMA beads. Current investigations are focusing on the efficacy of antimicrobial peptide-loaded PMMA beads in an *in vivo* osteomyelitis model.

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