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Comparable Efficacies of the Antimicrobial Peptide Human Lactoferrin 1-11 and Gentamicin in a Chronic Methicillin-Resistant *Staphylococcus aureus* Osteomyelitis Model

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The therapeutic efficacy of an antimicrobial peptide, human lactoferrin 1-11 (hLF1-11), was investigated in a model of chronic methicillin-resistant *Staphylococcus aureus* (MRSA) (gentamicin susceptible) osteomyelitis in rabbits. We incorporated 50 mg hLF1-11/g or 50 mg gentamicin/g cement powder into a calcium phosphate bone cement (Ca-P) and injected it into the debrided tibial cavity, creating a local drug delivery system. The efficacy of hLF1-11 and gentamicin was compared to that of a sham-treated control (plain bone cement) ($n = 6$) and no treatment (infected only) ($n = 5$). The results were evaluated by microbiology, radiology, and histology. MRSA was recovered from all tibias in both control groups ($n = 11$). On the other hand, hLF1-11 and gentamicin significantly reduced the bacterial load. Furthermore, no growth of bacteria was detected in five out of eight and six out of eight specimens of the hLF1-11- and gentamicin-treated groups, respectively. These results were confirmed by a significant reduction of the histological disease severity score by hLF1-11 and gentamicin compared to both control groups. The hLF1-11-treated group also had a significantly lower radiological score compared to the gentamicin-treated group. This study demonstrates the efficacy of hLF1-11 incorporated into Ca-P bone cement as a possible therapeutic strategy for the treatment of osteomyelitis, showing efficacy comparable to that of gentamicin. Therefore, the results of this study warrant further preclinical investigations into the possibilities of using hLF1-11 for the treatment of osteomyelitis.

Osteomyelitis causes major morbidity and remains the most feared and difficult infection to treat in orthopedic surgery. Radical surgical debridement with local administration of antibiotics e.g., gentamicin-loaded polymethyl methylacrylate (PMMA) beads in combination with systemic antibiotics, has been the treatment of choice (7, 27, 28). The major disadvantage of PMMA beads is the need to remove them after they have eluded their antibiotics, requiring an additional surgical procedure (7, 27, 28). Numerous investigations have been performed to develop other resorbable/biodegradable carriers for several antibiotics (1, 9).

Gentamicin has long been the primary choice of orthopedic surgeons for local treatment of osteomyelitis (13, 28). This aminoglycoside is a broad-spectrum antimicrobial agent, which is active against both gram-positive and gram-negative bacteria (5). The long-term release profiles of gentamicin from PMMA and calcium phosphate bone cements have been demonstrated in kinetic release studies (9, 26). Overall, the use of gentamicin-loaded PMMA beads results in high local antibiotic bone and soft tissue concentrations versus low systemic concentrations, reducing systemic side effects (26). On the contrary, the high systemic dosage of gentamicin needed to reach sufficient tissue penetration would create serious toxic side effects (2).

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Over the past few years, studies have shown an increase in antibiotic-resistant bacteria such as gentamicin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) (30). The prevalence of MRSA in nosocomial infections has exceeded 30% in some countries, e.g., southern Europe and the United States (8, 30). Several authors have also demonstrated the presence of gentamicin-resistant staphylococci after gentamicin-loaded PMMA bead therapy, raising concern about the efficacy of this treatment option (14, 25). Furthermore, reports on staphylococcal infections with reduced susceptibility or resistance to vancomycin are emerging (29). As current antibiotic therapy options are becoming limited for staphylococcal infections, there is an urgent need for new antimicrobial agents to combat these resistant pathogens.

Antimicrobial peptides are a new and promising class of antimicrobial agents derived from naturally occurring peptides (4). These peptides are found on epithelial surfaces, in secretion fluids, and in neutrophils and thus form a first line of host defense as part of the innate immune system (31). Furthermore, they are thought to have a diminished tendency to induce resistance because of the evolutionary difficulty in changing bacterial membrane structure (4, 31). These properties together with the antimicrobial activity against a broad variety of pathogens have made antimicrobial peptides attractive candidates for antimicrobial drug development (24).

The antimicrobial peptide human lactoferrin 1-11 (hLF1-11) is derived from the active domain of human lactoferrin (N-

terminal amino acids 1 to 11) (12). hLF1-11 has a broad antimicrobial spectrum in vitro against both bacteria and fungi (11, 12, 16). Furthermore, Nibbering et al. demonstrated the efficacy of hLF1-11 in vivo, reducing the number of bacteria (MRSA) after systemic administration in the mouse thigh infection model (16).

In the light of antimicrobial resistance and the urgent need for new antimicrobial agents, we previously investigated the efficacy of using calcium phosphate bone cement as a carrier for hLF1-11 (22). In general calcium phosphate bone cements showed a high and prolonged in vitro release of hLF1-11 in its biologically active form (22). Furthermore, we also showed that hLF1-11 incorporated into calcium phosphate bone cement can prevent *S. aureus* osteomyelitis in rabbits (21). Therefore, the aim of the present investigation was to evaluate and compare the performance of hLF1-11 and gentamicin incorporated into calcium phosphate bone cement to treat MRSA osteomyelitis in rabbits.

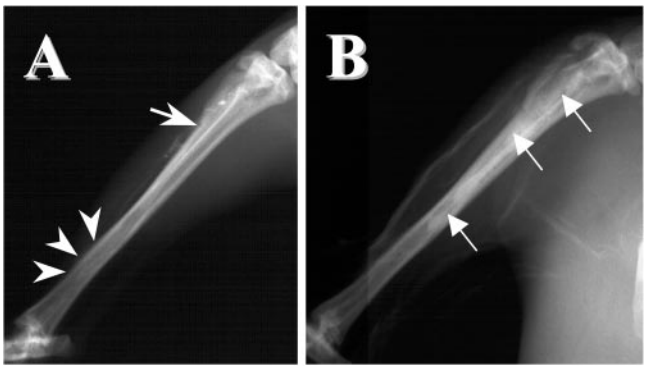

FIG. 1. Preoperative lateral radiograph (A) of the right tibia from a sham-treated rabbit. Periosteal reaction is present (arrowheads), and the drill hole can still be seen as a slight shadow (arrow). The postoperative lateral radiograph (B) of the same rabbit shows the cement in situ (arrows).

MATERIALS AND METHODS

Experimental design. The study was set up according to the well-described osteomyelitis model of Norden et al. (18). The proximal right tibias of 27 New Zealand White rabbits were inoculated with approximately 3×10^6 CFU MRSA. After 3 weeks, on day 21 treatment was initiated, except for five control animals which were sacrificed at this time point. Six animals were assigned to the sham-treated control group ($n = 6$), receiving a thorough debridement and plain calcium phosphate bone cement (Calcibon, Biomet Merck, Darmstadt, Germany). The remaining 16 animals were also thoroughly debrided and received either 50 mg hLF1-11 ($n = 8$) or 50 mg gentamicin sulfate ($n = 8$) per gram of bone cement powder (approximately 20 mg/kg body weight). On day 42, 3 weeks after treatment, all treated animals were sacrificed and treatment outcome was assessed by radiology, microbiology, and histology.

Animals. Female New Zealand White rabbits (3.5 to 4 kg) were obtained 3 weeks prior to surgery and allowed to acclimatize to the Clinical Animal Laboratory, Vrije Universiteit Medical Center. The animals were housed in groups of 10 and were allowed water and antibiotic-free rabbit diet ad libitum. The experimental protocol was approved by the Animal Ethics Committee of the Vrije Universiteit Medical Center.

Bacterial strain. The strain which was used for this study was a methicillin-resistant *Staphylococcus aureus* (MRSA) strain W234 isolated from a patient with chronic osteomyelitis. Besides resistance to all β-lactam antibiotics, the strain was also resistant to erythromycin, tetracycline, trimethroprim-sulfamethoxazole, and quinolones, but was susceptible to clindamycin, rifampin, aminoglycosides, and glycopeptides (not shown).

An overnight culture was grown in brain heart infusion (BHI) broth and washed in phosphate-buffered saline (PBS). Subsequently, aliquots were prepared and stored at −80°C. The numbers of viable CFU were determined in the defrosted aliquots by serial dilution and plating on blood agar. At the day of surgery a fresh aliquot was defrosted, and a bacterial suspension of approximately 3×10^7 CFU/ml was prepared in PBS. The tibial canal was inoculated by injection of 0.1 ml bacterial suspension (3×10^6 CFU). After surgery, the inoculates were serially diluted, plated on blood agar, and cultured to confirm the number of viable bacteria.

Surgery. The anesthesia was exactly the same for both the inoculation and the treatment of infection. After weighing, the animals were given a subcutaneous injection of xylazine, 2.5 mg/kg (Xylalin, CEVA Sante Animale, Maassluis, The Netherlands), and ketamine, 62.5 mg/kg (Aescoket, Aesculaap, Boxtel, The Netherlands), between the shoulder blades. Once sedated, the right knee area was shaven and washed with iodine solution. Subsequently, lidocaine 5 mg/kg (Pharmacy Vrije Universiteit Medical Center, Amsterdam, The Netherlands) was injected around the tuberculum and tibial plateau of the right tibia. Preoperative blood samples were drawn from the auricular vein for the determination of white blood cell count (WBC) and erythrocyte sedimentation rate (ESR). The animals were then placed on their back, the skin painted again with iodine and the surgical area isolated with sterile drapes. During surgery, the anesthesia was maintained by free-flow inhalation of a gas mixture containing 45% oxygen; 53.5% air and 1.5% isoflurane (Forene, Abbot BV, Hoofddorp, The Netherlands). Postoperative pain relief was provided by subcutaneous injection of 0.15 mg buprenorphine, which was repeated if necessary.

Inoculation of the tibia. A small stab incision was made over the tuberculum of the right tibia, and subsequently a 1.8-mm drill hole was made with a K-wire mounted on a hand-held drill. Then a 16-gauge needle was inserted into the tibial cavity. Subsequently, 100 μl of 5% sodium morrhuate (Scleromate, Glenwood, Englewood, New Jersey) was injected, followed by the MRSA inoculum (3×10^6 CFU in 100 μl) through the same needle. The needle was then flushed with 100 μl of 0.9% NaCl to ensure all bacteria reached the tibial cavity. The skin was subsequently closed with Vicryl 4/0.

Debridement and bone cement injection. The tibias were debrided 21 days after the first operation. A 3-cm incision was made over the pattello-tibial tendon, parallel to the tibial shaft. Subsequently, access to the tibal cavity was gained through the pattello-tibial tendon with a small drill (2.0-mm diameter), which was gradually increased to 3.5-mm diameter. The tibial canal was then debrided by reaming with stainless steel brushes (Abrasives Center, Maastricht, The Netherlands) increasing in size up to 4.0-mm diameter. It was attempted to remove as much infected and necrotic tissue as possible. Tissue remnants were whipped from the drills and brushes with conventional swabs and sent for culture. The canal was then flushed with 100 ml 0.9% saline solution to remove any loose debris and remaining bone marrow. A suction cuvette was placed in the tibial canal until the cement was injected.

The calcium phosphate bone cement (2.5 g) (Calcibon, Biomet Merck, Darmstadt, Germany) was prepared under strict sterile conditions on the surgical table according to the manufacture's instructions. The cement paste was injected into the tibial cavity through a stainless steel cannula with an inside diameter of 2.4 mm while slowly retracting the syringe. The syringe was weighed before and after injection to determine the amount of injected cement. Hereafter, the fascia and skin were closed with Vicryl 4/0. Pre- and postoperative X-rays were also taken to confirm the correct position of the bone cement and for the initial assessment of radiological osteomyelitis signs (Fig. 1).

Follow-up. All animals were monitored daily and examined at preset time points, days 1, 2, 4, and 7 and thereafter every 7 days. After the second surgical procedure on day 21 they were again examined more frequently, days 22, 24, and 28 and subsequently every 7 days until autopsy on day 42. During the examinations, the wound was inspected for signs of infection, the animals were weighed, and blood was drawn from the auricular vein for WBC and ESR determination.

Autopsy and sample acquisition. On day 42, the animals were euthanized, first by sedation with a subcutaneous injection of 2.5 mg/kg xylazine and 62.5 mg/kg ketamine, and then sacrificed by an intravenous injection of 75 mg/kg pentobarbital (Nembutal, Ceva Sante Animale BV, Naaldwijk, The Netherlands). The right tibias were excised under strict aseptic conditions. After excision, radiographs were taken to assess radiological disease severity.

A high-speed dental drill was used to saw a standardized square bone sample (±1 g) from the proximal medial tibia for quantitative microbiological analyses. The saw was cooled with physiological saline, and care was also taken not to damage the bone cement. Subsequently, the saw-cut surfaces and interior of the bone sample were cultured with conventional swabs. A transverse section adjacent to the removed bone sample was processed for histological analyses. A strictly standardized protocol was enforced to insure that comparable samples

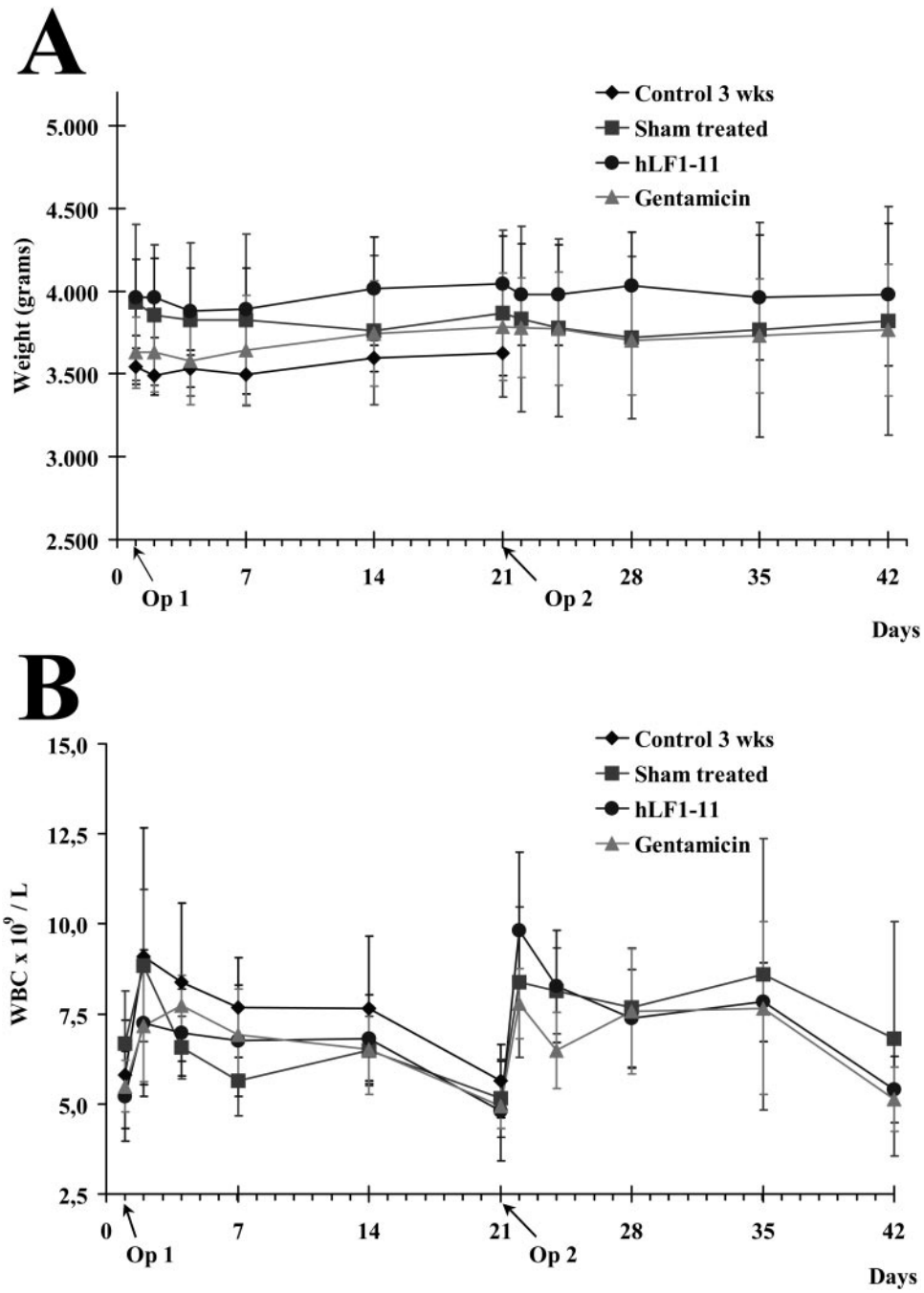

FIG. 2. Changes in body weight and white blood cell (WBC) count are plotted over time in A and B, respectively. The first and second surgical procedure are indicated as OP1 and OP2, respectively. There were no significant differences between groups. Values are the means ± standard deviation.

were acquired. The samples for quantitative microbiological analysis were then further processed by a technician blinded to the assigned groups.

Radiology. The radiographs obtained at the second procedure and at autopsy were randomized, scored by an independent orthopedic surgeon. The standard osteomyelitis disease severity score of Norden et al. was used; this score is based on the presence of sequestra, periosteal new bone formation, and destruction of bone (18). Involvement of the proximal, mid-, and distal tibia was also scored, resulting in a maximum score of seven points. Animals were considered to have radiological osteomyelitis when the severity score was 3 or more.

Microbiology. The conventional swabs were plated on horse blood agar and incubated for at least 48 h at 37°C. To further increase the sensitivity of this method, the tips were also incubated in 10 ml brain heart infusion (BHI) broth on a shaker for 7 days at 37°C.

The quantitative assay consisted of weighing the standardized bone sample and then homogenizing it in 50 ml PBS at high speed (15,000 rpm) for 2 min (Sorval Omnimix, Dupont Instruments, Newton, Connecticut). Bacterial load was determined by serially diluting the homogenate and plating a standard volume (10 μl in triplicate) on blood agar, followed by incubation for 48 h at 37°C. The colonies were counted and bacterial load was calculated as the number of CFU per gram of bone tissue. After each run, the Omnimixer was thoroughly cleaned and subsequently sterilized to prevent any possible cross contamination between samples. The detection limit of this method was approximately 2.5×10^3 CFU/g bone depending on the mass of the specific homogenized bone section. For statistical analysis, the negative samples (without growth of microorganisms) of the quantitative assay were considered to have the detection limit value ($\approx 2.5 \times 10^3$ CFU/g bone) of the bacterial load. Additionally, a 1-ml sample of

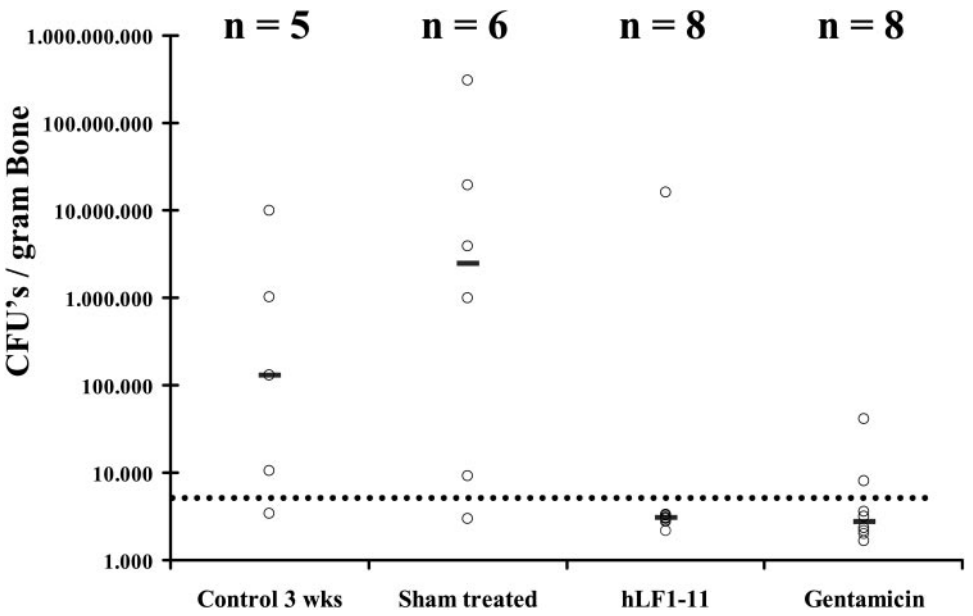

FIG. 3. Quantitative microbiological analyses of the cultured bone homogenate. The individual data points (circles) and the medians (black bars) are indicated. The dotted line represents the approximate detection limit (≈2.5×10^3 CFU/g bone) of the assay; animals with values below this limit had negative cultures with the quantitative method (no growth). Both the 3-week control and the sham-treated animals had a significantly higher bacterial load than the hLF1-11- ($P = 0.01$) or gentamicin-treated animals ($P = 0.004$). There was no significant difference in bacterial load between the 3-week controls and the sham-treated animals ($P = 0.6$), nor was there a difference between the gentamicin- and hLF1-11-treated animals ($P = 0.6$).

bone homogenate was cultured in 10 ml BHI broth for 7 days at 37°C in a shaker to detect bacteria below the detection limit of the quantitative method.

Identification of *S. aureus* was based on the coagulase test (Staphytect, Oxoid Ltd., Basingstoke, Hamshire, United Kingdom). To genotype the strains, SmaI digests of total bacterial DNA were resolved by a pulsed-field gel electrophoresis performed as previously described by Ichiyama et al. (6). Strains were considered clonal if less than two bands varied on a gel.

Histology. Bone slices were fixed in buffered formalin for two days, decalcified in EDTA, embedded in paraffin, mounted on slides, and stained with hematoxylin and eosin. The disease severity score described by Smeltzer and coworkers was used to rate signs of infection (20). This score is divided into four categories that are scored with zero to four points: intra-osseous acute inflammation, intra-osseous chronic inflammation, periosteal inflammation, and bone necrosis. The diagnosis of osteomyelitis was considered positive when the Smeltzer score was at least 4. The maximum score of 16 signifies severe osteomyelitis with intramedullar abscesses, fibrosis, and multiple foci of sequestra. Slides were randomized and subsequently scored by an independent pathologist.

Statistical analysis. The results were analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.1 for Windows. The Mann-Whitney tests for independent samples were performed for comparison of the CFU count between groups, as these data were not normally distributed. This test was also used to test the ordinal radiological and histological score between different groups. The Wilcoxon signed rank test was performed for comparison of radiological scores within groups. Furthermore, analysis of variance tests using Bonferroni's posthoc test were performed for comparison of blood parameters between groups and paired Student *t* tests were performed for comparison of different time points within groups. Differences were considered significant at $P \leq 0.05$.

RESULTS

General. All animals tolerated both operations well, displaying no signs of systemic infection. One rabbit in the gentamicin-treated group had wound dehiscence after the second procedure. This wound was debrided, flushed with physiological saline, and subsequently closed subcutaneously, after which the rabbit recovered well. None of the rabbits developed soft tissue swelling or fistulae, and clinically the infection was maintained in the tibia.

The rabbits did display a tendency to slight weight loss after each procedure, recovering to normal after 7 to 14 days (Fig. 2A). The initial weights were not significantly different from the weight at day 21 or day 42 except for the gentamicin group, which showed an increase at day 21. Furthermore, there were no significant differences in weight between the different treatment regimens.

The pattern of changes in weight after each procedure are also reflected in the WBC (Fig. 2B), which increased after each procedure and then returned to normal at day 21 and at day 42. The ESR showed a similar pattern, although none of the changes were significant (data not shown). Both the WBC and the ESR patterns were seen in all treatment regimens.

The sham-treated group had a mean of 1.8 ± 1.1 g of wet bone cement injected. The hLF1-11 had 2.2 ± 0.2 g of wet bone cement injected, representing a mean total dose of 19.0 ± 1.8 mg hLF1-11/kg body weight. The gentamicin-treated animals had a similar amount of wet cement injected, 2.4 ± 0.3 g, with a mean of 21.9 ± 2.3 mg gentamicin sulfate/kg body weight.

Microbiology. The bacterial load per gram of bone is plotted in Fig. 3. The median bacterial load of both the 3-week control and the sham group was 1.3×10^5 and 2.4×10^6 CFU/g bone, respectively. The hLF1-11 group had a significantly lower bacterial load (median of 3.0×10^3 CFU/g bone) compared to both the control and sham groups ($P = 0.01$). Seven out of eight animals did not have quantifiable bacteria using this method. Furthermore, the gentamicin-treated animals also had a significantly lower bacterial load (median of 2.7×10^3 CFU/g bone) compared to both the control and sham-treated groups ($P = 0.004$). Two animals out of the eight gentamicin-treated animals still had quantifiable bacteria in their tibia. There was

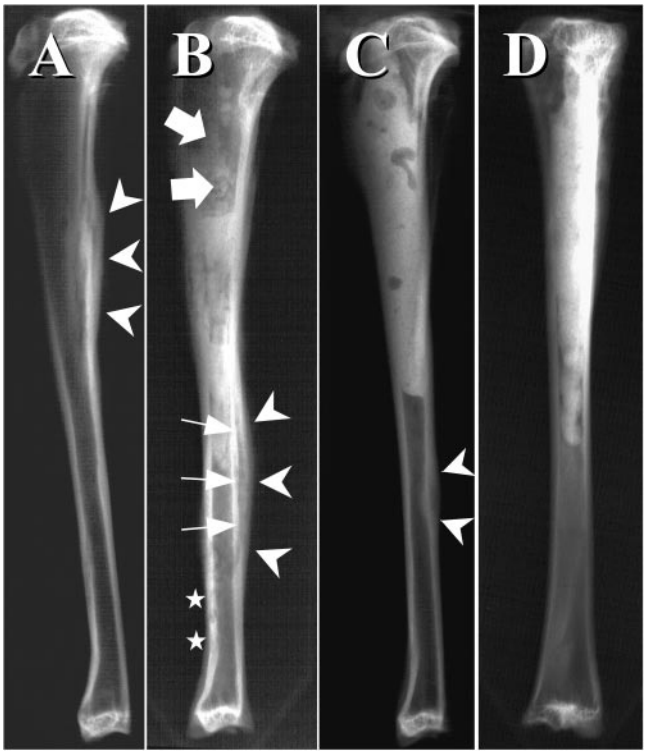

FIG. 4. Representative radiographs of excised tibias. In the 3-week control animal (A), periosteal reaction is visible (white arrowheads). More severe signs of chronic osteomyelitis are seen in the 6-week sham-treated animal (B). The periosteal reaction (arrowheads) is exacerbated and a sequestrum is present (arrows). Also, there is a moth-eaten appearance with bony destruction (stars). More proximal, there is evidence of bone cement resorption (block arrows). Slight periosteal elevation (arrowheads) was seen in the gentamicin-treated tibia (C) distal to the bone cement, whereas this hLF1-11-treated animal (D) did not show signs of osteomyelitis.

no significant difference in bacterial load between gentamicin- and hLF1-11-treated animals ($P = 0.6$).

At autopsy, two out of eight gentamicin-treated animals still yielded MRSA at autopsy. Similarly, MRSA was cultured in three out of eight animals in the hLF1-11 group. On the other hand, MRSA was cultured from all five control animals. Moreover, all six sham-treated animals yielded MRSA after 6 weeks.

All bacteria isolated at autopsy were found to be coagulase positive. These *S. aureus* strains were subsequently also shown to be clonal (and identical to the strain which was used to induce the osteomyelitis) by pulsed-field gel electrophoresis, indicating that there was no contamination with other bacterial strains during the operative procedures.

Radiology. The radiographs obtained at autopsy shown in Fig. 4 illustrate the variety of disease severity found in this study. The average radiographical scores are plotted in Fig. 5. The severity score for both the sham- and gentamicin-treated animals increased significantly from days 21 to 42 ($P = 0.039$ and $P = 0.011$, respectively). In contrast, the score for the hLF1-11-treated animals did not increase ($P = 0.29$). Furthermore, at autopsy the score of the hLF1-11 group was also significantly lower than that of both the sham- and gentamicin-treated groups, $P = 0.043$ and $P = 0.035$, respectively. In the control group four out of five and the sham-treated group five out of six animals had a score higher than 3, indicating apparent osteomyelitis. In the gentamicin group, five out of eight animals still had a score higher than 3 points, whereas in the hLF1-11-treated animals only three out of eight animals still had radiological signs of osteomyelitis.

Histology. Examples of the histological sections are displayed in Fig. 6, illustrating that disease severity varied considerably between animals. Some specimens displayed all signs of osteomyelitis; periosteal reaction, intramedullary or subperiosteal abscesses, sequestra, and fibrosis, as opposed to others that only had minor signs of osteomyelitis in the same group.

This variation is reflected in the disease severity scores, which are plotted in Fig. 7. However, both gentamicin and hLF1-11 significantly reduced the disease severity score compared to the sham and the control groups ($P = 0.04$ and $P = 0.01$, respectively). There was no significant difference in disease severity score between hLF1-11- and gentamicin-treated animals or between control and sham-treated animals.

A histological score of 4 or more points was indicative of osteomyelitis (20); this was seen in four out of five control animals and in five out of six sham-treated animals. In the gentamicin-treated animals three out of eight animals and in the hLF1-11 group only 2 out of eight still had osteomyelitis based on the histological score alone.

DISCUSSION

In this study, we induced chronic osteomyelitis in the tibia as described by Norden et al. (18). This model has been used extensively for evaluating experimental treatment protocols for osteomyelitis (17). We modified the treatment protocol by local debridement and subsequent injection of calcium phosphate cement containing the antimicrobial agent. The pathogen used in this study is an MRSA strain isolated from a

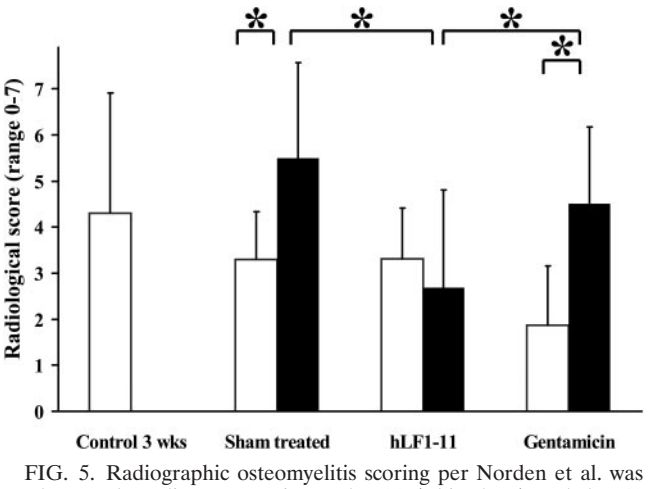

FIG. 5. Radiographic osteomyelitis scoring per Norden et al. was used to analyze disease severity at day 21 (white bars) and day 42 (black bars). The maximum score of 7 reflects severe chronic osteomyelitis in the entire tibia with periosteal new bone, sequestra, and foci of necrosis. The disease severity score increased from day 21 to day 42 in both the sham-treated group ($P = 0.04$) and the gentamicin group ($P = 0.011$). Furthermore, at day 42, the hLF1-11-treated animals had a significantly better score than both the sham-treated ($P = 0.043$) and the gentamicin-treated animals ($P = 0.035$). Given are the mean values and standard deviation.

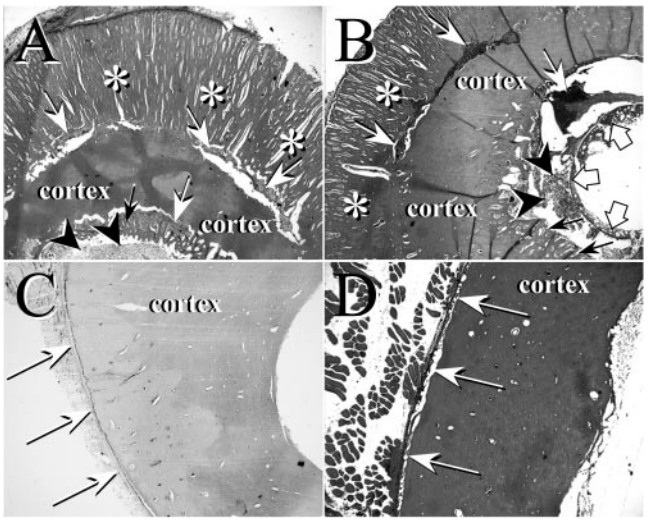

FIG. 6. Representative photomicrographs (hematoxylin and eosin stained) of transverse sections of the tibial cortex. Panel A depicts a 3-week control animal; note the marked periosteal reaction (stars) surrounding the largely necrotic cortex. Also, abscesses (white arrows) are present both intramedullarly and subperiosteally. The intramedullary canal is filled with fibrotic tissue (arrowheads) and some new bone formation also can be seen (black arrow). Panel B depicts a sham-treated specimen. Note the bone marrow versus bone cement interface (white block arrows). Similar pathology can be seen with periosteal bone formation (stars), abscesses (white arrows), intramedullary fibrosis (arrowheads), and intramedullary new bone formation (black arrows). Panel C shows an hLF1-11-treated specimen. Note the absence of periosteal inflammation (white arrows); no other pathology can be seen. A gentamicin-treated specimen without periosteal inflammation (white arrows) is depicted in panel D. Magnification: A and B, 25×; C and D, 50×.

patient with osteomyelitis that had been used in a previous osteomyelitis study and shown to be sufficiently virulent (U. Joosten, personal communication).

MRSA was recovered from all control and sham-treated animals. However, in both groups, one animal each had a low bacterial load, which was also reflected in their radiological and histological scores. Since the gold standard for osteomyelitis was a positive culture for MRSA, we concluded that our model was valid, with a 100% infection rate in both the control and the sham-treated groups (no antibiotic).

The data presented in this study clearly demonstrate the ability of hLF1-11 to treat MRSA osteomyelitis in this model. The hLF1-11 treatment not only significantly reduced bacterial load compared to controls, but also significantly reduced the radiological and histopathological score. In this study, the performance of hLF1-11 was comparable to that of gentamicin, even though the MRSA strain was gentamicin susceptible.

Regarding the hLF1-11 group, in five out of eight animals no growth was detectable. And, of the three MRSA-positive rabbits, only one rabbit was found to have a high bacterial load. This rabbit also had an initial high radiological score of 5.5 at the onset of treatment. The other two rabbits did not have quantifiable bacteria but showed growth of MRSA in the broth culture and also had radiological and histological signs of osteomyelitis.

In the gentamicin group, five out of eight animals were still found to have radiological osteomyelitis and three out of eight animals had histological signs of infection. Only two of these animals had positive cultures for MRSA. Since radiological signs were seen distal of the bone cement, it is suggested that the infection survived in the distal diaphysis, a location at which gentamicin levels could be substantially lower (26). Some osteomyelitis signs may have been missed on histology, as only sections at one level next to the bone cement were examined, whereas radiology picked up osteomyelitis signs of the entire femur. The two methods supplemented each other, allowing reliable interpretation of the data.

Nibbering et al. found a 2.5 log reduction of bacterial load in the mouse thigh model using very low systemic dosages of hLF1-11 (0.1 to 1 nmol) (16). An immune-inciting effect was presumed to be responsible for the reduction of bacterial load in this mouse thigh model (16), whereas the direct killing effect of higher concentrations of hLF1-11 is presumed to play a role in the efficacy of hLF1-11 in the prophylactic osteomyelitis rabbit model (21). In this study both mechanisms may have acted simultaneously, rapid bacterial killing occurring at the level of the tibia and due to the bone-serum gradient, low systemic concentrations of hLF1-11 could then incite an immune response (26). However, more pharmacokinetic studies are required to elucidate the mechanism of action in this model.

A disadvantage of local delivery of conventional antibiotics is the low-level subinhibitory release after the burst release has subsided. This phenomena is well-known to occur in the case of gentamicin release from PMMA, and release can even continue for years, thereby also inducing and selecting antimicrobial resistance of bacteria (14, 15, 23, 25). However, the advantage of antimicrobial peptides is their intrinsic low tendency to induce resistance, despite prolonged passages of bacteria through subinhibitory concentrations (3, 4, 31). Furthermore, we used a resorbable cement, which ensures that the antibiotic cannot be released indefinitely (19).

Over the past decade, numerous naturally occurring antimicrobial peptides have been identified, emphasizing their piv-

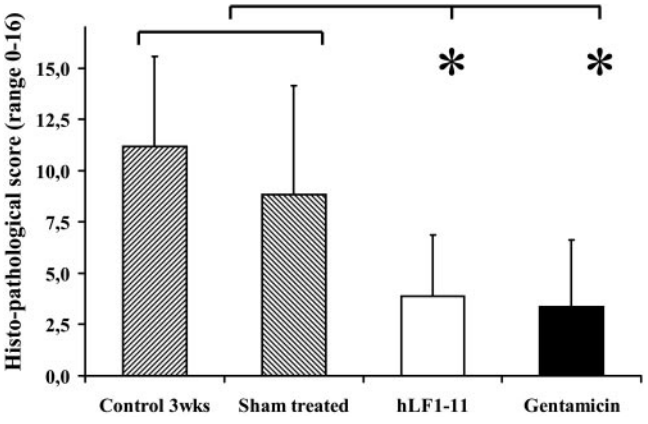

FIG. 7. Histopathological data as scored using the score of Smeltzer et al. (mean + standard deviation). The maximum score is 16, indicating severe osteomyelitis with intramedullary abscess and fibrosis, severe periosteal reaction with subperiosteal abscess, and multiple foci of necrotic bone. Both the hLF1-11- and gentamicin-treated animals have a significantly lower score than the 3-week control and the sham groups, $P = 0.04$ and $P = 0.01$, respectively.

otal role as part of the first line of defense in our innate immune system (4, 31). Several research groups have specifically described options for the development of antimicrobial peptides as new antimicrobial agents and investigated their efficacy in vivo. Van't Hof et al. have reviewed a large number of antimicrobial peptides currently under investigation (24). The $rBPI_{21}$ antimicrobial peptide has reached phase III clinical trials and has shown promising clinical results, improving the outcome of meningococcal sepsis in children (10). The rise of antimicrobial resistance has further emphasized the need for new antimicrobial agents. MRSA has already emerged in large areas of the world, and with the additional increase of infections due to *S. aureus* with reduced susceptibility or resistance to glycopeptides, concern about the development of strains resistant to all available antibiotics has arisen (29).

This study demonstrates the ability of hLF1-11 incorporated into calcium phosphate cement to treat MRSA osteomyelitis in rabbits. hLF1-11 improved the clinical outcome radiologically, microbiologically, and histologically compared to control animals. Furthermore, the efficacy of hLF1-11 was comparable to that of gentamicin in this model. The results of this study thus warrant further preclinical investigations into the use of hLF1-11 for the treatment of osteomyelitis.

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