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Award Number: W81XWH-10-1-0872

TITLE: Inhibition of Orthopaedic Implant Infections by Immunomodulatory Effects of Host Defense Peptides

PRINCIPAL INVESTIGATOR: Edward Greenfield, PhD

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland OH 44106-1712

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2012		2. REPORT TYPE Annual		3. DATES COVERED 15 September 2011- 14 September 2012	
4. TITLE AND SUBTITLE Inhibition of Orthopaedic Implant Infections by Immunomodulatory Effects of Host Defense Peptides				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0872	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Edward Greenfield, PhD E-Mail: emg3@cwru.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Case Western Reserve University Cleveland OH 44106-1712				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT- Host defense peptides represent a promising new approach to inhibit infection. The anti-infective actions of these peptides are primarily due to their immunomodulatory effects. Since they regulate multiple aspects of the mammalian immune system, host defense peptides are also less likely to induce bacterial resistance than are traditional antibiotics. The local delivery of anti-bacterial agents allows for both high local concentrations to increase efficacy and low systemic levels to reduce toxicity. A promising strategy for the local delivery of anti-bacterial agents is to bind them directly to the surfaces of orthopaedic implants. The progress in the second year of this project has demonstrated: 1. Development of a quantitative and reproducible murine model of orthopaedic implant infection. 2. Preliminary results indicate that the soluble host defense peptide reduces orthopaedic implant infection in the murine model. 3. Preliminary results indicate that the soluble host defense peptides increases osseointegration in mice that were not inoculated with bacteria. The host defense peptides have the potential to substantially reduce infections of fractures sustained on the battlefield and in civilian settings. If the synthetic peptide reduces infections in the studies proposed in this application, more extensive pre-clinical testing would precisely determine its potential benefits and risks and determine whether the peptide is a high priority for human trials.					
15. SUBJECT TERMS- Orthopaedic infections, Host Defense Peptides, Murine model, Staphylococcus aureus, Acinetobacter baumannii					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 7	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

Host defense peptides represent a promising new approach to inhibit infection. The anti-infective actions of these peptides are primarily due to their immunomodulatory effects. Since they regulate multiple aspects of the mammalian immune system, host defense peptides are also less likely to induce bacterial resistance than are traditional antibiotics. The local delivery of anti-bacterial agents allows for both high local concentrations to increase efficacy and low systemic levels to reduce toxicity. A promising strategy for the local delivery of anti-bacterial agents is to bind them directly to the surfaces of orthopaedic implants. Our hypotheses are:

1. Soluble host defense peptides reduce infection of orthopaedic implants.
2. Host defense peptides bound to orthopaedic implant surfaces reduce infection.

BODY:

One of the primary areas of progress in this year has been the development of the murine model of orthopaedic implant infection. Appropriate concentrations of *Staph. aureus* were identified that reproducibly provide chronic implant infection without causing any signs of systemic sepsis. In mice without bacteria, BLI was low at all time points (Fig. 1) and no bacteria were detectable either adherent to the implants or in the bones by counting colony-forming units (Fig. 2) or by quantitative PCR of a marker gene (Fig. 3). Importantly, bacterial inoculation dose-dependently increased all of those measures of infection (Figs. 1-3).

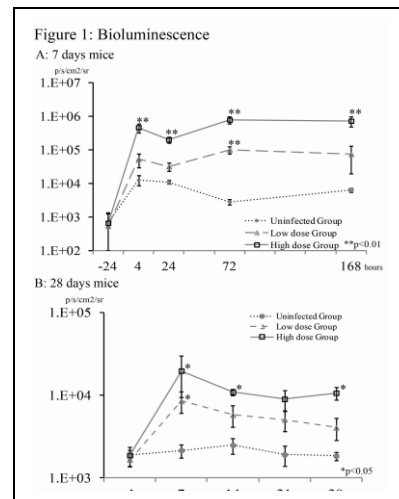


Figure 2. Colony forming units at 7 days

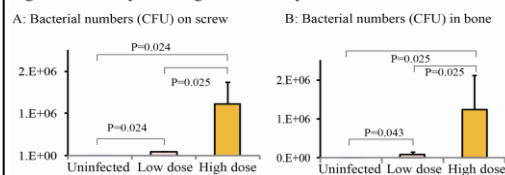
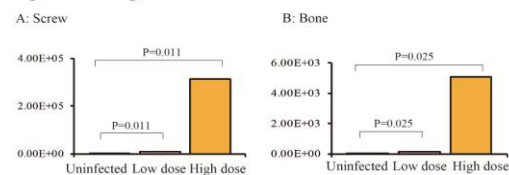
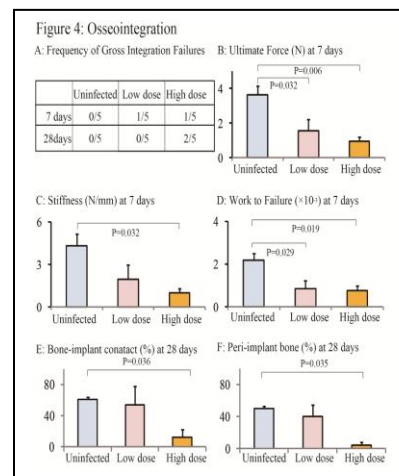


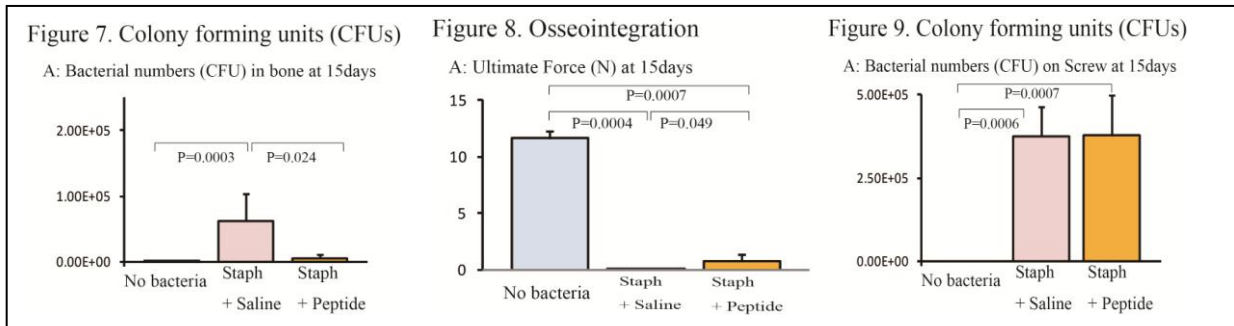
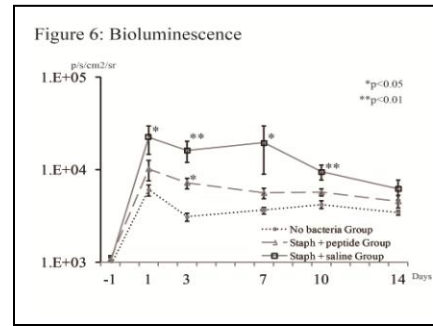
Figure 3: *Lux A* gene based real-time PCR results



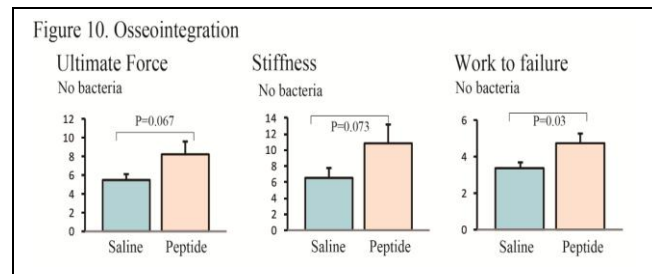
The long-term ability to detect BLI is likely due to the use of *S. aureus* with the *lux* operon in a stable plasmid as shown by other investigators [1]. Osseointegration was significantly decreased in the groups with the higher dose of bacteria. Thus, there was a higher rate of gross integration failure (Fig. 4A) and reduced biomechanical (Figs. 4B-D) and histomorphometric (Fig. 4E-F) measures of osseointegration. Representative histological images of the impaired osseointegration are shown in Figure 5. Taken together the results described in this paragraph demonstrate that the higher bacterial inoculation dose provides a quantitative and reproducible murine model of orthopaedic implant infection that includes impaired osseointegration.



With the model described in the previous paragraph, we are beginning to test hypothesis #1 that soluble host defense peptides reduce infection of orthopaedic implants. Preliminary results indicate that three daily injections of the soluble host defense peptide reduces BLI (Fig. 6) and CFUs in the bone (Fig. 7) and modestly increases biomechanical measures of osseointegration (Fig. 8). Interestingly, there was no effect on CFUs adherent to the implant (Fig. 9).



Preliminary results also indicate that the soluble host defense peptide increases osseointegration in mice that were not inoculated with bacteria (Fig. 10). Since osseointegration is impaired by inflammation, this result may be due to the ability of the host defense peptide to reduce inflammation [2-3].



We are currently confirming the results described in this paragraph in additional mice and determining whether the effects of the soluble host defense peptide can be increased, for example, by increasing the number of injections of the peptide.

KEY RESEARCH ACCOMPLISHMENTS:

1. Development of a quantitative and reproducible murine model of orthopaedic implant infection (Figs. 1-5).
2. Preliminary results indicate that the soluble host defense peptide reduces orthopaedic implant infection in the murine model (Figs. 6-9).
3. Preliminary results indicate that the soluble host defense peptides increases osseointegration in mice that were not inoculated with bacteria (Fig. 10).

REPORTABLE OUTCOMES:

Development of the murine model was reported in a poster presented at the Military Health System Research Symposium, Ft Lauderdale, Florida, 13-16 August 2012 (see Appendix)

CONCLUSION:

The host defense peptides have the potential to substantially reduce infections of fractures sustained on the battlefield and in civilian settings. If the synthetic peptide reduces infections in the studies proposed in this application, more extensive pre-clinical testing would precisely determine its potential benefits and risks and determine whether the peptide is a high priority for human trials.

REFERENCES:

- [1] Bernthal et al, 2010 PLoS ONE 5:e12580
- [2] Wieczorek et al Chem Biol. 2010 17:970-80
- [4] Achtman et al, Sci Trans Med 2012 4:135ra64

APPENDIX:

Poster presented at the Military Health System Research Symposium, Ft Lauderdale, Florida, 13-16 August 2012



Hyonmin Choe, Joscelyn Tatro, David Corn, Steve Marshall, Lindsay A Bonsignore, Zhenghong Lee, Robert A Bonomo, and Edward M Greenfield
Case Western Reserve University and Louis Stokes Cleveland VA Medical Center
Cleveland, OH

INTRODUCTION

Infection is the most difficult complication of orthopaedic implant surgery and often impairs osseointegration of the implant. Treatment typically requires two-stage revisions and extended intravenous antibiotic therapy. The increasing prevalence of multidrug-resistant (MDR) bacteria has made antibiotic treatment especially difficult. Development of novel therapeutic agents is therefore required. Murine models are extremely useful for this purpose due to the availability of transgenic and knockout strains. Moreover, the reduced cost of mice compared to larger animals facilitates screening of novel therapeutic strategies.

A murine infection model that assesses implant osseointegration has not yet been established. In this study, we developed murine model of osseointegration to compare sterile implants and implants with adherent bacteria based on our previously validated murine model of osseointegration [1,2]. We quantified the bacterial burden by bioluminescence imaging (BLI) and counting of colony forming units (CFUs) and measured osseointegration by histomorphometry and biomechanical pull-out testing.

METHODS

1.0×10^5 or 3×10^6 CFUs/implant of bioluminescent *Staph. aureus* (Xen36 strain, Caliper Life Sciences) were adhered to rigorously cleaned titanium alloy screw shaped implants (1 mm diameter, 3.2 mm length) immediately before insertion into pilot holes in the mid-diaphysis of the femur of 6-7 week old male C57BL/6 mice [1-2]. BLI was measured 24 hours before surgery as a baseline and at the indicated time-points after implant placement using a Xenogen IVIS 200 system (Caliper Life Sciences). BLI results are reported as photons/second/cm²/steradian in the region of interest with subtraction of the background signal. Mice were euthanized 7 or 28 days after implant placement and osseointegration was measured by histomorphometry and biomechanical pull-out testing [1,2]. After pull-out testing, implants were sonicated and femurs were homogenized and bacterial CFUs were determined in the sonicates and the homogenates [3].

RESULTS

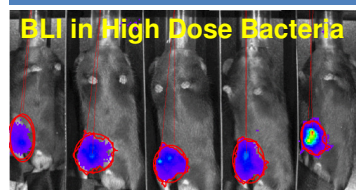
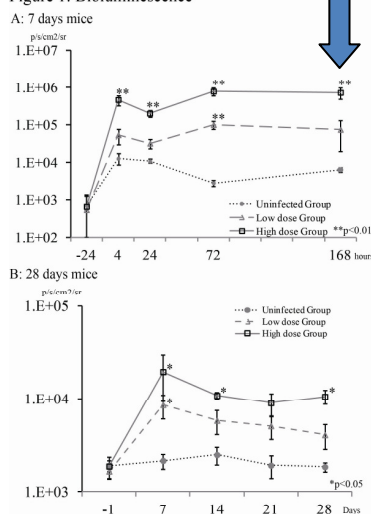


Figure 1: Bioluminescence



Dissected Femur



Biomechanical analysis

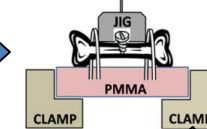
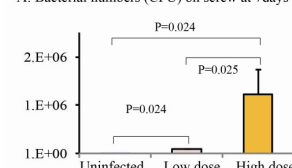


Figure 2: Colony forming units (CFUs)

A: Bacterial numbers (CFU) on screw at 7days



B: Bacterial numbers (CFU) in bone at 7days

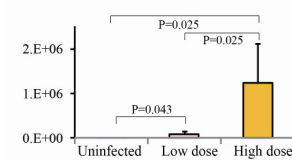
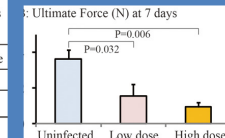


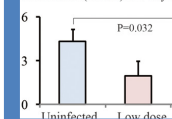
Figure 3: Osseointegration

A: Frequency of Gross Integration Failures

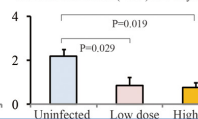
	Uninfected	Low dose	High dose
7 days	0/5	1/5	1/5
28days	0/5	0/5	2/5



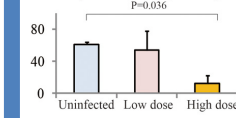
C: Stiffness (N/mm) at 7 days



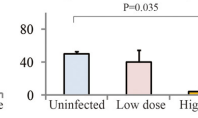
D: Work to Failure (x10⁻³) at 7 days



E: Bone-implant contact (%) at 28 days



F: Peri-implant bone (%) at 28 days



Histomorphometry

DISCUSSION

- Signs of systemic sepsis were not observed in any of the mice and the BLI imaging demonstrated that the infection was localized to the implant site.
- In mice without bacteria, BLI was low at all time points (Fig 1).
- Bacterial inoculation increased both BLI (Fig 1) and CFUs on the implants and in the bones (Fig 2).
- Osseointegration was significantly decreased in the groups with the higher dose of bacteria (Fig 3).
- The higher bacterial inoculation dose provides a quantitative and reproducible murine model of orthopaedic implant infection that includes impaired osseointegration.
- The long-term ability to detect BLI is likely due to the use of *S. aureus* with the *lux* operon in a stable plasmid as shown by other investigators [3].
- With this model, we are now in a position to assess novel methods of preventing/treating infections.

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ACKNOWLEDGEMENT

USAMRAA
Peer Reviewed Orthopaedic Research Program
Idea Development Award