Comparative Medicine
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Vol 51, No 1
February 2001
Pages 75-79

Therapeutic Effect of a Pig-Derived Peptide Antibiotic on Porcine Wound Infections

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Purpose: **We investigated the therapeutic potential of the pig-derived antimicrobial peptide protegrin-1 (PG-1) against porcine skin wounds infected with *Pseudomonas aeruginosa*.**

***Materials and Methods*: Using a porcine skin wound model, PG-1 was added to the wound fluid either at the time of *P. aeruginosa* inoculation, four hours after inoculation or 24 hours after inoculation. Wound fluids were analyzed 20-24 hours later by use of colony-forming unit (CFU) assays, semiquantitative immunoblot analysis for PG-1, and radial diffusion assays (RDA) for residual in vitro activity.**

Results: **Results of the CFU assays indicated a 10,000-fold decrease in the number of bacteria when PG-1 was added at the time of inoculation, a 120-fold decrease when added 4 hours after inoculation and a 10-fold decrease when added 24 hours after inoculation. Results of immunoblot analysis and RDA indicated that PG-1 concentrations for each of the three conditions remained increased in wound fluid 20 to 24 hours after treatment, and correlated with increased residual in vitro antimicrobial activity.**

Conclusions: **These results document that the endogenous antibiotic PG-1 significantly prevented the colonization of *P. aeruginosa* in wounds and reduced the in vivo bacterial concentration in established wound infections. Therapeutics used in the same animal species from which they were derived are a promising means for preventing and treating localized infections.**

Unfettered antibiotic use in agriculture to treat illnesses, prevent infections, and increase weight gain in animals has been implicated in the emergence of an increased number of antibiotic-resistant pathogens. Although resistant "superbugs" cause problems for the farm industry, recent evidence also indicates that human pathogens can acquire resistance to drugs that are similar to antibiotics used for livestock (1). Misuse and overuse of antibiotics in human and veterinary medicine, as well as in animal husbandry, have also been implicated in the emergence of resistant strains of microbes (2-5). Human and veterinary medicine would understandably benefit from a new class of broad-spectrum antibiotics that do not induce resistance in target organisms.

Antimicrobial peptides are naturally produced antibiotics that have been isolated from myeloid cells and the epithelial surfaces of most animal species tested to date (6). These peptides act in the first line of immune defense when the host is challenged by microbes, prior to activation of acquired immunity. Many antimicrobial peptides are broadly active against gram-positive and gram-negative bacteria, as well as fungi and some enveloped viruses (6-8). Antimicrobial peptides share common properties, such as low molecular weight and cationic charge at physiologic pH, that facilitate peptide binding and insertion into microbial membranes. Peptides then aggregate into pores, and it is thought that microbial lysis and ensuing death occur once a critical number of pores have formed (9, 10). Unlike many conventional antibiotics, most antimicrobial peptides do not readily induce resistance in target microorganisms (11). Many highly active peptides have 20 or fewer residues and can be readily produced either synthetically or in bioreactors. These combined features make antimicrobial peptides a class of novel antibiotics with potential veterinary and clinical applications (12).

One particularly active group of antimicrobial peptides, the protegrins, are found in the granules of porcine neutrophils (13). The five known protegrins (protegrins 1-5) are potent and broad antimicrobials with 16 to 18 residues, that are stored in the granules as inactive propeptides. Cleavage by the serine protease, neutrophil elastase, is required to release the active fragment in vitro (14, 15) and in vivo (16), and this cleavage occurs on neutrophil activation.

We previously documented that protegrins are the principal antibacterial substances in porcine wound fluids (15). Additionally, we reported that blocking the activity of elastase decreased in vivo activation of protegrins, which resulted in impaired clearance of *Staphylococcus epidermidis* and *Escherichia coli* from wound fluids (16). Supplementing the inhibited protegrin with a synthetic peptide that was identical to the endogenous peptide partially restored the microbicidal activity against *E. coli* in vitro, and *S. epidermidis* in vitro and in vivo. These observations motivated the study reported here, in which we investigated whether supplementing the concentrations of antimicrobial peptides with synthetic peptide can prevent and treat localized infections. It was recently reported that local application of IB-367, a congener of protegrins, reduced microbial densities in experimentally induced oral mucositis in hamsters (17) and that protegrin-1 had in vivo activity (18).

In this study, we used a porcine skin wound model to explore the therapeutic potential of topically administered protegrin-1 on wounds experimentally infected with *Pseudomonas aeruginosa*.

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Materials and Methods

Reagents: Protegrin-1 (PG-1), provided by Dr. Robert I. Lehrer (UCLA Department of Medicine), is a synthetic peptide based on its natural sequence. Synthetic protegrin was purified to > 96.5% homogeneity by use of reverse-phase high-performance liquid chromatography (RP-HPLC).

Microbe and culture conditions: A pathogenic strain of *P. aeruginosa*, H103, was provided by Dr. Robert E. W. Hancock (University of British Columbia, Vancouver, BC, Canada). The strain was first grown for 18 h at 30°C. Prior to use, it was subcultured in 50 ml 3% trypticase soy broth (TSB) at 1:100 dilution for 2.5 h at 37°C in an environmental shaking incubator (250 rpm) to obtain microbes in mid-logarithmic growth phase. Subcultures were centrifuged at 1,400× *g* for 10 min, washed in Hanks' balanced salt solution without phenol red (HBSS; Life Technologies, Gaithersburg, Md.), re-suspended in 5 ml of HBSS, and diluted to the desired concentration in HBSS. *Staphylococcus epidermidis* strain EGD, *E. coli* ML-35p, *C. albicans*, and *S. aureus* are laboratory test strains that were treated as described (19). For each bacterial strain, OD_{625} = 1.0 was equivalent to 2.5 x 10^8 colony-forming units (CFU)ml.

Housing and maintenance of pigs: Female Yorkshire pigs (40 to 60 lbs) were housed individually in 2-m^2 pens with automatic watering systems, and fed a minipig porcine diet (LabDiet 5P94, ProLab, Brentwood, Mo.). Room temperature was maintained at 19 to 24°C, with relative humidity between 40 and 60%, and the light cycle was 12/12 h light/dark throughout the study.

Porcine skin wound fluid generation: Under strict guidelines of the UCLA Animal Research Committee, porcine wound chambers were created as described by Gallo and colleagues, with slight modifications (20). Briefly, in pigs under general anesthesia with tiletamine/zolazepam combination (Telazol, Fort Dodge, Cherry Hill, N.J.; 5 mg/kg of body weight) and xylazine (AnaSed, Lloyd Laboratories, Shenandoah, Iowa; 2 mg/kg), their skin was cleansed and disinfected with 7.5% Povidone iodine scrub, followed by 70% isopropanol. Using a scalpel, four partial excisional wounds (2 cm^2, 1 to 1.5 mm deep) were created in the dorsum immediately caudal to the scapula, two at each side, equidistant to the midline. Polyvinyl chambers (P.A. Medical Inc, Columbia, Tenn.) were attached and secured by tape over each wound. Special padding was mounted around each chamber to ensure that the pig did not injure itself or dislodge the chamber. Infection was then induced in the wounds.

Protegrin treatments: Three sets of experiments were performed. First, *P. aeruginosa* in 2 ml of HBSS (2 × 10^6 CFU/ml) was introduced into the wound chamber in the presence or absence of 20 or 200 mg of PG-1/ml. After 24 h incubation, the wound fluid from each chamber was collected to determine CFU counts. Second, *P. aeruginosa* in 2 ml of HBSS (2 × 10^6 CFU/ml) was added to each of four chambers. After 4 h incubation, the fluids were collected for CFU assays and chambers were removed. Medium-viscosity carboxymethylcellulose (50 mg/chamber; Sigma Chemical Co., St. Louis, Mo.), completely dissolved in 0.5 ml of de-ionized water to create a thick paste, was used as a vehicle to deliver saline or 200 μg of PG-1 directly to the infected wound. The wounds were subsequently covered with sterile chambers. After 20 h additional incubation, the chambers were thoroughly washed with 2 ml of HBSS, and the entire fluid volume was recovered for analysis. Third, *P. aeruginosa* in 2 ml of HBSS (2 × 10^6 CFU/ml) was added to each of four chambers. After 24 h incubation, the fluids were collected for CFU assays and chambers were removed. Medium-viscosity carboxymethylcellulose (50 mg/chamber) was applied directly to the infected wound with or without 200 μg or 1 mg of PG-1 and the wounds were covered with new sterile chambers. After 24 h additional incubation, the chambers were thoroughly washed with 2 ml of HBSS, and the entire fluid volume was recovered for analysis. Aliquots of each set of wound fluids were clarified by centrifugation at 20,800× *g* for 3 min, and were immediately used for immunoblot analyses and radial diffusion assays or were stored at -20°C for further study.

Colony-forming unit assay: To determine bacterial concentration, 100 μl of porcine wound fluid was diluted in HBSS and hand spread on trypticase soy agar (TSA) plates. After 18 h incubation at 37°C, the colonies were counted and CFU/ml concentrations were calculated.

Radial diffusion assay: Radial diffusion assays were performed as described (21). The underlay consisted of 1% agarose and 1:100 dilution of TSB in 10 m*M* sodium phosphate, pH 7.4. Overlay consisted of 6% TSB and 1% agarose in phosphate-buffered saline (PBS). Bacteria (4×10^6) were mixed with 10 ml of underlay gel solution, kept molten at 48°C, and poured into 100-cm^2 square petri dishes. After the agarose solidified, a series of 3.2-mm diameter wells were punched and 5 μl of wound fluid or PG-1 was added to designated wells. Plates were incubated for 3 h at 37°C to allow peptide diffusion. The microbe-laden underlay was then covered with 10 ml of molten nutritive overlay, and the plates were allowed to harden. Antimicrobial activity was identified as a clear zone around the well after 18 h incubation at 37°C, and was represented in radial diffusion units (RDU): (diameter of clear zone [in mm] – 3.2 mm) × 10.

Immunoblot analysis for protegrins: Immunoblot analysis assays were performed for identification and semiquantitative analysis of PG-1. Briefly, wound fluids were subjected to acid-urea polyacrylamide gel electrophoresis (AU-PAGE). Proteins that migrated in the gel were immediately electroblotted to an Immobilon-P PVDF membrane in 0.7% acetic acid. Detection involved use of a monoclonal anti-PG antibody (1:1,000), alkaline phosphatase-conjugated goat anti-mouse IgG (1:2,000), and the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT). Concentrations of PG-1 in wound fluids were determined by comparing band intensities with those of synthetic PG-1 standards.

Statistics: Assays were performed in at least triplicate for each experiment and, when applicable, results from duplicate chambers were averaged for each animal. Sets of independent experiments were compared, using a paired *t*-test analyzing protegrin-treated against non-treated wound fluids (SigmaStat, SPSS Inc., Chicago, Ill.). Error bars represent standard errors of the mean (SEM).

Results

Selection of a bacteria that infects porcine wound fluids: We first sought a microbe that would cause persistent infection of porcine wound fluids. The in vivo antimicrobial activity of the naturally produced wound fluid was determined by CFU analysis against an inoculum (2 × 10^6 CFU/ml) of *P. aeruginosa* H103 (n = 8), *E. coli* ML-35p (n = 2), *S. aureus* (n = 2), *S. epidermidis* (n = 18) or *C. albicans* (n = 4), at 24 h after creation of the wound chamber. Wound fluid inhibited the growth of four of the five microbes tested (Fig. 1). Conversely, *P. aeruginosa* proliferated in porcine wound fluid to four logarithms above the

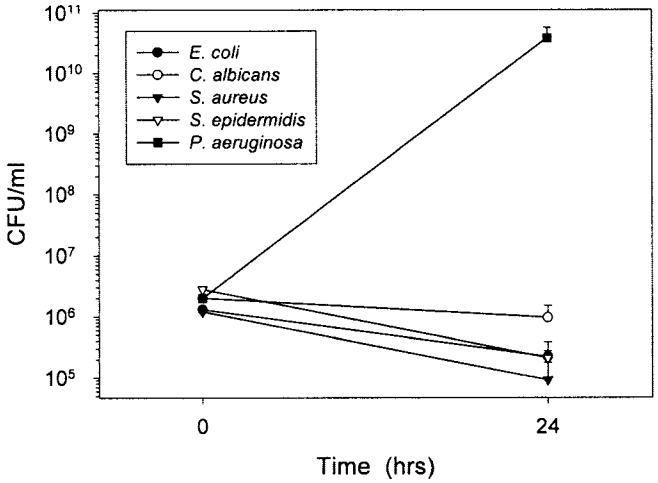

Figure 1. Growth rate of five microbes incubated in wound fluid in vivo. *Eshcherichia coli, Candida albicans, Staphylococcus aureus, S. epidermidis*, and *Pseudomonas aeruginosa* colony-forming units (CFUs) were counted 24 h after wound chamber preparation (n = 2 to 8). Only *P. aeruginosa* infected the wounds, growing to greater than four logarithms above the inoculum numbers. Error bars represent SEM.

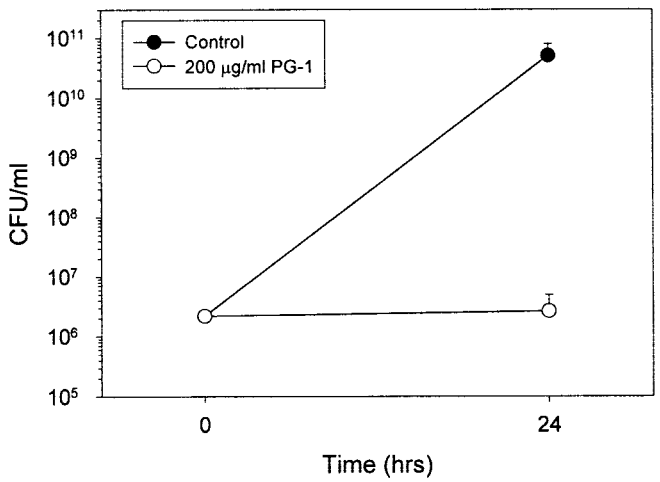

Figure 2. Protegrin 1 (PG-1) prevented infection of wounds by *P. aeruginosa*, which had been added to wound chambers in the presence or absence of 200 μg of PG-1/ml. The PG-1 prevented colonization of P. aeruginosa, compared with results for untreated fluids (n = 3; P < 0.003).

inoculum dose. Since it could infect our porcine wound model and commonly infects cutaneous lesions in humans and animals (22), *P. aeruginosa* was, therefore, used in the remaining experiments.

Protegrin treatments: In the first set of experiments (Fig. 2), PG-1 added at the same time as the inoculum resulted in a 10,000-fold decrease of bacterial CFU in treated, compared with non-treated fluids at 24 h (n = 3; $P < 0.003$). In the second set of experiments, fluids recovered after 4 h incubation in wound chambers without added PG-1 had 10-fold increase in CFU/ml (Fig. 3A). Wounds treated with PG-1 4 h after initial infection and examined 20 h after treatment contained 120-fold fewer CFU, compared with that in untreated wounds (n = 4; $P < 0.023$). In the third set of experiments, fluids recovered at 24 h had an approximate 10,000-fold increase in bacteria over numbers in the inoculum. Twenty-four hours after treatment, a 10-fold reduction of CFU observed in the PG-1-treated chambers was not significantly different from numbers in the control chamber, but followed the trend of the first two experiments (Fig. 3B).

Immunoblot analysis: For each of the three sets of experiments (PG-1 added simultaneously with inoculum, 4 h after the inoculum, or 24 h after the inoculum), the concentration of PG-1 remained significantly higher in the treated fluid than did concentration of naturally produced PG-1 in untreated fluid (Fig. 4; n = 3 or 4; $P < 0.01$).

Radial diffusion assays: There was a noticeable increase in the in vitro antimicrobial activity in the samples collected from all three sets of experiments when treated and untreated chambers were compared. However, statistical significance was reached only in samples where PG-1 was added simultaneously or 24 h after the inoculum (n = 3; $P \leq 0.013$) as shown in Fig. 5.

Discussion

Protegrin prevented *P. aeruginosa* wound infection. *Pseudomonas aeruginosa* (2×10^6 CFU/ml) was incubated in a porcine wound chamber in the presence or absence of 200 μg of PG-1/ml to determine whether protegrins could prevent bacterial colonization. Fluids from PG-1-treated and non-treated chambers

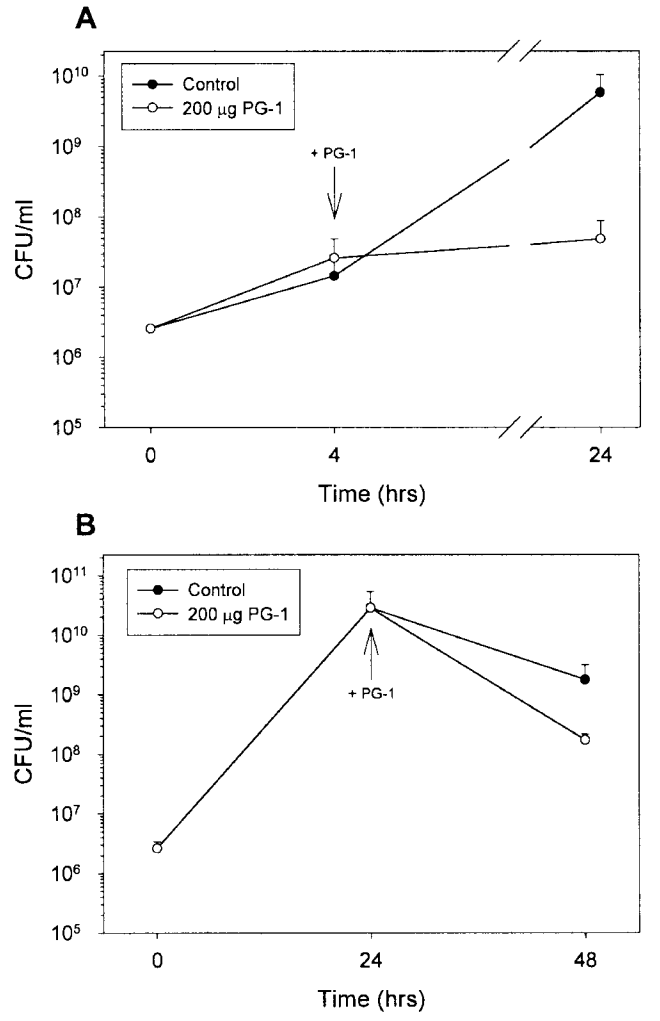

Figure 3. Protegrin 1 enhanced clearance of *P. aeruginosa* from established wound infections. The PG-1 (200 μg), in a carboxymethylcellulose paste, was added to wounds that were infected with *P. aeruginosa* for: (A) 4 h (n = 4) or (B) 24 h (n= 3). Bacterial CFUs, measured at (A) 24 or (B) 48 h, indicated that PG-1 treatment suppressed bacterial growth by 120- and 10-fold, respectively.

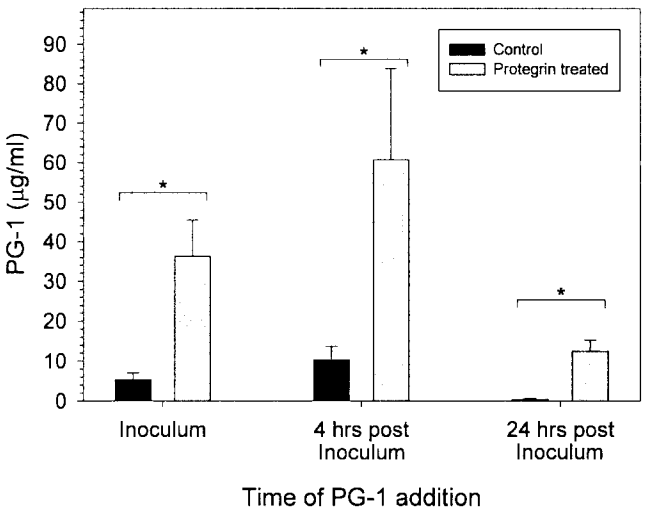

Figure 4. The PG-1 concentration in treated wound fluids was significantly higher than that in non-treated fluids. The PG-1 was added at the time of *P. aeruginosa* inoculation, 4 h after inoculation, or 24 h after inoculation, and wound fluids were assayed 20 to 24 h later by use of semiquantitative immunoblot analysis for PG-1. For each set of experiments, the concentration of PG-1 was significantly higher in PG-1-treated, compared with untreated fluids (n = 3 or 4; P < 0.010, indicated by asterisks). Error bars represent SEM.

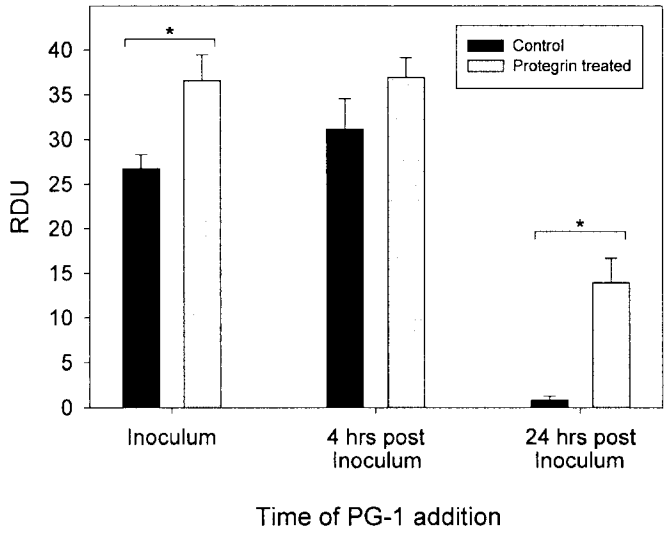

Figure 5. The effect of PG-1 treatment on antimicrobial activity of wound fluids in vitro. Wound fluids, collected either 24 h (PG-1 added simultaneously with inoculum or PG-1 added 4 h after the inoculum) or 48 h (PG-1 added 24 h after the inoculum) after *P. aeruginosa* inoculation, were subjected to radial diffusion assays (RDAs) against a laboratory strain of *S. epidermidis* under higher ionic (10 mM NaP/ 100 mM NaCl) buffer conditions. Antimicrobial activity, expressed as RD units (RDUs), was significantly increased in PG-1-treated fluids (shaded bars) in pairwise comparison with untreated fluids (P < 0.012, indicated by asterisks). Error bars represent SEM (n = 3 or 4).

were collected after 24 h and subjected to CFU assays. A marked greater than four logarithmic decrease of bacterial CFU was observed in the treated fluids. In additional experiments, we appreciated that PG-1 concentration as low as 20 μg/ml could also reduce colonization of *P. aeruginosa* by greater than one logarithm (data not shown). This would indicate that protegrins are effective in inhibiting bacterial colonization of the wound, and activity is directly correlated with PG-1 concentration.

Protegrins enhanced clearance of *P. aeruginosa* from established wound infections. To determine the therapeutic effect of PG-1 on established wound infections, wound chambers were inoculated with *P. aeruginosa* and allowed to incubate in vivo for 4 h. Fluids recovered at 4 h had an approximate 10-fold increase in CFU/ml. Carboxymethylcellulose paste with 200 μg of PG-1 applied to the wounds for an additional 20 h resulted in a significant 120-fold decrease in the CFU counts of treated, compared with untreated wounds (n = 4; $P < 0.023$). In a separate set of experiments, *P. aeruginosa* were incubated in vivo for 24 h. Fluids recovered at 24 h had a four logarithmic increase in bacteria over numbers in the inoculum.

Carboxymethylcellulose paste with or without 200 μg of PG-1 was applied to the wounds for an additional 24 h. Although there was a 10-fold reduction in CFU in the PG-1-treated chambers at 48 h, these values were not significantly different from those in the control chamber. One additional experiment involving use of 1 mg of PG-1, although documenting antimicrobial activity, did not prove more beneficial in reducing *P. aeruginosa* CFU for the PG-1-treated fluid compared with use of 200 μg of PG-1 (data not shown). These results indicate that a "ceiling effect" was reached, and treating wound infections with higher doses of PG-1 would not be substantially beneficial. However, increasing the dosing frequency could prove advantageous in future pharmacokinetic studies.

Higher concentrations of PG-1 were detected in treated wound fluids. Semiquantitative immunoblot analyses were performed to determine the concentration of PG-1 that persisted in wound fluid 20 or 24 h after treatment. For each of the three conditions (PG-1 added simultaneously with inoculum, 4 h after the inoculum, or 24 h after the inoculum), the concentration of PG-1 remained significantly higher in the treated fluid than did the concentration of naturally produced PG-1 in untreated fluid. However, for each chamber, the total amount of recovered PG-1 was severalfold less than the original treatment concentration of 200 μg/ml. This is not surprising since protegrins avidly interact with bacterial membranes (23) and immunoblot analysis was performed on fluid from which cells and bacteria had been removed.

The in vitro antimicrobial activity of porcine wound fluid was correlated with PG-1 concentration. Radial diffusion assays were performed on wound fluid, collected either 24 h (PG-1 added simultaneously with inoculum or PG-1 added 4 h after the inoculum) or 48 h (PG-1 added 24 h after the inoculum) after *P. aeruginosa* inoculation, to measure the residual antimicrobial activity and to determine whether the level of activity correlated with PG-1 treatment. For two of the three conditions (PG-1 added simultaneously with inoculum or 24 h after the inoculum), there was a significant increase in antimicrobial activity against our laboratory test strain, *S. epidermidis* EGD (Fig. 5; n= 3; $P \leq 0.013$). Although there was also a noticeable increase in antimicrobial activity in samples where PG-1 was added 4 h after the inoculum, it did not reach statistical significance (n = 4; $P > 0.1$).

Results of these experiments taken together indicate that: PG-1 can reduce the bacterial concentration in infected wounds, using either HBSS or carboxymethylcellulose paste as vehicles; PG-1 remains in the wound fluid even 24 h after treatment; and 24 h after treatment, the wound fluids still retained in vitro antimicrobial activity.

Use of conventional antibiotics to topically treat skin and

mucosal infections caused by *P. aeruginosa* and other bacteria is frequently accompanied by adverse effects, such as allergic reactions or microbial resistance (24). Therefore, our finding that the pig's own peptide can be used to supplement its innate immune system has important implications for antibiotic discovery. Pigs, as an example, have a number of other antimicrobial peptides in addition to PG-1, including four additional protegrins, prophenins, PR-39, porcine myeloid antimicrobial peptides (PMAPs), and the β-defensin PBD-1 (13, 25-32). Each peptide has a broad spectrum of activity; however, certain peptides are more effective against specific microbes, and some peptides synergize with one another. It remains to be seen whether appropriate mixtures of these peptides could affect a wider range of pathogens, using lower peptide concentrations.

Acknowledgments

We thank Timothy Lawson, DVM, Director, Division of Laboratory Animal Medicine, UCLA, for his guidance and support of these studies. Additionally, we thank Robert I. Lehrer, MD (UCLA Department of Medicine) for generously donating PG-1, permitting use of his labspace and equipment, and his continued enthusiasm and support of our projects. This work was supported by United States Department of Agriculture National Research Initiative Grant 98-35204-6594 (T.G.), by grant HL46809 from the National Institute of Health (T.G.) and by a postdoctoral fellowship from the American Lung Association (A.M.C.). Animal husbandry was partially subsidized by the Department of Laboratory Animal Medicine, UCLA.

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