ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1997, p. 1738–1742 Vol. 41, No. 8
0066-4804/97/$04.00+0

Protegrin-1: a Broad-Spectrum, Rapidly Microbicidal Peptide with In Vivo Activity

DEBORAH A. STEINBERG,[1]* MALINDA A. HURST,[1] CRAIG A. FUJII,[1] ADA H. C. KUNG,[1] J. F. HO,[2] F.-C. CHENG,[2] DAVID J. LOURY,[1] AND JOHN C. FIDDES[1]

IntraBiotics Pharmaceuticals, Inc., Sunnyvale, California 94086,[1] and Panlabs, Taipei, Taiwan[2]

Received 21 January 1997/Returned for modification 7 April 1997/Accepted 4 June 1997

Protegrin-1 (PG-1) is a cysteine-rich, 18-residue β-sheet peptide isolated from porcine leukocytes with antimicrobial activity against a broad range of microorganisms. The MICs of PG-1 against representative gram-positive and gram-negative bacteria ranged from 0.12 to 2 μg/ml. At these levels, PG-1 was rapidly bactericidal in vitro, reducing the number of viable CFU of either methicillin-resistant *Staphylococcus aureus* (MRSA) or *Pseudomonas aeruginosa* by more than three log units in less than 15 min. Resistance to PG-1 did not develop after 11 subculturings of *P. aeruginosa* or 18 subcultures of MRSA in Mueller-Hinton broth containing PG-1 at one-half the MIC. Under similar conditions of serial passage, the MICs of norfloxacin and gentamicin against *P. aeruginosa* increased 10 and 190 times, respectively. Similarly, the MIC of norfloxacin against MRSA increased 85 times. Immunocompetent mice inoculated intraperitoneally (i.p.) with *P. aeruginosa* or *S. aureus* exhibited 93 to 100% mortality in the vehicle control group compared with 0 to 27% mortality in animals that received a single i.p. injection of PG-1 (0.5 mg/kg of body weight). Mice inoculated with *S. aureus* by intravenous (i.v.) injection and dosed 0 to 60 min later with a single i.v. injection of PG-1 (5 mg/kg) had a mortality of 7 to 33%, compared to a mortality of 73 to 93% in the vehicle controls. In leukopenic mice inoculated i.v. with vancomycin-resistant *Enterococcus faecium*, mortality was 87% in the vehicle control group and 33% in animals that received a single i.v. injection of PG-1 (2.5 mg/kg). Taken together, these data indicate that PG-1 has potential for use as an antimicrobial agent in the treatment of local or systemic infections caused by clinically relevant pathogens.

Antimicrobial peptides have been identified from extremely diverse organisms, including bacteria, vertebrates, invertebrates, and plants (3, 6, 7, 10, 17, 20). In mammals, such peptides are part of the host defense mechanisms and exist either in phagocytic cells, such as neutrophils, or on epithelial surfaces, such as those of the airways and the gastrointestinal tract. Evidence that the production of these peptides can be increased by local stimuli such as infection or lipopolysaccharides has been accumulating (19, 22). Many host defense peptides are broad-spectrum, microbicidal agents that act rapidly.

Antimicrobial peptides have been divided into the following four structural subclasses: α-helices, extended helices with one or more-predominant amino acids, β-sheets, and peptides with loop structures (6). Each subclass contains a variety of primary sequences (7). Representative α-helical peptides include the frog skin-derived magainins and the insect-derived cecropins; representative β-sheet peptides include the mammalian defensins and porcine protegrins (5, 8, 24, 28). The mammalian peptides PR-39 and prophenins are examples of peptides with extended helices (1, 9).

The protegrin family of antimicrobial peptides was identified in porcine leukocytes (12). Five native protegrin sequences (PG-1 to PG-5) have now been identified (29, 30). These native protegrins contain 16 to 18 amino acids, are highly homologous, and share the common feature of being cationic, amphipathic β-sheets. The molecules have disulfide bridges between cysteine residues at positions 6 and 15 as well as positions 8 and 13 (2, 4, 8). Members of the protegrin family have been shown to exhibit broad-spectrum antimicrobial and anti-human immunodeficiency virus type 1 activity in vitro (12, 18, 26, 27).

We report an evaluation of the in vitro antimicrobial activity of PG-1 (amino acid sequence, RGGRLCYCRRRF CVCVGR$_{CONH2}$) against a range of microorganisms and present data demonstrating the in vivo efficacy of PG-1 in murine models of local and systemic infection.

MATERIALS AND METHODS

Bacterial strains. Strains obtained from the American Type Culture Collection (ATCC; Rockville, Md.) included *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027 and ATCC 27853), methicillin-sensitive *Staphylococcus aureus* (MSSA; Smith type ATCC 19636, and ATCC 29213), methicillin-resistant *S. aureus* (MRSA; ATCC 33591), vancomycin-sensitive *Enterococcus faecalis* (ATCC 29212), and *Candida albicans* (ATCC 10231). Additional strains included *E. coli* 004 (10), *E. coli* BAS849 (21), and vancomycin-resistant *Enterococcus faecium* (VREF) 032 and VREF 033 (clinical isolates, kindly provided by G. Schoolnik, Stanford University, Calif.). All strains were analyzed with API test strips (BioMerieux, Hazelwood, Mo.) to confirm identity and were stored frozen in 10% glycerol at −85°C.

Media for in vitro assays. All media were prepared in distilled, deionized water. Mueller-Hinton broth (MHB), Trypticase soy broth (TSB), and Trypticase soy agar (TSA) were purchased from Becton-Dickinson, Cockeysville, Md. Blood agar plates (BAP) containing TSA with 5% sheep blood added were purchased from Hardy Diagnostics, Santa Maria, Calif. Liquid testing medium (LTM) contained the following: 10 mM phosphate buffer (pH 6.5), 1% TSB, and 100 mM NaCl. Agarose (Sigma Chemical Co., St. Louis, Mo.) was added to liquid testing medium at a final concentration of 1% for the zone diffusion assay.

Reagents. Norfloxacin, rifampin (both 95% pure by high-performance liquid chromatography (HPLC), nystatin (5,010 U/mg), amphotericin B (80% pure by HPLC), vancomycin (1,118 μg/mg), polymyxin B (7760 U/mg), and gentamicin (647 μg/mg) were obtained from Sigma Chemical Co. Piperacillin was kindly provided by Wyeth-Ayerst, Pearl River, N.Y. PG-1 was synthesized in-house with Rink-amide resins, 9-fluorenylmethoxycarbonyl solid-phase chemistry, and an automated peptide synthesizer (model 431A; Applied Biosystems, Foster City, Calif.). Following air oxidation, the refolded peptide was analyzed by reverse-phase HPLC (Vydac C18 [0.1% trifluoroacetic acid, 21 to 49% acetonitrile gradient in 30 min, flow rate of 0.8 ml, detection at 214 nm]). Unless otherwise noted, the amide form of PG-1 (≥96% purity as determined by area normalization of the HPLC peak) was used in all studies.

* Corresponding author. Mailing address: IntraBiotics Pharmaceuticals, Inc., 816 Kifer Rd., Sunnyvale, CA 94086. Phone: (408) 991-6439. Fax: (408) 991-6401. E-mail: dsteinb@ibm.net.

In vitro susceptibility testing. We noted two problems when using the standard microbroth dilution assay recommended by the National Committee for Clinical Laboratory Standards (NCCLS) to determine in vitro antimicrobial activities of peptides: (i) PG-1 precipitated in MHB at concentrations ≥128 μg/ml; and (ii) PG-1 adsorbed to glass and plasticware which caused significant reduction of available peptide at concentrations of ≤25 μg/ml (16). To compensate for these problems, we developed a slightly modified version of the NCCLS method in which we prepared 10× peptide solutions in 0.01% acetic acid containing 0.2% bovine serum albumin (BSA) as a carrier protein, rather than in MHB. In this modified NCCLS broth microdilution method, inocula were prepared with the appropriate McFarland standard and then diluted into fresh MHB to give 2×10^5 to 7×10^5 CFU/ml for bacteria or 2×10^3 to 7×10^3 CFU/ml for *Candida*. After 100-μl aliquots of the microbial suspension were dispensed into each well of a 96-well polypropylene microtiter plate, 11 μl of test compound was added. As described above, peptides were dissolved in 0.01% acetic acid containing 0.2% BSA (Intergen, Purchase, N.Y.) at 10× the desired test concentration in polypropylene minitubes. Vancomycin, polymyxin B, and gentamicin were dissolved in water, whereas norfloxacin, rifampin, nystatin, and amphotericin B were dissolved in 100% dimethyl sulfoxide and then serially diluted in water. The MIC was defined as the lowest concentration of drug which prevented visible turbidity after 18 to 20 h at 35°C. Minimum bactericidal concentrations were determined by transferring 50 μl from each clear well (≥MIC) to an individual well of a 24-well microtiter plate containing 1.5 ml of TSA with 10% fetal bovine serum (Cool Calf; Sigma). After incubation for 20 h, the minimum bactericidal concentration was identified as the lowest concentration that did not permit any visible growth on the surface of the agar.

Resistance studies. MRSA and *P. aeruginosa* were harvested from the well equal to one-half of the MIC, diluted to 1×10^5 to 5×10^5 CFU/ml in fresh MHB, and dispensed into microtiter plates as 100-μl aliquots. Compounds were added as described above, and MICs were determined daily for up to 18 serial passages.

Microbicidal assays. Bacteria were grown overnight in MHB (10 ml in a 50-ml Erlenmeyer flask) at 200 rpm and at 37°C to stationary phase. The stationary-phase cultures were centrifuged, resuspended in filter-sterilized spent medium (MRSA) or LTM (*P. aeruginosa*) at 4×10^5 CFU/ml, and then dispensed into polypropylene microcentrifuge tubes. Exponential-phase cultures were prepared by diluting the overnight culture 1:100 in fresh MHB and incubating at 200 rpm at 37°C until an A_{600} of 0.2 was reached. The culture was then diluted to 1×10^5 to 4×10^5 CFU/ml in MHB, reincubated to allow for two cell doublings, and then dispensed into polypropylene microcentrifuge tubes. After addition of the test compounds at a 1/10 volume, the tubes were incubated without aeration at the appropriate temperature. Viable CFU counts were determined by a pour plate method in which 20-μl aliquots of serial dilutions in PBS were mixed with approximately 20 ml of tempered (50°C) TSA. This method allowed for rapid sampling, minimized drug carryover, i.e., ≤0.01× the MIC, and precluded the need for washing the cells to remove the drug. After the agar solidified, the plates were incubated for 18 to 24 h at the appropriate temperature. Colonies were enumerated to determine the microbicidal effect of the drug.

Measurement of levels of PG-1 in plasma. Male ICR mice, 4 to 6 weeks old (Charles River Laboratories, Hollister, Calif.), were used to assess levels of PG-1 in plasma following intravenous (i.v.) and intramuscular (i.m.) dosing. Twelve mice received a 4-mg/kg of body weight dose of PG-1 in phosphate-buffered saline (PBS) via tail vein injections, and another 12 mice were administered an 8-mg/kg dose of PG-1 by i.m. injection. Blood samples were collected by cardiocentesis from groups of three mice, each at different intervals from 5 to 120 min after dosing. The mice were anesthetized with Metofane (Mallinckrodt Veterinary, Inc., Mundelein, Ill.), blood was collected by cardiocentesis, and the animals were euthanized by cervical dislocation. After transferring the blood to 2.0-ml vacutainers (Becton Dickinson Co., Franklin Lakes, N.J.) containing EDTA as an anticoagulant, plasma samples were prepared by centrifugation and stored frozen at −20°C. The plasma samples were analyzed for PG-1 content by a modification of an agarose diffusion assay previously reported (13). Briefly, *E. coli* BAS849 was cultured overnight at 37°C on BAP and then resuspended in PBS (pH 6.5) to an A_{600} of 0.2 (approximately 4×10^7 CFU/ml). A 100-μl aliquot of this suspension was added to 10 ml of LTM-agarose (tempered to 48°C), which was inverted three times to mix, and the contents were poured into a 10-cm^2 polystyrene petri dish (Nunc, Naperville, Ill.). After the agarose solidified, 3-mm plugs were removed in an evenly spaced array (4 by 4). An aliquot (5 μl) of plasma or control material was added to each well, the plate was incubated at 37°C for 3 h, and then the plate was overlaid with 10 ml of 1% agarose in double-strength (2×) TSB. Following further incubation at 37°C for 18 to 24 h, the diameter of the clear zone of growth inhibition was measured with a ×7 magnifier (Bausch and Lomb, Buffalo, N.Y.). The concentration of PG-1 in the test sample was calculated from a standard curve generated by measuring the zone diameters for known concentrations of compound diluted in plasma.

Murine infection assays. Male ICR mice (4 weeks of age; approximately 20 g) were obtained from the Animal Supply Center, Medical School of the National Taiwan University, Taipei, Taiwan. Bacteria were cultured in brain heart infusion broth (Difco, Detroit, Mich.) with aeration at 37°C for 8 h to obtain log-phase growth. Inocula containing a quantity of bacteria expected to result in 90 to 100% mortality were prepared by diluting the overnight cultures in PBS. The numbers of viable bacteria in the inocula were estimated based on the optical density at 550 nm and were verified by plating serial dilutions onto TSA. Depending on the test organism and the route of administration, inocula ranged from 4.5×10^6 to 1.4×10^9 CFU per mouse. Fifteen mice per dose level were infected and monitored for survival over a 7- to 10-day period after infection.

In the peritonitis model, mice were given 0.5 ml of vehicle (PBS) or PBS containing an antimicrobial agent by intraperitoneal (i.p.) injection immediately after the i.p. bacterial challenge. Bacteremia was established in immunocompetent mice by i.v. injection of MRSA at a level expected to result in 90 to 100% mortality. In order to establish bacteremia with VREF, mice were pretreated with 200-mg/kg cyclophosphamide (i.p.) 4 days prior to bacterial challenge. This treatment had been shown to reduce levels of circulating leukocytes from a predose level of approximately 8,000 to 750/μl 4 days after dosing. Leukopenic mice were infected by i.v. injection of VREF at a level expected to result in 90 to 100% mortality. Immediately following injection of the inoculum, the mice received a 0.2-ml i.v. injection of vehicle (PBS) or test formulation containing various concentrations of PG-1. In a second set of protection experiments, mice challenged i.v. with MRSA were given PG-1, PG-1 acid, or vancomycin by i.v. injection immediately after infection or were given PG-1 i.v. at various times between 15 and 120 min after infection.

TABLE 1. Effect of method on MIC

Organism and ATCC no.	Compound	MIC by the following methods (μg/ml):		Acceptable MIC range
		Modified method	NCCLS	
S. aureus 29213	PG-1	1.7	8	NA[a]
	Vancomycin	1	0.7	0.5–2.0
	Ciprofloxacin	0.5	0.3	0.12–0.5
	Tobramycin	0.125	0.42	0.12–1.0
E. coli 25922	PG-1	0.75	11	NA
	Vancomycin	>128	>128	None
	Ciprofloxacin	0.004	0.004	0.004–0.015
	Tobramycin	0.5	0.33	0.12–1.0
P. aeruginosa 27853	PG-1	0.5	14.7	NA
	Vancomycin	>128	>128	None
	Ciprofloxacin	0.25	0.19	0.25–1.0
	Tobramycin	0.25	0.25	0.25–1.0
E. faecalis 29212	PG-1	2.7	16	NA
	Vancomycin	2	2	1.0–4.0
	Ciprofloxacin	1	0.5	0.25–2.0
	Tobramycin	8	16	8.0–32.0

[a] NA, not applicable.

RESULTS

In vitro activity. MICs determined by the standard NCCLS broth microdilution method were compared to those obtained in our modified version (Table 1). Both methods provided acceptable MICs for conventional antimicrobial agents against the NCCLS reference strains, thus validating the modification. However, the MICs of PG-1 were approximately fivefold lower against *S. aureus* and *E. faecalis* and 15 to 30 times lower against *E. coli* and *P. aeruginosa* when measured by our modified method. By the modified NCCLS method, PG-1 exhibited excellent activity against representative strains of gram-positive and gram-negative bacteria and moderate activity against *C. albicans* (Table 2).

PG-1 was rapidly bactericidal against exponentially growing cultures of MRSA in MHB (Fig. 1A). Viable CFU decreased by 3 log units within 10 min after addition of PG-1 (5 μg/ml). In contrast, addition of gentamicin (4 μg/ml) or norfloxacin (8 μg/ml) required ≥4 h to effect the same decrease in CFU. Vancomycin (16 μg/ml) was not bactericidal in this time period but did show reduced CFU after 24 h (data not shown). When tested against stationary-phase MRSA in spent MHB, PG-1 reduced CFU by 4 log units within 4 min, whereas gentamicin, norfloxacin, and vancomycin were completely ineffective at 16 μg/ml, even after 4 h (Fig. 1B). Although the numbers below

TABLE 2. Antimicrobial activity of PG-1 by the modified NCCLS broth microdilution method

Compound	MIC (μg/ml)				
	E. coli 004	*P. aeruginosa* ATCC 9027	MRSA ATCC 33591	VREF 032	*C. albicans* ATCC 10231
PG-1	0.12	0.5	2	0.25	8
PG-1 acid	ND[a]	1.0	8.0	0.25	4.0
Piperacillin	>128	4	128	>128	>128
Gentamicin	0.5	0.5	1	>128	>128
Norfloxacin	<0.06	0.25	0.5	16	128
Vancomycin	>128	>128	1	>128	>128
Polymyxin B	0.25	0.5	>128	64	64
Nystatin	ND	ND	ND	ND	0.25
Amphotericin B	ND	ND	ND	ND	<0.06

[a] ND, not determined.

the dashed line in Fig. 1 are not accurate determinations, they suggest continued decrease in CFU over the time period.

Similar results were observed for exponentially growing cultures of *P. aeruginosa*. PG-1 (4 μg/ml) reduced CFU more rapidly than either norfloxacin or gentamicin (2 and 4 μg/ml, respectively), and the level of reduction was similar to that observed for polymyxin B (Fig. 2). In contrast, norfloxacin and gentamicin were relatively ineffective at reducing CFU of stationary-phase *P. aeruginosa*, whereas PG-1 was similar to polymixin B in reducing CFU (data not shown).

When cultures of MRSA were serially passaged 18 times in MHB at one-half of the MIC, resistance to norfloxacin (MIC increased by 85-fold) but not vancomycin developed. MICs for norfloxacin and gentamicin increased by 10 and 190 times after only 11 transfers of *P. aeruginosa*. In contrast, repeated exposure to one-half of the MIC levels of PG-1 did not engender resistance in either organism.

Concentrations of PG-1 in plasma. The average concentration of PG-1 in plasma 5 min after i.v. dosing with 4-mg/kg PG-1 was 28 μg/ml (Fig. 3). The concentrations of PG-1 declined rapidly and were below the limit of detection (0.25 μg/ml) by 120 min after dosing. No detectable levels of PG-1 were present in plasma prepared from blood samples collected 10, 20, 40, 60, and 120 min after i.m. injection at a dose of 8 mg/kg.

In vivo efficacy. The abilities of PG-1 to protect mice from a lethal challenge of bacteria from infections with MRSA, MSSA, VREF, and *P. aeruginosa* were determined (Table 3). For peritoneal infections with MRSA, MSSA, or *P. aeruginosa*, PG-1 administered i.p. at a dose of 0.5 mg/kg reduced mortality from 93 to 100% in the vehicle control group to 0 to 27%. For

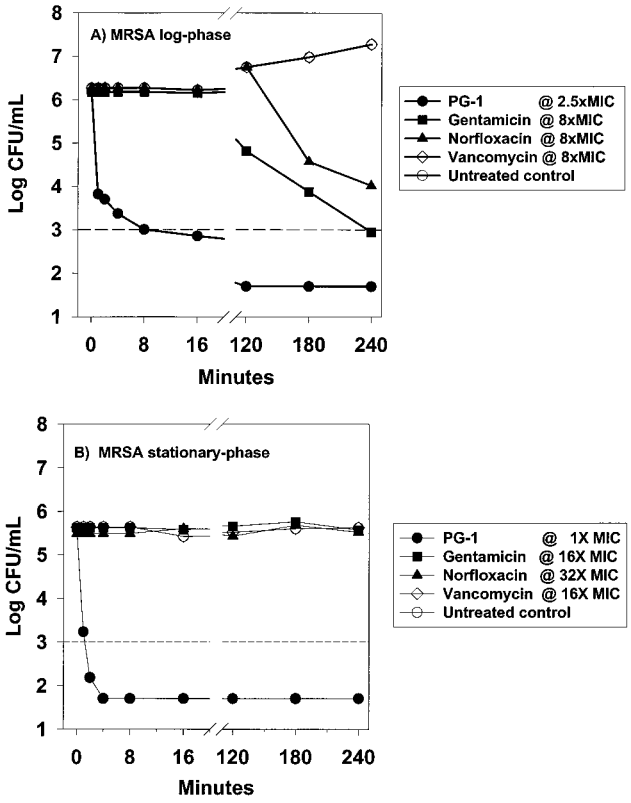

FIG. 1. Bactericidal activity of PG-1 against log- and stationary-phase MRSA. (A) MRSA was grown to log phase, and aliquots were treated with compounds at incremental concentrations relative to MICs. (B) MRSA was grown to stationary phase and resuspended in spent MHB at 5×10^5 CFU/ml and then treated as described for panel A. MICs (in micrograms per milliliter) were determined by the modified NCCLS broth microdilution method to be 2.0 for PG-1, 1.0 for gentamicin, 0.5 for norfloxacin, and 1.0 for vancomycin. At the indicated times, survivors were enumerated by the pour plate method. The dashed line represents the minimum number of CFU per milliliter which could be accurately determined.

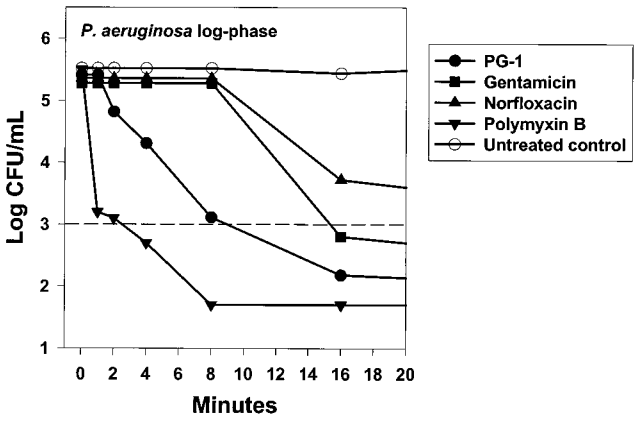

FIG. 2. Bactericidal activity of PG-1 against log-phase *P. aeruginosa*. Exponentially growing *P. aeruginosa* was treated with compounds at eight times the MICs. MICs (in micrograms per milliliter) were determined by the modified NCCLS method to be 0.5 for PG-1, 0.5 for gentamicin, 0.25 for norfloxacin, and 0.5 for polymyxin B. At the indicated times, survivors were enumerated by the pour plate method. The dashed line represents the minimum number of CFU that could be accurately determined.

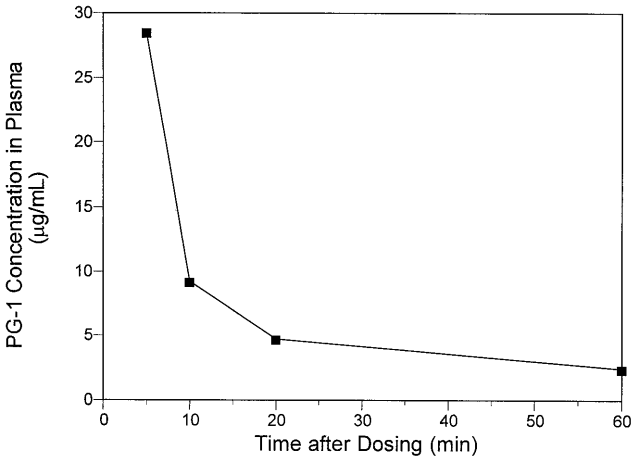

FIG. 3. Elimination of PG-1 from plasma. PG-1 (4 mg/ml) in PBS was administered to mice by i.v. injection at 0 min. Blood was obtained by cardiocentesis from three mice per sample time, and plasma samples were prepared by centrifugation. Concentrations of PG-1 in plasma were determined by microbiological assaying (the limit of detection was 0.25 μg/ml).

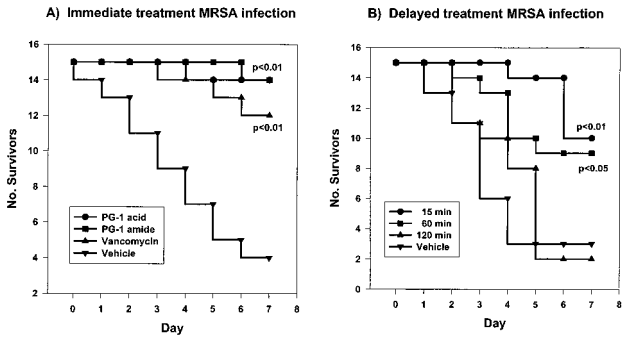

FIG. 4. Evaluation of PG-1 against MRSA in a bacteremia infection model. MRSA was administered to mice in 0.2 ml of PBS by i.v. injection. PG-1 acid, PG-1 amide, or vancomycin was administered at 5 mg/kg as a single i.v. injection immediately after challenge with 5.5×10^7 CFU (A). In a separate study, PG-1 (5 mg/kg) was delayed for up to 120 min after injection of 3.7×10^7 CFU (B). The numbers of surviving mice were determined daily for 7 days. Statistical significance was determined by Fisher's exact test.

i.v. infections with MRSA, a single i.v. injection of PG-1 at 2.5 mg/kg limited mortality to 33%, compared with 93% mortality in the vehicle-treated controls. Similarly, mortality in mice with i.v. infections caused by VREF was reduced from 87 to 33% in animals receiving a single i.v. injection of PG-1 at 2.5 mg/kg.

By the i.v. infection model, we compared the efficacies of both the acid and amide forms of PG-1 to vancomycin. At an i.v. dose of 5 mg/kg, mortality was 7% with either form of PG-1 and was 20% with vancomycin (Fig. 4A). In the concurrent vehicle control group, mortality was 73%. PG-1 was also capable of protecting mice even when it was administered up to 60 min after the bacterial challenge (Fig. 4B). However, mice were no longer protected from bacterial challenge when i.v. administration of PG-1 was delayed by 120 min.

DISCUSSION

Various antimicrobial activities have been reported previously for protegrins (2, 12, 18, 26). However, the methods used to determine microbicidal activities have varied. In one report, MICs of PG-1 against *S. aureus* and *E. coli* were as high as 64 μg/ml (2). Other studies have employed an agarose radial diffusion assay rather than conventional MIC methods and obtained microbicidal values in the 1- to 10-μg/ml range (12, 18). Significantly lower inhibitory concentrations were also reported for a related peptide, defensin, when it was assayed by the radial diffusion assay compared with the NCCLS broth microdilution method (25).

In this study, we report activity equivalent to that of conventional antimicrobial agents for PG-1 against strains representative of gram-positive and gram-negative bacteria. Although PG-1 showed moderate activity against *C. albicans*, the MIC may be higher than plasma levels that can be readily maintained in vivo. Modifications to the standard NCCLS broth microdilution assay were necessitated due to precipitation of the peptides in MHB and adsorption of the peptide to glass and polystyrene surfaces. The latter has been reported for other cationic peptides and was effectively blocked by the addition of surfactants or carrier protein (11, 23). These slight modifications of the standard NCCLS method decreased MICs by as much as 30 times in our study but had no demonstrable effect on the activity of conventional antimicrobial agents, thus validating the assay modifications.

The rapid bactericidal activity observed for PG-1 against both log- and stationary-phase cells was consistent with previous studies suggesting that cationic peptides disrupt the integrity of the bacterial cell membrane (4, 6). Similarly, the rapid fungicidal activity observed at four to eight times the MIC (i.e., 32 to 64 μg/ml) against *C. albicans* (data not shown) was in agreement with the ability of PG-1 to increase the conductance of eukaryotic membranes at concentrations of ≥20 μg/ml (15). However, this effect of PG-1 on conductance was reversible at concentrations of as much as 200 μg/ml, suggesting that cytotoxic doses may be much higher than those required to induce a disturbance in the electrical potential of the eukaryotic mem-

TABLE 3. In vivo activity of PG-1 in mice

Organism	Strain	Route[a]	Inoculum (CFU)[b]	Effective dose of PG-1 (mg/kg)	Mortality (%)	
					PG-1	Vehicle[c]
MSSA	ATCC 19636	i.p./i.p.	5.0×10^7	0.5	0	100
MRSA	ATCC 33591	i.p./i.p.	3.2×10^7	0.5	13	93
P. aeruginosa	ATCC 9027	i.p./i.p.	4.5×10^6	0.5	27	100
MRSA	ATCC 33591	i.v./i.v.	7.0×10^7	5.0	33	93
VREF	Clinical isolate 033	i.v.[d]/i.v.	1.4×10^9	2.5	33	87

[a] Administration of inoculum/administration of test compound.
[b] Total CFU administered in PBS per mouse.
[c] Mortality in infected animals treated with vehicle (PBS). Vehicle alone exhibited no effect on noninfected animals.
[d] Mice were treated with cyclophosphamide to induce neutropenia prior to administration of VREF inoculum.

brane. The rapid microbicidal activity of PG-1, especially against stationary-phase cells, may offer significant advantages over conventional antimicrobial agents that require longer exposure periods in vivo.

Repeated subculture of bacteria in the presence of PG-1 at one-half of the MIC did not result in the development of resistance. In parallel studies with the fluoroquinolone norfloxacin, resistance was readily engendered. The inability to engender protegrin resistance under laboratory conditions may be related to the speed of action of the peptide and the nature of its electrostatic interaction with the negatively charged phospholipid of the bacterial cell membrane.

Administration of PG-1 i.v. protected mice from a lethal challenge of *S. aureus* or VREF. In these studies, PG-1 was as effective as vancomycin in treating MRSA-induced bacteremias and could be delivered up to 60 min after infection. The lack of protection by PG-1 when administered 120 min after infection may be due to limited tissue penetration by the peptide. Pharmacokinetic experiments performed in mice in which PG-1 was administered by the i.m. route indicated that the peptide does not translocate appreciably from the site of injection. Bacteria localizing in tissues following i.v. dosing may, therefore, not be exposed to effective concentrations of PG-1.

When PG-1 was given i.p. to treat peritoneal infections caused by either *S. aureus* or *P. aeruginosa*, the protective dose was significantly lower (0.5 mg/kg) than that required for protection against systemic infections. i.p. administration of PG-1 either immediately or up to 60 min after i.p. infection with MSSA was equally protective (data not shown). This route of administration is preferred for antimicrobial therapy of peritonitis in patients receiving chronic ambulatory peritoneal dialysis for treatment of renal failure (14). In these patients, a combination of vancomycin and an aminoglycoside administered i.p. is recommended as an initial empirical regimen. The broad spectrum and rapid microbicidal activity of PG-1 might allow for single-agent therapy of such patients.

Overall, these studies demonstrate that PG-1 is a potent antimicrobial agent with in vivo activity against clinically relevant, antibiotic-resistant bacteria. Currently, clinical trials with an analog of PG-1 to prevent oral mucositis in cancer patients resulting from polymicrobial infections associated with the oral flora are ongoing.

ACKNOWLEDGMENT

We thank Robert Lehrer for his continual encouragement of this work and his critical reading of the manuscript.

REFERENCES

1. **Agerberth, B., J. Y. Lee, T. Bergman, M. Carlquist, H. G. Boman, V. Mutt, and H. Jornvall.** 1991. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline, arginine-rich antibacterial peptides. Eur. J. Biochem. **202:**849–854.
2. **Aumelas, A. A., M. Mangoni, C. Roumestand, L. Chiche, E. Despaux, G. Grassy, B. Calas, and A. Chavanieu.** 1996. Synthesis and solution structure of the antimicrobial peptide protegrin-1. Eur. J. Biochem. **237:**575–583.
3. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:**61–92.
4. **Fahrner, R. L., T. Dieckmann, S. S. L. Harwig, R. I. Lehrer, D. Eisenberg, and J. Feigon.** 1996. Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes. Chem. Biol. **3:**543–550.
5. **Ganz, T., and R. I. Lehrer.** 1995. Defensins. Pharmacol. Ther. **66:**191–205.
6. **Hancock, R. E. W.** 1997. Peptide antibiotics. Lancet **349:**418–422.
7. **Hancock, R. E. W., T. Falla, and M. Brown.** 1995. Cationic bactericidal peptides. Adv. Microb. Physiol. **37:**135–175.
8. **Harwig, S. S. L., K. M. Swiderek, T. D. Lee, and R. I. Lehrer.** 1995. Determination of disulphide bridges in PG-2, an antimicrobial peptide from porcine leukocytes. J. Pept. Sci. **3:**207–215.
9. **Harwig, S. S. L., V. N. Kokryakov, K. M. Swiderek, G. M. Aleshina, C. Zhao, and R. I. Lehrer.** 1995. Prophenin-1, an exceptionally proline-rich antimicrobial peptide from porcine leukocytes. FEBS Lett. **362:**65–69.
10. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram positive bacteria. Microbiol. Rev. **59:**171–200.
11. **Joosten, H. M. L. J., and M. Nunez.** 1995. Adsorption of nisin and enterocin 4 to polypropylene and glass surfaces and its prevention by Tween 80. Lett. Appl. Microbiol. **21:**389–392.
12. **Kokryakov, V. N., S. S. L. Harwig, E. A. Panyutich, A. A. Shevchenko, G. M. Aleshina, O. V. Shamova, H. A. Korneva, and R. I. Lehrer.** 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. **327:**231–236.
13. **Lehrer, R. I., M. Rosenman, S. S. L. Harwig, R. Jackson, and P. Eisenhauer.** 1991. Ultrasensitive assays for endogenous antimicrobial peptides. J. Immunol. Methods **137:**167–173.
14. **Levison, M. E., and L. M. Bush.** 1995. Peritonitis and other intra-abdominal infections, p. 705–740. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases, 4th ed. Churchill Livingstone, New York, N.Y.
15. **Mangoni, M. E., A. Aumelas, P. Charnet, C. Roumestand, L. Chiche, E. Despaux, G. Grassy, B. Calas, and A. Chavanieu.** 1996. Change in membrane permeability induced by protegrin 1: implication of disulphide bridges for pore formation. FEBS Lett. **383:**93–98.
16. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved standard M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
17. **Nicolas, P., and A. Mor.** 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. **49:** 277–304.
18. **Qu, X.-D., S. S. L. Harwig, A. Oren, W. M. Shafer, and R. Lehrer.** 1996. Susceptibility of *Neisseria gonorrhea* to protegrins. Infect. Immun. **64:**1240–1245.
19. **Russell, J. P., G. Diamond, A. P. Tarver, T. F. Scanlin, and C. L. Bevins.** 1996. Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. Infect. Immun. **64:**1565–1568.
20. **Sahl, H.-G.** 1994. Gene-encoded antibiotics made in bacteria. CIBA Found. Symp. **186:**27–52.
21. **Sampson, B., R. Misra, and S. A. Benson.** 1989. Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. Genetics **122:**491–501.
22. **Schonwetter, B. S., E. D. Stoltzenberg, and M. A. Zasloff.** 1995. Epithelial antibiotics induced at sites of inflammation. Science **267:**1645–1648.
23. **Scotti, R., J. K. Dulworth, M. T. Kenny, and B. P. Goldstein.** 1993. Effect of protein on ramnoplainin broth microdilution minimum inhibitory concentrations. Diagn. Microbiol. Infect. Dis. **17:**209–211.
24. **Steiner, H., D. Hultmark, A. Engstrom, H. Bennich, and H. G. Boman.** 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature **292:**246–248.
25. **Takemura, H., M. Kaku, S. Kohno, Y. Hirakata, H. Tanaka, R. Yoshida, K. Tomono, H. Koga, A. Wada, T. Hirayama, and S. Kamihira.** 1996. Evaluation of susceptibility of gram-positive and -negative bacteria to human defensins by using radial diffusion assay. Antimicrob. Agents Chemother. **40:** 2280–2284.
26. **Tamamura, H., T. Murakami, S. Horiuchi, K. Sughara, A. Otaka, W. Takada, T. Ibuka, M. Waki, N. Yamamoto, and N. Fujii.** 1995. Synthesis of protegrin-related peptides and their antibacterial and anti-human immunodeficiency virus activity. Chem. Pharm. Bull. **43:**853–858.
27. **Yasin, B., S. S. L. Harwig, R. I. Lehrer, and E. A. Wagar.** 1996. Susceptibility of *Chlamydia trachomatis* to protegrins and defensins. Infect. Immun. **64:** 709–713.
28. **Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from Xenopus laevis skin: isolation, characterization of two active forms and partial DNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:**5449–5553.
29. **Zhao, C., L. Liu, and R. I. Lehrer.** 1994. Identification of a new member of the protegrin family by cDNA cloning. FEBS Lett. **346:**285–288.
30. **Zhao, C., T. Ganz, and R. I. Lehrer.** 1995. The structure of porcine protegrin genes. FEBS Lett. **368:**197–202.