Bactericidal Activity of Synthetic Peptides Based on the Structure of the 55-Kilodalton Bactericidal Protein from Human Neutrophils

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Received 13 October 1993/Returned for modification 16 December 1993/Accepted 11 April 1994

Short (10- to 11-mer) hydrophilic peptides based on the structure of the 55-kDa bactericidal protein (BP55, B/PI, and CAP57) from human neutrophil granules were identified from the hydropathy plot of the 456-amino-acid sequence predicted from the nucleotide sequences of cDNA clones for BP55 and B/PI. Peptides corresponding to amino acid residues 90 to 99 (peptide #90–99), 86 to 99, or 90 to 102 of BP55 were bactericidal toward 5×10^6 *Pseudomonas aeruginosa* cells at 0.6×10^{-5} to 1.5×10^{-5} M and killed an *Escherichia coli* rough strain at 3×10^{-5} M. The #90–99 peptide with a cysteine added at the amino terminus (C#90–99) was approximately 10 times more active than #90–99, killing *P. aeruginosa* at 1.5×10^{-6} M. Peptides representing amino acid residues 27 to 37, 118 to 127, and 160 to 170 and the first 10 amino acids of the signal sequence for BP55 were not bactericidal. When coupled to either keyhole limpet hemocyanin or ovalbumin protein carriers through the thiol group, the C#90–99 peptide was not diminished on a molar basis in its capacity for killing of *P. aeruginosa*. Two other relatively hydrophilic peptides with an added amino-terminal cysteine, peptides C#227–236 and C#418–427, were not bactericidal at 1.2×10^{-4} M or at 100 times the effective bactericidal concentration of C#90–99. The C#90–99 peptide killed *E. coli* at 1.5×10^{-5} M, or at 10 times the concentration required to kill an equal number of *P. aeruginosa* cells. Although *Pseudomonas cepacia* and *Staphylococcus aureus* were resistant to killing by the parent BP55 molecule, they were susceptible to the C#90–99 and #90–99 peptides in the same concentration range as was *E. coli*. When all peptides were compared for the ability to neutralize *E. coli* O55:B5 endotoxin in a *Limulus* amoebocyte lysate assay, the C#227–236, C#418–427, and #160–170 peptides completely inhibited gelation at a 10^{-4} M concentration. All other synthetic peptides, including bactericidal peptide #90–99 and its congeners, lacked endotoxin-neutralizing activity at the highest concentration tested (4.5×10^{-4} M). A hybrid of the C#227–236 and #90–99 peptides (CHybrid) was identical to the C#227–236 peptide component in effectiveness for carrying out endotoxin neutralization and was fivefold better than the #90–99 peptide in its capacity for killing *P. aeruginosa*.

The 55-kDa bactericidal protein from human neutrophils is most well known as the bactericidal/permeability-increasing factor (B/PI) (2, 37) but is also known as the cationic antibacterial protein of 57 kDa (CAP57) (27, 32), and, more simply, as the bactericidal protein of 55-kDa molecular mass (BP55) studied in our laboratory (12, 36). Evidence that all are the same protein is convincing and has been recently reviewed (32, 36). The amino-terminal amino acid sequences of BP55, B/PI, and CAP57 are identical (24, 32, 36). This sequence corresponds to the amino acid sequence deduced from the nucleotide sequence of a full-length cDNA clone for B/PI (6). We have substantially confirmed the predicted amino acid sequence for B/PI (6) by the nucleotide sequencing of a cDNA clone for BP55, which showed 99% identity of the 456 residues (unpublished data). A monoclonal antibody to CAP57 was shown to cross-react with B/PI (32). BP55, B/PI, and CAP57 are primarily bactericidal toward gram-negative bacteria (2, 32) but whereas studies of CAP57 and B/PI have concentrated on *Escherichia coli* and *Salmonella typhimurium*, our studies of BP55 evolved from the demonstration that *Pseudomonas aeruginosa* was killed by an oxygen-independent mechanism (12). Our studies showed that, although there was no evidence

of permeability increase, the inner membrane functions of *P. aeruginosa*, such as amino acid transport, ceased immediately upon exposure to BP55 (11). Studies of *E. coli* (18) have recently confirmed the loss of amino acid transport function in the lethal effects of B/PI at pH 6.0.

An important persistent difference in the studies of *P. aeruginosa* with BP55 is that whether smooth or rough, serum resistant or serum sensitive, all strains thus far studied are equally sensitive to killing by BP55 (31). By contrast, studies of *S. typhimurium* and *E. coli* have consistently shown that resistance to CAP57 and B/PI increases dramatically with the length of the O polysaccharide chains on the lipopolysaccharide (LPS) of the bacterial outer membranes (3, 39).

Studies of B/PI have turned to its ability to neutralize the endotoxin effects of *E. coli* LPS and the localization of this function, as well as the bactericidal function, to the amino-terminal half-molecule of 199 amino acids. The native B/PI molecule has been shown to neutralize both the pyrogenicity of LPS for rabbits and the ability of LPS to initiate the coagulation pathway of the *Limulus* amoebocyte lysate or to cause tumor necrosis factor (TNF) release from human monocytes (19, 20). Neutralization of the ability of LPS to activate in the *Limulus* assay has also been demonstrated for a 25-kDa amino-terminal proteolytic fragment of native B/PI (nBPI₂₅) and for a 23-kDa recombinant form (rBPI₂₃) of the same region of B/PI (23, 38). The nBPI₂₅ fragment and rBPI₂₃ product consist of the first 199 amino acids of the amino

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terminus of B/PI. Both are capable of killing *E. coli* and of binding to LPS or lipid A (2) while neutralizing the ability of endotoxin to upregulate the expression of complement receptors on neutrophils and to cause TNF release from whole blood (38). The carboxyl-terminal half of native B/PI (nBPI₅₅) inhibits the ability of LPS to cause TNF release from whole blood and is weakly active in inhibition of the effects of LPS on *Limulus* amoebocyte lysate (23). High-affinity binding of LPS by rBPI₂₃ has been demonstrated (5, 8). In vivo studies of nBPI₅₅ and rBPI₂₃ have shown that both are effective in treatment of gram-negative pneumonia (13).

The synthesis of overlapping 15-mer peptides based on the amino acid sequence of rBPI₂₃ was recently achieved (17). Only the peptide corresponding to amino acid residues 85 to 99 (peptide #85–99) was bactericidal for rough-strain *E. coli*. Approximately 50% of the 47 synthetic peptides, including #85–99, had significant endotoxin-neutralizing capacity. We have independently investigated the bactericidal capacity and the endotoxin-neutralizing ability of several short hydrophilic synthetic peptides based on the structure of BP55 and B/PI. Only the peptide representing residues 90 to 99 of the parent molecule and its congeners were capable of killing bacteria. None of the bactericidal peptides were capable of inhibiting the ability of *E. coli* LPS to coagulate the *Limulus* amoebocyte lysate. Two of the three peptides capable of inhibiting endotoxin in the *Limulus* assay were located in the carboxyl-terminal half-molecule of BP55 (B/PI). A hybrid of one of these endotoxin-neutralizing peptides with the #90–99 bactericidal peptide retained full activity for both biological functions. Our studies suggest that the bactericidal function of BP55 may be separated from the endotoxin-neutralizing region in the primary sequence of the molecule. Alternative explanations based on the crystalline structure of another LPS-binding protein (9) are discussed.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* type 1 is a clinical isolate maintained in the laboratory since 1972. The isolate remains a smooth strain and was serotyped by the scheme of Homma (10). Rough strain *E. coli* B and *Staphylococcus aureus* 502A were obtained from Paul Quie, University of Minnesota. The characteristics of the *S. aureus* strain have been described (29). *Pseudomonas cepacia* ATCC 25608 was purchased from the American Type Culture Collection in 1980. *S. aureus* and *E. coli* were maintained on nutrient agar plates, and the *Pseudomonas* strains were maintained on blood agar plates.

Bactericidal assay. Pyrogen-free solutions were used throughout the bactericidal assay and all methods discussed below. Log-phase bacteria were prepared from a culture in brain heart infusion broth as previously described (12) except that the bacteria were washed and resuspended in saline with adjustment to an optical density at 650 nm which would yield 3×10^8 CFU/ml. The bacteria were then diluted 1:10 in 0.08 M citrate-phosphate buffer, pH 5.6 or pH 7.0 (prepared by mixing 0.08 M citric acid with 0.08 M dibasic sodium phosphate). *S. aureus* rapidly lost viability in the pH 5.6 buffer and was studied only at pH 7.0. Bactericidal activity was determined by dose response, and when a 50% lethal dose (LD₅₀) is indicated, it was determined by linear regression. Bacteria (0.15 ml) were incubated with peptide or BP55 in a final volume of 1.0 ml of buffer for 30 min in optically matched tubes in a reciprocal water bath shaker at 37°C. Five milliliters of growth medium was then added, and growth was monitored by optical density at 650 nm. Data were collected when bacteria were at mid-log phase. The growth medium for *P. aeruginosa*, *S. aureus*, and *E.*

coli was nutrient broth. The growth medium for *P. cepacia* was nutrient broth containing 0.2% glucose. Bactericidal tests were also performed in the presence of human plasma, which was prepared by centrifugation of blood containing 1.5 mg of potassium EDTA per ml. Plasma was heat inactivated at 56°C for 30 min. Inhibition of bacterial killing by LPS from *P. aeruginosa* ATCC 27312 (List Biological Laboratories, Campbell, Calif.) was determined with LPS suspended in 0.1% triethylamine. LPS and peptide were incubated at pH 5.6 in 0.85 ml of buffer for 15 min at which time 0.15 ml of bacteria were added. Where a 50% inhibitory dose (ID₅₀) is indicated, it was determined by linear regression analysis after dose-response experiments.

Purification of BP55. BP55 was purified in three column chromatography steps as previously described (36). In the final step the sample was applied to a molecular sieving column (1 by 180 cm) of Toyopearl HW55S (TosoHaas, Philadelphia, Pa.) which had been equilibrated with 0.05 M glycine buffer, pH 2.5, containing 0.5 M NaCl. Protein concentration was determined according to Hartree (7). Purity was confirmed by visualization of a sodium dodecyl sulfate (SDS)-polyacrylamide gel (15) following electrophoresis of 1 µg of purified BP55 protein and silver staining of the gel (22), also as previously described.

Peptide preparation. Peptides were synthesized at the University of Minnesota Microchemical Facility using a Millgren/Biosearch 9600 peptide synthesizer. All peptides were solid phase synthesized by use of fluorenylmethoxycarbonyl chemistry. The cleaved-peptide profile was obtained by analytical high-performance liquid chromatography (HPLC) (Model HP1090; Hewlett Packard). When necessary, lyophilized crude peptides were purified by preparative reversed-phase HPLC on a C18 column with an elution gradient of 0 to 60% acetonitrile with 0.1% trifluoroacetic acid in water. Crude and pure peptides were subjected to gel filtration using a Sephadex G25 column (1 by 9 cm) and pyrogen-free saline buffered with 0.008 M citrate-phosphate buffer, pH 7.0, as the elution buffer and used to confirm results obtained at every step of the study. The purity and composition of the peptides were verified by HPLC (Beckman Model 6300) analysis of amino acid composition of hydrolysates prepared by treating the peptides under argon in 6 N HCl for 24 h at 110°C. The amino acid sequence of peptide #90–99 was confirmed by gas-phase Edman degradation, using an Applied Biosystems 470A gas-phase sequencer with a reverse-phase C18 column (2.1 by 250 mm). That the thiol groups of all peptides containing a cysteine residue were fully reduced was ascertained with Ellman's reagent, using reduced glutathione as a standard.

Conjugation of peptides to protein carriers. Peptides were conjugated to keyhole limpet hemocyanin (KLH) or ovalbumin (OVA) carrier proteins which had been activated with the heterobifunctional sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate reagent according to the manufacturer's instructions (monograph 77108; Pierce Chemical Company, Rockford, Ill.). Cross-linking of activated carrier to peptide was due to the reaction of the maleimide group of the carrier with the sulfhydryl group of the peptide. Conjugates were separated from free peptide with a Sephadex G25 column (1 by 9 cm) rather than the column provided by the manufacturer. Conjugates and unconjugated protein carriers were hydrolyzed, and the amino acid compositions were determined as described above. By using the amino acid composition of the carrier compared with that of the conjugate, the stoichiometry of peptide to carrier was calculated. By using a molecular mass of 70 kDa for KLH and of 45 kDa for OVA, the molar concentration of peptide in each conjugate and the peptide/

carrier ratio were calculated by difference. For example, the amino acid compositions of the peptide #90-99-KLH conjugate and the KLH used to prepare the conjugate were determined, and the composition of the latter was subtracted from the former. The differences of amino acid content were zero for all but E (14.8), A (15.6), L (15.1), F (15.3), K (63.3), and R (15.6). The molar excess is shown in parentheses and was used to calculate the ratio of peptide to carrier of 15:1 for the #90-99 peptide, with the confirmed sequence of KWK AQKRFLK. The glutamine (Q) is accounted for in the composition as glutamic acid (E), and the tryptophan was destroyed by hydrolysis in HCl.

Limulus amoebocyte lysate assay. The ability of synthetic peptides to neutralize endotoxin was detected with the E-TOX-ATE kit manufactured by Sigma Chemicals, St. Louis, Mo. The concentration of peptide required to completely inhibit the coagulation of *Limulus* amoebocyte lysate driven by *E. coli* O55:B5 LPS was determined by dose response and as outlined in technical bulletin 210 of Sigma Chemicals. Peptide was incubated with LPS at room temperature for 5 min in 100 μ l of a 1:2 dilution of pyrogen-free saline, pH 6.4. The reaction was started by addition of 100 μ l of amoebocyte lysate, and the final volume was 200 μ l.

Toxicity of synthetic peptides for eukaryotic cells. Human lung fibroblasts were grown in monolayers in Dulbecco's modified Eagles's medium (DMEM) with 10% fetal calf serum (FCS) and originated as primary cultures of lung mesenchymal cells (1). The cells were trypsinized, washed with either DMEM plus FCS or phosphate-buffered saline (PBS), pH 7.4, and incubated with peptide at a final cell concentration of 10^7 /ml. Peptides were added at 10 times the effective bactericidal dose in PBS, pH 7.4. At the same doses, 90 to 95% of *P. aeruginosa* cells were killed in DMEM plus 10% FCS. Nine volumes of cell suspension were diluted with 1 volume of 0.4% trypan blue, and the cells were counted with a hemocytometer, while the fraction of cells which were increased in permeability to dye was also determined. There was no significant loss of cell number due to exposure of the fibroblasts to peptide.

RESULTS

Bactericidal activity of synthetic peptides. All peptides studied are shown in Table 1 and were synthesized according to the amino acid sequence deduced from the nucleotide sequences of cDNA for B/PI (6) and BP55 (unpublished data). The peptides represent hydrophilic stretches of BP55 and were selected from a Kyte-Doolittle (14) hydropathy plot (data not shown) which we averaged over five amino acids. Of those peptides shown in the Table 1, only #90-99 and its relatives had bactericidal activity. The bactericidal activity of the #90-99 peptide toward *P. aeruginosa* was linear with dose (Fig. 1) and is compared in the figure with the dose response for BP55 and the dose response of the most active of the bactericidal peptides, which was the #90-99 peptide with an added cysteine (C) at the amino terminus (C#90-99). The LD_{50} of C#90-99 for *P. aeruginosa* was achieved at a peptide concentration 11 times lower than the LD_{50} of peptide #90-99 (8.2×10^{-6} M) and 37.7 times higher than the LD_{50} of BP55 (3.4×10^{-9} M). The #90-99 peptides were equally active towards two mucoid variants of *P. aeruginosa* (isolates 1M and 10M of reference 31) and towards the *P. aeruginosa* ATCC 27312. The bactericidal activities of the C#90-99 peptide towards four different bacteria are compared in Table 2 with the activity of two other peptides, #227-236 and #418-427 of the mature BP55 molecule. The last two peptides were synthesized with an amino-terminal cysteine for comparison with C#90-99 and so

TABLE 1. Synthetic peptides studied for bactericidal and endotoxin-neutralizing activities

Synthetic peptide	Sequence ^a	Net hydropathy Index ^b
#90-99	<i>KWKAQKRFLK</i>	-16.1
C#90-99	<i>CKWKAQKRFLK</i>	-13.6
#90-102	<i>KWKAQKRFLKMSG</i>	-15.4
#86-99	<i>KISGKWAQKRFLK</i>	-16.7
C#86-99	<i>CKISGKWAQKRFLK</i>	-14.2
Hybrid	<i>EFYSENHHNPKWKAQKRFLK</i>	-31.7
CHybrid	<i>CEFYSENHHNPKWKAQKRFLK</i>	-34.2
Signal	MRENMARGPC	-9.9
#27-37	KELKRIKIPDY	-13.3
#118-127	KLGSNPTSGK	-12.2
#160-170	KKIESALRNKM	-12.0
C#227-236	CEFYSENHHNP (G)	-19.2
C#418-427	CNEKLQKGFPL	-7.4

^a Peptides which were bactericidal toward 5×10^6 *P. aeruginosa* cells are in boldface italics. All other peptides had no bactericidal activity at doses up to 1.2×10^{-4} M.

^b The summation of Kyte-Doolittle hydropathy indices for each amino acid of the sequence.

that they could be coupled to protein carrier molecules through the thiol group. The C#227-236 sequence occurs at the halfway point of the molecule of BP55. It was synthesized with a carboxyl-terminal glycine (shown in Table 1 in parentheses) to facilitate cleavage from the resin after synthesis of the peptide.

The C#227-236 and C#418-427 peptides were not bactericidal for any of the four species shown in Table 2. The highest dose of these peptides tested was 1.2×10^{-4} . As previously reported (12, 36) and shown in Table 2, BP55 was equally bactericidal toward *P. aeruginosa* and rough strain *E. coli* B at 4.1×10^{-9} M (approximately 0.2 μ g of protein per ml). There was a small but reproducible loss of activity at pH 7.0 compared with that at pH 5.6. *P. cepacia* and *S. aureus* resisted bactericidal activity at 9.1×10^{-8} M BP55, or approximately a 20-fold-higher protein concentration than that which killed *P. aeruginosa*. By comparison with BP55, the C#90-99 peptide killed *P. aeruginosa* when tested at 1.5×10^{-6} M (2.1 μ g of peptide per ml). *E. coli* B and *P. cepacia* were killed at a peptide concentration of C#90-99 which was 10-times higher than the peptide concentration which killed *P. aeruginosa*.

FIG. 1. Dose response for the bactericidal effects of BP55 (Δ) and of peptides C#90-99 ($\circ$) and #90-99 ($\bullet$) toward 5×10^6 CFU of *P. aeruginosa* type 1 incubated at pH 5.6. Values for C#90-99 are the means of three experiments $\pm$ one standard error of the mean. Values for six experiments with #90-99 are shown, and three of these were performed in parallel with BP55.

TABLE 2. Bactericidal activities of BP55 and synthetic peptides related to BP55

Species	% Killing of 5×10^6 bacteria ^a							
	BP55 (4.1×10^{-9} M)		C#90-99 (1.5×10^{-6} M)		C#227-236 (1.2×10^{-4} M)		C#418-427 (1.2×10^{-4} M)	
	pH 5.6	pH 7.0	pH 5.6	pH 7.0	pH 5.6	pH 7.0	pH 5.6	pH 7.0
<i>P. aeruginosa</i>	50 ± 5	35 ± 3	83 ± 13	83 ± 15	0	0	0	0
<i>P. cepacia</i>	0	0 ^b	0	0 ^c	0	0	0	0
<i>E. coli</i>	35 ± 4	25 ± 5	0	0 ^d	0	0	0	0
<i>S. aureus</i>		0 ^b		0 ^e		0		0

^a Values shown are the means ± one standard error for three experiments.

^b *P. cepacia* and *S. aureus* were not killed at a concentration 20-fold higher (approximately 5 µg of protein per ml).

^c Seventy-eight percent of *P. cepacia* bacteria were killed at 1.5×10^{-5} M at pH 7.0, and 80% were killed at pH 5.6.

^d Ninety-seven percent of *E. coli* bacteria were killed at 1.5×10^{-5} M at pH 7.0, and 98% were killed at pH 5.6.

^e Ninety-five percent of *S. aureus* bacteria were killed at 3×10^{-5} M at pH 7.0.

Killing of *S. aureus* by C#90-99 occurred at 20-times the concentration which killed *P. aeruginosa*.

When compared with C#90-99 (Fig. 1), approximately 10- to 12-fold more of the peptide without the added cysteine (#90-99) was required for equal killing of *P. aeruginosa*. When rough-strain *E. coli* organisms were tested at pH 5.6 with 3×10^{-5} M #90-99, 31% of 5×10^6 bacteria were killed (Table 3). The #90-99 peptide concentration required to kill *P. cepacia* at pH 5.6 and *S. aureus* at pH 7.0 was four times higher than the effective concentration of C#90-99 (data not shown).

The hydrophilic peptides #27-37, #118-127, and #160-170 are also shown in Table 1 and were not bactericidal at 1.2×10^{-4} M for any of the bacteria of this study (data not shown). A peptide corresponding to the first 10 amino acids of the signal sequence for BP55 also was not bactericidal, and data are not shown for studies of this peptide.

Two elongated amino acid sequences of the #90-99 peptide were synthesized (Table 1). The molar doses of peptides #86-99 and #90-102 required to kill *P. aeruginosa* were 2.5 times lower than the effective dose of the #90-99 peptide (Table 3, Fig. 1). At pH 5.6, neither the activity of the #90-102 peptide nor that of the #86-99 peptide was substantially improved over the bactericidal activity of peptide #90-99 toward *E. coli* (Table 3).

Bactericidal activity of peptide-protein conjugates. The bactericidal activity of C#90-99 coupled to KLH (KLH-C#90-99) was also tested with *P. aeruginosa* and compared with those of the KLH conjugates of the inactive peptides C#227-236 and C#418-427 (Table 4). Based on the results for the amino acid composition of each purified conjugate and unconjugated carrier protein, the ratio of peptide to carrier and the final concentration of peptide were calculated (Table 4). Approximately 90 to 95% of 5×10^6 *P. aeruginosa* cells were killed by

the KLH-C#90-99 conjugate at a peptide concentration calculated at 10^{-6} M. The bactericidal molar concentration of conjugate was very close to the 1.5×10^{-6} M free C#90-99 peptide which caused 83% killing of this microorganism (Table 2, Fig. 1). Neither KLH-C#227-236 nor KLH-C#418-427 was bactericidal for *P. aeruginosa* at five and nine times higher concentrations of peptide, respectively (Table 4). Closely similar results were obtained with the OVA conjugates of these three peptides (data not shown). For example, at pH 5.6, 70% of *P. aeruginosa* cells were killed by the OVA-C#90-99 conjugate at 4.5×10^{-8} M. This represents a peptide concentration of approximately 6×10^{-6} M and a molar ratio of C#90-99 peptide to OVA carrier of 9:1, both determined by calculation from the amino acid composition of the conjugate compared with that of the unconjugated carrier.

Endotoxin-neutralizing capacity of synthetic peptides. Although they were bactericidal for *E. coli*, synthetic peptides C#90-99, #90-99, C#86-99, #86-99, and #90-102 (Table 1) did not neutralize the procoagulant effects of *E. coli* endotoxin on *Limulus* amoebocyte lysate. The highest concentration of each peptide tested was 4.5×10^{-4} M, and the concentration of peptide tested in the *Limulus* assay ranged from 120 µg/0.2 ml for the #90-99 peptide to 164 µg/0.2 ml for the C#86-99 peptide. We also observed that, at 120 µg of the #90-99 peptide per 0.2 ml, the gelation of amoebocyte lysate by 0.005 ng of *P. aeruginosa* LPS was unimpaired (data not shown). As shown in Fig. 2, the C#90-99 and #90-99 peptides will bind to purified LPS from *P. aeruginosa*, which inhibits killing of *P. aeruginosa* by either of these peptides. The ID₅₀ of LPS with

TABLE 3. Bactericidal activities of synthetic peptides and BP55 at pH 5.6

Peptide	% Killing of 5×10^6 bacteria (molar concn)	
	<i>P. aeruginosa</i>	<i>E. coli</i> B
C#90-99	83 ± 13 (1.5×10^{-6})	98 ± 3 (1.5×10^{-5})
#90-99 ^a	72 ± 3 (1.5×10^{-5})	31 ± 3 (3.0×10^{-5})
C#86-99	90 ± 5 (3.0×10^{-6})	91 (1.5×10^{-5})
#86-99	79 ± 3 (6.0×10^{-6})	90 ± 1 (3.0×10^{-5})
#90-102	67 ± 4 (6.0×10^{-6})	77 ± 6 (3.0×10^{-5})
CHybrid	74 ± 3 (3.0×10^{-6})	71 (3.0×10^{-5})
Hybrid	79 ± 5 (3.0×10^{-5})	72 (6.0×10^{-5})
BP55	87 ± 8 (5.5×10^{-9})	71 (5.5×10^{-9})

^a n = 6; three experiments were performed in parallel with BP55.

TABLE 4. Bactericidal activities of BP55 peptides when conjugated to a protein carrier

Conjugate (concn) ^a	pH	% Killing of <i>P. aeruginosa</i>	Peptide concn (ratio) ^b
KLH-C#90-99 (9×10^{-8} M)	5.6 7.0	95 ± 3 90 ± 5	1×10^{-6} M (15:1)
KLH-C#227-236 (3×10^{-7} M)	5.6 7.0	0 0	5×10^{-6} M (16:1)
KLH-C#418-427 (5×10^{-7} M)	5.6 7.0	0 0	9×10^{-6} M (18:1)

^a 5×10^6 bacteria were incubated with a conjugate of peptide with KLH. The final concentration of conjugate is shown and was based on quantitation by amino acid composition.

^b The final concentration of peptide was calculated from the amino acid composition of each conjugate compared with that of unsubstituted KLH. The ratio of peptide to carrier is shown and was also derived from the amino acid composition.

FIG. 2. Percent inhibition by purified LPS from *P. aeruginosa* 27312 (List Biological Laboratories) of the bactericidal activity of 2.3 µg of peptide C#90-99 per ml (○) ($n = 3$) or 23 µg of peptide #90-99 per ml (●). Three of the six experiments with #90-99 were performed in parallel with BP55 (Δ). Peptides were incubated at pH 5.6 with LPS for 15 min before the addition of 5×10^6 *P. aeruginosa* cells.

BP55 was 0.23 µg/ml, while the ID_{50} for C#90-99 was approximately 21 µg/ml and the ID_{50} for peptide #90-99 was approximately 42 µg/ml. Killing of *P. aeruginosa* was also inhibited by LPS from *E. coli* O111:B4 (data not shown), but inhibition of bacterial killing by both C#90-99 and #90-99 peptides required 40% more *E. coli* LPS (data not shown) than was required with LPS from *P. aeruginosa* (Fig. 2).

Three peptides with 11 amino acid residues were capable of neutralizing endotoxin in the *Limulus* assay. The concentration of peptide required for 100% inhibition of LPS-induced gelation of the amoebocyte lysate was determined by dose response. The endotoxin-neutralizing activity of the peptides was comparatively weak and is shown in Table 5. The most active endotoxin-neutralizing peptide was C#227-236, which neutralized 0.04 endotoxin unit of *E. coli* O55:B5 LPS (0.04 endotoxin unit = 0.01 ng of LPS; personal communication, Sigma) at a concentration of 1.2×10^{-4} M. The C#418-427 peptide was closely similar and neutralized endotoxin at 1.5×10^{-4} M. The third peptide with neutralizing capacity (#160-170) was less active than the aforementioned peptides and neutralized endotoxin at 4.5×10^{-4} M.

A hybrid peptide of the C#227-236 and #90-99 peptides (CHybrid, Table 1) was synthesized and tested for endotoxin neutralization (Table 5) and killing of *P. aeruginosa* and *E. coli* (Table 3). All results shown in Table 5 were reproduced without variation in three different experiments. The CHybrid molecule completely neutralized endotoxin at the same concentration (1.2×10^{-4} M) as did the original C#227-236 peptide. The hybrid molecule without the N-terminal cysteine

TABLE 5. Endotoxin-neutralizing activities of BP55 and active synthetic peptides

Synthetic peptide or BP55	Sequence	Effective dose	
		M (10^{-4})	(µg/0.2 ml) ^a
#160-170	KKIESALRNKM	4.5	119
C#227-236	CEFYSENHHNP (G)	1.2	34
C#418-427	CNEKLQKGFLP	1.5	38
Hybrid	EFYSENHHNPKWKAQKRFLK	3.0	155
CHybrid	CEFYSENHHNPKWKAQKRFLK	1.2	81
BP55		0.00015	0.165

^a The dose of peptide required to inhibit clotting of *Limulus* amoebocyte lysate by 100%. Each tube contained 0.04 endotoxin unit of *E. coli* LPS.

TABLE 6. Killing of *P. aeruginosa* by BP55 and synthetic peptides C#86-99 and #86-99

Medium	Peptide	% Killing	
		pH 5.6	pH 7.0
CPB ^a	C#86-99	90 ± 5 (3.0×10^{-6})	88 ± 8 (3.0×10^{-6})
	#86-99	81 ± 3 (6.0×10^{-6})	79 ± 3 (6.0×10^{-6})
	BP55	87 ± 8 (5.5×10^{-9})	80 ± 3 (1.1×10^{-8})
CPB plus heat-inactivated human plasma	C#86-99	91 ± 5 (3.0×10^{-6})	88 ± 5 (1.5×10^{-6})
	#86-99	44 ± 8 (1.2×10^{-5})	75 ± 7 (6.0×10^{-6})
	BP55	81 ± 10 (1.1×10^{-8})	66 ± 8 (2.2×10^{-8})
CPB plus fresh human plasma	C#86-99	66 ± 4 (1.5×10^{-6})	91 ± 6 (1.5×10^{-6})
	#86-99	51 ± 10 (1.2×10^{-5})	73 ± 7 (6.0×10^{-6})
	BP55	56 ± 6 (5.5×10^{-9})	63 ± 7 (2.2×10^{-8})

^a CPB, citrate phosphate buffer.

(Hybrid, Table 5) required two times more peptide for endotoxin neutralization than was required in the case of CHybrid.

At pH 5.6, the CHybrid molecule killed 74% of 5×10^6 *P. aeruginosa* cells at 3×10^{-6} M. This is a concentration of CHybrid fivefold lower than the concentration of #90-99 required to kill 75% of the bacteria (Table 3, Fig. 1) and two times higher than the concentration of C#90-99 which killed 83% of the bacteria (Tables 2 and 3). The Hybrid molecule killed 79% of the *P. aeruginosa* cells at 3×10^{-5} M, which is approximately two times the concentration of #90-99 which would kill the same fraction of bacteria. At pH 5.6, 71% of *E. coli* cells were killed by CHybrid at 3×10^{-5} M. This is equal to the concentration of #90-99 required to kill *E. coli* and two times higher than the concentration of C#90-99 required to kill *E. coli* (Table 3).

Bactericidal peptides C#86-99 and #86-99 were also studied in buffer containing heat-inactivated or fresh human plasma and compared with BP55 for killing of *P. aeruginosa* (Table 6). The most consistent plasma-induced change of the effective dose was in the case of BP55, for which the effective dose was increased twofold with heat-inactivated plasma at pH 5.6 and 7.0 and with fresh plasma at pH 7.0. The requirement for #86-99 in the presence of heat-inactivated or fresh plasma at pH 5.6 was increased twofold over the buffer control. The effective dose of C#86-99 was unchanged or moderately reduced by the presence of plasma. Similar results (not shown) were obtained with the peptides and a balanced salt solution containing 10% heat-inactivated or fresh-frozen normal human serum. However, the effective dose of BP55 was increased five times at pH 5.6 and 10 times at pH 7.0. The toxicities of four of the bactericidal peptides for mammalian cells were tested by incubation of human lung fibroblasts with 10 times the bactericidal peptide concentration effective with *P. aeruginosa* in PBS and at the effective bactericidal dose in DMEM plus FCS. There was no significant increase in permeability to trypan blue (Table 7) as a result of exposure to either of the peptides tested.

DISCUSSION

While this report was in preparation, the crystal structure of an endotoxin-neutralizing protein from horseshoe crab was published by Hoess et al. (9). The *Limulus* anti-LPS factor (LALF) is cationic, consists of 101 amino acids, and is bactericidal towards rough-strain *Salmonella minnesota* and toward Re, Rd2, Rc, and Rb strains of *S. typhimurium*, but not toward *S. aureus* (21). Hoess et al. describe a single-domain high-

TABLE 7. Effect of synthetic peptides on viability of human lung fibroblasts

Synthetic peptide	Sequence	% Viability ^a	
		DMEM	PBS
Control		98.5	93.1
#90-99	KWKAQKRFLK^b	98.0	89.4
C#90-99	CKWKAQKRFLK^c	98.4	94.7
Hybrid	EFYSENHHNPKWKAQKRFLK^b	98.8	95.6
CHybrid	CEFYSENHHNPKWKAQKRFLK^c	99.2	92.3

^a Viability was determined by trypan blue exclusion following incubation of cells with peptide for 30 min in DMEM plus 10% FCS or in PBS, pH 7.4.

^b Final concentration of 1.5×10^{-4} M.

^c Final concentration of 1.5×10^{-5} M.

resolution structure of a recombinant LALF and propose that LPS is bound by an amphipathic loop which includes residues 32 to 50 of LALF. The loop was suggested as the LPS-binding structure by a comparison with the sequence of polymyxin B (PMB) arrived at by substituting lysine for the α - γ -diaminobutyric acids not involved in the formation of the cyclic structure. The resulting linearized 10-amino acid motif of PMB was composed of positively charged amino acids alternating with hydrophobic amino acids. A pair of lysines were located at residues 9 and 10 when a gap was inserted for alignment with LALF. There are no negatively charged amino acids. This motif is present in LALF starting at residue 32 and extending well beyond the alignment with PMB to amino acid residue 50 of LALF. A pair of arginines is present at positions 9 and 10 of the #32-50 sequence and are in the location of the turn of the amphipathic loop. The investigators cite as supporting evidence that this is the putative LPS-binding motif of LALF that one of the exposed tryptophans (either residue 44 or 51) is buried when the LALF molecule is bound to LPS (35). The motif was then located by inspection of the amino acid sequence of B/PI (6) and the human serum LPS-binding protein (LBP) sequence (30). An amphipathic sequence was located in both amino acid sequences starting at residue 86 and ending at 104. As in the PMB and LALF molecules, a pair of positively charged amino acids are present, but at positions 10 and 11 of the #86-104 sequence of B/PI (residues 95 and 96) and at positions 9 and 10 (residues 94 and 95) of the #86-104 sequence of LBP.

Although not stated by Hoess et al. (9), the 19-amino-acid motif should also identify a bactericidal structure for BP55 (B/PI), since the PMB antibiotic and LALF molecule have both LPS-binding and bactericidal functions (9, 26) and since we have shown that the #90-99 (10-mer), #86-99 (14-mer), and #90-102 (13-mer) peptides are bactericidal for *P. aeruginosa*. However, these peptides were bactericidal in the micromolar range, and this is 3 orders of magnitude higher than the effective bactericidal concentration of BP55. We will test the activity of the 19-amino-acid sequence (#86-104), but it is difficult to believe that an additional five amino acids would increase bactericidal activity by 1,000-fold or more. We are in the process of testing a cyclic structure of #90-99 for the purpose of examining the influence of a rigid structure on bactericidal activity. The cyclic structure was achieved by adding cysteine to both ends of the #90-99 sequence and then oxidizing the sulfhydryl groups as was done by Rustici et al. (26) in studies of synthetic peptides modeled after PMB. It may be that the "loop" structure will amplify the bactericidal activity of the linear #90-99 peptide sequence. We will also investigate the impact of cyclizing peptide #86-104 on bactericidal function. The fact that the cyclic peptides modeled after

PMB were not bactericidal (26) somewhat quenches our optimism that a simulated loop structure will achieve the desired amplification of bactericidal activity of peptides #90-99 and #86-104.

The very interesting biology of LBP has been reviewed recently by Ulevitch (34). The activity of LBP depends on the ability of LBP-LPS complexes to trigger TNF release by monocytes through interaction of the complexes with CD14 of the cell surface. Because LBP is not bactericidal (33), the alternating motif of 19 amino acids between residues 86 and 104 cannot be the only important issue. Perhaps, here again, the actual conformation of the loop is the key to understanding the differences in biological function of BP55 (B/PI) and LBP. There is a difference in motif and location of the positively charged amino acid pair of LBP and B/PI which might alter the location of the turn in the peptide loop of LBP and also the final conformation of the loop.

The most promising aspect of the investigation of cyclic peptides stimulated by the prediction of Hoess et al. (9) may be the influence of the more rigid structure on the endotoxin-neutralizing capacity of the synthetic peptides. Cyclic synthetic peptides modeled after PMB had potent endotoxin-neutralizing ability but were not bactericidal (26). We found that synthetic peptides C#90-99, #90-99, #86-99, and #90-102 did not neutralize the procoagulant effects of *E. coli* endotoxin on *Limulus* amoebocyte lysate but were bactericidal. We have demonstrated that the C#90-99 and #90-99 peptides will bind to purified LPS from *P. aeruginosa* and *E. coli* by showing that the bactericidal activity of both peptides is inhibited by preincubation with LPS. However, the concentration of LPS required for inhibition is at least 90 times that required in our studies with native BP55. This suggests that the interaction of synthetic peptide with LPS is of considerably lower affinity than the interaction of B/PI (BP55) with LPS, which has been measured at a K_d of 4 nM (5). This may also suggest that the structural requirements for binding to and killing of *P. aeruginosa* may be separable from the structural requirement for binding to LPS and neutralizing its toxic effects. On the other hand, a synthetic peptide extending the above sequences, such as #86-104, may have endotoxin-neutralizing activity and that activity may improve remarkably when the cyclic structure is imposed. Indeed, Little et al. (17) have shown that peptide #85-99 of B/PI will neutralize endotoxin in a colorimetric *Limulus* assay. Seven other synthetic peptides with equal endotoxin-neutralizing activity were distributed throughout the amino-terminal half of the B/PI molecule (17). We may also find that the cyclized #90-99 peptide will acquire the ability to neutralize endotoxin. A careful examination of the endotoxin-neutralizing capacity of #90-99 with the quantitative colorimetric assay may also reveal an interaction which we have missed using the semiquantitative *Limulus* assay.

The synthetic peptides which were shown to inhibit endotoxin in the *Limulus* assay had very weak activity. The two most active peptides were C#227-236 and C#418-427, the former located in the center of the linear sequence of BP55 (B/PI) immediately following the 199 amino acids of rBPI₂₃ and the latter near the carboxy terminus of the 456-amino-acid sequence (6). Ooi et al. (23) have shown that nanomolar amounts of a 30-kDa carboxyl-terminal fragment of B/PI has the capacity to neutralize LPS from *E. coli* O55:B5 in a colorimetric *Limulus* assay. Although much less active, the C#227-236 and C#418-427 peptides may be, at least in part, responsible for the activity of the 30-kDa fragment.

Two other synthetic peptides with bactericidal activity toward *P. aeruginosa* were recently reported. Shafer et al. (28) have achieved the consecutive synthesis of 20-mer stretches of

the 223-amino-acid sequence of human neutrophil cathepsin G. They found that the #117–136 peptide was most active and killed *S. aureus*, as well as *P. aeruginosa* (500 µg of peptide killed 5×10^7 CFU). There are no lysines in the sequence but 4 of 20 of the amino acids are arginine. There are no negatively charged glutamic or aspartic acid residues. Inspection of the sequence does not reveal the alternating motif of the #86–104 peptide of LALF and BP55 (B/PI), but a pair of arginines is present at residues 134 and 135 of the sequence. The investigators suggest that the peptide may be within the β -sheet structure of native cathepsin G (28).

The second antipseudomonal peptide of recent description was identified after overlapping peptide synthesis of the 165-amino-acid sequence of the human neutrophil CAP37 molecule by Pieria et al. (25). Peptide #20–44 was bactericidal toward 5×10^2 CFU of *P. aeruginosa* at 2.5×10^{-5} M and was also active toward *S. aureus*. The peptide contained two arginines and no lysines. The alternating motif is not evident in the #20–44 peptide, but there are no aspartic acid or glutamic acid residues in the sequence. The investigators showed that this peptide was inactive if the sulfhydryl groups of the two cysteines were blocked or if the cysteines at residues 26 and 42 were substituted with serines. This is reminiscent of results of studies of defensins (4, 16), which are also bactericidal toward *P. aeruginosa*. The peptides from human neutrophils are 30 or 31 amino acids in length and contain six cysteine residues (16). The three intramolecular disulfide bonds of defensin molecules are essential for bactericidal activity (16). The #117–136 antibacterial peptide of Shafer et al. (28) also contains a cysteine (residue 122), but its importance for bactericidal function is unknown. We have shown that the C#90–99 peptide was 10 times more active in bactericidal reactions with *P. aeruginosa* than was the #90–99 peptide. The addition of cysteine to the #86–99 peptide increased bactericidal activity only twofold, suggesting that there is something unique about the C#90–99 peptide. We have determined that the cysteine of the C#90–99 peptide was 100% reduced but that bactericidal activity was not diminished when the C#90–99 peptide was coupled to KLH or OVA protein carriers, thus eliminating the reactive reduced sulfhydryl group. The free sulfhydryl group is lost through bond formation with the *N*-maleimidomethyl group of the activated carrier, and this suggests that retention of antibacterial activity is not dependent on a disulfide bond, as it might be in the #20–44 peptide of CAP37 (25) and as it is in the defensin molecules (16).

Studies of the CHybrid peptide (C#227–236 plus #90–99) showed that the bactericidal activity of #90–99 was improved fivefold, and this suggests that the cysteine can be some distance from the bactericidal peptide and continue to influence biologic activity. It is of interest that endotoxin neutralization by the CHybrid peptide was not dramatically diminished when a hybrid of #227–236 and #90–99 was tested which did not have the amino-terminal cysteine (Hybrid). This peptide was only twofold reduced in its capacity to inhibit endotoxin compared with that of the CHybrid peptide.

Our working hypothesis has been that binding of BP55 to the bacterial surface and to purified LPS (with neutralization of its toxic effect) is a single function and perhaps separated in the molecule from the bacterial killing function. We and others (3, 6, 11, 28, 36) have postulated a hydrophilic interaction for the binding function and a hydrophobic interaction for the second, killing function. The existence of the C#90–99, #90–99, #86–99, and #90–102 peptides with bactericidal function at micromolar concentrations suggests that binding and killing functions can reside in a single peptide as small as 10 amino acids which is hydrophilic overall. However, because these

same peptides do not neutralize the endotoxin effects on the *Limulus* amoebocyte lysate, we must conclude that this function of the peptides, if it exists in these structures, is more dependent on conformation than is the bactericidal function. It is also possible that the endotoxin-neutralizing region of rBPI₂₃ and the parent molecule is distant in the linear sequence from the #90–99 bactericidal peptide or that endotoxin neutralization is achieved by a combinatorial structure composed of more than one sequence separated in the linear amino acid sequence. A choice from these alternative possibilities seems near.

ACKNOWLEDGMENTS

This work was supported by Cystic Fibrosis Foundation Grant CFF/NIH RFA 5578 and, in part, by National Institutes of Health Grant AI 26159 and the Graduate School of the University of Minnesota.

We are grateful to Ron Jemmerson for assistance with peptide selection and to Eric Eccleston and Dinesha Walik of the Microchemical Facility for their patient cooperation during the synthesis of peptides. Our thanks to Rebecca Savoie for assistance in preparation of the manuscript.

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