Antimicrobial activity of bovine bactericidal permeability-increasing protein-derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis

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Objective—To evaluate antimicrobial activity of bovine bactericidal permeability-increasing protein (bBPI)-derived synthetic peptides against mastitis-causing gram-negative bacteria.

Sample Population—Bacterial isolates from the milk of cows with clinical mastitis.

Procedures—Three peptides were synthesized with sequences corresponding to amino acids 65 to 99 (bBPI65-99), 142 to 169 (bBPI142-169), or the combination of amino acids 90 to 99 and 148 to 161 of bBPI. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these peptides against bacterial isolates from cows with mastitis were determined by use of a standardized broth microdilution assay. The ability of these peptides to retain their antimicrobial activity in serum and milk was also evaluated. Finally, bacterial lipopolysaccharide (LPS)-neutralizing activity of these peptides was assayed with the Limulus amebocyte lysate test.

Results—Of the 3 peptides tested, bBPI90-99148-161 had the widest spectrum of antimicrobial activity, with MIC and MBC values ranging from 16 to 64 μg/mL against Escherichia coli, Klebsiella pneumoniae, and Enterobacter spp and from 64 to 128 μg/mL against Pseudomonas aeruginosa. The antimicrobial activity of bBPI90-99148-161 was inhibited in milk, but preserved in serum. Finally, bBPI90-99148-161 completely neutralized LPS.

Conclusions and Clinical Relevance—bBPI90-99148-161 is a potent neutralizer of the highly proinflammatory molecule bacterial LPS and has antimicrobial activity against a variety of gram-negative bacteria. The ability of bBPI90-99148-161 to retain antimicrobial activity in serum suggests a potential therapeutic application for this peptide in the management of gram-negative septicemia. (Am J Vet Res 2007;68:1151-1159)

Mastitis continues to be among the most costly diseases to the dairy industry, and annual economic losses attributed to this disease in the United States are estimated to approach $2 billion.1 Worldwide, mastitis is associated with economic losses of $35 billion annually.2 Decreased milk production and quality, as well as veterinary expenses, all contribute to these economic losses. Further, because mastitis is one of the leading reasons for culling cows,3 animal replacement costs contribute to the financial burden that this disease imposes on producers.

From an animal health perspective, mastitis is one of the most frequent diseases of dairy cows and a leading cause of death and culling.4 Although implementation of preventative measures, including before-and-after teat disinfection, has been effective at reducing intramammary infection caused by contagious pathogens, these measures have had little impact on controlling mastitis caused by environmental pathogens.7 As a result, the proportion of cows with clinical mastitis caused by environmental bacteria has increased.

Gram-negative bacteria are among the most common environmental pathogens to cause mastitis and are responsible for approximately a third of all cows affected with clinical mastitis.10,11 Nearly 25% of the most severe instances result in culling or death of the cow.12 Of the gram-negative bacteria that cause mastitis, Escherichia coli spp are the most prevalent.13,14 Other common gram-negative isolates include bacteria from the Klebsiella, Serratia, Enterobacter, and Pseudomonas genera. An inverse relationship between the incidence
of gram-negative infections and bulk-tank milk somatic cell counts has been reported. Thus, the number of cows with clinical mastitis caused by these infections is expected to increase as dairymen continue to strive for lower milk somatic cell counts. Unfortunately, current antimicrobials for the treatment of intramammary gram-negative bacterial infections remain suboptimal. It has been estimated that 10% to 30% of cows with mastitis caused by gram-negative pathogens develop severe peracute mastitis, and bacteremia is associated with a substantial number of these affected cows. Systemic complications and deleterious outcomes associated with gram-negative infection are the result of an exaggerated inflammatory response elicited largely by a highly proinflammatory component of the gram-negative bacterial envelope known as endotoxin or bacterial LPS. The bovine mammary gland is highly susceptible to low doses of LPS, and LPS is detectable in the milk of cows with coliform mastitis. Absorption of LPS into blood following LPS injection into healthy mammary glands and during naturally occurring E. coli mastitis has been reported. In affected cows in which bacteremia develops, LPS is directly introduced into the circulation. It is established that the systemic inflammatory response that accompanies peracute coliform mastitis is mediated, in part, by LPS; however, treatment to counteract the excessive inflammatory response elicited by LPS remains lacking. Conventional antimicrobials do not target the LPS molecule and increase its circulating concentrations by inducing bacterial death and corresponding LPS shedding, thereby exacerbating the deleterious inflammatory response. In addition to mastitis, gram-negative bacteria are responsible for several other economically important diseases of cattle, including enteric colibacillosis, coliform septicaemia, brucellosis, metritis, salmonellosis, and campylobacteriosis. In fact, most of the clinical infections and mortality associated with food-animal neonates result from gram-negative organisms and the ensuing host inflammatory response. Therefore, development of novel interventions that can moderate the inflammatory response elicited by LPS remains an important animal health goal.

Synthetic congeners corresponding to the functional domains of naturally occurring endogenous antimicrobial and LPS-neutralizing proteins may have promising potential for the management of gram-negative infections in cattle and other agriculturally relevant animals. Human bactericidal permeability–increasing protein is a 55-kd cationic protein expressed in the primary (azurophilic) granules of human neutrophils, where it constitutes nearly 0.5% to 1% of the total cellular protein content. Human bactericidal permeability–increasing protein possesses bactericidal, LPS binding, and opsonic activity, all of which contribute to its role in host defense. In a variety of studies on experimental animals and humans, recombinant hBPI is therapeutically efficacious in the treatment of gram-negative infections and the attendant complications associated with such infections.

The amino-terminal region spanning amino acids 1 to 199 of hBPI contains the functional domains that confer bactericidal and LPS-neutralizing activity. These activities are governed by short regions within the amino-terminus, including regions corresponding to amino acids 65 to 99 and 142 to 169. Synthetic peptides corresponding to sequences within these regions, as well as fusion peptides containing combinations of sequences of amino acids from both regions, retain antibacterial and LPS-neutralizing properties.

The cDNA sequence corresponding to mature bBPI is found to be moderately conserved at the nucleotide (75%) and amino acid (63%) level with hBPI. In contrast to hBPI, little is known about the functional activity of bBPI or peptides corresponding to sequences within this protein that are homologous to regions governing functional activity in the human ortholog. The objective of the study reported here was to evaluate the bactericidal and LPS-neutralizing activity of 3 synthetic peptides, with sequences corresponding to amino acids 65 to 99 or 142 to 169 or the combination of amino acids 90 to 99 and 148 to 161 of bBPI.

Materials and Methods

Peptides—Peptides corresponding to the sequence of bBPI amino acids 65 to 99 (bBPI161-199) or the combination of amino acids 90 to 99 and 148 to 161 of bBPI were commercially synthesized. The synthetic peptides, with sequences corresponding to amino acids 65 to 99 and 142 to 169, or the combination of amino acids 90 to 99 and 148 to 161 of bBPI, were used to assess the bactericidal activity of 3 synthetic peptides in broth microdilution assay. Peptides were reconstituted in citrate-buffered saline solution (20 mM sodium citrate, 150 mM sodium chloride, 0.1% pluronic F-68, and 0.002% polysorbate 80 [pH, 5.0]) up to a final concentration of 10 mg/mL, placed in aliquots, and stored at –20°C. All aliquots were thawed only once. Immediately prior to use, aliquots were diluted to a 2-fold higher concentration than the highest concentration used in any given assay.

Bacterial strains—Bacterial isolates of E. coli, Klebsiella pneumonia, Serratia marcescens, Pseudomonas aeruginosa, Enterobacter cloacae, and Enterobacter aerogenes, which were obtained from the milk of cows with clinical mastitis, were used to assess the bactericidal activity of peptides in a broth microdilution assay. Strains were obtained from repositories at Cornell University and the USDA Beltsville Agricultural Research Center. Escherichia coli strain P4, which was originally isolated from a cow with clinical mastitis, was also used to assess the bactericidal activity of the peptides in biological fluids. Reference strains of E. coli (ATCC 29212) and Staphylococcus aureus (ATCC 29213) were obtained from a commercial source. To obtain fresh inocula for evaluation, a sterile loop of each bacterial glycerol stock was streaked on a blood agar plate. After an overnight incubation at 35°C, a single uniform colony was picked and incubated on a blood agar plate. After an overnight incubation at 35°C, a single uniform colony was picked and incubated on a blood agar plate. After an overnight incubation at 35°C.
Broth microdilution assay for antimicrobial susceptibility—To evaluate the antimicrobial activity of the peptides against various clinical mastitis bacterial isolates, the MIC and MBC of these peptides against the isolates were determined by use of a standardized broth microdilution assay. In addition to the bacterial isolates from cows with mastitis, *E coli* (ATCC 25922) and *S aureus* (ATCC 29213) reference strains were evaluated. Tetracycline MIC and MBC values for the various bacteria were determined as well. Assays were performed in accordance with the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) guidelines.46-47 First, a single colony of each fresh bacterial inoculum was diluted in cation-adjusted Mueller-Hinton broth, and the suspension was adjusted to achieve a transmittance equivalent to a 0.5 McFarland standard (10^4 CFUs/mL) by use of a colorimeter. The bacterial suspension was then diluted 10-fold in cation-adjusted Mueller-Hinton broth to reach a final inoculum concentration of 10^4 CFUs/mL. Fifty microliters of the inoculum was added to the wells of a 96-well round bottom plate containing 50 μL of either broth alone or broth containing serially diluted peptide or tetracycline. A negative control column was included in the plate that included 100 μL of broth alone. Plates were incubated for 18 hours at 35°C with ambient air circulation and without shaking. Wells were subsequently inspected for cloudiness and pellet formation by use of a magnifying glass plate reader. The lowest concentration at which no pellet or cloudiness was apparent in a given well was identified as the MIC. The MBC values were determined by plating 10-μL aliquots from the 96-well plates used to determine MIC values onto blood agar plates. Plates were incubated overnight at 35°C, and colonies were subsequently enumerated. The MBC values were determined as the lowest concentration at which ≥ 99.9% of inoculated bacteria were killed. All assays were performed in triplicate, and the MIC and MBC range was reported.

Serum and whey preparation—Milk and blood samples were aseptically collected from 8 healthy primiparous Holstein cows in midlactation with milk somatic cell counts of < 150,000 cells/mL. All cows were determined to be free of intramammary pathogens by the absence of growth on blood agar plates spread with milk samples aseptically collected from each cow on 3 days. For the preparation of whey, milk samples were centrifuged at 4,000 × g at 4°C for 30 minutes and the fat layer was removed with a spatula. The skim milk was transferred into another sterile tube and centrifuged for an additional 30 minutes, and the translucent supernatant was collected. Milk and whey samples were pasteurized by heating at 63°C for 30 minutes. For the preparation of serum, blood was collected from the coccygeal vein into evacuated tubes containing gel and clot activator for serum separation. The tubes were inverted 5 times, the blood was allowed to clot for 30 minutes, and the tubes were centrifuged at 1,500 × g for 15 minutes. Clear serum supernatants were aseptically transferred into sterile tubes and heat inactivated by incubating at 56°C for 30 minutes. An aliquot of each sample was plated on blood agar plates, and samples that were free of detectable bacterial growth were stored at –20°C.

Assay of antimicrobial activity in serum, milk, or whey—Overnight bacterial cultures of *E coli* strain P4 grown in tryptcase soy broth were diluted 1:1,000 in brain-heart infusion broth and incubated at 37°C for 2 hours while shaking at 225 revolutions/min. The log-phase bacteria were diluted 1:10 in brain-heart infusion broth to obtain a final concentration of 1 × 10^8 CFUs/mL. A 0.01-mL aliquot of the bacterial inoculum was added to a 1.5-mL sterile microcentrifuge tube containing 0.04 mL of serum, milk, or whey. A 0.05-mL aliquot of either peptide or citrate-buffered saline solution alone was added to each tube. Samples were then shaken at 100 revolutions/min for 6 hours at 37°C. Sample mixtures were subsequently serially diluted in sterile PBS solution and plated on MacConkey agar plates. Plates were incubated overnight at 37°C, and the colonies were enumerated.

Assay for neutralization of LPS—The ability of the peptides to neutralize LPS was determined by use of a commercially available LAL assay.8 As a positive control sample, polymyxin B was evaluated in parallel with the peptides. Increasing concentrations of peptides or polymyxin B were incubated with 1 ng of highly purified bacterial LPS,9 derived from *E coli* strain O111:B4, in a 500-μL reaction volume of endotoxin-free water for 30 minutes at 37°C while shaking at 100 revolutions/min. Following incubation, the amount of free LPS was determined in each sample according to the instructions of the manufacturer. Briefly, 50 μL of the mixture was placed into a 96-well microtiter plate, and the reaction was initiated by the addition of an equal volume of amoebocyte lysate. Following incubation at 37°C for 10 minutes, 100 μL of chromogenic substrate was added and the plate was incubated for 5 to 10 minutes at 37°C. The reaction was stopped by the addition of 50 μL of glacial acetic acid, and the absorbance was measured at a wavelength of 405 nm on a microplate reader.8 The amount of nonbound LPS was extrapolated from a standard curve, and the percent inhibition was calculated according to the following formula:

\[
\text{(amount of free LPS in control samples - amount of free LPS in test samples)} / \text{amount of free LPS in control samples}) \times 100
\]

Results

Bacteriostatic and bactericidal activity of bBPI peptides—The spectrum of activity of 3 bBPI peptides against various gram-negative bacteria isolated from cows with clinical mastitis was assessed by use of the broth microdilution susceptibility assay (Table 1). The range of MIC and MBC values from 3 independent assays was determined. At the concentrations tested, bBPI_p5_69 had no growth-inhibitory or bactericidal activity against any of the bacterial isolates. A limited spectrum of isolates was susceptible to bBPI_p42_169 at concentrations ≥ 128 μg/mL. Within the range of concentrations tested, all strains of *E coli* were susceptible, whereas only 1 isolate of *K pneumoniae* and *E cloacae* had repeatable susceptibility to bBPI_p42_169 in all 3 independent experiments. The third peptide, bBPI_p40_99_149_161.
had the lowest MIC and MBC values against the widest spectrum of bacteria. With the exception of S. marcescens, bBPI14169 had both growth-inhibitory activity on and bactericidal activity against all bacteria tested. The MIC and MBC of the peptide values were all ≤ 64 μg/mL for all isolates of E. coli, K. pneumoniae, and Enterobacter spp and ≤ 128 μg/mL for all P. aeruginosa isolates. Initial assays of susceptibility of S. marcescens revealed that it was refractory to the antimicrobial effects of any of the bBPI peptides tested at concentrations ≤ 256 μg/mL. Thus, the dose-response range of the peptides was increased so as to assay the efficacy of these peptides up to a concentration of 512 μg/mL. Even at the higher concentration assayed, none of the peptides had any ability to inhibit S. marcescens growth. In addition to the gram-negative isolates, bBPI142-169 and bBPI30-99,148-161 had growth-inhibitory and bactericidal activity against the quality-control reference strain of S. aureus.

Each bacterial isolate was also evaluated for its susceptibility to tetracycline to enable comparison with the MIC and MBC values determined for bBPI peptides (Table 1). Tetracycline inhibited the growth of all isolates. Tetracycline MIC values for the 2 reference strains, E. coli ATCC 25922 and S. aureus ATCC 29213, were within the established quality-control ranges of 0.5 to 2 μg/mL and 0.12 to 1 μg/mL, respectively. With the exception of 3 isolates of Enterobacter spp and 1 isolate of K. pneumoniae, MIC values of tetracycline were lower than those of the most efficacious bBPI peptide, bBPI142-169. In contrast to MIC values, MBC values of tetracycline were almost universally higher than those of bBPI142-169. The MBC values for the bBPI peptide were all equivalent to or only 1-fold higher than corresponding MIC values, whereas the fold difference in tetracycline MBC values relative to those of its MIC usually were higher.

**Bactericidal activity of bBPI peptides in serum and milk**—To determine whether bBPI142-169 and bBPI30-99,148-161 retained their bactericidal activity in physiological fluids, peptides were incubated with E. coli strain P4 (1 × 10⁵ CFUs) in the presence of serum (Table 2) or

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**Table 1—Antimicrobial activity of bBPI peptides against gram-negative bacterial isolates from cows with mastitis.**

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>bBPI142-169</th>
<th>bBPI30-99,148-161</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIC</strong></td>
<td><strong>MBC</strong></td>
<td><strong>MIC</strong></td>
<td><strong>MBC</strong></td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The MIC and MBC concentrations are reported as μg/mL. The range of values from 3 broth microdilution assays evaluating peptide antimicrobial activity is shown.
Table 2—Antimicrobial activity of bBPI peptides against *Escherichia coli* strain P4 in serum.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>bBPI&lt;sub&gt;13-99&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;16-161&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;10-148-161&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>0</td>
<td>8.40 ± 0.05</td>
<td>8.40 ± 0.05</td>
<td>8.40 ± 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>8.27 ± 0.03</td>
<td>8.42 ± 0.05</td>
<td>8.25 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>8.28 ± 0.04</td>
<td>8.38 ± 0.03</td>
<td>8.15 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>8.23 ± 0.03</td>
<td>8.29 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>8.07 ± 0.05</td>
<td>7.96 ± 0.07</td>
<td>0</td>
</tr>
</tbody>
</table>

Mean ± SE log<sub>10</sub> CFUs are reported. No. = Number of replicates.

Table 3—Antimicrobial activity of bBPI peptides against *E. coli* strain P4 in milk.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>bBPI&lt;sub&gt;13-99&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;16-161&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;10-148-161&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>0</td>
<td>8.69 ± 0.05</td>
<td>8.69 ± 0.05</td>
<td>8.69 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>8.69 ± 0.06</td>
<td>8.67 ± 0.03</td>
<td>8.65 ± 0.08</td>
</tr>
<tr>
<td>200</td>
<td>8.54 ± 0.10</td>
<td>8.55 ± 0.07</td>
<td>7.89 ± 0.12</td>
</tr>
<tr>
<td>2,000</td>
<td>8.06 ± 0.12</td>
<td>7.99 ± 0.07</td>
<td>6.55 ± 0.27</td>
</tr>
</tbody>
</table>

See Table 2 for key.

Table 4—Antimicrobial activity of bBPI peptides against *E. coli* strain P4 in whey.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>bBPI&lt;sub&gt;13-99&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;16-161&lt;/sub&gt;</th>
<th>bBPI&lt;sub&gt;10-148-161&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>0</td>
<td>8.28 ± 0.24</td>
<td>8.32 ± 0.23</td>
<td>8.27 ± 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>8.47 ± 0.14</td>
<td>8.29 ± 0.18</td>
<td>8.17 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>8.26 ± 0.18</td>
<td>8.44 ± 0.14</td>
<td>7.97 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>8.16 ± 0.16</td>
<td>8.10 ± 0.20</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>7.36 ± 0.30</td>
<td>7.45 ± 0.21</td>
<td>0</td>
</tr>
</tbody>
</table>

See Table 2 for key.

milk (Table 3). Because bBPI<sub>13-99</sub> had no bactericidal activity in broth, this peptide was assayed in parallel as a negative control sample. In the presence of serum, bBPI<sub>10-148-161</sub> had bactericidal activity against *E. coli* at concentrations ≥ 10 µg/mL. In contrast, neither bBPI<sub>16-161</sub> nor bBPI<sub>16-161</sub> had bactericidal activity in serum at concentrations ≤ 100 µg/mL.

Initial evaluation of the peptides in milk revealed no bactericidal activity against *E. coli* (data not shown); therefore, a more extended dose-response range was used to evaluate higher concentrations of the peptides (Table 3). Even at concentrations up to 2 mg/mL, none of the peptides had bactericidal activity against *E. coli* in milk. However, bBPI<sub>10-148-161</sub> was able to inhibit > 99% of the growth of *E. coli* in milk. Because milk appeared to have an inhibitory effect on the bactericidal activity of bBPI<sub>10-148-161</sub>, the peptides were subsequently evaluated for their ability to retain activity in whey, which is the casein-depleted, protein-rich liquid fraction of milk (Table 4). In the presence of whey, bBPI<sub>10-148-161</sub> had bactericidal activity against *E. coli* at concentrations ≥ 10 µg/mL. In contrast, neither bBPI<sub>13-99</sub> nor bBPI<sub>14-161</sub> had bactericidal activity at any concentration tested in whey.

**LPS-neutralizing activity of bBPI peptides**—To determine whether the bBPI peptides were able to neutralize LPS, increasing concentrations of the pep-

Figure 1—Mean ± SE percentage neutralization of bacterial LPS bioactivity as determined with the Limulus amebocyte lysate assay. Increasing concentrations of peptides or polymyxin B were incubated with 1 ng of *Escherichia coli*-derived LPS for 30 minutes. Following incubation, the amount of free LPS was determined by extrapolation from a standard curve and the percentage of bound (neutralized) LPS calculated.
As a positive and negative control sample, respectively, polymyxin B and a non–BPI-derived peptide were also assayed. At the lowest concentration tested (1 µg/mL), bBPI 65-99 and bBPI 142-169 inhibited approximately 20% of LPS activity, whereas bBPI 90-99,148-161 had no inhibition. Within the dose–response range tested, bBPI 65-99 maximally inhibited 50% of LPS activity. In contrast, bBPI 142-169 and bBPI 90-99,148-161 were able to neutralize ≥ 93% of LPS activity at peptide concentrations of 30 and 100 µg/mL, respectively. Polymyxin B inhibited LPS activity in a dose-dependent manner, whereas the control peptide had no inhibitory effect even at the highest concentration assayed (100 µg/mL).

Discussion

In the present study, the bacteriostatic, bactericidal, and LPS-neutralizing activity of peptides corresponding to amino acid sequences within bBPI were evaluated. Two peptides were synthesized that had sequences corresponding to amino acids 65 to 99 or 142 to 169 of mature bBPI. These sequences were chosen on the basis of reports establishing that homologous sequences of functional domains within the human ortholog possess antimicrobial and LPS-binding activity.41 Within amino acids 65 to 99 of hBPI, bactericidal activity is conferred by a subset region within amino acids 85 to 99. A synthetic peptide corresponding to amino acids 90 to 99 of hBPI has bactericidal, but not LPS-neutralizing, activity.40 Another report41 has established that a peptide corresponding to amino acids 148 to 161 of hBPI can neutralize LPS. Peptides composed of fused sequences of amino acid regions from different functional domains within hBPI confer bactericidal and LPS-neutralizing properties.40,42 Thus, a third peptide corresponding to hBPI amino acids 90 to 99 and 148 to 161 was evaluated to determine whether the fusion of sequences of amino acid regions from 2 hypothesized functional domains of bBPI could confer bifunctionality.

The first objective of the present study was to evaluate whether any of the bBPI peptides had growth-inhibitory activity and bactericidal activity against gram-negative bacteria isolates from cows with clinical mastitis. Among the 3 peptides tested, bBPI 90-99,148-161 had the lowest MIC and MBC values against the widest spectrum of clinical isolates. Escherichia coli, K pneumoniae, and Enterobacter spp were the pathogens most susceptible to the bacteriostatic and bactericidal effects of the peptide, whereas P aeruginosa had an intermediate susceptibility. All isolates of S marcescens were completely resistant to the growth-inhibitory and bactericidal activity of this peptide. Because others have similarly reported that S marcescens is resistant to the bactericidal effects of hBPI and rBPI or their derivatives,42,48,49 it is not surprising that this bacterium may be resistant to bBPI and thus its peptide derivatives. Serratia marcescens resistance to a variety of antimicrobials, including β-lactams, aminoglycosides, and quinolones, has been reported.30,52 In addition, S marcescens has resistance to common disinfectants such as chlorhexidine, which is used in some teat dips.30,54 Mechanisms for resistance to these antimicrobials include the production of enzymes that destroy the antimicrobials53,56; expression of efflux pumps that decrease accumulation of antimicrobials within the cell57,59; and alteration of porin expression, which reduces membrane permeability to antimicrobials.39,60 Whether these effector mechanisms of S marcescens confer resistance to the antimicrobial effects of bBPI or its peptide derivatives remains unknown.

For those bacteria that were susceptible to bBPI 90-99,148-161, the MIC values of the peptide were usually higher than those of tetracycline. The MBC values of the peptide were generally lower than those of tetracycline, as was the MBC:MIC ratio, consistent with the respective bactericidal and bacteriostatic actions of bBPI and tetracycline. In accordance with quality-control guidelines,46 2 reference strains of bacteria, E coli and S aureus, were evaluated and the tetracycline MIC values were within established quality-control ranges. Interestingly, the S aureus reference strain had susceptibility to the bactericidal effects of bBPI 142-169 and bBPI 90-99,148-161. Although S aureus is reportedly resistant to the bactericidal activity of full-length hBPI, peptides corresponding to either amino acids 90 to 99 of this protein or the combination of regions 90 to 99 and 148 to 161 effectively kill S aureus at concentrations comparable to those that are bactericidal against E coli.40,42,61 Thus, the finding that bBPI 90-99,148-161 had bactericidal activity against S aureus is consistent with results from studies of peptides derived from hBPI.

Despite findings that the bactericidal activity of bBPI can be localized to amino acids 65 to 99 and that synthetic peptides corresponding to amino acids 90 to 99 of the human protein are bactericidal,40,61 bBPI 65-99 had a complete lack of bactericidal activity against all of the bacterial isolates tested. One explanation may be simply that the differences in amino acids between hBPI and bBPI within this homologous region are responsible for the differential antimicrobial activity between synthetic human and bovine peptides. However, although bBPI 65-99 lacked activity, bBPI 90-99,148-161 had bactericidal activity against a wide spectrum of isolates. The finding that the other peptide tested, bBPI 142-169, had modest antimicrobial activity against a limited number of isolates at MIC and MBC values higher than those of bBPI 90-99,148-161 suggests that the bactericidal activity of bBPI 90-99,148-161 is largely governed by amino acids 90 to 99 of bBPI. Because these amino acids are contained within bBPI 65-99, it is surprising that this peptide did not have similar antimicrobial activity to that of bBPI 90-99,148-161. This finding may suggest that amino acids N- or C-terminal to a region of amino acids with functional activity can affect the properties governed by that sequence. Evidence supporting this contention comes from studies with hBPI and includes the following: addition of a single cysteine amino acid to the amino-terminus of an hBPI synthetic peptide enhances bactericidal activity by approximately 10-fold, and peptides corresponding to sequences within hBPI, but not the whole protein, exert bactericidal activity against P aeruginosa.46 In a study62 with another peptide corresponding to the C-terminus of the protein chemerin, addition of a single amino acid decreased activity by several orders of magnitude. Thus, the amino acids N-terminal to the region corresponding to amino acids 90 to 99 of bBPI 65-99 may impair the activity governed by this region and ablate corresponding bactericidal activity relative to that observed for the bBPI 90-99,148-161 peptide.
Because constituents of physiologic fluids can inhibit the activity of various antimicrobial compounds, the ability of bBPI to retain its activity in serum and milk was evaluated. The bactericidal activity of this peptide in these fluids was evaluated against the E coli P4 strain, which was isolated from a cow naturally affected with mastitis and that has well-characterized pathogenesis. The bBPI peptide retained bactericidal activity against the E coli P4 strain in serum, but not in milk. In whey, which is the protein-rich, liquid fraction of milk devoid of cells, casein, and fat, the peptide had bactericidal activity that was equivalent to that in serum. Cations, including magnesium and calcium, have been reported to impair the activity of or susceptibility to an array of antimicrobials as well as hBPI and rBPl. Because calcium and magnesium are present in bovine milk at approximately 11- and 3-fold higher concentrations, respectively, than those in blood, the increased concentrations of one or both of these cations may be responsible for the inability of the peptide to exert its bactericidal effect in milk. In bovine milk, approximately 25% and 65% of total magnesium and calcium, respectively, are associated with casein. Thus, the finding that the bBPI peptide retained bactericidal activity in whey, which is depleted of casein and contains correspondingly decreased concentrations of calcium and magnesium, compared with milk, is consistent with the hypothesized impairment of the activity of the peptide by the presence of these cations at increased concentrations. These data, however, do not rule out the possibility that other constituents of milk contribute to or are solely responsible for the impairment of bactericidal activity.

The finding that bBPI retains antimicrobial activity in serum suggests that additional studies are warranted to investigate the therapeutic potential of this peptide in the treatment of septicemia. The lack of bactericidal activity of the peptide in milk may dampen enthusiasm for further evaluation of its efficacy in the localized treatment of intramammary infections. However, during experimentally induced and naturally occurring mastitis, the concentrations of calcium and magnesium in milk have been reported to decrease by > 50%. Thus, alterations in the composition of milk during the course of mastitis may enable bBPI to exert its bactericidal effect. Treatment of intramammary gram-negative infections routinely is in response to the development of severe clinical signs, a time that often coincides with the greatest change in milk composition. Therefore, it is during this critical time of intervention when bBPI may be able to exert a therapeutic effect when infused into the mammary gland.

In addition to bactericidal activity, bBPI and its peptide derivatives bind and neutralize LPS. Peptide domain mapping has identified several regions within bBPI, including amino acids 65 to 103 and 137 to 171, which enable inhibition of LPS bioactivity. Synthetic peptides corresponding to sequences within these regions neutralize LPS in the LAL assay. Consistent with homologous human peptides, all 3 bBPI peptides had at least a partial ability to neutralize LPS. As determined by the lowest peptide concentration at which LPS was completely neutralized, bBPI appeared to be the most effective of the peptides. The bactericidal permeability-increasing protein has the ability to target both the pathogen and LPS. Both bBPI and its peptide derivatives improve survival during gram-negative infection and endotoxic shock.

To our knowledge, no previously published studies exist assessing the functional activity of bBPI. Results from the present study evaluating peptides corresponding to regions within this protein suggest that endogenous bBPI contains both LPS-neutralizing and bactericidal properties similar to its human and rabbit orthologs. The combined bactericidal and LPS-neutralizing activity of bBPI and its derivatives indicates potential for their therapeutic applicability to the treatment of gram-negative infections. The life-threatening systemic complications that arise in response to these infections are attributable, in part, to the excessive inflammatory response elicited by LPS. Current antimicrobials only target the pathogen, whereas a bactericidal permeability-increasing protein has the ability to target both the pathogen and LPS. Both bBPI and its peptide derivatives have therapeutic potential for the management of gram-negative infections in cows and other animals. Additional studies investigating the activity of the peptide in vivo will be needed to further evaluate its therapeutic potential.

References


47. NCCLS. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 2nd ed. Wayne, PA: The National Committee for Clinical Laboratory Standards (NCCLS), 2002.


