Effect of Lipopolysaccharide (LPS) Chain Length on Interactions of Bactericidal/Permeability-Increasing Protein and Its Bioactive 23-Kilodalton NH₂-Terminal Fragment with Isolated LPS and Intact Proteus mirabilis and Escherichia coli

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The target-specific cytotoxicity for gram-negative bacteria and the endotoxin-neutralizing activity of the 55-kDa bactericidal/Permeability-increasing protein (BPI) and its bioactive 23-kDa NH₂-terminal fragment depend on the strong attraction of BPI for the lipid A region of lipopolysaccharides (LPS). We have shown before that smooth gram-negative bacteria with long-chain LPS are more resistant to BPI (especially holo-BPI) than are rough strains. It has been suggested that the high BPI resistance of some gram-negative bacteria, such as Proteus mirabilis, might also reflect the structural diversity of lipid A. To explore this possibility, we compared the antibacterial activity and binding of natural and recombinant holo-BPI and a recombinant NH₂-terminal fragment (rBPI-23) to an isogenic rough (Re-LPS chemotype) and a smooth (S-LPS chemotype) strain of P. mirabilis and to LPS isolated from the two strains. Holo-BPI and rBPI-23 were both potent against the Re strain of P. mirabilis (96% lethal dose, 20 nM). In contrast, the smooth strain was ≤100 times more resistant to holo-BPI but only 10 times more resistant to rBPI-23. rBPI-23 was also more potent against several Escherichia coli strains from clinical bacteremia isolates. Differences in the antibacterial potency of BPI toward the Re and S strains of P. mirabilis correlated with differences in the binding of holo-BPI and rBPI-23 to these bacteria. In contrast, the binding of biosynthetically (in vitro transcribed and translated) 35S-labeled holo-BPI and NH₂-terminal fragment to isolated Re- and S-LPS from P. mirabilis in solution was similar. Moreover, in the Limulus amebocyte lysate assay, holo-BPI and rBPI-23 potent neutralized both forms of LPS with equal effectiveness. Together, these results strongly suggest that BPI recognizes Proteus lipid A and that the relative resistance of (smooth) P. mirabilis to holo-BPI is due to the inhibitory effect of long polysaccharide chains of tightly packed LPS in the envelope.

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growth-inhibitory activities against rough (Re-LPS chemotype) *P. mirabilis* and bind equally well to LPS isolated from both the Re and the S strains of *P. mirabilis*. In contrast, holo-BPI shows only weak binding and growth-inhibitory activity toward smooth *P. mirabilis*. However, rBPI-23 is ca. 30-fold more active than the holo-protein against this strain and binds more avidly to it. These results demonstrate that BPI recognizes *P. mirabilis* lipid A and further demonstrate that differences in BPI resistance reflect mainly differences in the barrier function of the envelope LPS that depend on the length of the polysaccharide chains.

**MATERIALS AND METHODS**

**Bacteria.** The bacteria used in this study included *E. coli* J5, a rough UDP-galactose-4-epimerase-deficient mutant of the smooth strain O111:B4 (32). This strain produces short-chain LPS (Re chemotype) when grown in medium without galactose and long-chain LPS (S chemotype) when the growth medium is supplemented with 2 mM galactose. Clinical isolates of various other strains of *E. coli* representing strains frequently recovered from human *E. coli* bacteremia cases were kindly provided by S. Warren (Department of Medicine, Massachusetts General Hospital). The strains of *P. mirabilis* used were the smooth strain S1959 and its rough (Re) mutant R45 (12, 14). All bacteria were grown overnight at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) except for *E. coli* J5, which were grown in triethanolamine-buffered minimal salts medium (30). A 50-μl aliquot of the overnight culture was added to 5 ml of fresh medium and grown for 4 h to the late logarithmic phase. The bacteria were harvested and resuspended in sterile physiological saline to the desired concentration.

**LPS.** LPS was extracted from the R45 mutant of *P. mirabilis* with phenol-chloroform-petroleum ether (5) and purified by electrodialysis (4). LPS from the smooth strain S1959 was extracted by the phenol-water method (39) and purified as described previously (11). Purified LPS was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and showed the expected differences for LPS purified from *P. mirabilis* R45 and S1959. The purified LPS was sonicated and diluted in a 10 mM sodium phosphate buffer (pH 7.0) for the *Lamalus* amoebocyte lysate (LAL) assay or in phosphate-buffered saline (PBS, pH 7.4) for the LPS binding assay (see below).

**BPI and BPI NH2-terminal fragment.** Native human BPI (nBPI) was purified from crude polymorphonuclear leukocyte extracts as described previously (19). Recombinant forms of human holo-BPI (rBPI) and its NH2-terminus-derived fragment (rBPI-23) were generously provided by Xoma Corporation, Berkeley, Calif. (6). In all bioassays used, the activities of nBPI and rBPI were virtually identical.

**In vitro transcription and translation of BPI cDNA.** The cDNA for human BPI (8) cloned downstream of the T3 polymerase promoter in plasmid pT7T3 (Pharmacia Biotech Inc., Piscataway, N.J.) was kindly provided by G. Theofan (Xoma Corporation, Santa Monica, Calif.). Since an in vitro translation system without microsomes was used, the signal sequence was deleted by excising the cDNA, in frame, between two DraII restriction sites (−25 to +12), as described by Ooi and Weiss (22). The N terminus of the resulting translation products contained either Met−31...Arg−26...Gly−13...Leu−14 or Met−27...Arg−26...Gly−13...Leu−14. To express full-length mRNA and protein species, the plasmid was digested with the restriction enzyme BamHI, yielding, after translation, BPI species terminating at the natural C terminus, codon 456 (BPI-456). A truncated form of BPI, roughly corresponding to the bioactive NH2-terminal fragment, was produced by digestion with the restriction enzyme Rsal, yielding, after translation, BPI species terminating at residue 221 (BPI-221). Restriction enzymes were purchased from Boehringer Mannheim, Indianapolis, Ind.

In vitro transcription was performed with an Mcap in vitro transcription kit from Stratagene (La Jolla, Calif.), for which 20 U of T7 polymerase was used for 1 μg of linearized DNA. The mRNA was purified, and 7 μl (0.25 μg/μl) was added to an in vitro translation mixture that contained 49 μl of rabbit reticulocyte lysate, 2 μl of RNasin (40 U/μl), 2 μl of a 1 mM amino acid mixture (without methionine), 4 μl of [35S]methionine (Dupont/New England Nuclear, Boston, Mass.) (10 mCi/ml), and 5 μl of diethylpyrocarbonate-treated H2O. The reaction mix was incubated at 30°C. After 60 min, the samples were placed on ice to stop the reaction.

To monitor incorporation of [35S]methionine into translation products, a 1-μl aliquot of the incubation mixture was evaluated by trichloroacetic acid precipitation by the procedure described in the Promega technical manual. In addition, translation products were analyzed by SDS-PAGE and autoradiography to ensure that a single major product of the expected size was produced. An aliquot of the translation products was boiled in sample buffer containing SDS (16), run on 10 to 15% polyacrylamide gradient gels with the Pharmacia Phast Gel System, and visualized by autoradiography. T 3 polymerase and RNasin were obtained from Promega Biotech (Madison, Wis.).

**Assay of BPI binding to immobilized LPS.** The BPI binding assay was based on a protocol by Paquette et al. (25) and Wood et al. (40). LPS derived from a rough (Ra) *E. coli* mutant, purchased from Sigma Chemical Company (St. Louis, Mo.), was sonicated and diluted in methanol (250 ng/ml) and applied in 50-μl aliquots to plastic wells (Immulon 2 Removable Strips; Dynatech Laboratories, Inc., Chantilly, Va.). Methanol, without LPS, was added to parallel wells. The methanol was allowed to evaporate overnight at 37°C, after which the wells were blocked with 0.1% bovine serum albumin (United States Biochemical Corp., Cleveland, Ohio) in PBS (pH 7.4) at 37°C for 6 h. The wells were washed three times with PBS containing 0.2% Tween 20 (PBST). A solution (50 μl) containing 50 ng of nBPI per ml and 1 μl of [35S]methionine-labeled translated products (BPI-456 or BPI-221) in the presence of either increasing concentrations of protein, nBPI, or rBPI-23 or increasing amounts of dispersed LPS from *P. mirabilis* R45 or S1959 was placed in the blocked wells and allowed to incubate overnight at 4°C. The next morning, the wells were washed seven times with PBST, and radioactivity was counted in a scintillation counter (Beckman, Fullerton, Calif.). Each assay was carried out in triplicate. LPS-dependent binding was calculated as the difference between binding of [35S]BPI to LPS-coated wells and control (no LPS) wells. In the absence of competing unlabeled BPI or dispersed LPS, binding of [35S]BPI to control wells was ≤20% of BPI binding to LPS-coated wells.

**Chromogenic LAL assay.** The ability of nBPI and rBPI-23 to inhibit the activity of isolated dispersed LPS (25 to 1,000 pg/ml) in a chromogenic LAL assay was measured as described previously (23).

**Assay of bacterial viability.** Bacteria (10⁶/ml) were incubated for 30 min at 37°C alone or with nBPI, rBPI, or rBPI-23 in a reaction mixture containing 0.8% (wt/vol) nutrient broth in physiological saline buffered with 20 mM sodium phos-
phate, pH 7.4. After termination of the incubation, an aliquot of the reaction mixture was serially diluted in sterile physiological saline and then plated (25 μl) in 5 ml of molten (48°C) 1.3% (wt/vol) Bacto-agar (Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) NaCl. Bacterial colonies were counted after incubation at 37°C for 18 to 24 h.

**Assay of BPI binding to intact bacteria.** Bacteria (4 × 10^7) were incubated with 6 or 30 μg of either rBPI or rBPI-23 in a final volume of 0.5 ml of PBS (pH 7.4). The samples were incubated for 20 min at 37°C, after which the bacteria were sedimented at 12,000 rpm in a centrifuge (model 5415; Eppendorf, Madison, Wis.) and washed twice with 250 μl of PBS. The pellets were resuspended in 3 × 25 μl of triple-distilled H₂O and transferred to a new tube. The resuspended pellets were then dried down in a Speed Vacuum Concentrator (Savant, Farmingdale, N.Y.). Samples were boiled in sample buffer containing SDS (16), run on 10 to 15% polyacrylamide gradient gels with the Pharmacia Phast Gel System, and visualized by Coomassie blue staining.

**RESULTS**

**Comparison of Re and S. P. mirabilis as targets for holo-BPI and rBPI-23.**

(i) **Growth-inhibitory activity.** Figure 1A shows that *P. mirabilis* R45 is highly sensitive to the growth-inhibitory effects of nBPI, rBPI, and rBPI-23. For each BPI species, the 50% lethal dose (LD₅₀) is ca. 5 to 10 nM for this bacterial strain. In contrast, the smooth parent strain (*P. mirabilis* S1959) displayed the high level of resistance to holo-BPI (nBPI and rBPI; LD₅₀ >1 μM; Fig. 1B) reported previously (27). However, the recombinant BPI fragment (rBPI-23) exhibited potent antibacterial activity (LD₅₀ = 70 nM) against this more resistant strain of *P. mirabilis.*

(ii) **Binding.** We have shown previously that the antibacterial potency of BPI against various strains and species of bacteria correlates closely with binding of BPI to the bacteria (32, 34, 36). Figure 2 shows the extent of binding of rBPI and rBPI-23 to *P. mirabilis* R45 and S1959. This binding parallels the antibacterial activity of the two proteins against these strains (Fig. 1). Whereas rBPI-23 binds avidly to the deep rough strain of *P. mirabilis* and somewhat less well to the smooth strain, holo-BPI binds avidly to the rough strain but not appreciably to the smooth strain, even when high concentrations of holo-BPI are added (compare Fig. 2A and B, lanes 3 and 4 and lanes 6 and 7).

**Comparison of antibacterial activity of holo-BPI and rBPI-23 against E. coli strains from clinical isolates.** The same pattern of BPI activity was also observed among a wide range of *E. coli* strains, many representing strains frequently isolated from human *E. coli* bacteremia cases (1, 10). Thus, holo-BPI and rBPI-23 displayed similar potent growth-inhibitory activity against a strain producing short-chain LPS (i.e., J5) (LD₅₀ >10 nM; Table 1), whereas all of the strains that produce long-chain LPS were more resistant to holo-

**FIG. 1.** Comparison of growth-inhibitory activity of holo-BPI (nBPI and rBPI) and BPI fragment (rBPI-23) against *P. mirabilis* R45 and S1959. Increasing concentrations of nBPI, rBPI, or rBPI-23 were incubated with either *P. mirabilis* R45 (A) or S1959 (B). Bacterial viability was measured as described in Materials and Methods. Each value represents the mean of three or more independent determinations, and the results are expressed as a percentage of the number of bacterial CFU for cultures incubated without BPI.

**FIG. 2.** Potency of BPI correlates with binding to *P. mirabilis.* *P. mirabilis* R45 (A) or S1959 (B), 4 × 10^7/sample, was incubated with either 6 or 30 μg of rBPI or rBPI-23 in a final volume of 0.5 ml of PBS for 20 min at 37°C. Bound and unbound BPI were separated by sedimentation and washing of the bacteria. The equivalent of 4 × 10^6 bacteria were applied to each lane. Gels were stained with Coomassie brilliant blue R. Lanes: 1, bacteria alone; 2, 250 ng of rBPI-23; 3 and 4, bacteria incubated with 30 and 6 μg of rBPI-23, respectively; 5, 500 ng of rBPI; 6 and 7, bacteria incubated with 30 and 6 μg of rBPI, respectively.
TABLE 1. Growth-inhibitory activity of rBPI against various strains of E. coli

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>LD₉₀(nM) rBPI-23</th>
<th>LD₉₀(nM) rBPI</th>
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<tbody>
<tr>
<td>J5</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>J5 + galactose</td>
<td>50</td>
<td>200</td>
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<tr>
<td>O1</td>
<td>200</td>
<td>400</td>
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<tr>
<td>O2</td>
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<tr>
<td>O4</td>
<td>200</td>
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<td>O6</td>
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<td>300</td>
</tr>
<tr>
<td>O75</td>
<td>150</td>
<td>600</td>
</tr>
</tbody>
</table>

* Bacteria were incubated with increasing concentrations of rBPI-23 and rBPI. "+Galactose" indicates that galactose was present in the growth medium. Bacterial viability was measured as described in Materials and Methods. The values shown represent the doses of rBPI-23 and rBPI needed to produce 90% inhibition of bacterial colony formation (LD₉₀). All strains were tested a minimum of two times.

BPI than to rBPI-23. The potency of rBPI-23 was two to six times greater than that of holo-BPI against these more-resistant bacteria.

**Binding of in vitro-translated holo-BPI and N-terminal fragment to immobilized LPS: inhibition by added Re- and S-LPS from P. mirabilis.** In contrast to the differences in the interaction of BPI with bacteria containing long- or short-chain LPS and the differences between holo-BPI and the BPI fragment against smooth strains of gram-negative bacteria, no appreciable effect of LPS polysaccharide chain length on the interaction of either holo-BPI or the BPI fragment with isolated LPS has been observed (6, 23). Because of the very great differences in the sensitivity of P. mirabilis R45 and S1959 to holo-BPI and in the relative activities of holo-BPI and rBPI-23 against P. mirabilis S1959 (Fig. 1), we also compared the interaction of holo-BPI and the N-terminal fragment with LPS isolated from these two strains.

Physical interaction between purified P. mirabilis LPS and BPI species corresponding to holo-BPI and the bioactive N-terminal fragment was measured indirectly by testing the ability of added P. mirabilis LPS to inhibit the binding of in vitro-translated ³⁵S-labeled BPI-456 and BPI-221 to immobilized E. coli Ra LPS (see Materials and Methods). In vitro-translated ³⁵S-labeled BPI-456 and BPI-221 bind to immobilized LPS in a manner indistinguishable from that of nBPI and rBPI-23 (unpublished data), and binding of both ³⁵S-labeled in vitro-translated species is competitively inhibited in a nearly identical fashion by nBPI and rBPI-23 (Fig. 3). Both the Re- and S- forms of purified P. mirabilis LPS, added in dispersed form, also competitively inhibited the binding of ³⁵S-labeled BPI-456 and BPI-221 to immobilized LPS (Fig. 4). Since the inhibitory effects of the two forms of dispersed Proteus LPS on the binding of the two species of BPI to immobilized E. coli Ra LPS were virtually identical, we conclude that the interaction of BPI-456 and BPI-221 with LPS isolated from P. mirabilis R45 and S1959 is not significantly different.

**Comparison of LPS-neutralizing activity of nBPI-55 and rBPI-23 for P. mirabilis LPS.** The ability of holo-BPI and rBPI-23 to inhibit the bioactivity of purified dispersed LPS from Re and S P. mirabilis strains was also tested. In the LAL assay, the two proteins were equipotent against the two chemotypes of LPS (Fig. 5). Thus, in contrast to interactions with intact P. mirabilis, the interaction of the two proteins with either S- or Re-LPS was nearly the same.

**DISCUSSION**

The primary target of BPI is the highly conserved lipid A region of LPS (3, 6, 19, 20, 23), enabling BPI to be effective...
FIG. 4. Effect of added Re- or S-LPS from *P. mirabilis* on binding of in vitro-translated ³⁵S-labeled BPI-456 and BPI-221 to immobilized LPS. LPS-dependent binding of ³⁵S-labeled in vitro-translated BPI-456 and BPI-221 was measured in the presence of increasing amounts of dispersed Re- (A) or S- (B) *P. mirabilis* LPS. Each value represents the mean ± standard error of the mean for three independent determinations. Data are expressed as a percentage of the ³⁵S-labeled BPI bound in samples without added dispersed LPS.

Figure 5. Comparison of LPS-neutralizing activities of rBPI and rBPI-23 against purified Re- and S-LPS from *P. mirabilis* in the LAL assay. The activity of Re-LPS (A) and S-LPS (B) was measured after incubation with increasing concentrations of nBPI or rBPI-23. Each value represents the mean of three independent determinations, and results are expressed as a percentage of the activity of LPS incubated without BPI.
or micelles, than readily of BPI

... surface
... because of tight packing of LPS in the bacterial envelope. The smaller N-terminal fragment of BPI presumably penetrates this barrier more easily than does holo-BPI. Purified LPS is dispersed as aggregates (e.g., micelles) or as single molecules. In this form, the polysaccharide chain does not impede the access of BPI to the lipid A region, and binding of holo-BPI and rBPI-23 to LPS is essentially identical regardless of polysaccharide chain length.

This study (Fig. 4 and 5) and previously (6, 23) that holo-BPI and the N-terminal fragment interact with isolated dispersed LPS similarly regardless of polysaccharide chain length. We speculate that it is the looser packing of LPS in these assemblies (Fig. 6) that permits equal access of holo-BPI and its N-terminal fragment to the target site(s) when LPS is presented in this manner.

The systematic comparison of the action of holo-BPI and BPI fragment on intact bacteria and purified LPS from isogenic Re and S strains of P. mirabilis has clarified the basis of resistance (and sensitivity) of these gram-negative bacteria to BPI. Similar studies comparing the interaction of BPI with apparently highly resistant bacteria, such as S. marcescens and P. cepacia, and the interaction of BPI with isolated LPS from the same organism may aid in revealing the basis of resistance of these organisms to BPI.

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