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Human Lipopolysaccharide-Binding Protein Potentiates Bactericidal Activity of Human Bactericidal/Permeability-Increasing Protein

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Human bactericidal/permeability-increasing protein (BPI) from neutrophils and a recombinant amino-terminal fragment, $rBPI_{23}$, bind to and are cytotoxic for gram-negative bacteria both in vitro and ex vivo in plasma or whole blood. To function in vivo as an extracellular bactericidal agent, $rBPI_{23}$ must act in the presence of the lipopolysaccharide-binding protein (LBP), which also binds to but has no reported cytotoxicity for gram-negative bacteria. LBP, which is present at 5 to 10 μg/ml in healthy humans and at much higher levels in septic patients, mediates proinflammatory host responses to gram-negative infection. On the basis of these previous observations, we have examined the effect of recombinant LBP (rLBP) on the bactericidal activity of $rBPI_{23}$ against *Escherichia coli* J5 in vitro. Physiological concentrations of rLBP (5 to 20 μg/ml) had little or no bactericidal activity but reduced by up to ~10,000-fold the concentration of BPI required for bactericidal or related activities in assays which measure (i) cell viability as CFUs on solid media or growth in broth culture and (ii) protein synthesis following treatment with BPI. LBP also potentiated BPI-mediated permeabilization of the *E. coli* outer membrane to actinomycin D by about 100-fold but had no permeabilizing activity of its own. Under optimal conditions for potentiation, fewer than 100 BPI molecules were required to kill a single *E. coli* J5 bacterium.

Bactericidal/permeability increasing protein (BPI) is a 55-kDa basic protein found in azurophilic granules of polymorphonuclear leukocytes (5) and, more recently, on the surface of neutrophils (15, 30), in plasma (3, 35) and in inflammatory fluids (14, 31). BPI is highly cytotoxic for many gram-negative bacteria but has no reported cytotoxicity towards gram-positive bacteria or eukaryotic cells (5). BPI binds to lipopolysaccharide (LPS) and disrupts the gram-negative bacterial outer membrane, initiating events leading ultimately to cell death. BPI is thought to act in two stages (13). The first stage is rapid and is initiated by binding of BPI to the bacterial outer membrane. This action results in sublethal effects, including growth arrest, permeabilization of the outer membrane to small molecules, and selective activation of bacterial enzymes that hydrolyze phospholipids and peptidoglycan (5, 13). Bacteria in this early stage can be rescued by growth in the presence of serum albumin (13). The second, later stage occurs after continued bacterial exposure to BPI and is characterized by physiologic and structural changes associated with cytoplasmic membrane damage leading to cell death (5, 13). At this stage, growth inhibition is refractory to rescue by serum albumin.

A 25-kDa amino-terminal proteolytic fragment of BPI retains full bactericidal activity (20). We cloned cDNA encoding the first 199 amino acids of BPI and expressed the protein in CHO-K1 cells (7). This recombinant N-terminal fragment ($rBPI_{23}$) also retains the bactericidal activity of neutrophil-derived BPI both in vitro (7) and in ex vivo assays with whole blood (32). $rBPI_{23}$ binds LPS with high affinity (7), inhibits several LPS-mediated responses (6, 7, 18, 19), and protects animals against challenge to both endotoxins (2, 8) and live bacteria (1).

To be effective as an extracellular bactericidal agent for treatment of gram-negative sepsis, $rBPI_{23}$ must function in plasma in the presence of an excess of LPS-binding protein (LBP), an ~60 kDa glycoprotein found in serum at 5 to 10 μg/ml in healthy humans and at up to 200 μg/ml in septic patients (25, 28). LBP binds LPS (26, 27), and the resulting complex binds to CD14 on macrophages to stimulate cytokine synthesis (22, 25, 28, 29, 36). LBP also binds to (37) but has no reported cytotoxicity for gram-negative bacteria when tested at physiologic concentrations (36).

These properties of LBP stimulated our interest in examining its possible effects on the bactericidal activity of BPI. To facilitate our studies, we have expressed recombinant LBP (rLBP [24]) and confirmed that purified rLBP binds to LPS (6, 24) and that rLBP-LPS complexes stimulate cytokine synthesis by cells which express its receptor, CD14 (24). In this paper, we describe experiments which demonstrate that while rLBP alone at physiologic concentrations has little or no bactericidal activity, it reduces by, up to 10,000-fold, the BPI concentration required to kill the gram-negative bacterium *Escherichia coli* J5.

MATERIALS AND METHODS

Purification of $rBPI_{23}$ and rLBP. The $rBPI_{23}$ and rBPI proteins used for these experiments were purified as previously described (7). The rLBP and $rLBP_{25}$ proteins were purified as previously described (24). Proteins were diluted in 20 mM citrate (pH 5.0)–150 mM NaCl prior to use.

Reagents. Bovine serum albumin (BSA, fraction V, >98% pure) was obtained from United States Biochemicals (Cleveland, Ohio). Ovalbumin was obtained from Sigma (St. Louis, Mo.). Bacteriological medium was obtained from Difco (Detroit, Mich.).

Bactericidal assays. *E. coli* J5, a rough UDP-galactose-4-epimerase-negative mutant of the smooth strain O111-B4 commonly used for studies on bactericidal activities of BPI (13), was grown overnight in TYE broth (7), and then a 1/200 dilution was subcultured in TEA medium (23). Bacteria were harvested at mid-logarithmic phase, suspended at $\sim 8 \times 10^8$ cells per ml in sterile physiological saline, and diluted 20-fold in a bactericidal assay medium consisting of 10% Hanks' balanced salts solution (Gibco), 40 mM Tris-HCl (pH 7.4), 0.1% Casamino Acids (Difco), and 0.9% NaCl (final cell density, $\sim 4 \times 10^7$ cells per ml). Cells were then incubated for 45 min at 37°C with various concentrations of $rBPI_{23}$ (added at a 1/100 dilution of concentrated stock solutions diluted in 20 mM citrate [pH 5.0]–150 mM NaCl) and/or rLBP (added at a 1/20 dilution of concentrated stock solutions prepared by diluting in the same buffer as described

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above) or control buffer in a total assay volume of 0.2 ml in sterile 15-ml polypropylene tubes. Following incubation, cells were either diluted in sterile physiological saline and plated on nutrient agar containing 0.9% NaCl or supplemented with 10 volumes (2 ml) of nutrient broth containing 0.9% NaCl and incubated at 37°C with shaking for up to 6 h.

Outer membrane permeability-increasing assay. The permeability of the outer membrane of *E. coli* J5 to actinomycin D was determined essentially as described by Ooi et al. (21). Cells were grown and prepared as described above, except that 0.4 mg of BSA per ml was included in the bactericidal assay medium. Cells at ~4 × 10^7/ml were mixed in a total volume of 200 μl with various concentrations of $rBPI_{23}$ with or without 20 μg of rLBP per ml followed by 50 μg of actinomycin D (Sigma) per ml. As a control, the same experiment was performed without actinomycin D. Following a 10-min incubation at 37°C, the cells were diluted in sterile physiological saline containing 4 mg of BSA per ml and plated on nutrient agar containing 0.9% NaCl and 1 mg of BSA per ml.

Protein synthesis assay. The effects of BPI and LBP on protein synthesis were assessed for *E. coli* J5 cells grown and prepared as described above for the bactericidal assays. The cells were incubated at 37°C in the bactericidal assay medium described above for 45 min with rLBP alone or for 45 or 90 min with $rBPI_{23}$ alone or with 20 μg of rLBP per ml plus various concentrations of $rBPI_{23}$. Following incubation with BPI and/or LBP, 0.4 μCi of ^{14}C-amino acids (New England Nuclear, Boston, Mass.) was added, and incubation was continued for an additional 20 min at 37°C. Incorporation of the ^{14}C-amino acids was linear as a function of time for at least 20 min. Cells were treated with 3 ml of cold 10% trichloroacetic acid to arrest protein synthesis and release free ^{14}C-amino acids from cells, which were then applied to a 0.45 μm-pore-size HA Millipore filter and washed once with 3 ml of 10% trichloroacetic acid and then with 5 ml of water. The filters were dried and counted in 10 ml of Ready Flow III scintillation cocktail (Beckman Instruments, Fullerton, Calif.) in a Beckman 7500 scintillation counter.

RESULTS

Bactericidal activities of $rBPI_{23}$ and rLBP on *E. coli* J5 cells. The bactericidal activities of $rBPI_{23}$ and rLBP were examined for the rough gram-negative bacterium *E. coli* J5 by using assays in which bacteria were first incubated with BPI, LBP, or buffer and then either diluted and plated on nutrient agar or grown in nutrient broth. Bactericidal activity was measured as either a reduction in CFUs on agar or reduced growth in broth. The addition of $rBPI_{23}$ at 1 μg/ml reduced the number of CFUs by ~95%; no bactericidal activity was detected at or below 0.1 μg/ml (Fig. 1A). By comparison, up to 10 μg of rLBP per ml caused no significant reduction in the number of CFUs; 20 and 50 μg of rLBP per ml caused an ~25 and ~50% CFU reduction, respectively, suggesting only slight bactericidal-like activity at elevated LBP concentrations (Fig. 1A). Similar results were obtained when the cells were grown in broth following treatment (Fig. 1B).

rLBP potentiates the bactericidal activity of $rBPI_{23}$. When rLBP was examined in combination with sublethal concentrations of $rBPI_{23}$, the bactericidal activity of $rBPI_{23}$ was significantly potentiated as measured by the reduction of CFUs on solid medium (Fig. 2A) and reduced growth in broth following treatment (Fig. 2B). In the CFU assay, maximum potentiation, approaching 10,000-fold, was observed with 20 μg of rLBP per ml in combination with 0.1 ng of $rBPI_{23}$ per ml, which had bactericidal activity (~90% CFU reduction) similar to that of 1 μg of $rBPI_{23}$ alone per ml (~95% CFU reduction). Potentiation of $rBPI_{23}$ bactericidal activity of approximately 100- and 1000-fold was observed with normal physiological concentrations of 5 and 10 μg of rLBP per ml, respectively; potentiation was also observed with as little as 2.5 μg of LBP per ml (Fig. 2A). A similar degree of potentiation (~10,000-fold) was observed in the broth assay since cells pretreated with 0.1 ng of $rBPI_{23}$ per ml and 20 μg of rLBP per ml were growth inhibited to the same extent as those pretreated with 1 μg of $rBPI_{23}$ alone per ml (Fig. 2B). Broth growth inhibition of cells pretreated with 1 ng of $rBPI_{23}$ per ml plus 20 μg of rLBP per ml exceeded that for those treated with 1 μg of $rBPI_{23}$ alone per ml and cells pretreated with either 20 μg of rLBP per ml or 0.1 μg of $rBPI_{23}$ alone per ml were not significantly inhibited in comparison to buffer-treated cells (Fig. 2B).

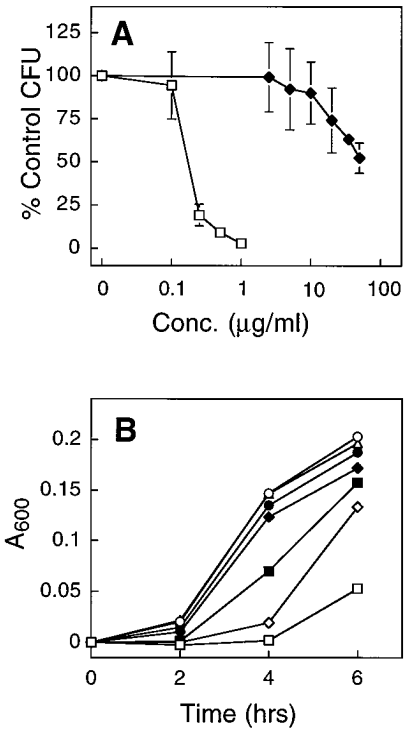

FIG. 1. Bactericidal activity of $rBPI_{23}$ and rLBP. *E. coli* J5 cells were grown and prepared for treatment, and the assays were performed as described in Materials and Methods. For these experiments, the order of addition was cells followed by buffer, rLBP, or $rBPI_{23}$. (A) Plating experiment. Control CFU (%) = CFU protein-treated cells/CFU buffer-treated cells × 100. □, $rBPI_{23}$; ◆, rLBP. Each point represents an average of at least three separate experiments. Standard errors of the means are shown as error bars on the graph. (B) Representative broth growth experiment. A_{600} was measured at 2-h intervals for 6 h. Open symbols represent control (△) or $rBPI_{23}$ at 1 (□), 0.5 (◇), or 0.1 (○) μg/ml. Closed symbols represent rLBP at 50 (■), 20 (◆), or 10 (●) μg/ml.

The bactericidal activity of rBPI was also potentiated by 20 μg of rLBP per ml to approximately the same extent as that of $rBPI_{23}$ (data not shown), and a recombinant fragment of rLBP corresponding to the first 197 amino acids of the protein ($rLBP_{25}$) potentiated the bactericidal activity of $rBPI_{23}$ (data not shown). Treatment of rLBP at 56°C for 30 min, a condition known to denature LBP, completely abolished its potentiating activity (data not shown). Ovalbumin at 20 μg/ml did not substitute for rLBP in potentiating $rBPI_{23}$ bactericidal activity (data not shown), suggesting that rLBP did not act by protecting low concentrations of BPI from nonspecific adsorption or denaturation.

rLBP potentiates the later stage of $rBPI_{23}$ bactericidal activity. Prolonged exposure to BPI results in progression to the second stage of action at which cells become refractory to rescue by serum albumin. To examine the effect of rLBP on this stage, *E. coli* J5 cells were incubated with $rBPI_{23}$, rLBP, or $rBPI_{23}$ plus rLBP for 15, 45, or 90 min and plated on nutrient agar with or without serum albumin supplement. On plates without serum albumin, treatment with $rBPI_{23}$ alone at 1 or 5 μg/ml or at 1 ng/ml in combination with 20 μg of rLBP per ml caused a 95 to 98% reduction in CFUs after only 15 min of incubation (Fig. 3A, −BSA); rLBP alone at 20 μg/ml caused an ~25% reduction in CFUs (Fig. 3A, −BSA) as also observed in Fig. 1A. For cells plated on agar medium supplemented with serum albumin (Figure 3B, +BSA), treatment with 20 μg of

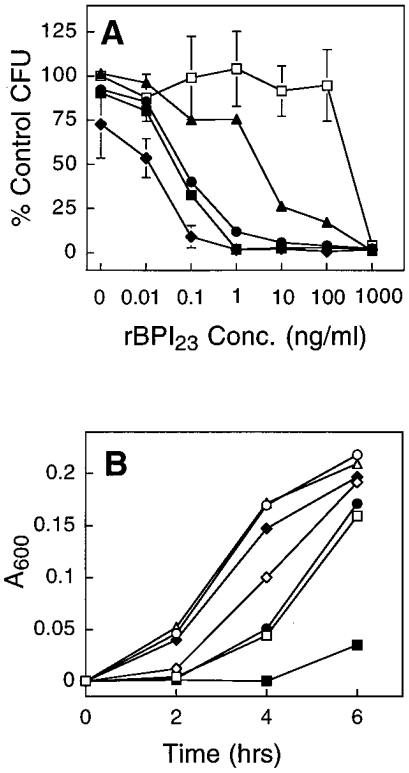

FIG. 2. Effect of rLBP on bactericidal activity of $rBPI_{23}$. *E. coli* J5 cells were grown and prepared for treatment, and the assays were performed as described in Materials and Methods. For these experiments, the order of addition was buffer, rLBP, and $rBPI_{23}$. (A) Plating experiment. Each point represents an average from at least three separate experiments. □, $rBPI_{23}$ with no rLBP. Closed symbols represent rLBP at 20 (◆), 10 (■), 5 (●), and 2.5 (▲) μg/ml in combination with various concentrations of $rBPI_{23}$. Standard errors of the means are shown as error bars on the graph only for the $rBPI_{23}$ alone and with rLBP at 20 μg/ml. The standard error did not exceed ±15% for points determining the other curves. (B) Representative broth experiment. Open symbols represent control (△) or $rBPI_{23}$ at 1 (□), 0.5 (◇), or 0.1 (○) μg/ml. Closed symbols represent rLBP at 20 μg/ml alone (◆) or plus $rBPI_{23}$ at 1 (■) and 0.1 (●) ng/ml.

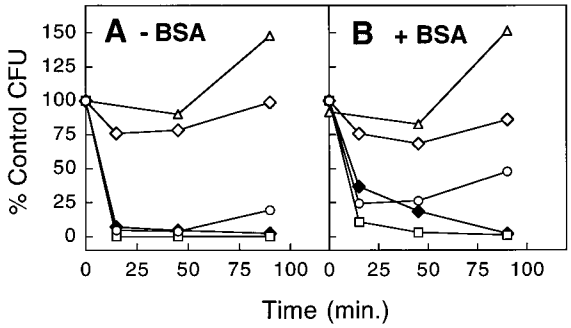

FIG. 3. Serum albumin rescue of *E. coli* J5 cells from LBP-potentiated killing by $rBPI_{23}$. *E. coli* J5 cells were grown and prepared for treatment and incubation with $rBPI_{23}$ and/or rLBP as described in Materials and Methods. Following the addition of the various components, the cells were incubated at 37°C for the times shown, diluted in sterile physiological saline, and plated on nutrient agar with 0.9% NaCl and without (A) or with (B) 1 mg of BSA (Fraction V; United States Biochemical) per ml. Percent viability was calculated as described in the legend to Fig. 1 by using untreated cells at time zero as the 100% value. Each point represents an average from at least three experiments. Open symbols represent control (△) or $rBPI_{23}$ at 5 (□) or 1 (○) μg/ml or rLBP at 20 μg/ml (◇). The closed symbol represents rLBP at 20 μg/ml plus 1 ng of $rBPI_{23}$ per ml.

rLBP per ml plus 1 ng of $rBPI_{23}$ per ml caused a progressive reduction in CFUs at 15, 45, and 90 min (~65, ~80, and >95% reductions, respectively), demonstrating that rLBP potentiates the later stage of BPI action. When measured after 90 min of incubation, the potency of the rLBP plus $rBPI_{23}$ mixture (98% CFU reduction) was nearly equivalent to that of 5 μg of $rBPI_{23}$ per ml (99% CFU reduction) and substantially exceeded that of 1 μg of $rBPI_{23}$ alone per ml (53% CFU reduction) in this assay. These data are consistent with the extent to which rLBP potentiated $rBPI_{23}$ action on cells plated on agar without serum albumin (Fig. 2A). Treatment with 20 μg of rLBP per ml plus 1 ng of $rBPI_{23}$ per ml required a longer incubation time than with 5 μg of $rBPI_{23}$ alone per ml (45 versus 90 min) to achieve >95% killing (Fig. 3B).

rLBP potentiates $rBPI_{23}$-mediated protein synthesis inhibition. Treatment with BPI ultimately leads to inhibition of incorporation of radiolabeled amino acids into trichloroacetic acid-precipitable protein (5, 13). The effect of rLBP on $rBPI_{23}$-mediated protein synthesis inhibition was measured following a 45- or 90-min incubation of *E. coli* J5 cells with rLBP (20 μg/ml) plus various concentrations of $rBPI_{23}$, using conditions similar to those described above for the experiments for which the results are shown in Fig. 3. The results of these experiments demonstrated that while incubation for 45 min with rLBP alone at concentrations up to 50 μg/ml had no effect on protein synthesis (Fig. 4A), rLBP at 20 μg/ml enhanced $rBPI_{23}$-mediated inhibition of protein synthesis by about 1,000-fold following a 45-min incubation and 10,000-fold following a 90-min incubation (Fig. 4B).

rLBP potentiates $rBPI_{23}$-mediated permeabilization to actinomycin D. BPI rapidly permeabilizes the outer membrane of gram-negative bacteria to hydrophobic agents such as actinomycin D (5, 13). To determine if rLBP also potentiates outer membrane permeabilization, cells were incubated for 10 min with various concentrations of $rBPI_{23}$ alone or with 20 μg of rLBP per ml in the presence or absence of actinomycin D and then plated on serum albumin-supplemented nutrient agar. Under these conditions, cells are protected by serum albumin from direct effects of BPI but not from the combined effects of BPI plus actinomycin D (5, 13). Treatment of cells with 1 μg of $rBPI_{23}$ per ml plus actinomycin D caused a >95% reduction in CFUs on serum albumin-supplemented agar relative to treat-

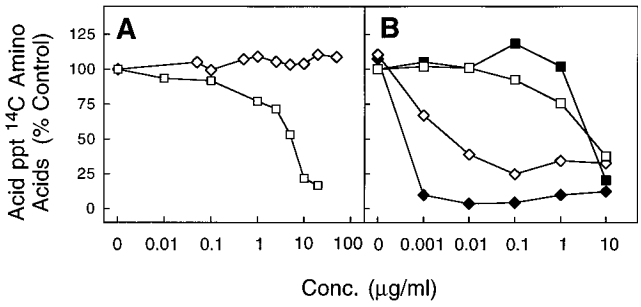

FIG. 4. LBP potentiation of protein synthesis inhibition by $rBPI_{23}$. *E. coli* J5 cells were grown and prepared for treatment as described in Materials and Methods. The cells were incubated for 45 min with various concentrations of rLBP alone or for 45 and 90 min with various concentrations of $rBPI_{23}$ alone or with rLBP at 20 μg/ml plus various concentrations of $rBPI_{23}$. Following incubation, the cells were labeled for 20 min with ^{14}C-amino acids, and the incorporation of the amino acids into the protein was determined as described in Materials and Methods. Each point represents an average from at least two (BPI with or without LBP, 90 min) or three (all others) experiments. (A) Effect of $rBPI_{23}$ (□) and rLBP (◇) on protein synthesis. (B) Effect of rLBP on protein synthesis inhibition by $rBPI_{23}$. Treatment was done with $rBPI_{23}$ for 45 (□) or 90 (■) min or with rLBP plus $rBPI_{23}$ for 45 (◇) or 90 (◆) min.

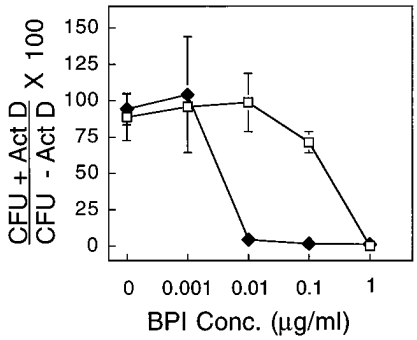

FIG. 5. LBP potentiation of $rBPI_{23}$ activity in actinomycin D (Act D)-assisted killing. *E. coli* J5 cells were grown and prepared for treatment as described in Materials and Methods. The cells were incubated for 10 min with or without 50 μg of actinomycin D per ml and with various concentrations of $rBPI_{23}$ alone (□) or with 20 μg of rLBP per ml (◆). The cells were diluted and plated on nutrient agar as described in Materials and Methods. Actinomycin D-assisted killing was the ratio of (CFU + actinomycin D/CFU − actinomycin D) × 100 determined for each BPI concentration in the assay. The results represent an average of three separate experiments.

TABLE 1. Concentrations of $rBPI_{23}$ required to kill ~95% of 4×10^7 *E. coli* J5 cells per ml in the presence of various concentrations of rLBP[a]

Protein	Concn	
	μg/ml	Molecules/cell[b]
rLBP	0	0
	5	~1,200,000
	10	~2,400,000
	20	~4,800,000
$rBPI_{23}$		~600,000
	0.01	~6,000
	0.001	~600
	0.0001[c]	~60

[a] Approximately 95% reduction in CFU (except as noted below) was achieved with the following rLBP-plus-$rBPI_{23}$ combinations (in micrograms per milliliter): 0 + 1, 5 + 0.01, 10 + 0.001, and 20 + 0.0001, respectively.

[b] Ratios obtained by dividing the number of rLBP or $rBPI_{23}$ molecules/ml by 4×10^7.

[c] ~90% reduction in CFUs.

ment with $rBPI_{23}$ alone (Fig. 5). Lower concentrations of $rBPI_{23}$ did not substantially facilitate actinomycin D-assisted killing, and actinomycin D alone was not bactericidal in this assay (Fig. 5). The addition of 20 μg of rLBP per ml reduced by almost 100-fold the $rBPI_{23}$ concentration required to achieve ~95% actinomycin D-assisted killing, though rLBP alone did not permeabilize cells to actinomycin D (Fig. 5). These results demonstrate that rLBP also potentiates the initial stage of $rBPI_{23}$ action on the outer membrane.

DISCUSSION

We have demonstrated that physiologic concentrations of LBP potentiate the bactericidal activity of BPI by reducing its 90% effective dose against the gram-negative bacterium *E. coli* J5, by up to 10,000-fold. Potentiation was observed in assays which measured (i) a reduction in CFUs on agar media (Fig. 2A and 3A and B) and attenuation of growth in broth culture (Fig. 2B) following treatment with BPI plus LBP, (ii) protein synthesis inhibition that increased as a function of incubation time with both agents (Fig. 4B), and (iii) permeabilization of the outer membrane to actinomycin D. Since rLBP had little or no bactericidal or outer membrane permeabilization activity at the concentrations used for the potentiation assays, we conclude that its primary role in these experiments is to facilitate more efficient action by BPI.

Elevated concentrations of LBP (>20 μg/ml) appeared to have slight bactericidal-like activity on *E. coli* J5 cells as measured by reduction in CFUs on solid media (Fig. 1A and 3A and B) or change in absorbance in broth (Fig. 1B). However, the reduction in CFUs for cells treated solely with rLBP may not represent true bactericidal activity since rLBP at concentrations up to 50 μg/ml (~1 μM) failed to inhibit *E. coli* protein synthesis while $rBPI_{23}$ at 10 μg/ml (~0.45 μM) inhibited this activity by about 85% (Fig. 4A). By contrast, results obtained for cells treated with LBP plus BPI represent true bactericidal activity since 20 μg of rLBP per ml potentiated $rBPI_{23}$-mediated action to the same extent (~10,000-fold) in assays measuring both protein synthesis inhibition (Fig. 4B) and reduction in CFUs (Fig. 2A and 3A and B) or growth in broth (Fig. 2B). The reduction in the number of CFUs caused by >20 μg of rLBP per ml more likely results from the formation of bacterial aggregates that are not completely dispersed by agitation prior to plating (unpublished observations).

It has been estimated that 1 or 2 million BPI molecules can bind to the surface of *E. coli* J5 cells (6, 12). Weiss et al. (33) suggested that as few as 5% of the bound BPI molecules are responsible for gram-negative bacterial growth inhibition since the removal of up to 95% of bound BPI by high concentrations of Mg^{2+} or Ca^{2+} failed to attenuate bactericidal activity. On the basis of these estimates, only 50,000 to 100,000 BPI molecules per cell would be required for cell killing. The results presented here suggest an even lower BPI molecule per cell threshold for killing. Thus, optimal LBP potentiation of BPI killing activity was achieved with 20 μg of rLBP per ml plus 0.1 ng of $rBPI_{23}$ per ml, which had a potency close to that of 1 μg of $rBPI_{23}$ alone per ml on 4×10^7 cells per ml (Fig. 2A and B). Under these conditions, a stoichiometry of only about 60 BPI molecules per cell (in the presence of ~4.8 million rLBP molecules) was sufficient to kill a single *E. coli* J5 bacterium compared with about 600,000 molecules per cell for $rBPI_{23}$ alone at 1 μg/ml (Table 1). These results suggest that only a very small number of BPI molecules may be directly responsible for lethal activity in the presence of an excess of rLBP or $rBPI_{23}$. The site(s) at which lethal BPI activity occurs is not known but might be quite different from the majority of sites to which BPI (or LBP) binds on the outer membrane of gram-negative bacteria.

The results from the present study suggest at least one possible mechanism for LBP potentiation of BPI bactericidal activity. Thus, since both BPI and LBP bind to the surface of *E. coli* J5 cells (6), LBP may function by occupying the large number of available LPS sites on the outer membrane surface (6, 12) that are normally bound by the majority of BPI molecules. Binding of LBP (or an excess of BPI) to these sites could promote subsequent interaction of a smaller number of BPI molecules at a second class of sites at or below the outer membrane surface. BPI interaction at these second sites could then lead first to breaching of the barrier presented by the outer membrane (Fig. 5) and ultimately to growth arrest and the later stage of BPI action as defined by the lack of serum albumin rescue and inhibition of protein synthesis. Since rLBP by itself is not bactericidal, it presumably could not interact at sites or perform functions in which BPI exerts its lethal effects. The discovery that $rBPI_{23}$ is composed of three biologically

active domains (11) raises the possibility that the irreversibly lethal action might be mediated by a processed portion of BPI rather than the intact protein. Further studies will be required to test this model.

While rLBP potentiated $rBPI_{23}$ bactericidal activity by up to ~10,000-fold in assays which measured the reduction of CFUs on solid medium (Fig. 2A), the inhibition of growth in broth (Fig. 2B), or the inhibition of protein synthesis (Fig. 4), it only potentiated the outer membrane permeability-increasing activity by ~100-fold (Fig. 5). This difference may reflect limitations in permeabilization to, or killing by, actinomycin D under the conditions of these experiments. Alternatively, more BPI molecules may be required to permeabilize the outer membrane than to cause growth arrest or the later expressed lethal activity in which cells become damaged beyond rescue by serum albumin.

LBP also potentiated the later stage of BPI action as seen in assays that measure both reduction of CFUs on serum albumin-supplemented agar (Fig. 3B) and inhibition of protein synthesis (Fig. 4B). However, the mixture of 1 ng of $rBPI_{23}$ per ml plus 20 μg of rLBP per ml appeared to act more slowly than 5 μg of $rBPI_{23}$ alone per ml. Thus, only a 45-min incubation was required to achieve 95% reduction in CFUs for cells treated with $rBPI_{23}$ alone, while 90 min was needed to achieve the same degree of killing for cells treated with rLBP plus $rBPI_{23}$ (Fig. 3B). By comparison, cells treated with 1 μg of $rBPI_{23}$ alone per ml never achieved greater than 75% CFU reduction on albumin-supplemented plates even after a 90-min incubation. Similarly, the extent of protein synthesis inhibition was greater at 45 min for cells treated with 10 μg of $rBPI_{23}$ alone per ml than for those treated with 1 ng of $rBPI_{23}$ per ml plus 20 μg of rLBP per ml (Fig. 4B). However, similar protein synthesis inhibition was achieved for both $rBPI_{23}$ plus rLBP and $rBPI_{23}$ alone after a 90-min incubation, confirming the results of the serum albumin rescue experiment. The reason for these differences is not known, although it would appear that LBP functions less rapidly than an excess of $rBPI_{23}$ in mediating progression by a small number of BPI molecules to the later, lethal stage. This effect may be due to affinity differences between BPI and LBP for excess LPS sites on the bacterial outer membrane surface (6) and/or to the availability, in samples treated with 5 μg of $rBPI_{23}$ per ml, of additional BPI molecules that facilitate the later, lethal stage.

Other proteins also potentiate BPI action. One of these is a basic 15-kDa protein (p15) isolated from rabbit polymorphonuclear leukocytes (10, 21). In contrast to LBP, p15 potentiates only the early phase of BPI activity and, at higher doses, actually inhibits progression to the later, lethal stage (21). Moreover, p15 potentiates BPI activity by up to 50-fold (9), compared with almost 10,000-fold by LBP. These results suggest different modes of action for p15 and rLBP.

LBP is thought to function primarily in facilitating LPS-stimulated cytokine synthesis by monocytes and macrophages (22, 25, 28, 36). This activity, which requires LBP concentrations well below those found in serum (4, 16, 17, 25), is intimately linked to the adverse consequences of gram-negative infection that can result in a cytokine cascade leading to septic shock and death. The BPI-LBP synergy described here, together with the observation that LBP can opsonize gram-negative bacteria (37), suggests that, at normal physiological concentrations, LBP may play other, more-direct roles in host defense against gram-negative infection. For example, LBP-opsonized bacteria might be more efficiently killed by BPI within polymorphonuclear phagocytes or perhaps by BPI in the bloodstream. This latter possibility is supported by a recent observation that BPI is present at 0.2 to 2 ng/ml in plasma of healthy humans (35) and at up to >200 ng/ml in plasma from septic patients (34), levels which are bactericidal in the presence of physiologic concentrations of LBP (Fig. 2). Another possible function is suggested by the observation that extravascular LBP levels can increase in conjunction with elevated LBP levels in serum (16). Under these conditions, LBP might potentiate bactericidal activity by BPI released through degranulation of polymorphonuclear phagocytes that have migrated to the site of focal infections. Future experiments will extend the studies described here with *E. coli* J5 (a rough gram-negative bacterium) to clinically relevant gram-negative bacterial strains.

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