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Competition between $rBPI_{23}$, a Recombinant Fragment of Bactericidal/Permeability-Increasing Protein, and Lipopolysaccharide (LPS)-Binding Protein for Binding to LPS and Gram-Negative Bacteria

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Lipopolysaccharide (LPS)-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) are two structurally related lipid A-binding proteins with divergent functional activities. LBP mediates activation of macrophage and other proinflammatory cells. In contrast, BPI has potent bactericidal and LPS-neutralizing activities. A recombinant fragment of BPI ($rBPI_{23}$) retains the potent biological activities of the holo protein and may represent a novel therapeutic agent for the treatment of gram-negative infections, sepsis, and endotoxemia. For therapeutic effectiveness in many clinical situations, $rBPI_{23}$ will have to successfully compete with high serum levels of LBP for binding to endotoxin and gram-negative bacteria. The relative binding affinities of $rBPI_{23}$ and human recombinant LBP (rLBP) for lipid A and gram-negative bacteria were evaluated. The binding of both proteins to lipid A was specific and saturable with apparent K_ds of 2.6 nM for $rBPI_{23}$ and 58 nM for rLBP. $rBPI_{23}$ was approximately 75-fold more potent than rLBP in inhibiting the binding of ^{125}I-rLBP to lipid A. The binding affinity of $rBPI_{23}$ (K_d = 70 nM) for *Escherichia coli* J5 bacteria was also significantly higher than that of rLBP (K_d = 1,050 nM). In addition, $rBPI_{23}$ at 0.2 μg/ml was able to inhibit LPS-induced tumor necrosis factor release from monocytes in the presence of 20 μg of rLBP per ml. These results demonstrate that $rBPI_{23}$ binds more avidly to endotoxin than does rLBP and that, even in the presence of a 100-fold weight excess of rLBP, $rBPI_{23}$ effectively blocks the proinflammatory response of peripheral blood mononuclear cells to endotoxin.

Lipopolysaccharide (LPS) or endotoxin, a component of the outer membrane of gram-negative bacteria, is an important mediator in the pathogenesis of gram-negative septic shock (20, 24), one of the major causes of death in intensive-care units in the United States (23). LPS does not cause cellular injury directly. Rather, it is generally accepted that the LPS molecule activates monocytes and macrophages to secrete inflammatory cytokines (tumor necrosis factor [TNF] and interleukin-1, etc.) and other potent mediators. These mediators, in turn, act on additional target cells to produce cardiovascular shock, multisystem organ failure, and death (2, 8). The proinflammatory bioactivities exhibited by LPS are known to reside in the lipid A region of the molecule (25).

In recent years, two lipid A-binding proteins that are believed to have important roles in mediating the host response to endotoxin have been identified in mammals: lipopolysaccharide-binding protein (LBP) (27, 31) and bactericidal/permeability-increasing protein (BPI) (4, 36). Human LBP shares a 45% amino acid sequence identity with human BPI (26). Despite the sequence homology, the two lipid A-binding proteins have very different functional activities. LBP is a serum protein synthesized by hepatocytes that binds LPS and initiates the proinflammatory host response to gram-negative infection (33). LBP markedly potentiates the sensitivity of the host to LPS by a mechanism which involves binding of the LBP-LPS complex to CD14 receptors on monocytes, neutrophils, and endothelial cells (42). LBP is not cytotoxic against gram-negative organisms. In contrast, BPI is a neutrophil granule protein with bactericidal and LPS-neutralizing properties (4, 15, 16, 21, 28, 34, 39). Polymorphonuclear leukocytes have an essential role in host defense against bacterial infections, and BPI is believed to be a critical element in this cell's potent antimicrobial activity against gram-negative bacteria and endotoxin. The divergent functional properties of these two LPS-binding proteins may be explained by the inability of BPI-LPS complexes to bind to cell surface CD14 receptors.

A proteolytically derived fragment of BPI, corresponding to the amino-terminal region of BPI, has been shown to retain the potent bactericidal and endotoxin-neutralizing activities of the holo molecule (21, 22). This observation led to the cloning, expression, and purification of a recombinant protein ($rBPI_{23}$) corresponding to the amino-terminal 23-kDa fragment of human BPI. The binding affinity and specificity of BPI and $rBPI_{23}$ for lipid A and LPS were shown to be essentially identical (7). Furthermore, $rBPI_{23}$ was shown to be bactericidal against a broad spectrum of gram-negative organisms (37), to neutralize LPS activity in a variety of clinically relevant in vitro assays (9, 18, 35), and to prevent the lethal effects of LPS in animal models of sepsis (1, 12). Because of these potent activities, $rBPI_{23}$ is being considered as a potential therapeutic agent for the treatment of gram-negative infections, sepsis, and endotoxemia.

For therapeutic effectiveness in many clinical situations,

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$rBPI_{23}$ will have to compete successfully with serum LBP for binding to endotoxin and gram-negative organisms. LBP is a relatively abundant serum protein and is present in normal human plasma at concentrations of around 5 to 10 μg/ml (13). LBP is known to be an acute-phase protein in rabbits (31) and presumably also in humans since the levels of LBP in the plasma of six septic patients ranged from 18 to 64 μg/ml with an average of 41 μg/ml (40). In contrast to LBP, BPI is primarily associated with circulating polymorphonuclear leukocytes and is not an abundant serum protein. In 20 healthy adults, BPI concentrations in plasma ranged from 0.2 to 2.1 ng/ml as determined by using a sensitive enzyme-linked immunosorbent assay (ELISA) (41). The ability of $rBPI_{23}$, at therapeutically desirable dose levels, to compete successfully with relatively high plasma levels of LBP will depend, in part, upon the relative affinities of LBP and $rBPI_{23}$ for LPS.

In the present study, we have used in vitro binding assays to determine the binding affinities of recombinant human LBP (rLBP) and $rBPI_{23}$ for lipid A and gram-negative bacteria. The results demonstrate that $rBPI_{23}$ has a significantly higher affinity for lipid A and whole bacteria than does rLBP. Consistent with these in vitro binding results, we find that $rBPI_{23}$ blocks the LPS-mediated release of TNF by monocytes, even in the presence of 100-fold weight excess of rLBP over $rBPI_{23}$.

MATERIALS AND METHODS

Reagents. Recombinant $rBPI_{23}$, corresponding to the N-terminal fragment (amino acids 1 to 199) of human BPI was cloned, expressed, and purified to homogeneity (7). rLBP was cloned and expressed as described previously (29). *Escherichia coli* O113 smooth LPS and *E. coli* J5 lipid A were obtained from RIBI, ImmunoChem Research, Inc., Hamilton, Mont. *Salmonella minnesota* R60 RaLPS (lipid A with complete core oligosaccharide) was obtained from List Biological Laboratories, Inc., Campbell, Calif. *E. coli* J5 bacteria were a gift from Lowell Young, Kuzell Institut for Arthritis and Infectious Diseases, Medical Research Institut, California Pacific Medical Center, San Francisco. Bovine serum albumin (very low endotoxin, fatty acid-free) was purchased from Miles, Kankakee, Ill.

PBMC preparations. Peripheral blood mononuclear cells (PBMC) were prepared from buffy coat from blood of healthy human donors by separation over Ficoll-Hypaque (3). The percentage of monocytes (15 to 25%) in different PBMC preparations was determined by fluorescence-activated cell sorter analysis on the basis of expression of CD14 antigen as described previously (5). For experimentation, the PBMC (2×10^6) were incubated in 1 ml of Dulbecco's modified Eagle medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 1% (vol/vol) HB101 supplement (Irvine Scientific, Santa Ana, Calif.) at 37°C and 7.5% CO_2 for 4 h. After incubation, the cells and media were separated by centrifugation. The PBMC supernatants were assayed for TNF by using a commercial immunoassay kit as described by the manufacturer (Biokine ELISA; T Cell Sciences, Cambridge, Mass.).

Bacterial growth conditions. *E. coli* J5 cells were grown in triethanolamine-buffered medium (38). Overnight stationary-phase cultures were transferred to fresh medium (diluted 1/50) and grown for about 3 h at 37°C to the mid-logarithmic phase. The bacteria were sedimented by centrifugation at 3,000 rpm ($2,000 \times g$) for 5 min in a J-6M Beckman centrifuge and washed twice in Dulbecco's phosphate-buffered saline (D-PBS). The bacteria were resuspended in 10 ml of D-PBS, and the concentration was determined by reading the A_{570} ($1\ A_{570} = 1.25 \times 10^9$ cells per ml).

Radiolabeling of $rBPI_{23}$ and rLBP. Protein radiolabeling was performed as described previously (7) with modifications. Briefly, iodination was initiated by the addition of 4 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Wilmington, Del.) and two IODO-BEADS to samples containing 100 to 200 μg of protein in a total volume of 500 μl of D-PBS (pH 7.0). After 15 min of incubation at room temperature, free ^{125}I was separated from ^{125}I-labeled protein by gel filtration on a Sepharose G-25 column and the iodinated proteins were exchanged into 20 mM sodium citrate–0.15 M NaCl–0.1% Pluronic F68 (poloxamer 188)–0.002% polysorbate 80 (pH 5.0). Specific activities of ^{125}I-$rBPI_{23}$ and ^{125}I-rLBP varied from 2 to 8 μCi/μg in different preparations.

Binding of $rBPI_{23}$ or rLBP to immobilized *E. coli* J5 lipid A. Binding assays were conducted by using a modification of a previously described protocol (7). In brief, *E. coli* J5 lipid A suspension was sonicated and diluted in methanol to a concentration of 0.1 μg/ml, and 100-μl aliquots were allowed to evaporate in wells (Immulon 2 Removawell Strips; Dynatech, Inc., Chantilly, Va.) overnight at 37°C. The wells were then blocked with 215 μl of D-PBS–1% bovine serum albumin (BSA) for 3 h at 37°C. The blocking buffer was discarded, and the wells were washed twice with D-PBS–0.1% BSA (D-PBS–BSA) and then incubated overnight at 4°C with increasing amounts of ^{125}I-$rBPI_{23}$ or ^{125}I-rLBP in 100 μl of D-PBS–BSA. After three washes in D-PBS–BSA, bound radioactivity remaining in the wells was counted with an LKB gamma counter, and the results were depicted as the mean of six replicate samples. The binding of ^{125}I-labeled protein to wells treated with D-PBS–1% BSA only was taken to represent nonspecific binding; specific binding of ^{125}I-labeled protein was defined as the difference between total and nonspecific binding. Nonspecific binding represented between 5 and 36% of total binding. The resulting data were fitted to a standard binding equation by computerized nonlinear curve fitting (GraFit; Erathacus Software Ltd., Staines, United Kingdom). K_d values and capacity were taken directly from the program outputs. Control experiments demonstrated that neither $rBPI_{23}$ nor rLBP at the concentrations used in binding assays was able to strip immobilized ^{125}I-labeled LPS from the surface of wells (results not shown).

Inhibition of ^{125}I-rLBP binding to lipid A. *E. coli* J5 lipid A was diluted in methanol to a concentration of 1 μg/ml, and 100-μl aliquots were dispensed into wells and allowed to evaporate overnight at 37°C. The wells were blocked, washed, and incubated overnight at 4°C in 100 μl of D-PBS–BSA containing a fixed amount of ^{125}I-rLBP (1.45×10^6 cpm; specific activity, 1.74 μCi/μg), equivalent to a concentration of 10 nM, and increasing concentrations of unlabeled $rBPI_{23}$ or rLBP. The wells were then treated as described above.

Binding of $rBPI_{23}$ or rLBP to bacteria. *E. coli* J5 bacteria (2×10^7) were incubated in 1.5 ml of D-PBS containing a fixed amount of ^{125}I-$rBPI_{23}$ (187,000 cpm; specific activity, 1.47 μCi/μg) or ^{125}I-rLBP (3×10^6 cpm; specific activity, 1.74 μCi/μg) and increasing concentrations of unlabeled $rBPI_{23}$ or rLBP. After 60 min of incubation at room temperature, the bacteria were sedimented by centrifugation at $13,000 \times g$ for 15 min at 4°C in an Eppendorf model 1217 microfuge and washed twice with 1.5 ml of D-PBS. Bound ^{125}I-protein was measured by counting the radioactivity in the resulting pellets.

Chromogenic LAL assay. Increasing concentrations of unlabeled rLBP or ^{125}I-rLBP (specific activity, 2 μCi/μg) were incubated in the presence of 2 ng of *E. coli* O113 LPS (60-μl volume) per ml for 3 h at 37°C. The samples were then diluted

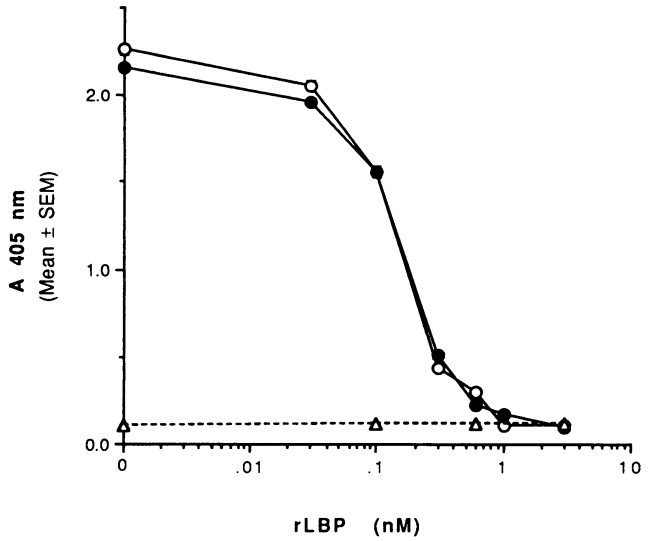

FIG. 1. Inhibition of LPS activity by rLBP in the chromogenic LAL assay. Two nanograms of *E. coli* O113 LPS per ml was preincubated with increasing doses of ^{125}I-rLBP (closed circles) or unlabeled rLBP (open circles). The mixtures were then assayed for LPS activity in the chromogenic LAL assay. As a control (open triangles), rLBP was incubated in the presence of D-PBS only. Data are expressed as the mean A_{405} ± standard error of the mean (SEM) of six replicates.

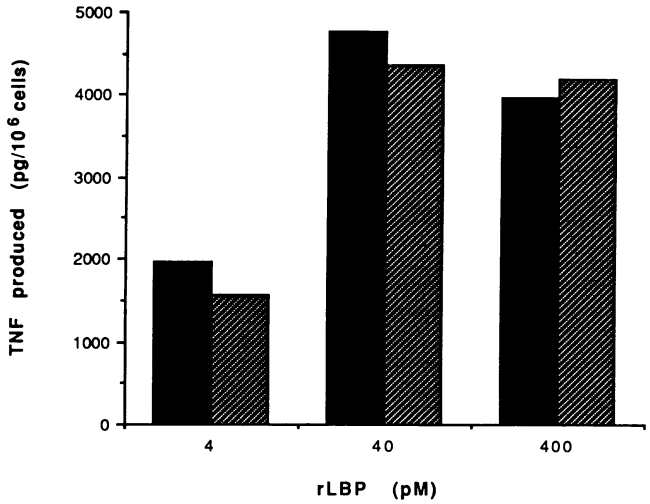

FIG. 2. Effect of rLBP on LPS-mediated TNF release by THP-1 cells. THP-1 cells were stimulated by increasing concentrations of radiolabeled (■) or unlabeled (▨) rLBP in the presence of 20 ng of RaLPS per ml for 2 h at 37°C. The cells were then sedimented by centrifugation, and the supernatants were assayed for TNF by an ELISA. Background level (no LBP) was subtracted from each datum point.

with D-PBS to bring the LPS concentration to 333 pg/ml, and the amount of LPS activity was determined in the chromogenic *Limulus* amebocyte lysate (LAL) assay (Whittaker M. A. Bioproducts, Inc.) as described previously (7).

LPS-induced TNF production by THP-1 cells. THP-1 cells were obtained from the American Type Culture Collection Tumor Immunology Bank, Rockville, Md. Cells were grown to a density of 3.5 × 10^5/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 1 mM glutamine, 1 mM pyruvate, 10 U of penicillin per ml, and 10 U of streptomycin per ml (all from GIBCO). They were then transferred at the same density into fresh medium supplemented with 100 nM 1,25-dihydroxyvitamin D_3 (Biomol Research Laboratories, Plymouth Meeting, Pa.) and incubated for another 72 h for induction of surface CD14 (11). THP-1 cells (2 × 10^6 cells) were incubated in 1 ml of RPMI 1640 medium containing 0.1% BSA, 20 ng of *S. minnesota* RaLPS per ml, and various concentrations of unlabeled rLBP or ^{125}I-rLBP (specific activity, 2 μCi/μg) for 2 h at 37°C. Following this incubation, the cells were sedimented by centrifugation for 7 min at 1,200 × *g*, and the supernatants were assayed for TNF by ELISA.

Every experiment detailed in this manuscript was conducted at least three times, and representative results are given.

RESULTS

Properties of unlabeled and ^{125}I-labeled proteins. In a previous study (7), we demonstrated that the procedures used to prepare ^{125}I-$rBPI_{23}$ did not impair the functional activities of this protein. However, to our knowledge, ^{125}I-rLBP has never been used in a published study. Therefore, control studies were performed to determine whether the radiolabeling procedure altered the functional activities of rLBP. rLBP inhibits LPS activity in the LAL assay, and Fig. 1 demonstrates that ^{125}I-rLBP and rLBP had essentially identical inhibitory activities. LBP is also known to stimulate the LPS-mediated release of TNF by the human monocytic cell line, THP-1 (19), and Fig. 2 demonstrates that ^{125}I-rLBP and rLBP had comparable stimulatory activities in this assay.

The results described above demonstrate that the oxidation procedure used to radiolabel rLBP did not impair the functional activities of a significant fraction of the rLBP molecules. However, these results do not exclude the possibility that rLBP molecules with covalently linked ^{125}I have reduced functional activity since the radiolabeling procedure resulted in the labeling of only a small fraction of the rLBP molecules (0.05 mol of iodine per mol of protein). To address this possibility, a binding experiment was performed in which lipid A-coated wells were incubated overnight at 4°C with a constant amount of rLBP (250 ng per well) prepared by mixing unlabeled and labeled preparations of rLBP at different ratios. After three washes, the amount of rLBP bound per well was then calculated on the basis of the counts per minute bound to each well and the specific radioactivity of rLBP in the incubation mixture. The results were as follows: 28, 33, 29, and 29 ng of rLBP bound to each well at ratios (percent ^{125}I-rLBP/percent rLBP) of 100:0, 20:80, 40:60, and 60:40, respectively (values are averages of six replicates). The results show that essentially the same amount of rLBP (28 to 33 ng) was bound to lipid A-coated wells for all ratios of labeled rLBP/unlabeled rLBP tested. These results demonstrate that the covalent attachment of ^{125}I described did not impair the binding activity of rLBP.

Binding of $rBPI_{23}$ and rLBP to lipid A. To evaluate the relative affinities of $rBPI_{23}$ and rLBP for lipid A, we conducted equilibrium binding studies using the iodinated proteins. Results from a representative study are depicted in Fig. 3 and demonstrate that there was approximately a 22-fold difference in the relative affinities of rLBP and $rBPI_{23}$ for lipid A. In this study, rLBP had an apparent K_d of 58 nM (Fig. 3B) compared with a K_d of 2.6 nM for $rBPI_{23}$ (Fig. 3A), indicating that $rBPI_{23}$ has a higher affinity than rLBP for lipid A. In addition, the two proteins also differed in terms of binding capacities. Almost

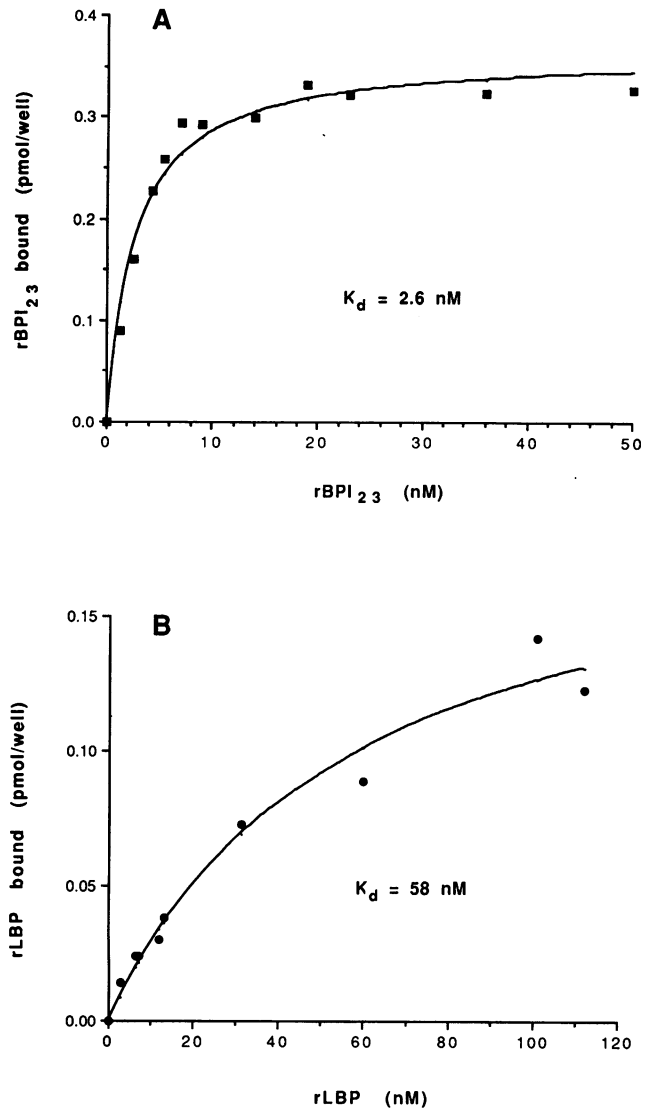

FIG. 3. Binding $rBPI_{23}$ and rLBP to *E. coli* J5 lipid A. Lipid A-coated wells were incubated overnight at 4°C with increasing amounts of ^{125}I-$rBPI_{23}$ (A) or ^{125}I-rLBP (B) as described in Materials and Methods. Each point represents the mean of six replicates.

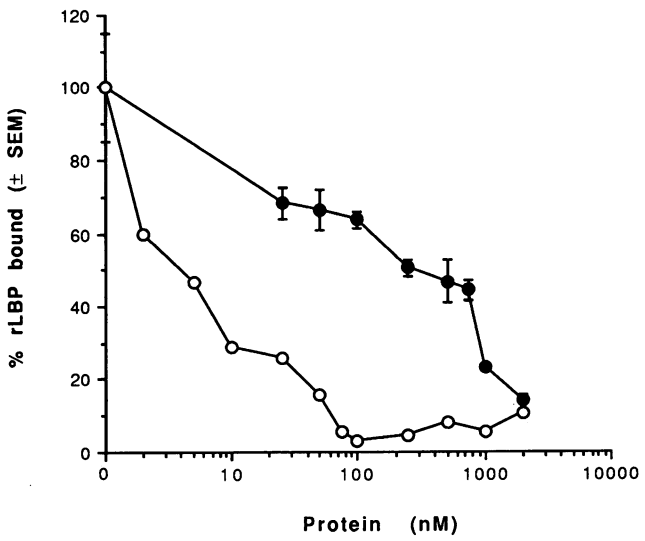

FIG. 4. Competition by $rBPI_{23}$ and rLBP for ^{125}I-rLBP binding to lipid A. Immobilized lipid A was incubated overnight at 4°C in the presence of ^{125}I-rLBP (10 nM) and increasing concentrations of $rBPI_{23}$ (○) or rLBP (●) as described in Materials and Methods. Each point represents the mean of six replicates ± standard error of the mean (SEM).

twice as many molecules of $rBPI_{23}$ as rLBP were bound to lipid A at saturation (0.36 versus 0.20 pmol per well, respectively).

Competition by $rBPI_{23}$ and rLBP for binding of ^{125}I-rLBP to lipid A. To further characterize the binding activity of $rBPI_{23}$ and rLBP, a direct competition assay was performed in which the abilities of $rBPI_{23}$ and rLBP to inhibit the binding of ^{125}I-rLBP to immobilized lipid A was determined. The results shown in Fig. 4 demonstrate that both proteins were able to inhibit ^{125}I-rLBP binding in a dose-dependent manner. However, the concentration of $rBPI_{23}$ required to inhibit binding of ^{125}I-rLBP to lipid A by 50% (IC_{50}) was 4 nM compared with an IC_{50} of 300 nM for rLBP. Therefore, $rBPI_{23}$ was approximately 75-fold more potent than rLBP in this assay. These competition results further support our finding that $rBPI_{23}$ has a significantly higher affinity for lipid A than does rLBP.

Binding of $rBPI_{23}$ and rLBP to *E. coli* J5 bacteria. In many clinical situations, the predominant presentation of LPS to the host may be as an envelope component of invading bacteria. Therefore, it was important to compare the relative affinities of $rBPI_{23}$ and rLBP to intact gram-negative bacteria. As shown in Fig. 5, both rLBP and $rBPI_{23}$ demonstrated saturable binding to *E. coli* J5 bacteria. In this representative study, rLBP had an apparent K_d of 1,050 nM (Fig. 5B) compared with 70 nM for $rBPI_{23}$ (Fig. 5A). Once again, the two proteins also differed in terms of binding capacities. Approximately twice as many $rBPI_{23}$ molecules as rLBP molecules bound to each *E. coli* J5 bacterium at saturation (1.17×10^6 $rBPI_{23}$ molecules bound per bacterium versus 0.54×10^6 rLBP molecules bound per bacterium).

Effect of $rBPI_{23}$ on the LPS-induced production of TNF by human PBMC mediated by rLBP. Results from our in vitro binding studies suggested that $rBPI_{23}$ would be a potent inhibitor of the LPS activation of monocytes mediated by LBP. To test this prediction, we measured the ability of $rBPI_{23}$ to inhibit TNF production by human PBMC incubated with LPS in a serum-free system containing various concentrations of rLBP. As shown in Fig. 6, rLBP enhanced the LPS-mediated production of TNF. However, low concentrations of $rBPI_{23}$ potently inhibited TNF production even in the presence of high rLBP levels. For example, at all three LPS concentrations tested, when 400 nM (20 μg/ml) rLBP was used, an almost complete inhibition of TNF release was observed with 8 nM (0.2 μg/ml) $rBPI_{23}$.

DISCUSSION

BPI and LBP are evolutionarily related proteins, and both contain a binding site specific for the lipid A region of bacterial LPS (30). Although both proteins have been shown to bind to a variety of bacterial LPSs (7, 32), there is no information about the relative binding affinities of these two proteins. In this study, we have compared the binding affinities of rLBP and $rBPI_{23}$, a biologically active recombinant fragment of BPI, to lipid A and gram-negative bacteria. We found that $rBPI_{23}$ has a significantly higher affinity for lipid A than does rLBP (K_ds of

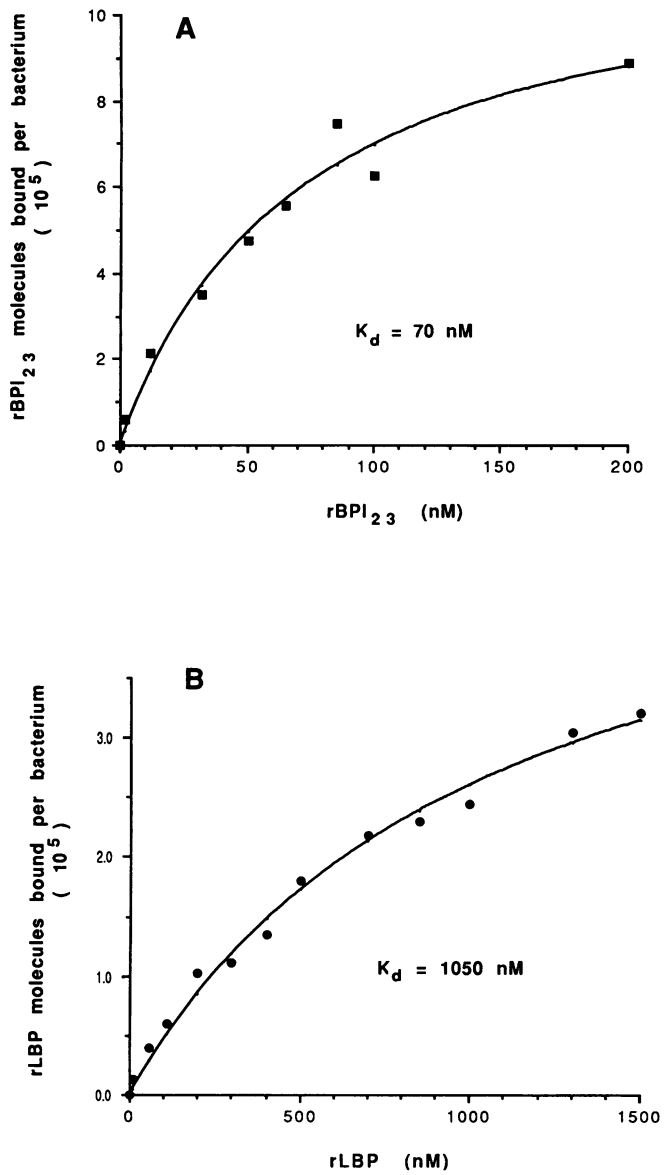

FIG. 5. Binding of $rBPI_{23}$ and rLBP to *E. coli* J5 bacteria. *E. coli* J5 bacteria (2×10^7) were incubated in 1.5 ml of D-PBS in the presence of ^{125}I-$rBPI_{23}$ (2 nM) and various concentrations of unlabeled $rBPI_{23}$ (A) and ^{125}I-rLBP (10 nM) and various concentrations of unlabeled rLBP (B) as described in Materials and Methods.

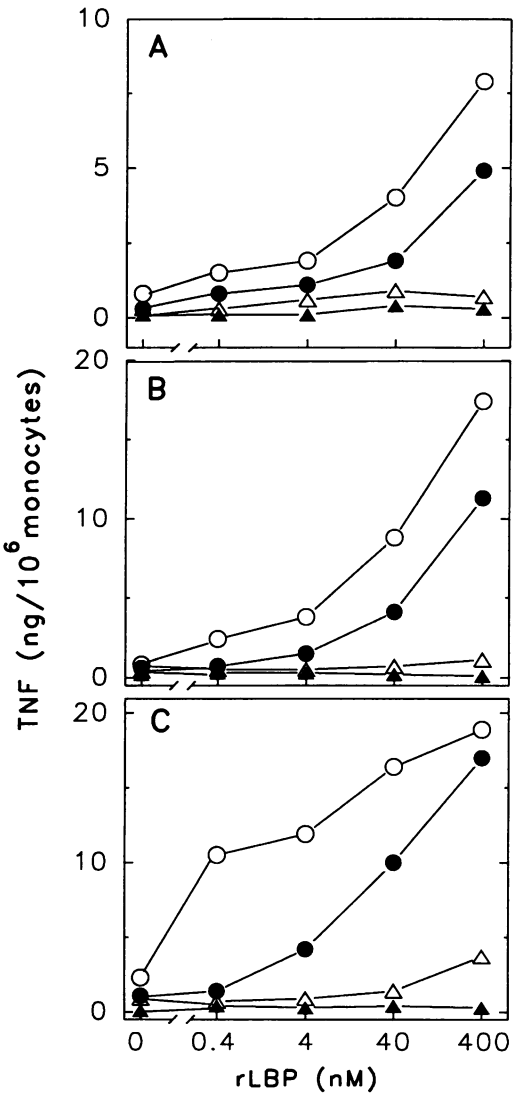

FIG. 6. Effect of $rBPI_{23}$ on the rLBP-dependent production of TNF by LPS. Human PBMC were incubated with *E. coli* O113 LPS (40 pg/ml [A], 130 pg/ml [B], or 400 pg/ml [C]) and various concentrations of human rLBP in the presence of 0 (○), 0.8 (●), 8 (△), or 80 (▲) nM $rBPI_{23}$ as described in Materials and Methods.

2.6 and 58 nM, respectively). Consistent with the binding specificity of these proteins for bacterial LPSs, $rBPI_{23}$ also has a higher affinity for *E. coli* J5 bacteria than does rLBP (K_ds of 70 and 1,050 nM, respectively). The affinity of $rBPI_{23}$ for *E. coli* J5 bacteria is similar to that previously reported for holo BPI (14).

LBP and $rBPI_{23}$ also differed in terms of saturation values. Almost twice as many molecules of $rBPI_{23}$ as rLBP molecules were bound to lipid A or *E. coli* J5 bacteria at saturation. A single *E. coli* cell has been estimated to contain around 2×10^6 LPS molecules (24), which is a value comparable to the number of $rBPI_{23}$ molecules bound to each *E. coli* J5 cell at saturation (1.17×10^6). This observation is consistent with identification of LPS as the binding site for $rBPI_{23}$ (7). Furthermore, since LPS is the major lipid component of the outer monolayer of the outer membrane of gram-negative bacteria (24), these results suggest that the packing density of bound $rBPI_{23}$ on the surface of the bacterial cell at saturation is very high. Perhaps the smaller mass of $rBPI_{23}$ relative to that of rLBP allows it to pack more densely onto immobilized lipid A micelles or bacterial outer membranes. This interpretation is consistent with our previous report that, at saturation, twice as many molecules of $rBPI_{23}$ versus holo BPI molecules are bound to immobilized lipid A, although the binding affinities of $rBPI_{23}$ and holo BPI were essentially identical (7). Since LBP and BPI are similar in mass, these results suggest that the enhanced saturation values for $rBPI_{23}$ are again related to its smaller mass.

To further evaluate the relative binding activities of these two proteins, we performed competition studies in which the concentration of rLBP or $rBPI_{23}$ required to inhibit the

binding of ^{125}I-rLBP to immobilized lipid A by 50% (i.e., IC_{50}) was determined. The IC_{50} value for $rBPI_{23}$ was approximately 75-fold lower than the IC_{50} for rLBP in this assay. Therefore, $rBPI_{23}$ was even more potent against rLBP in this competition assay format than would be predicted solely on the basis of the 22-fold difference in the binding affinities of these two proteins. However, the two proteins also differ in terms of their binding capacities to lipid A, and $rBPI_{23}$ may be more effective than rLBP in coating the surface of lipid A micelles, thus enhancing the ability of $rBPI_{23}$ to compete with rLBP. In a reciprocal experiment, in which ^{125}I-labeled $rBPI_{23}$ competed with $rBPI_{23}$ and rLBP for binding to lipid A, similar results were obtained; i.e., rLBP was an 80-fold-less-potent competitor (data not shown).

In a study by Tobias et al. (32), the binding affinity of rabbit LBP for ReLPS (lipid A with attached 3-keto-2-deoxyoctonate) was estimated to be 1 nM, which represents an affinity more than 50-fold higher than we have obtained for the binding of human rLBP to lipid A. The inhibition binding assay used in the previous study involved incubation of a fixed concentration of LBP with increasing concentrations of ReLPS in solution, and the affinity calculation depended upon the assumption that ReLPS exists in a monomolecular form in aqueous solution. However, LPS is an amphipathic molecule and exists primarily as macromolecular aggregates or micelles in aqueous suspension (6, 27). Because of the solubility characteristics of lipid A and LPS, we used a different binding assay format in our affinity studies in which a fixed amount of lipid A immobilized onto plastic was incubated with increasing concentrations of rLBP or $rBPI_{23}$. In preliminary studies using purified rabbit LBP and our binding assay format, we found that rabbit LBP and human rLBP bound lipid A with similar affinities.

The results of our in vitro binding and competition studies demonstrate that $rBPI_{23}$ has a significantly higher affinity than rLBP for LPS and gram-negative bacteria. These results indicated that $rBPI_{23}$ would be a very potent inhibitor of LPS-induced release of cytokines by monocytes, macrophages, and neutrophils even in the presence of high LBP levels. This was confirmed by our finding that low concentrations of $rBPI_{23}$ effectively prevented the LPS-mediated induction of TNF production by human PBMC incubated in the presence of high rLBP levels. For example, TNF production by PBMC incubated in the presence of physiologically relevant LPS concentrations (40 to 400 pg/ml) and 400 nM (20 μg/ml) rLBP was almost completely inhibited by 8 nM (0.2 μg/ml) $rBPI_{23}$. These results show that $rBPI_{23}$ can successfully compete with a 100-fold weight excess of rLBP.

The results presented here are consistent with other reports that low concentrations of $rBPI_{23}$ antagonize a variety of LPS-mediated cellular effects despite the presence of serum or plasma, which presumably contained relatively high levels of LBP. These LPS-mediated effects include release of proinflammatory cytokines (TNF and interleukin-1β, -6, and -8) and oxygen-derived free radicals by cells in human blood (18, 37), synthesis of tissue factor by monocytes (17), and synthesis of adhesion molecules by human endothelial cells (10). Furthermore, $rBPI_{23}$ has been shown to inhibit the binding and internalization of LPS by monocytes mediated by LBP (9). Results from our in vitro binding studies suggest that the potent inhibitory activity of $rBPI_{23}$ in these clinically relevant functional assays is related to the ability of $rBPI_{23}$ to successfully compete with LBP for binding to LPS. Furthermore, our results suggest that $rBPI_{23}$ may be an effective therapeutic agent even in the presence of high endogenous levels of LBP in human serum.

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