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High-Affinity Binding of the Bactericidal/Permeability-Increasing Protein and a Recombinant Amino-Terminal Fragment to the Lipid A Region of Lipopolysaccharide

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Bactericidal/permeability-increasing protein (BPI) is a 55-kDa cationic protein ($nBPI_{55}$) elaborated by polymorphonuclear neutrophils (PMN). BPI has potent bactericidal activity against a wide variety of gram-negative organisms and neutralizes endotoxin activities. An N-terminal fragment of $nBPI_{55}$ exhibits the bactericidal and antiendotoxin properties of the holoprotein. To further characterize the biological activities of the N-terminal fragment, a recombinant protein ($rBPI_{23}$) corresponding to the first 199 amino acids of human BPI was produced and purified. $rBPI_{23}$ had antibacterial activity equivalent to that of $nBPI_{55}$ against *Escherichia coli* J5. Furthermore, both $rBPI_{23}$ and $nBPI_{55}$ bound identically to a broad range of R- and S-form lipopolysaccharides (LPS) and to natural and synthetic lipid A. Binding of radiolabeled $nBPI_{55}$ to LPS was inhibited in an identical fashion by either $nBPI_{55}$ or $rBPI_{23}$. The binding of both proteins to immobilized *E. coli* J5 lipid A was inhibited in a comparable fashion by long- or short-chain LPS or lipid A. The binding of both $rBPI_{23}$ and $nBPI_{55}$ was specific, saturable, and of high affinity, with an apparent K_d of approximately 2 to 5 nM for all ligands tested. These results demonstrate that BPI recognizes the highly conserved lipid A region of bacterial LPS via residues contained within the amino-terminal portion of the BPI molecule.

Lipopolysaccharide (LPS), a major component of the outer membranes of gram-negative bacteria, consists of serotype-specific O-side-chain polysaccharides linked to a conserved region of core oligosaccharide and lipid A (1, 26). LPS is an important mediator in the pathogenesis of gram-negative septic shock (19), one of the major causes of death in intensive-care units in the United States (24).

In recent years, a number of LPS-binding proteins have been identified in various mammalian tissues (18, 20, 28, 33). Among the most extensively studied is bactericidal/permeability-increasing protein (BPI), a basic protein found in the azurophilic granules of polymorphonuclear leukocytes (5, 32, 37, 38, 42). This protein is cytotoxic against many gram-negative bacteria but has no cytotoxic activity toward gram-positive bacteria, fungi, or mammalian cells (4, 38). Two proteins isolated from human polymorphonuclear leukocytes with potent antibacterial activity have been called bactericidal protein (BP) and cationic antimicrobial protein (CAP57) (9, 31, 32). On the basis of N-terminal amino acid sequence identity, CAP57 has been recognized to be BPI, and it seems likely that BP is also BPI or a closely related isoform (32).

Ooi et al. (22) have shown that a proteolytic fragment corresponding to the N-terminal half of human BPI (7) possesses all of the antibacterial activities exhibited by the 55-kDa naturally derived human holoprotein ($nBPI_{55}$). To further characterize the biological activities of the N-terminal fragment, the DNA sequence encoding the first 199 amino acids of the human holoprotein was cloned and expressed and the recombinant protein was purified to homogeneity. This recombinant protein has a molecular mass of approximately 23 kDa and is referred to as $rBPI_{23}$.

The specificity of BPI for gram-negative bacteria is thought to be due to the selective binding of BPI to LPS in the bacterial outer membrane (12, 37, 41). This conclusion is further supported by recent reports that BPI has potent LPS-neutralizing activity in a variety of in vitro assays (14, 15, 21). However, little is known about the specificity and avidity of the interactions of BPI with purified LPS, and the influence of core and O-antigen polysaccharides on the binding of BPI to isolated LPS has not been evaluated.

With a variety of binding-assay formats, the interaction of BPI with purified LPS was investigated. We report here that both $rBPI_{23}$ and $nBPI_{55}$ bind specifically and with high affinity to natural and synthetic lipid A, as well as to a wide spectrum of LPS isolated from clinically relevant smooth bacterial strains. Our results support the concept that BPI recognizes the highly conserved lipid A region of bacterial LPS via residues contained within the amino-terminal portion of the BPI molecule.

MATERIALS AND METHODS

LPS and lipid A preparations. All lipid A preparations were obtained from commercial sources (List Biological Laboratories, Inc., Campbell, Calif.; Ribi ImmunoChem Research, Inc., Hamilton, Mont.; and ICN Biomedical, Inc., Costa Mesa, Calif.). Synthetic lipid A compounds were designated as follows: synthetic diphosphoryl *Escherichia coli*-type lipid A, LA-15-PP, and synthetic diphosphoryl *Salmonella*-type lipid A, LA-16-PP. Rough-form (R-form) LPSs from *Salmonella minnesota* were obtained from List Biological Laboratories, Inc. Smooth-form (S-form) LPSs were either obtained from commercial sources (*Klebsiella pneumoniae*, *E. coli* O113, *Yersinia enterocolitica*, and *Salmonella abortus equi*) or isolated by the aqueous phenol procedure described by Westphal and Jann (43) from wild-

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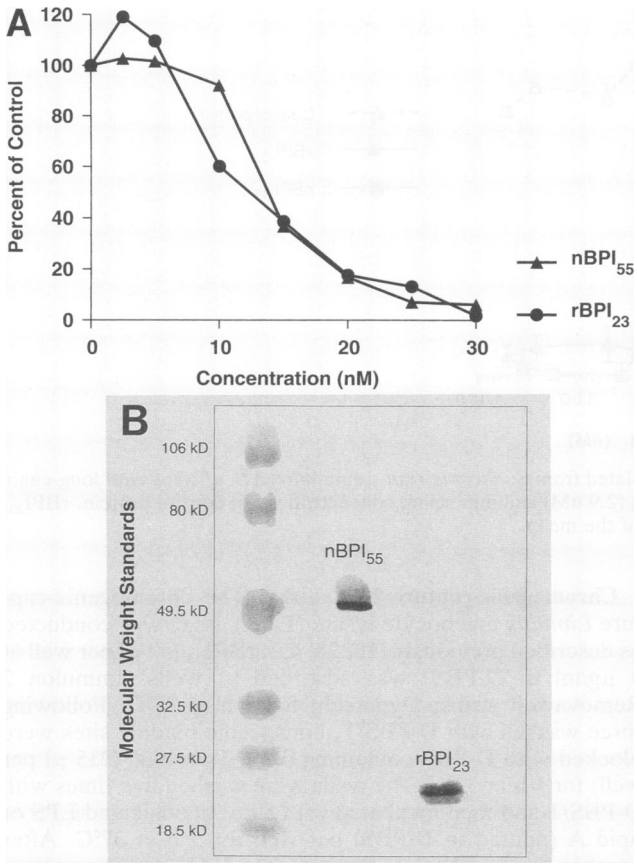

FIG. 1. (A) Comparison of the antibacterial activities of $rBPI_{23}$ and $nBPI_{55}$ on *E. coli* J5. The bacteria were incubated in the presence of increasing concentrations of either $rBPI_{23}$ or $nBPI_{55}$, and bacterial viability was measured as described in Materials and Methods. The results are shown as percentages of values for an untreated control. (B) Coomassie brilliant blue-stained SDS-PAGE profiles of $rBPI_{23}$ and $nBPI_{55}$. A 5-μg amount of each BPI preparation was separated by SDS-PAGE under reducing conditions, and separated proteins were visualized by Coomassie blue staining.

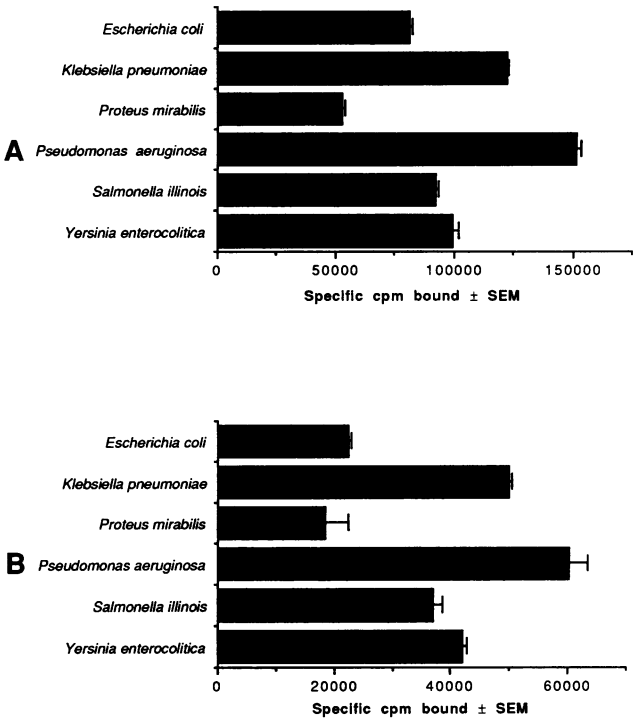

FIG. 2. Binding of radiolabeled BPI to S-form LPSs isolated from various gram-negative bacteria. Immobilized LPS antigens were incubated in the presence of ^{125}I-labeled $rBPI_{23}$ (21 nM) (A) or ^{125}I-labeled $nBPI_{55}$ (18 nM) (B) as described in Materials and Methods. Each point represents the means ± standard errors of the means (SEM) of six replicates.

type bacteria (*Salmonella illinois*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) obtained from the American Type Culture Collection, Rockville, Md. (ATCC catalog numbers 11646, 25933, and 19960, respectively).

BPI. Naturally derived BPI ($nBPI_{55}$) was isolated and purified as previously described (12). To generate $rBPI_{23}$, DNA encoding the signal sequence and the first 199 amino acids of human $nBPI_{55}$ was obtained by polymerase chain reaction of $nBPI_{55}$ cDNA (7) with the GeneAmp polymerase chain reaction kit from Perkin-Elmer Cetus (Norwalk, Conn.) according to the manufacturer's protocols. A stop codon was inserted after amino acid 199, and the BPI DNA was cloned into a mammalian expression vector (27) to generate plasmid pING4502. CHO-K1 cells were transfected with pING4502 as described by Wigler et al. (44). Transfectants which secreted $rBPI_{23}$ were identified by sandwich enzyme-linked immunosorbent assay (36). $rBPI_{23}$ was purified from CHO culture medium by cation-exchange chromatography. Thaumatin, used as a control protein in some binding assays, has a molecular weight and isoelectric point similar to those of $rBPI_{23}$ and was cloned, expressed, and purified as previously described (11).

Assay of bacterial viability. *E. coli* J5 cells were grown overnight in TYE broth (1.5% tryptone [Difco], 1.0% yeast extract [Difco], 0.5% NaCl) and then in galactose-free triethanolamine-buffered medium (40) at 5×10^8 to 10×10^8 cells per ml corresponding to mid- to late-logarithmic phase. The culture was harvested and adjusted to an A_{600} of 0.5 in physiological saline. The bacterial suspension (10 μl ≈ 5×10^6 cells) was incubated for 30 to 40 min at 37°C with various concentrations of $rBPI_{23}$, $nBPI_{55}$, or buffer control in a medium consisting of 10% Hanks balanced salt solution (GIBCO), 40 mM Tris-HCl (pH 7.5), and 0.1% Casamino Acids in a total volume of 200 μl. $MgCl_2$ was added, when desired, at a final concentration of 100 mM to the mixture described above. The effect of BPI on bacterial viability was assessed by plating dilutions of the cells on nutrient agar plates.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the buffer system of Laemmli (10) with an 8 to 18% gradient acrylamide resolving gel and a 4% stacking gel. The protein bands were visualized by Coomassie blue staining.

Preparation of ^{125}I-labeled $rBPI_{23}$ and $nBPI_{55}$. Protein radiolabeling was performed with IODO-BEADS (Pierce Chemical Co., Rockford, Ill.) according to the procedure described by Markwell (13), with modifications. Briefly, iodination was initiated by the addition of 4 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Wilmington, Del.) and 2 IODO-BEADS to samples containing 75 μg of protein in a total volume of 500 μl of Dulbecco's phosphate-buffered saline (D-PBS) containing 0.05% Tween 20 (D-PBS/T; pH 7.0). The reaction was allowed to continue for 15 min at

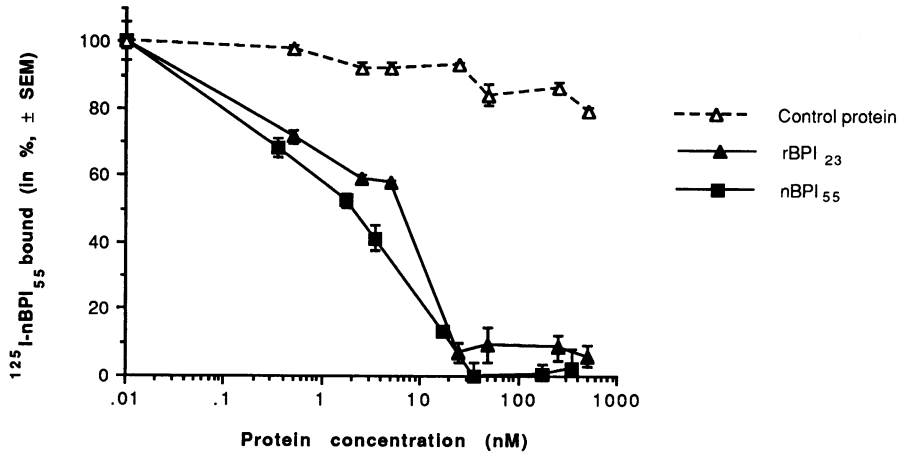

FIG. 3. Inhibition of radiolabeled $nBPI_{55}$ binding to long-chain LPS isolated from *S. abortus equi*. Immobilized *S. abortus equi* long-chain LPS was incubated overnight at 4°C in the presence of ^{125}I-labeled $nBPI_{55}$ (2.9 nM) and increasing concentrations of control protein, $rBPI_{23}$, or $nBPI_{55}$ as described in Materials and Methods. SEM, standard error of the mean.

room temperature with occasional shaking. Free ^{125}I was separated from ^{125}I-labeled BPI by gel filtration on a Sephadex G-25 column (PD-10 column; Pharmacia Fine Chemicals, Piscataway, N.J.). The specific activities of $rBPI_{23}$ and $nBPI_{55}$ varied between 10 and 30 μCi/μg in different preparations.

Binding assay with ^{125}I-labeled $rBPI_{23}$ or ^{125}I-labeled $nBPI_{55}$. Binding of radiolabeled BPI to LPS was determined by a modification of a previously described protocol (23, 45). R-form LPS or lipid A suspensions were sonicated, diluted to 5 μg/ml in absolute methanol, dispensed in 50-μl aliquots into wells (Immulon 2 Removawell strips; Dynatech, Inc., Chantilly, Va.), and evaporated overnight at 37°C. S-form LPSs were plated in D-PBS at the same concentration (5 μg/ml), and the mixtures were incubated overnight at 4°C. The wells were then blocked with 215 μl of D-PBS–0.1% bovine serum albumin (BSA) for 3 h at 37°C. The blocking solution was discarded, and the wells were washed twice with D-PBS/T. ^{125}I-labeled $nBPI_{55}$ or $rBPI_{23}$ (10^6 cpm/50 μl) equivalent to a concentration of 18 and 21 nM, respectively, was then added directly to each well and further incubated overnight at 4°C. After three washes with D-PBS/T, the radioactivity remaining in the wells was counted in an LKB gamma counter. The binding of ^{125}I-labeled BPI to wells treated with D-PBS–0.1% BSA was taken to represent nonspecific binding; specific binding of ^{125}I-labeled BPI was defined as the difference between total and nonspecific binding. Nonspecific binding represented between 10 and 40% of total binding.

Fractionation of LPS by gel filtration chromatography. *S. abortus equi* S-form LPS was separated by size exclusion column chromatography into long-chain and short-chain fractions as previously described (23, 25). The average numbers of O-repeat units per LPS molecule in the two fractions were 16.3 and 1.3, respectively (23).

Inhibition of radiolabeled $nBPI_{55}$ binding to long-chain LPS by unlabeled $rBPI_{23}$ or $nBPI_{55}$. Long-chain LPS purified from *S. abortus equi* was diluted in D-PBS to a concentration of 2 μg/ml, and 50-μl aliquots were placed into wells overnight at 4°C. The wells were blocked and washed, and ^{125}I-labeled $nBPI_{55}$ (400,000 cpm/50 μl) equivalent to a concentration of 2.9 nM was then incubated overnight at 4°C in the presence of increasing amounts of $nBPI_{55}$, $rBPI_{23}$, or thaumatin. The wells were then treated as described above.

Chromogenic-capture LAL assay. The chromogenic-capture *Limulus* amebocyte lysate (LAL) assay was conducted as described previously (16, 23, 45). $rBPI_{23}$ (50 μl per well at 5 μg/ml in D-PBS) was adsorbed to wells (Immulon 2 Removawell strips; Dynatech) for 5 h at 37°C. Following three washes with D-PBS/T, nonspecific binding sites were blocked with D-PBS containing 0.2% Tween-20 (215 μl per well) for 1 h at 37°C. The wells were washed five times with D-PBS/T and then incubated with 50 μl of sonicated LPS or lipid A (diluted in D-PBS) per well for 5 h at 37°C. After another five washes, a chromogenic LAL assay was performed by using a modification of the standard protocol described by the manufacturer (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, Md.). Thus, sequential incubations were carried out with 50 μl of the *Limulus* lysate per well for 20 min at room temperature and then with 100 μl of chromogenic substrate per well for 20 to 60 min, depending on the activities of the reagents as determined with the LPS standard supplied with the kit. A 100-μl volume of 25% acetic acid per well was then added to terminate the chromogenic reaction, and the A_{405} was measured. Values are the means ± standard errors of the mean of six replicates.

Inhibition of radiolabeled BPI binding to lipid A by lipid A or its analogs. *E. coli* J5 lipid A was diluted in methanol to a concentration of 0.5 μg/ml, and 50-μl aliquots were adsorbed to wells overnight at 37°C. Following incubation, the wells were blocked with D-PBS–0.1% BSA for 3 h at 37°C and washed twice with D-PBS/T, and ^{125}I-labeled $nBPI_{55}$ or $rBPI_{23}$ (200,000 cpm/50 μl) equivalent to 1.45 and 12.6 nM, respectively, was then incubated overnight at 4°C in the presence of increasing concentrations of lipid A or its analogs. After three washes with D-PBS/T, the radioactivity remaining in the wells was counted in an LKB gamma counter.

Determination of BPI affinity constants. For the determination of affinity constants, lipid A or R-form LPS was plated at 0.1 μg/ml while S-form LPS was plated at 1 μg/ml. The wells were then blocked, washed, and incubated overnight at 4°C with D-PBS/T containing a fixed amount of ^{125}I-labeled $nBPI_{55}$ or $rBPI_{23}$ (100,000 cpm/50 μl) equivalent to 1.3 and 3 nM, respectively, and increasing concentrations of unlabeled BPI. Bound radioactivity was then determined as previously described (23, 45).

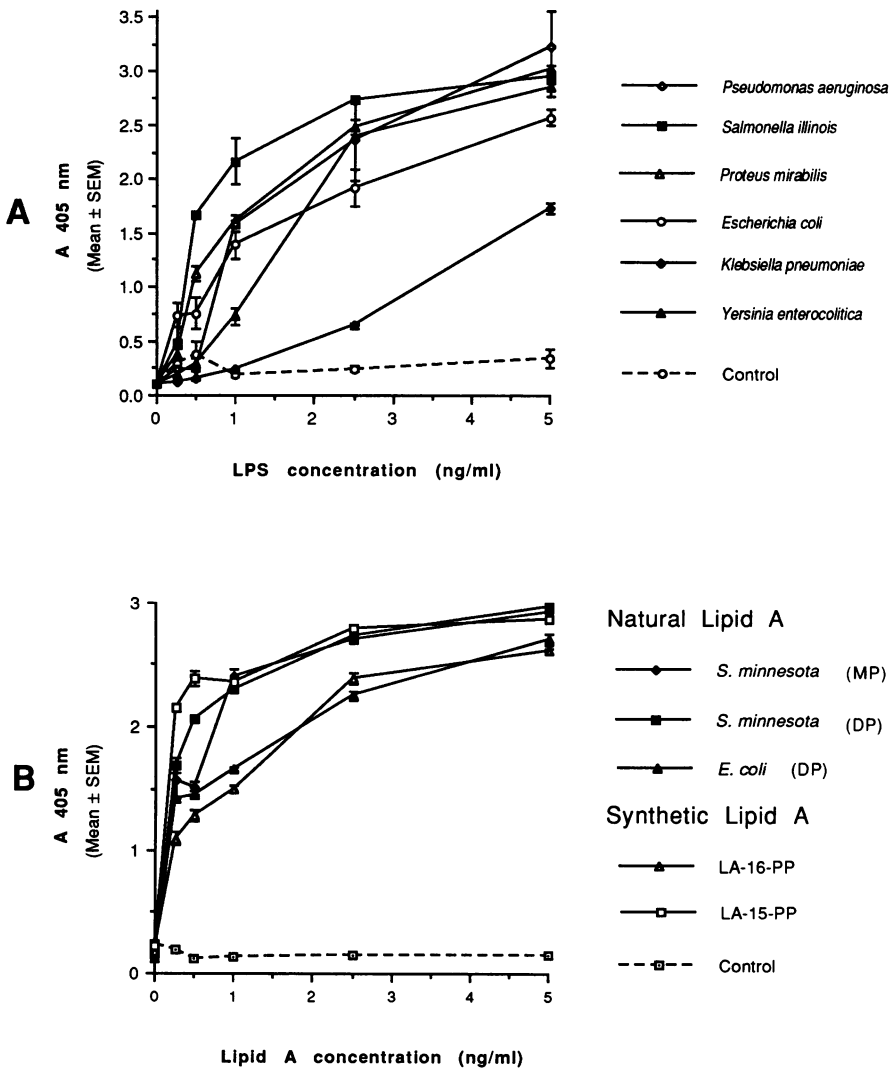

FIG. 4. Binding of $rBPI_{23}$ to S-form LPS and lipid A in the capture LAL assay. Solutions containing increasing concentrations of S-form LPS isolated from gram-negative bacteria (A) or lipid A preparations (B) were incubated in microtiter wells containing immobilized $rBPI_{23}$. The amount of bound lipid A or LPS was determined by chromogenic LAL assay as described in Materials and Methods. Control values show binding of *E. coli* O113 LPS (A) or of *E. coli* J5 lipid A (B) to wells which did not contain $rBPI_{23}$ but were blocked and washed as described in Materials and Methods. SEM, standard error of the mean; MP, monophosphoryl; DP, diphosphoryl.

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

RESULTS

Antibacterial activity of $rBPI_{23}$ and $nBPI_{55}$ against *E. coli* J5. Recombinant ($rBPI_{23}$) and natural ($nBPI_{55}$) BPIs each migrated as single bands on an SDS-PAGE gel (Fig. 1B). To assess the functional activity of the recombinant protein, $rBPI_{23}$ was compared with $nBPI_{55}$ in the antibacterial assay with *E. coli* J5 (22, 40). The results shown in Fig. 1A demonstrate that $rBPI_{23}$ has the same potent antibacterial activity as does $nBPI_{55}$.

Binding of $rBPI_{23}$ and $nBPI_{55}$ to LPS. To better understand the interactions of BPI and LPS, a critical step in the antibacterial actions of BPI, binding studies with highly purified LPS and ^{125}I-labeled $rBPI_{23}$ and $nBPI_{55}$ were carried out. Figure 2 shows the binding of radiolabeled $rBPI_{23}$ and $nBPI_{55}$ to immobilized LPS isolated from clinically relevant smooth strains of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. illinois*, and *Y. enterocolitica*. While it should be noted that radiolabeled $rBPI_{23}$ had a higher specific activity than radiolabeled $nBPI_{55}$ (24 and 13.4 μCi/μg, respectively), thus influencing the total counts bound per minute, both proteins showed nearly an identical spectrum of binding to immobilized S-form LPS.

To confirm that the LPS-binding site resided in the amino-terminal half of BPI, the binding of radiolabeled $nBPI_{55}$ to *S. abortus equi* long-chain LPS was measured in the presence of increasing amounts of unlabeled $rBPI_{23}$, $nBPI_{55}$, or a control protein. As seen in Fig. 3, unlabeled $rBPI_{23}$ and $nBPI_{55}$ had equivalent activities in inhibiting the binding of radiolabeled $nBPI_{55}$ to LPS, whereas the control protein had no significant effect. The reciprocal experiment, with radiolabeled $rBPI_{23}$, gave comparable results (data not shown).

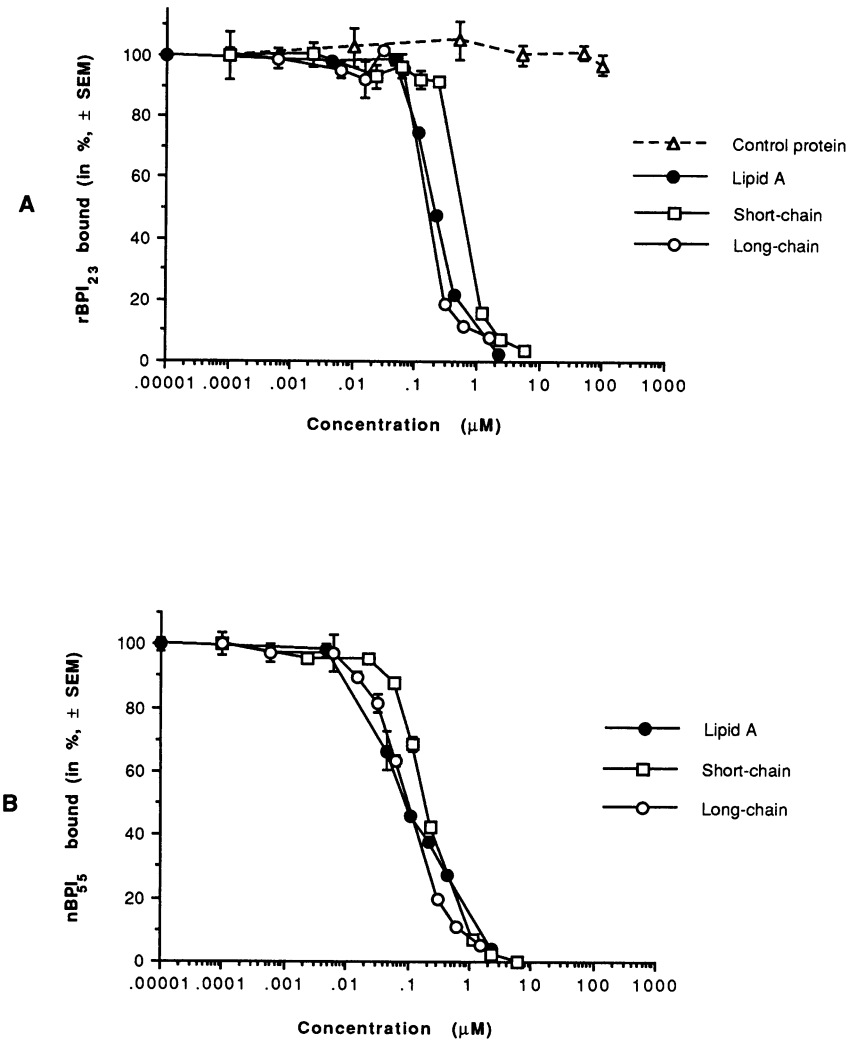

FIG. 5. Inhibition of radiolabeled BPI binding to *E. coli* J5 lipid A. Immobilized lipid A antigens were incubated overnight at 4°C in the presence either of ^{125}I-labeled $rBPI_{23}$ (12.6 nM) and increasing concentrations of control protein, *E. coli* J5 lipid A, or *S. abortus equi* short- or long-chain LPS (A) or of ^{125}I-labeled $nBPI_{55}$ (1.45 nM) and increasing concentrations of *E. coli* J5 lipid A or *S. abortus equi* short- or long-chain LPS (B) as described in Materials and Methods. SEM, standard error of the mean.

Binding of immobilized $rBPI_{23}$ to smooth LPS and lipid A. LPS is an amphipathic molecule, and its physicochemical state can influence exposure of hydrophilic and hydrophobic regions in the molecule (2). To evaluate the influence of the physical state of LPS on the binding specificity of BPI, an additional assay format, the capture LAL assay, in which LPS was presented in aqueous suspension rather than immobilized onto microtiter wells, was used. As seen in Fig. 4A, a variety of S-form LPS showed dose-dependent binding to $rBPI_{23}$ in this assay. No detectable LPS reactivity was measured in control wells preincubated with buffer alone (Fig. 4A) or with a control protein (data not shown). Taken together, the results shown in Fig. 2A and 4A demonstrate that $rBPI_{23}$ binds to a broad range of S-form LPS irrespective of whether the LPS was in aqueous suspension or immobilized on microtiter wells.

Since BPI is an inhibitor of the LPS-mediated activation of the LAL cascade (15, 21), it was possible that the LPS-neutralizing activity of BPI would affect detection of bound LPS by the LAL assay. The fact that bound LPS can be detected by this assay suggests that only a fraction of molecules in LPS micelles is bound by BPI immobilized onto the plastic surfaces of microtiter wells. Indeed, similar results were obtained with ^{125}I-labeled Ra LPS in this assay format, regardless of whether the captured ^{125}I-labeled Ra LPS was quantitated by counts per minute or LAL reactivity (data not shown).

Lipid A is the most conserved structural domain in LPS, and the binding of $rBPI_{23}$ to isolated lipid A in aqueous suspension was next investigated by the LAL assay format. Figure 4B shows that immobilized $rBPI_{23}$ bound both natural and synthetic mono- and diphosphoryl forms of lipid A in a comparable dose-dependent fashion.

Inhibition of $rBPI_{23}$ and $nBPI_{55}$ binding to immobilized lipid A by lipid A or short- or long-chain LPS. The influence of O-polysaccharide chains on the interaction of BPI and LPS was evaluated by using competition experiments in which lipid A or short- or long-chain S-form LPS from *S. abortus equi* was examined for the ability to inhibit binding of radiolabeled $rBPI_{23}$ or $nBPI_{55}$ to immobilized lipid A. As

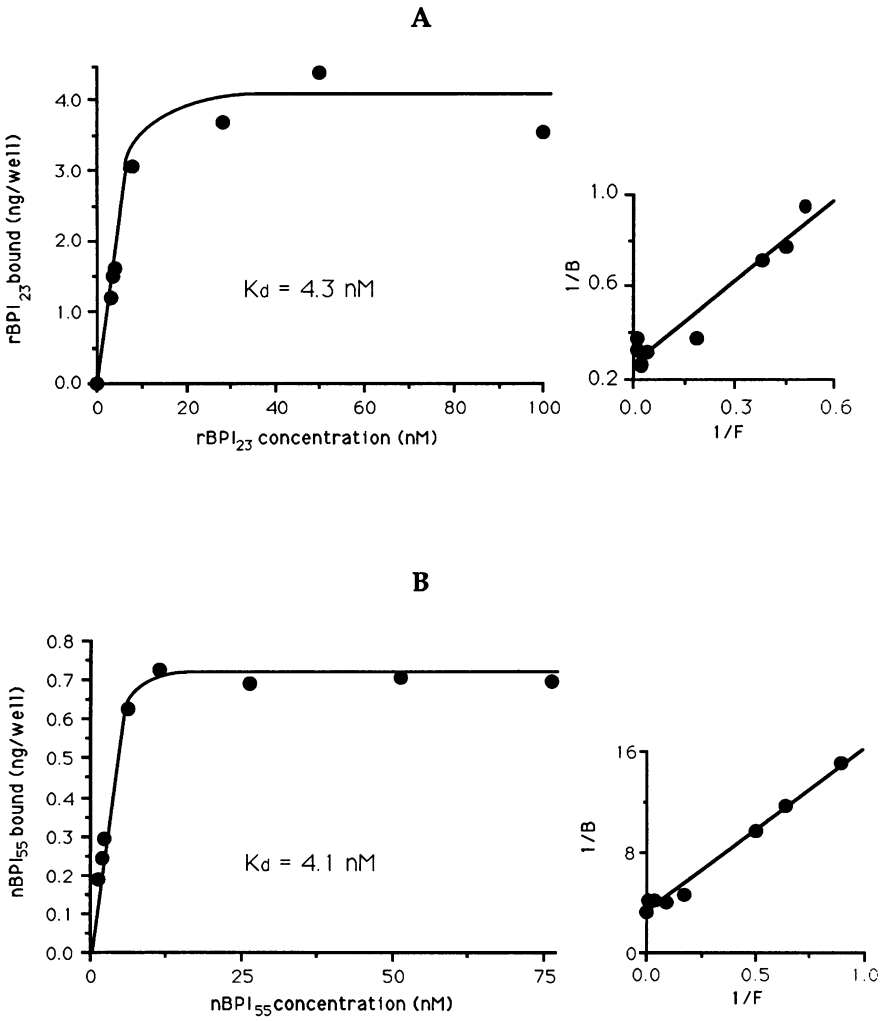

FIG. 6. Binding of BPI to lipid A. Immobilized *E. coli* J5 lipid A was incubated in the presence of ^{125}I-labeled $rBPI_{23}$ (3 nM) and various concentrations of unlabeled $rBPI_{23}$ (A) and ^{125}I-labeled $nBPI_{55}$ (1.3 nM) and various concentrations of unlabeled $nBPI_{55}$ (B) as described in Materials and Methods. Binding to wells without adsorbed antigen was subtracted to obtain specific counts bound per minute. Each point represents the mean ± standard error of the mean (SEM) of six replicates. The insets display data replotted by the double-reciprocal method.

shown in Fig. 5A, compared on a molar basis, all three samples had essentially eqivalent activities as competitive inhibitors of $rBPI_{23}$ binding to immobilized lipid A. Similar results were also obtained with $nBPI_{55}$ (Fig. 5B).

Binding affinity of $rBPI_{23}$ and $nBPI_{55}$ to lipid A and LPS. To further evaluate the effect of the core and O-antigen polysaccharides on BPI-LPS interactions, equilibrium-binding studies with lipid A and R-form, S-form, or long-chain LPS (isolated from *S. abortus equi*) were done. As seen in Fig. 6, binding of either $rBPI_{23}$ or $nBPI_{55}$ to *E. coli* J5 lipid A was saturable and of high affinity, with apparent K_ds of 4.3 and 4.1 nM, respectively. Similar K_d values for $rBPI_{23}$ were obtained with purified LPSs isolated from the rough bacterium *S. minnesota* Ra chemotype (3.9 nM) and the smooth bacterium *E. coli* O113 (5.2 nM) and the *S. abortus equi* long-chain LPS (1.7 nM), indicating that the presence of core plus or minus O-antigen polysaccharide had little or no effect on BPI binding to LPS.

DISCUSSION

BPI is known to bind isolated LPS (12, 14, 15, 21, 37, 41); however, the quantitative aspects of the interaction between BPI and LPS have not been reported. In the present study, $rBPI_{23}$, a recombinant protein corresponding to the biologically active N-terminal portion of the natural human holoprotein ($nBPI_{55}$), as well as $nBPI_{55}$ itself, was used to investigate the specificity and affinity of the BPI binding site for various lipid A's and LPSs.

The evidence presented herein demonstrates that $rBPI_{23}$ contains an intact, functional LPS binding site, since both $rBPI_{23}$ and $nBPI_{55}$ bind to the lipid A component of LPS in a saturable manner and with similar binding affinities (apparent K_ds of approximately 2 to 5 nM). Moreover, the binding of radiolabeled $nBPI_{55}$ to purified S-form LPS was inhibited in an identical fashion by unlabeled $nBPI_{55}$ and $rBPI_{23}$. Both $rBPI_{23}$ and $nBPI_{55}$ display a similar broad spectrum of reactivity to S-form LPS isolated from *Escherichia*, *Klebsi-*

ella, *Proteus*, *Pseudomonas*, *Salmonella*, and *Yersinia* species. This binding appears to be functional, because $rBPI_{23}$ has the same potent antibacterial activity as does $nBPI_{55}$. We have also found that $rBPI_{23}$ retains LPS-neutralizing activities comparable to those of the holoprotein in a variety of in vitro assays (17, 39).

Several lines of evidence presented in this study suggest that the LPS-binding site on BPI recognizes the lipid A region of LPS and that the presence of long O-specific polysaccharide chains on purified LPS have little or no effect on BPI reactivity. We found that $rBPI_{23}$ binds to natural and synthetic lipid A preparations and to both monophosphoryl and diphosphoryl forms of lipid A. Because a high percentage of unfractionated preparations of LPS from smooth strains is represented by molecules with few or no O repeats (8), the effect of O antigen on BPI-LPS interactions was assessed by using fractionated, highly enriched preparations of long-chain LPS. On a molar basis, lipid A and short- and long-chain LPSs were equally potent as competitive inhibitors of $rBPI_{23}$ binding to immobilized lipid A. Furthermore, the binding affinities of $rBPI_{23}$ for *E. coli* J5 lipid A, *S. minnesota* R-form LPS (Ra chemotype), *E. coli* O113 S-form LPS, and purified long chain S-form LPS isolated from *S. abortus equi* are similar, suggesting that BPI recognizes the conserved lipid A region in these molecules. These results confirm and also extend results reported by Marra et al. (15), in which it was demonstrated that the binding of $nBPI_{55}$ to immobilized *S. minnesota* R595 (Re) LPS was inhibited by preincubation with lipid A, R595 (Re) LPS, and *E. coli* O111:B4 LPS.

Although the length of carbohydrate chains attached to lipid A does not alter the interaction of $rBPI_{23}$ or $nBPI_{55}$ with purified LPS, a different picture emerges in studies with intact bacteria. The sensitivity of gram-negative bacteria to the cytotoxic activity of BPI is related to the length of the saccharide chain of the bacterial-envelope LPS (5, 6, 30–32, 37). Rough strains bearing LPS with short polysaccharide chains are the most sensitive, and smooth strains may require up to 20-fold higher doses of BPI for growth inhibition (37, 40). The differences in bacterial sensitivity appear to parallel differences in affinity of BPI for the bacterial envelope (40). LPS molecules in the outer leaflet of the hydrophobic outer bacterial membrane are more tightly packed than molecules in purified LPS preparations (1). Therefore, long polysaccharide chains hinder access of BPI to the core and lipid A region of LPS in intact bacteria but have little influence on binding to purified LPS preparations.

BPI is structurally related to an LPS-binding protein (LBP) of comparable size first isolated from acute-phase rabbit serum (29, 33–35). Human BPI shares a 45% amino acid sequence identity with human LBP, and the identity is randomly dispersed throughout the two sequences (29). LBP has been shown to bind to lipid A, R-form LPS, and S-form LPS isolated from a variety of gram-negative organisms (35). Therefore, both BPI and LBP appear to have binding sites with a high degree of specificity for the lipid A region of LPS. Interestingly, BPI and LBP have strikingly different biologic activities. BPI has potent bactericidal activity and inhibits endotoxin responses, whereas LBP lacks significant bactericidal activity and potentiates the LPS-mediated release of tumor necrosis factor by monocytes and macrophages (3, 15, 21, 29).

The broad LPS-binding specificity of BPI, as well as its potent bactericidal action and its potent effect on endotoxin-mediated events, suggests that BPI may have potential as a therapeutic agent for the treatment of gram-negative sepsis in humans.

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