The Carboxyl-terminal Domain of Closely Related Endotoxin-binding Proteins Determines the Target of Protein-Lipopolysaccharide Complexes*

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The bactericidal/permeability increasing (BPI) and lipopolysaccharide (LPS)-binding (LBP) proteins are closely related two-domain proteins in which LPS binding is mediated by the NH2-terminal domain. To further define the role of the COOH-terminal domain of these proteins in delivery of LPS to specific host acceptors, we have compared interactions of LBP, BPI, LBP-NBPI (NH2-terminal domain of LBP, COOH-terminal domain of BPI), and BPI-N-LBP, with purified 3H-LPS and, subsequently, with purified leukocytes and soluble (s)CD14.

The COOH-terminal domain of LBP promotes delivery of LPS to CD14 on both polymorphonuclear leukocytes and monocytes resulting in cell activation. In the presence of Ca2+ and Mg2+, LBP and BPI each promote aggregation of LPS to protein-LPS aggregates of increased size (apparent Mr, > 2 × 10^6 Da), but only LPS associated with LBP and BPI-N-LBPc is disaggregated in the presence of CD14. BPI and LBP-N-LBPc promote apparently CD14-independent LPS association to monocytes without cell activation. These findings demonstrate that the carboxy-terminal domain of these closely related endotoxin-binding proteins dictates the route and host responses to complexes they form with endotoxin.

Bacterial invasion triggers sensitive, specific molecular alarms that result in the mobilization of host defenses designed to recognize and eliminate invading bacteria and their remnants. The major inducer of host responses to Gram-negative bacteria (GNB) is endotoxin, i.e. lipopolysaccharide, LPS, the unique glycolipid that comprises the bulk of the outer leaflet of the GNB outer membrane (1). These amphipathic molecules are composed of a unique, conserved hydrophobic moiety, the lipid A region, that is a disaccharide of N-acetylgalactosamine substituted with saturated fatty acids and attached to a highly charged acidic carbohydrate region of varying size and composition (1, 2). The chemical structure of endotoxin promotes the formation of highly ordered, but potentially malleable aggregated state(s). Interaction with host endotoxin-binding proteins alters the physical presentation of endotoxin and its ability to activate cell responses (2–5). The cumulative work of many laboratories has implicated lipopolysaccharide-binding protein (LBP), CD14, Toll-like receptor 4, and MD-2 as key factors in cell activation by LPS (3, 6–13). LBP facilitates delivery of LPS to both membrane-bound, GPI-linked (mCD14) and soluble CD14 (sCD14) (14–19). Through interaction with CD14, LPS activates cells via a transmembrane receptor capable of promoting signal transduction. This recognition/response cascade includes the Toll-like receptor family of proteins, most notably Toll-like receptor 4, that serve as the primary mediator of endotoxin signaling (20, 21).

The host also utilizes defense mechanisms that blunt endotoxin-triggered inflammatory responses by eliminating viable GNB and by neutralizing endotoxin. One potent host protein that blocks LPS activity is bactericidal/permeability increasing protein (BPI), a basic protein residing in azurophilic granules of polymorphonuclear (PMN) leukocytes and in the extracellular fluid of PMN-rich inflammatory exudates (22). BPI efficiently neutralizes LPS and is also potently cytotoxic and opsonic, especially toward GNB (22–25). Although the functional properties of LBP and BPI differ markedly, they share 45% amino acid identity, are encoded within the same region of chromosome 20, and belong to a family of lipid-binding proteins that include phospholipid transfer protein and cholesteryl ester transfer protein (26–30). The three-dimensional crystal structure for BPI reveals an unusual “hinged” two-domain boomerang-like molecular structure. The extensive sequence homology between LBP and BPI predicts a nearly superimposable three-dimensional structure for LBP (30) suggesting that despite their different bioactivities, BPI and LBP have a similar organization of structure and function.

In support of this view, studies on the interaction of LBP and BPI with LPS have demonstrated that the NH2-terminal domain of each protein is responsible for binding LPS (28, 31–34). In BPI, both affinity for LPS and antibacterial activity are concentrated in this portion of the protein (31–33, 35, 36). In contrast, promotion by LBP of CD14-dependent cell activation by LPS requires the complete LBP molecule implying that after association of LBP with LPS, the COOH-terminal domain of LBP is needed for transfer of LPS to CD14 (37, 38). Similarly, the ability of BPI to promote delivery of intact GNB to PMN depends upon both the NH2- and COOH-terminal domains of BPI (23). Thus, protein binding to aggregated endotoxin is

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† The abbreviations used are: GNB, Gram-negative bacteria; BPI, bactericidal/permeability increasing protein; HBSS+/+HBSS−, Hank’s balanced salt solution (+/− Mg2+ and Ca2+); HSA, human serum albumin; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; LPSagg, lipopolysaccharide aggregates; PMN, polymorphonuclear leukocytes; mAb, monoclonal antibody; MNC, monocyte-enriched cells; s/m CD14, soluble CD14 or membrane CD14.

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mediated by the NH₂-terminal domain of LBP and BPI, followed by delivery of LPS to distinct host acceptors via their COOH-terminal domains. We speculate that the functional properties of endotoxin-containing particles associated with BPI or LBP could be determined, in part, by differences in the identity and/or cellular localization of their downstream targets.

In this study, LBP, BPI, and previously described chimeric proteins of LBP and BPI (39) have been utilized with metabolically labeled preparations of Escherichia coli K12 LPS (40) to further define the role of the COOH-terminal domain of BPI and LBP in delivery of endotoxin to specific cellular and extracellular host targets. We compared the effects of these proteins on the binding of LPS to and activation of enriched populations of leukocytes and on the interaction of LPS with sCD14. A gel filtration chromatography system has been applied that permits isolation of a homogeneous population of LPS aggregates (LPSagg). Using this gel filtration system, we monitored changes in the physical state of LPSagg with LBP, BPI, or chimeric proteins and sCD14. Our results indicate that both the targeting of LPS to specific host acceptors and the subsequent functional effects depend mainly on the properties of the carboxyl-terminal domain of BPI and LBP.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following proteins were provided by Dr. Stephen Carroll, Xoma Corp. (Berkeley, CA): recombinant human BPI (rBPI), human BPI-21, the biotinyl NH₂-terminal fragment of BPI (residues 1–193), LBP, and sCD14, and two chimeric proteins, BPI–LPB (amino acids 1–197 of LBP and 200–456 of BPI) and BPI–LPB (+) (amino acids 1–200 of BPI and 199–456 of LBP), and rabbit polyclonal anti-LBP antibodies. Isolation of native human BPI and LBP and the design and purification of the recombinant proteins have been described previously (39). Antibodies against BPI were generated in goat as has previously been described (41). Antibodies against CD14 utilized were MEM-18, MY4, and FITC-MY4 from Coulter, Inc. Sephacryl HR S500 was obtained from Amersham Biosciences, Inc. (Piscataway, NJ). Lucigenin was purchased from Sigma. Human serum albumin (HSA) was endotoxin-free, 25% stock solution manufactured by Baxter Healthcare Corp., Glendale, CA.

*H*-LPS (purified from List Biological Laboratories, Campbell, CA) was dissolved in endotoxin-free water to a final concentration of 100 μg/ml and sonicated twice on ice for 10 min. Aliquots were stored frozen until needed or aggregates were immediately isolated by column chromatography on Sephacryl S500 as described below. Samples for isolation of a homogeneous *H*-LPS aggregate population were diluted 1:1 in Hanks' balanced salts buffer solution containing magnesium and calcium (HBSS–), 20 mM HEPES with 2% HSA and incubated at 37 °C for 15 min before application to the column and elution as described below. Essentially all (>98%) *H* cpm in purified LPSagg are present within the fatty acids specific to the lipid A region of LPS (42).

**Cell Preparation**—Venous blood was drawn from healthy human volunteers, after informed consent in accordance with the established guideline (IRB committee approved protocol). In addition to these samples, control samples of LBP and BPI of varying concentration were electrophoresed using an Amersham Biosciences, Inc. PhastGel System through either 12.5 (LBP) or 10–15% (BPI) acrylamide gels and transferred to nitrocellulose by semi-dry transfer using the same system. For immunoblotting, the nitrocellulose was washed with phosphate-buffered saline containing 0.05% Tween 20 and then blocked with 3% bovine serum albumin in the same buffer for 1 h at 25 °C. After washing, the blots were treated with the appropriate primary antibody (1:100 rabbit anti-LBP or goat anti-BPI serum) diluted in 1% bovine serum albumin, phosphate-buffered saline, 0.05% Tween 20 overnight at 25 °C. After washing with phosphate-buffered saline, 0.05% Tween 20, the blots were incubated with secondary antibody conjugated to horseradish peroxidase (either donkey anti-rabbit IgG or rabbit anti-goat IgG) for 1 h at 25 °C, washed with PBS containing 0.05% Tween 20, and the sheets were developed using the Pierce SuperSignal substrate system.

These cells were histologically >85% monocytes and >75% CD14+ as evaluated by fluorescence-activated cell sorter analysis using FITC-MY4.

**Cell Binding Assays**—*H*-LPS (specific activity 900 cpm/μg) from *E. coli K12 CD25 (List Biological Laboratories) was utilized in binding studies to human PMN or enriched MHC as has been previously described (23). Typical incubation mixtures contained cells (2.5–5 × 10⁶) and 100 ng/ml *H*-LPS in 0.5 ml HBSS++, 1% HSA ± the indicated protein in a dose range from 0.1 to 100 nm. Incubations were for 1 h at 37 °C. After incubation, cells were washed twice in cold saline, transferred in saline to a fresh tube, centrifuged at 500 × g for 4 min at 4 °C (42), and the pellet resuspended by boiling for 10 min in 0.05% SDS, 10 mM EDTA, and 1% HSA and eluted from columns in the same buffer. Samples applied to the columns were eluted at room temperature at a flow-rate of 0.5 ml/min. Fractions (1 ml) were collected, and aliquots evaluated for *H*-LPS content by liquid scintillation spectroscopy to determine elution profiles. A homogeneous population of *H*-LPS aggregates was prepared by chromatography on Sephacryl S500 HR of 20 μg of *H*-LPS reconstructed as described above. Peak fractions (LPSagg, M– 1–1.5 × 10⁸ Da) were combined and used as a source of *H*-LPS of a defined size range in subsequent experiments. Samples of *H*-LPS ± indicated proteins (LBP, BPI, BPI–LPB, LPB–BPI, or BPI–LPB) were indicated to confirm homogeneity of pooled fractions. Because of the low concentration of LPS in these fractions, binding assays typically were done in a total volume of 1 ml. Molecular size of aggregates was determined by comparing elution of the aggregates against that of the following standards run under the same conditions: HSA (amino acids 1–193, thyroglobulin, blue dextran (+/− HSA), cytochrome c (+/− HSA), aldolase, ferritin (+/− HSA).

To verify the presence of LBP or BPI in isolated LPSagg protein aggregates, samples of LPSagg + LBP or BPI were incubated as described and the aggregates isolated in the void volume of Sephacryl S500 columns equilibrated in Hanks' 10 mM HEPES (1.5 × 6 cm). Sephacryl S500 was an LS 6000 column (1.5 × 6 cm) purchased from Pharmacia. The purified aggregates either were used immediately or stored in Teflon vials. These samples were stable at 4 °C for at least 2 weeks. For binding assays of LPSagg, LBP–LPSagg, and BPI–LPSagg, the peak fractions obtained from Sephacryl S500 chromatography were pooled, evaluated for radioactivity, and an aliquot rerun to confirm homogeneity of pooled fractions. Because of the low concentration of LPS in these fractions, binding assays typically were done in a total volume of 1 ml. Molecular size of aggregates was determined by comparing elution of the aggregates against that of the following standards run under the same conditions: HSA (amino acids 1–193, thyroglobulin, blue dextran (+/− HSA), cytochrome c (+/− HSA), aldolase, ferritin (+/− HSA).

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Structure/Function of Endotoxin-binding Proteins

RESULTS

Delivery of [3H]LPS-mediated by LBP, BPI, and LBP/BPI Chimera to PMN and Monocytes—The ability of endotoxin-binding proteins to facilitate interaction and delivery of purified [3H]LPS to peripheral blood leukocytes was evaluated by measuring cell association of [3H]LPS in the presence of varied concentrations of LBP, BPI, BPI-21, and two chimeric LBP/BPI proteins, LBP<sub>N</sub>-LBPC<sub>C</sub> and LBPN-BPIC. Two cell populations were examined: PMN (>98% pure) and a purified MNC that contained ~50% monocytes. PMN or MNC (0.5–1 x 10<sup>7</sup>/ml) were incubated with [3H]LPS (100 ng/ml) at 37°C and varying concentrations of protein. The amount of [3H]-LPS that remained associated after extensive washing of the cells was evaluated. As seen in Fig. 1A, only LBP and BPI<sub>N</sub>-LBPC<sub>C</sub> promoted association of [3H]-LPS to PMN. BPI<sub>N</sub>-LBPC<sub>C</sub> was nearly equipotent and intermediate between LBP and BPI in promoting delivery of [3H]-LPS to MNC. Maximal [3H]-LPS cell association to PMN and MNC required between 1 and 10 nM endotoxin-binding protein.

Therefore, proteins containing the carboxyl-terminal domain of LBP (LBP<sub>N</sub>-LBPC<sub>C</sub>) promoted delivery of purified LPS to both PMN and MNC (MNC > PMN). Proteins containing the carboxyl-terminal domain of BPI (BPI<sub>N</sub>-LBPC<sub>C</sub>) only promoted LPS association with MNC. BPI-21 did not promote [3H]-LPS cell association to either PMN or MNC (Fig. 1B) indicating a requirement of the carboxyl-terminal domain of BPI for BPI-dependent delivery of endotoxin to MNC.

Both BPI as well as LBP-dependent [3H]-LPS binding observed in MNC are apparently to the monocytes in MNC. [3H]-LPS binding was increased when a more monocyte-enriched MNC (>85%) was used (data not shown). In contrast, there was little or no LPS binding to purified lymphocytes in the absence or presence of LBP or BPI. Fluorescence-activated cell sorter analysis of MNC incubated with BODIPY-endotoxin indicated that both LBP- and BPI-dependent binding of [3H]-LPS to blood mononuclear cells is restricted to monocytes.2

Role of CD14 in LBP, BPI, and Chimeric Protein-mediated Delivery of LPS to MNC—On monocytes and PMN, mCD14 is the major initial target of LPS exposed to LBP (9, 42, 43). Differences in delivery of [3H]-LPS to PMN and monocytes by LBP, BPI, and the two chimera (Fig. 1) could be explained by: 1) differences in mCD14 levels in monocytes and PMN (monocytes > PMN) (42, 44); or 2) involvement of different acceptor molecules for LPS associated with LBP or BPI<sub>N</sub>-LBPC<sub>C</sub> versus BPI or LBPN-BPIC. To test the role of mCD14, we measured the effects of two anti-CD14 monoclonal antibodies (mAb) on cell association of LPS. The two mAbs, MY-4 and MEM-18, are directed against different epitopes of CD14 (45, 46). They were preincubated with the cells prior to addition of [3H]-LPS and the associated endotoxin-binding proteins. As shown in Fig. 2A, both MY-4 and MEM-18 almost completely blocked delivery of LPS to MNC (or to PMN; data not shown) that was mediated by LBP and BPI<sub>N</sub>-LBPC<sub>C</sub>. These same mAbs had no (MY-4) or more limited (MEM-18) effect on LPS association mediated by BPI or LBPN-BPIC to MNC. These findings suggest that LPS binding to MHC class II restricted to monocytes in MNC is dependent on CD14 when mediated by proteins containing the COOH-terminal domain of LBP. In contrast, LPS binding to CD14 mediated by proteins containing the COOH-terminal domain of BPI is largely independent of mCD14.

The COOH-terminal Domain of LBP Is Needed for sCD14-dependent Disaggregation of LPS—An alternative interpretation of the above findings is that LPS complexes associated with proteins containing the COOH-terminal domain of BPI engage CD14 but in a way that is less susceptible to inhibition by the particular anti-CD14 mAb examined. To further test the specific role of the COOH-terminal domain of LBP and BPI in interactions of LPS with CD14, we monitored these interactions in solution using sCD14 and a gel filtration system (5) for the separation of protein-LPS complexes and aggregates. To facilitate these analyses, a subpopulation of [3H]-LPS aggregates was isolated by Sephacryl S500 HR gel filtration of commercial [3H]-LPS from E. coli K12 LCD25 (Fig. 3). Gel filtration chromatography was carried out using buffer conditions compatible with bioassays to permit a direct juxtaposition

2 J. Hume and J. Weiss, unpublished observations.
of the physical and functional effects of protein interactions with LPS. The recovered subpopulation of LPS\(_{agg}\) yielded a symmetrical peak with an apparent size \(M_r = 1-1.5 \times 10^6\) upon re-chromatography (Fig. 3). These aggregates were stable at 4 °C for at least 1 month.

Incubation of LPS\(_{agg}\) with LBP produced aggregates of LPS with an increased apparent size (\(\geq 20 \times 10^6\) Da) (fraction 12, Fig. 4A). The aggregates contained LBP as shown by immunoblot analysis of isolated LBP:LPS\(_{agg}\) (Fig. 4B). Incubation and re-chromatography of the purified LBP:LPS\(_{agg}\) showed some minor degree of disaggregation of LPS (fraction 20–24, Fig. 5A). However, treatment of isolated LBP:LPS\(_{agg}\) (fraction 12) with sCD14 produced extensive disaggregation to a complex with apparent mass \(\sim 1 \times 10^5\) Da (fraction 24–25) as monitored by S500 chromatography (Fig. 5A). The disaggregation of LBP-treated endotoxin aggregates by incubation with sCD14 is comparable with results that we have observed previously with endotoxin from Neisseria meningitidis (5).

Treatment of LPS\(_{agg}\) with BPI also resulted in aggregates of LPS of increased apparent size comparable with that observed with LBP (Fig. 4). These large aggregates contained BPI as shown by immunoblot analysis of isolated BPI:LPS\(_{agg}\) (Fig. 4C). In contrast to LBP:LPS\(_{agg}\), BPI:LPS\(_{agg}\) do not disaggregate to a smaller complex during incubation with sCD14 treatment (Fig. 6). Co-incubation of LBP or BPI and sCD14 with purified LPS\(_{agg}\) (\(\sim 1-2 \times 10^6\) Da) produced similar results, i.e. small complexes (\(\sim 1 \times 10^5\) Da) in the presence of LBP (Fig. 5B), but larger aggregates (\(\geq 20 \times 10^5\) Da) in the presence of BPI (Fig. 6B). Under the same conditions, incubation of the LPS aggregates with sCD14 alone had no detectable effect on the aggregation state of LPS\(_{agg}\) (data not shown).

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3 D. S. Zhang, A. Teghanemt, J. Weiss, and T. L. Gioannini, unpublished observations.
The contribution of the carboxyl-terminal domain of these endotoxin-binding proteins to CD14-promoted disaggregation of isolated LPS agg was monitored by changes in the physical state of isolated LPS agg promoted by interaction with BPI N-LBPC and LBPN-BPIC. As shown in Fig. 7, BPIN-LBPC facilitated sCD14-dependent disaggregation of LPS agg, whereas LBPN-BPIC did not. The results indicate that whereas the COOH-terminal domains of LBP and BPI each contribute to LPS association with MNC, only the COOH-terminal domain of LBP has the capacity to deliver LPS to (s)CD14 in such a manner that results in disaggregation.

Role of the COOH-terminal Domain of LBP in sCD14-independent Disaggregation of LPS in the Presence of EDTA—The findings described above demonstrate appreciable disaggregation of LPS agg, only when an endotoxin-binding protein containing the COOH-terminal domain of LBP and, in addition, sCD14 are both present. These results differ from many earlier studies that have suggested direct disaggregating effects of LBP on LPS in the absence of divalent cations (i.e. Mg$^{2+}$ and Ca$^{2+}$) (8, 19, 47, 48, 50) needed to maintain close packing of the highly anionic LPS (51–53). Therefore, we repeated the analysis in the presence of EDTA of the effects of LBP on the aggregation state of LPS as assessed by gel sieving. Incubation of the purified LPS agg with EDTA alone induced heterogeneity among the LPS aggregates but did not cause marked disaggregation (Fig. 8A). In contrast, under these divalent cation-free conditions, LBP produced dramatic disaggregating effects (Fig. 8A) whereas BPI still increased the size of LPS aggregates (Fig. 8B).
The contrasting CD14-independent effects of LBP and BPI on LPS aggregates seen under these conditions conform closely to previous observations made utilizing density gradient centrifugation and light scattering (47). Proteins containing the COOH-terminal domain of LBP produced greater disaggregation of LPS than the corresponding proteins containing the COOH-terminal domain of BPI (i.e. LBP/H11022 LBPN-BPIC (Fig. 8A); BPI N-LBPC/H11022 BPI (Fig. 8B)). These findings therefore indicate a role of the COOH-terminal domain of LBP in both sCD14-independent LPS disaggregation in the presence of EDTA as well as sCD14-dependent LPS disaggregation in the presence of Ca\(^{2+}\) and Mg\(^{2+}\).

**Effect of LBP and BPI on Activation of Monocytes by LPS**—Previous studies have demonstrated that the LPS-dependent lucigenin-enhanced chemiluminescence response of a mixed leukocyte population (comprised primarily of PMN) was stimulated by LBP and BPIN-LBPC, but inhibited by BPI, LBPN-BPIC, and rBPI 21 (23). The data presented here that demonstrate BPI and LBPN-BPIC dependent delivery of LPS to monocytes, but no delivery to PMN, prompted us to re-examine the effect of BPI on LPS-triggered cell activation using a monocyte-enriched cell population. Under identical conditions to those utilized in the binding assays, LBP enhanced cellular response to LPS. In contrast, BPI caused a dose dependent decrease in lucigenin-enhanced chemiluminescence (Fig. 9). At the protein concentrations tested here, neither LBP nor BPI in the absence of LPSagg, had any effect on lucigenin-enhanced chemiluminescence.

A more direct assessment of the bioactivities of LBP:LPSagg and BPI:LPSagg was carried out with the large protein-LPS aggregates (\(\geq 20 \times 10^6\) Da) isolated by gel filtration chromatography (Fig. 4). The isolated aggregates are free of any LBP or BPI unassociated with LPSagg precluding any possible effects of free protein on the functional behavior of the aggregates. The LBP- and BPI-coated LPSagg associated with MNC far more than protein-free LPSagg (Fig. 10A). Increased cell association of LBP:LPSagg, was accompanied by increased cell activation (Fig. 10B) whereas binding of BPI:LPSagg provoked little cell activation (Fig. 10B). These findings clearly demon-
structure the different functional consequences of the binding of LBP:LPS<sub>agg</sub> versus BPI:LPS<sub>agg</sub> to monocytes.

**DISCUSSION**

Although LBP and BPI are closely related by sequence and predicted three-dimensional structure, they often display opposing effects on host cell-endotoxin interactions (22, 23, 39, 47). Previous studies have strongly suggested that the stimulatory effects on CD14-dependent cell activation by LPS, produced by LBP but not by BPI, depend on the ability of LBP to promote delivery of LPS to and disaggregation by CD14 (5, 19, 48, 49). Comparison of the effects of native and variant forms of BPI and LBP, including BPI/LBP chimeras, have led to the speculation that the COOH-terminal domain of LBP plays an essential role in this process (23, 37–39). We now provide experimental data that support this contention. Independent of the origin (BPI versus LBP) of the NH<sub>2</sub>-terminal domain that initially engages LPS, delivery of LPS to cells via mCD14 (Fig. 2) and disaggregation of LPS by sCD14 (Figs. 5–7) depend upon the presence of the COOH-terminal domain of LBP and closely correlate with cell activation (5, 23). The <sup>3</sup>H-LPS containing complexes generated during incubation with LBP or BPI<sub>L</sub>-LBP<sub>C</sub> and sCD14 elute with an apparent size of ~1 x 10<sup>6</sup> Da during Sephacryl S500 gel filtration chromatography. These species closely resemble potent bioactive sCD14-endotoxin complexes formed under essentially identical experimental conditions with other endotoxin species (5). Although it is not yet possible to test for physical transformations of endotoxin after interaction with mCD14, similar changes may be necessary for maximal mCD14 as well as sCD14-dependent cell activation.

In contrast to sCD14-dependent disaggregation of LPS, incubation of LPS aggregates (~1–1.5 x 10<sup>6</sup> Da) with either LBP or BPI alone produced an increase in apparent aggregate size (Fig. 4). Both LBP:LPS<sub>agg</sub> and BPI:LPS<sub>agg</sub> are eluted in the void volume during Sephacryl S500 chromatography suggesting a molecular size of ~2 x 10<sup>6</sup> Da. Estimates of the amount of BPI and LBP bound to the LPS<sub>agg</sub> indicate that the bound protein accounts for ~30% of the apparent increase in size suggesting additional alterations in the aggregation state of LPS. BPI-induced alterations in LPS density and light scattering have been previously observed (47) that could reflect similar physical alterations. However, many earlier studies have observed direct disaggregating effects of LBP on LPS (8, 19, 47, 48, 50). These experiments typically lacked divalent cations (i.e. Mg<sup>2+</sup> and Ca<sup>2+</sup>) needed to maintain the close intermolecular packing of the highly anionic LPS (51–53). By comparing the effects of LBP in the presence (Figs. 4 and 5) and absence (Fig. 8) of divalent cations, we have directly demonstrated that when physiological extracellular levels of Mg<sup>2+</sup> and Ca<sup>2+</sup> are present, efficient disaggregation of LPS by LBP is only possible when (s)CD14 is also present (Figs. 4 and 5) (5). Both sCD14-dependent disaggregation of LPS in the presence of Mg<sup>2+</sup> and Ca<sup>2+</sup> and sCD14-independent disaggregation of LPS in the presence of EDTA are promoted by the COOH-terminal domain of LBP (Figs. 7 and 8). Together with the role of this domain of LBP in delivery of LPS aggregates to CD14 (Fig. 2), these findings suggest that the COOH-terminal domain of LBP may facilitate formation of small bioactive endotoxin-CD14 complexes by helping to destabilize LPS aggregates and promote contact with CD14. The ability of isolated LBP:LPS<sub>agg</sub> to interact with sCD14 (Fig. 5A) and cells containing mCD14 (Fig. 10A) in the same manner as the addition of a mixture of LPS<sub>agg</sub> and LBP indicates that these LBP:LPS<sub>agg</sub> represent functionally relevant intermediates.

Initial interactions of LBP and BPI with LPS are driven by electrostatic attractions between anionic moieties of LPS concentrated near the lipid A region and basic amino acids clustered at the extreme end of the NH<sub>2</sub>-terminal domain (22, 30, 54). In BPI, these sites are contiguous with an extended cationic protein surface whereas in LBP this adjacent surface is acidic (30, 55). These differences in the density and distribution of protein charges probably account for the stronger attraction of LPS for BPI than for LBP and could contribute to the greater facility of LBP:LPS<sub>agg</sub> to extract and release CD14-endotoxin complexes and the inability of BPI:LPS<sub>agg</sub> to release LPS (5). This hypothesis is supported by the observed greater instability of LBP:LPS<sub>agg</sub> versus BPI:LPS<sub>agg</sub> (Figs. 5 and 6) and greater efficiency of LBP versus BPI<sub>L</sub>-LBP<sub>C</sub> in sCD14-dependent (Figs. 5 and 7) and sCD14-independent (Fig. 8) disaggregation. Therefore, differences in interactions with LPS mediated by the NH<sub>2</sub>-terminal domain of LBP and BPI may further contribute to the efficiency of LBP-promoted LPS disaggregation and cell activation.

It has been generally presumed that the inhibitory effects of BPI on LPS signaling reflect the inability of BPI-endotoxin

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4. T. Cardozo, unpublished observations.
5. A. Teghanemt and J. Weiss, unpublished observations.
with endotoxin-responsive cells. The absence of detectable BPI or LBP-α-BP1ζ-dependent binding of LPS to PMN (Fig. 1A) is consistent with that view. However, an earlier study suggested BPI-dependent association of LPS with promonocyte-like THP-1 cells (56, 57). We have extended that observation to mature monocytes. The fact that BPI-dependent LPS association is promoted by BPI or LBP-α-BP1ζ, but not BP1ζ, reveals that the COOH-terminal domain of BPI is instrumental in BPI-dependent delivery of LPS to these cells. Delivery of BPI-endotoxin aggregates does not provoke the same degree of cellular response elicited by LBP-endotoxin aggregates. Differences in interactions with CD14 as specified by different properties of the COOH-terminal domains of LBP and BPI (see Figs. 2 and 7) could explain the different functional consequences of LPS delivery to monocytes via LBP and BPI. The partial inhibitory effect of one particular mAb to CD14, MEM18, on BPI or LBP-α-BP1ζ delivery of LPS to monocytes raises the possibility that BPI:LPSagg transiently engages CD14, but in a manner insufficient to generate the CD14-endotoxin complexes responsible for maximal cell activation. The effects of the MEM-18 mAb could be nonspecific, for example, sterically impeding BPI-dependent delivery of LPS to a neighboring acceptor molecule. The versatility of CD14 in its associations with diverse extracellular microbial and host ligands and with host plasma membrane constituents makes either scenario a possibility (15, 58–66). It is noteworthy that a region in CD14 essential for MEM18 binding (amino acids 57–64) (46) may represent a flexible bridge important in intracellular and intercellular interactions.

We have previously shown that BPI and, to a lesser extent, BPI-21, promote uptake of encapsulated Gram-negative bacteria by neutrophils (23). These data as well as the findings reported in this study indicate a potential role of BPI in clearance of bacteria and bacterial remnants, each dependent on the COOH-terminal domain of BPI, but directed to different host cells, i.e. bacteria to neutrophils, cell-free LPS to monocytes. Although the precise molecular determinants of these interactions are unknown, our findings clearly indicate that recognition by host cells of BPI-coated endotoxin-containing particles depend both on the properties of BPI (e.g. the COOH-terminal domain) and on the nature of the particle itself. The targeting of BPI-coated bacteria and cell-free LPS to different cells should benefit host defense. Neutrophils are best equipped to quickly eliminate rapidly multiplying and disseminating organisms, whereas monocyte-like cells are better endowed with a digestive apparatus possibly involved in detoxification or antigen presentation (42, 67). It should be noted, however, that the extent of cellular uptake of BPI:LPSagg even at relatively low LPS concentrations (Fig. 10), is limited suggesting the need for other, as yet unknown, potential targets of BPI:LPSagg and/or other functional consequences of the delivery of BPI: LPSagg to monocytes or monocyte-like cells.

The results presented here establish that the carboxyl-terminal domain is responsible for dictating the fate of the LPS engaged by LBP and BPI. Both proteins have the ability to interact with LPS to promote the formation of larger aggregates which are delivered to acceptor molecules on monocytes. After binding, the fate of the LPS aggregate diverges depending on the vehicle of delivery. For LBP promoted delivery, the immediate acceptor molecule is (m)CD14. LPS is transferred via interaction with the carboxyl-terminal domain of LBP to CD14 which subsequently activates a receptor molecule, most likely Toll-like receptor 4, to transmit signals that result in proinflammatory responses. For BPI promoted delivery, the molecule immediately engaged upon delivery of LPS is unknown as is the ultimate fate of the delivered LPS and recipient cells. Whatever the identity of the acceptor molecule, however, it requires an interaction with the carboxyl-terminal domain of BPI since similar delivery is not seen with BPI-21. Studies are in progress to better define the cellular and subcellular targets of BPI-endotoxin complexes and the host responses they engender.

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The Carboxyl-terminal Domain of Closely Related Endotoxin-binding Proteins Determines the Target of Protein-Lipopolysaccharide Complexes
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