Membrane Expression of Soluble Endotoxin-binding Proteins Permits Lipopolysaccharide Signaling in Chinese Hamster Ovary Fibroblasts Independently of CD14*

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The activation of phagocytes by lipopolysaccharide (LPS) has been implicated in the pathogenesis of Gram-negative sepsis. Although the interaction between CD14 and LPS is a key event in the signaling cascade, the molecular mechanism by which cellular activation occurs remains obscure. We hypothesized that the main function of CD14 was to bind LPS and transfer it to a second receptor, which then initiates the subsequent signal for cellular activation. Thus, surface binding of LPS to the cell membrane would be the critical step that CD14 carries out. To test this hypothesis, we examined the activity of two other proteins known to bind LPS, lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein. We found that when these normally soluble proteins were expressed in Chinese hamster ovary-K1 fibroblasts as glycosylphosphatidylinositol-anchored proteins, both could substitute for CD14 in initiating LPS signaling. Pharmacological studies with synthetic lipid A analogues demonstrated that these surface expressed LPS-binding proteins had characteristics that were qualitatively identical to membrane CD14. These data support the hypothesis that a receptor distinct from CD14 functions as the actual signal transducer and suggest that surface binding of LPS to the cell membrane is the crucial first step for initiating downstream signaling events.

Endotoxic shock is an acute septic syndrome caused by the overproduction of pro-inflammatory mediators during a bloodstream infection with Gram-negative bacteria. The pathogenesis of the shock is presumed to be secondary to excessive stimulation of host cells by bacterial lipopolysaccharide (LPS, endotoxin), leading to the synthesis and release of cytokines, arachidonic acid metabolites, and various other mediators (1–3). The identification of CD14 as a mediator of LPS-inducible signal transduction was a crucial event in understanding the mechanism by which LPS-induced cellular activation occurs. CD14, a 55-kDa glycosylphosphatidylinositol (GPI)-linked protein present on the surface of phagocytic leukocytes, has been shown to bind LPS and to mediate cellular activation (4–6). In addition, a soluble form of CD14 (sCD14) is also capable of binding LPS and activating some CD14-deficient cells, such as endothelial cells (7–10).

Although it is generally agreed that the interaction between lipid A and CD14 is central to cellular activation by LPS, details of the downstream events, which follow, remain obscure. For example, because CD14 lacks a transmembrane domain, it is unlikely that CD14 alone is responsible for directly transmitting a signal across the plasma membrane. Many groups have hypothesized that CD14 and LPS must interact, either directly or indirectly, with a second transmembrane receptor, which would be the actual LPS signal transducer (reviewed in Ref. 11). Alternatively, CD14 could mediate cellular activation via a physical mechanism. Wright and colleagues (12, 13) observed that CD14 could move LPS into lipid bilayers that resemble mammalian cell membranes. Furthermore, they observed that trafficking of LPS from the membrane to the Golgi correlated with the ability to induce signaling.

The CD11/CD18 (β2) integrins are a second group of LPS receptors (14). The observation that CD11/CD18 could enable LPS responsiveness independently of CD14 when expressed on the surface of CHO cells (15, 16) suggested that the ability of CD14 to participate in the LPS signaling cascade was not unique. We hypothesized that the common function shared by these two LPS receptors was their ability to bind LPS and bring it in close proximity to the plasma membrane, where it could then interact with a signal transducer. To further investigate the role of LPS binding in cellular signaling, we decided to exploit the activity of two other proteins known to bind LPS, lipopolysaccharide-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI).

LBP and BPI are members of the lipid transfer/lipopolysaccharide binding gene family, which includes cholesteryl ester transfer protein and phospholipid transfer protein (reviewed in Ref. 17). LBP and BPI share approximately 45% homology at the amino acid level and are encoded in the same region of human chromosome 20, suggesting that they arose by gene duplication (18, 19). However, the two proteins appear to have quite different biological functions. LBP, a soluble protein secreted by hepatocytes into the blood stream, accelerates the binding of LPS monomers to CD14 (20, 21). Thus, it enhances the sensitivity of cells to LPS, and may be important in the host recognition of and response to Gram-negative bacteria (11). In contrast, BPI, which is found in the azurophilic granules of neutrophils, is bactericidal toward Gram-negative bacteria, and inhibits the biologic activity of LPS (22, 23).

To test the hypothesis that cellular binding alone of LPS...
would be sufficient to initiate cellular signaling, we constructed chimeric proteins consisting of LBP or BPI attached to the GPI anchor of decay accelerating factor (DAF) (24, 25). Expression of these constructs would thus create an artificial LPS receptor on the surface of the transfected cell. We found that CHO-K1 fibroblasts expressing GPI-anchored LBP or BPI could bind both LPS and Gram-negative bacteria. Furthermore, they could initiate the LPS signaling cascade in a CD14-independent manner, similar to CD11/CD18-transfected cell lines. We conclude from this that the ability of an LPS binding protein to focus LPS on the surface membrane of a cell, where it can interact with a second receptor, is sufficient to activate the specific LPS-signaling apparatus.

**EXPERIMENTAL PROCEDURES**

**Reagents—**PBS, Ham's F-12, and Trypsin-Verseae mixture were obtained from Bio-Whittaker (Walkersville, MD). Ex-Cell 301 serum-free medium was obtained from J.R.H. Biosciences (Lenexa, KS), fetal calf serum (FCS) (LPS less than 10 pg/ml) from Summit Biotechnology (Greeley, CO), G418 from Life Technologies, Inc., ciprofloxacin from Miles Pharmaceuticals (West Haven, CT), and trypsin blue solution (0.4%) from Sigma. Human LBP and soluble CD14 were gifts of Henry Lichi-Francisco, University of Pennsylvania, Philadelphia, PA. Purified recombinant human BPI was a gift of Terje Espevik and Egil Lien (Norwegian University of Science and Technology, Trondheim, Norway). LPS from *Salmonella minnesota* R595 (ReLPS) was a gift from Nilo Qureshi and Kun Takayama (University of Wisconsin, Madison, WI). LPS was protein-free by Bio-Rad protein assay (Bio-Rad). Compound B287 (26) and compound B1287 (patent reference number WO-9639411-A1) were prepared at Eisai Research Institute (Andover, MA). Lipids were prepared at Eisai Research Laboratory, Berlin, Germany. The PCR primers (5'-primer GCATGATCATGCGCTTTGGATAG, 3'-primer GCTCTAGATCTCAATTCTGCAAGTTGTTCTATTTTCA) under the following conditions: (94° 1:00, 55° 1:00, 72° 2:30) 15 cycles; (52° 1:00, 72° 2:30) 1 cycle. This DAF fragment was inserted into pCDNA3 (Invitrogen, San Diego, CA); the cDNAs for LBP, BPI, and CD4 were then subsequently ligated to the DAF GPI fragment. The human LBP cDNA was cloned from human LBP cDNA in the pCMV vector (gift of Ralf Schumann, Molecular Sepsis Research Laboratory, Berlin, Germany). The BPI cDNA fragment was obtained from the CDNA of vitamin D3-treated THP-1 cells using PCR primers (5'-primer GCATGATCATGCGCTTTGGATAG, 3'-primer GCTCTAGATCTCAATTCTGCAAGTTGTTCTATTTTCA) under the following conditions: (94° 1:00, 54° 0.45, 72° 2:00) 40 cycles; (72° 5:00, 35° 5:00) 1 cycle. This DAF fragment was inserted into pCDNA3 (Invitrogen, San Diego, CA); the cDNAs for LBP, BPI, and CD4 were then subsequently inserted into this vector 5' to the DAF GPI fragment.

Human LBP was cloned from a plasmid containing human LBP cDNA in the pCMV vector (gift of Ralf Schumann, Molecular Sepsis Research Laboratory, Berlin, Germany). Human CD4 was cloned from a plasmid containing human CD4 cDNA in the pCMV vector (gift of Ellis Reinherz, Ref. 27). The PCR primers (5'-primer CAAGGATCTGAGGCGAGGAGT, 3'-primer CAGCATAGTGCGCTTGGAG) were used under the following conditions: (94° 1:00, 55° 1:00, 72° 2:30) 10 cycles; (94° 1:30, 57° 1:00, 72° 2:30) 15 cycles; (72° 5:00, 28° 5:00) 1 cycle. Human BPI was cloned from a human bone marrow cDNA library (CLONTECH, Palo Alto, CA). The PCR primers (5'-primer GCCAGTCTCAGGCAAGGACAT, 3'-primer TCTTGCGACGCTTGATGCA) were used under the following conditions: (94° 1:00, 52° 1:00, 72° 2:30) 5 cycles; (94° 0.30, 57° 1:00, 72° 2:30) 15 cycles; (72° 5:00, 28° 5:00) 1 cycle.

All three chimeric proteins have two additional amino acids at the site of fusion, as shown in Fig. 1. The in-frame fusion of all constructs was confirmed by DNA sequencing using the dideoxy chain termination method (28). In addition, the LBP and BPI portions were sequenced in each construct.

**Cell Lines—**Chinese hamster ovary fibroblasts (CHO-K1) cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Ham's F-12 supplemented with 10% FCS and 10 µg of ciprofloxacin/ml (complete medium). Cell lines were grown as adherent monolayers in tissue culture dishes at 37 °C in 5% CO2, and passaged twice a week to maintain logarithmic growth. The following stably transfected cell lines have been described: CHO/Neo, CHO-K1 transfected with the pCDNA1/Neo vector (29); and, CHO/CD14, human CD14 transfected CHO-K1 (29). CHO/CD4, CHO/LBP, and CHO/CD14 were engineered by stably transfected CHO-K1 with the DAF constructs by calcium phosphate precipitation (29). Stable transfected were selected in medium supplemented with G418 (1 mg active drug/ml), and surface expression of the transfected genes was confirmed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). Bulk transfected cells were subjected to one round of positive selection using a Becton Dickinson FACScan Plus fluorescence activated cell sorter in enrichment mode to select for cells with the highest levels of the transfected receptor. A clonal cell line was then selected from this population by limiting dilution cloning. The following antibodies were used for flow cytometry: goat anti-human LBP antibody (1:100 dilution; gift of Ralf Schumann, Molecular Sepsis Research Laboratory, Berlin, Germany) and fluorescein isothiocyanate-conjugated anti-goat IgG (1:100 dilution; Sigma); rabbit anti-human BPI (1:100 dilution; gift of Russell Dedrick, SOMA Corp., Berkeley, CA) and fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100 dilution; Sigma); fluorescein isothiocyanate-conjugated anti-CD4 antibody (Leu-3a; 2.5 dilution; Becton Dickinson).

**Cell Culture and Stimulation Conditions—**One day before stimulation, cells were plated in 6-well tissue culture dishes at a density of 5 × 10^5 cells/well. Before the addition of LPS stimulation, cells were washed three times with PBS to remove FCS, and medium was replaced with 1 ml of Ham's F-12 with 2% FCS or Ex-Cell serum-free medium. When LBP and soluble CD14 were used in assays, they were added to the Ex-Cell medium for a final concentration of 200 ng/ml or 100 ng/ml, respectively. For antibody experiments, cells were preincubated with 3C10 (20 ng/ml) on ice for 20 min before the addition of LPS.

**Fluorescent Membrane Staining and Flow Cytometry by Shift Assay (EMSA)—**After stimulation, cells were washed with PBS, 2% FCS, harvested using a rubber policeman, and pelleted in a microcentrifuge (Beckman Microfuge 11). Nuclear proteins were isolated as described in detail previously (30). The extracted proteins were then assayed for the presence of nuclear factor-κB (NF-κB) as described using a NF-κB labeled oligonucleotide containing the consensus sequence for NF-κB binding from the murine immunoglobulin κ light chain gene enhancer. The DNA-protein binding reactions were analyzed by nondenaturing gel electrophoresis. Gels were transferred to filter paper, dried, and exposed to x-ray film (30).

**LPS Binding and Phagocytosis Assays—**LPS binding assays with boron dipyrromethane (BODIPY)-labeled LPS were performed as described (31) except that the assay was adapted for whole cell binding. Complexes of BODIPY-LPS (100 ng/ml; gift of Rolf Thieringer, Merck Research Laboratories) and soluble CD14 (2 µg/ml) were preformed overnight at 37 °C. CHO-K1 transfecants were plated at a density of 2 × 10^5/well in a 1-well volume, and allowed to grow overnight. BODIPY-LPS/BS/CD14 complexes were then added to adherent monolayers in the presence or absence of chimeric human lipopolysaccharide binding protein (200 ng/ml) for 30 min at 37 °C. Following the incubation period, wells were washed with PBS containing 1% feral bovine serum, and trypan blue (0.4%) was added for a 1-min incubation period to quench extracellular fluorescence. Cells were detached using trypsin-verseene, and analyzed for fluorescence using flow cytometry (29).

For whole bacteria phagocytosis assays, cells were treated as above except that BODIPY-conjugated *Escherichia coli* (R-12 strain) BioPar-
articles (Molecular Probes, Eugene, OR) were added to the CHO-K1 monolayers to give a final concentration of $3 \times 10^7$/ml. Binding and internalization of the BioParticles by the CHO cells were allowed to proceed for 1 h. Before analysis, fluorescent extracellular bacteria were quenched using trypan blue.

**RESULTS**

**CHO-K1 Cells Expressing LBP or BPI Bind LPS and Gram-negative Bacteria**—Shown in Fig. 1 are the DNA and amino acid sequences of LBP, BPI, and CD4 at the site of fusion with DAF. CHO-K1 fibroblasts were stably transfected with the three DAF plasmids to create the two experimental cell lines, CHO/LBP and CHO/BPI, and the control cell line, CHO/CD4. Surface expression of the chimeric proteins in clonal cell lines was confirmed by flow cytometry (Fig. 2). Whereas FACS is semi-quantitative, relative protein expression between cell lines could not be calculated from this analysis, because each cell line was stained using different primary and secondary antibodies. All cell lines, however, did express significant immunofluorescent signals compared with the isotype-stained control cells.

The ability of LBP and BPI to bind LPS as soluble proteins is well described (32, 33). We wanted to be certain that surface-expression of these proteins did not interfere with this function. To assess the ability of the transfectants to bind LPS, CHO/LBP, CHO/BPI, CHO/CD4, and CHO/CD14 were incubated with preformed complexes of BODIPY-LPS and soluble CD14, an experimental technique that is thought to dissociate LPS aggregates into monomers (31). Cells were then analyzed by flow cytometry for fluorescence. Only the cell lines expressing the LPS-binding proteins (LBP, BPI, and CD14) bound BODIPY-LPS (Fig. 3). Co-incubation of soluble LBP had no effect on the observed binding of LPS-sCD14 complexes to the CHO/LBP and CHO/BPI lines. In contrast, binding to CHO/CD14 was greatly enhanced when LPS-sCD14 complexes were added in the presence of soluble LBP, consistent with previous observations that LBP specifically promotes movement of LPS onto CD14 (31).

Previous reports by Tobias and colleagues (34) demonstrated that the affinity of BPI for LPS was higher than that for LBP. However, our data suggests that binding to the CHO/LBP line is better than that of CHO/BPI. In part, this may be due to changes in the conformation of the protein in its anchored state. In addition, Tobias’ data was obtained through a careful titration of the molar ratios of LPS to the binding proteins, something which would be more difficult to accomplish in our cell lines, because we cannot control for the exact level of surface expression of LBP, BPI and CD14. However, the data does demonstrate, at least in a qualitative manner, that LBP and BPI function as effective endotoxin-binding proteins when expressed as chimeric GPI-linked proteins on the surface of CHO cells.

Other groups (35, 36) have described the ability of CD14 to enable the internalization of Gram-negative bacteria, presumably through its interaction with a transmembrane receptor. We wanted to know whether the GPI-anchored LBP and BPI were also capable of enhancing bacterial uptake. When CHO/LBP, CHO/BPI, CHO/CD4 and CHO/CD14 were incubated with fluorescent *E. coli* BioParticles, we found, again, that only...
the lines expressing the LPS-binding proteins were capable of binding and internalizing the bacteria (data not shown).

CHO-K1 Cells Expressing LBP or BPI Respond to LPS—To assess the ability of the membrane anchored LBP and BPI to mediate LPS activation, cells were incubated with increasing doses of LPS, and nuclear extracts were analyzed for the immediate LPS activation, cells were incubated with increasing doses of LPS under serum-free conditions. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. Only the band representing NF-κB bound to the κB site-containing probe is shown. Similar results were found in the presence of 2% FCS.

The time course of NF-κB translocation also differed between the CD14, LBP, and BPI lines. The response to LPS in CHO/CD14 is rapid; it begins within 15–30 min, peaks at 1 h, and rapidly returns to baseline by 3 h. For the CHO/LBP and CHO/BPI lines, however, the signaling peaked between 30 min and 1 h, and was sustained even after 3 h (Fig. 5). This is similar to the time course observed previously with the CD11/CD18 expressing CHO cells (15, 16). The more transient activation seen with CD14 in comparison with the other receptors suggests that it alone may activate a divergent pathway involved in down-regulation of the signal.

LPS Signaling in CHO/LBP and CHO/BPI Is Independent of Soluble CD14 and Soluble LBP—Despite the use of strict serum-free conditions, we wanted to be certain that the signaling we observed in CHO/LBP and CHO/BPI was not being mediated through soluble CD14 contaminating our cell culture system. Historical evidence suggested that this was not the case. First, CHO cells do not express endogenous CD14 mRNA by Northern blot analysis, making it unlikely that they would produce and secrete the protein into the supernatant (29). In addition, unlike endothelial cells and several other non-CD14 expressing cell types, neither soluble CD14 nor serum has been shown to enable wild-type or mock transfected CHO-K1 cells to respond to low doses of LPS (29, 30).

Further evidence came from the use of 3C10, an anti-CD14 monoclonal antibody which has been shown to specifically inhibit LPS signaling (5). We found that 3C10 inhibited LPS signaling in CHO/CD14, whereas the response to LPS by CHO/LBP and CHO/BPI was not diminished in the presence of this antibody (Fig. 6). Finally, we found that the addition of soluble CD14 or soluble CD14 to the culture medium did not shift the sensitivity of the CHO/LBP line to LPS. This was in contrast to what has been described with CD14-mediated signaling, further demonstrating that the activity that we observed was independent of CD14 (Fig. 7).

The Effects of Lipid A Analogues in CHO/LBP and CHO/BPI Are Identical to That of CHO/CD14—Pharmacologic studies with biologically derived lipid A antagonists have led to the hypothesis that CD14 activates cells, not directly, but via an ancillary signaling molecule. The first line of data comes from careful binding studies by Kitchens and colleagues (37, 38) demonstrating that these compounds inhibit the ability of LPS to activate cells at concentrations that are too low to inhibit binding of LPS to CD14. In addition, the pharmacological effects of the antagonists suggest a complex signal transduction apparatus for LPS. For example, Rhodobacter sphaeroides lipid A (RSLA) and lipid IVα are potent LPS antagonists in LPS-responsive human cells (39–41), whereas in hamster and mouse cells these compounds have very different effects. In hamsters, both lipid IVα and RSLA are LPS mimetics (42). In mice, lipid IVα is an LPS mimic and RSLA is an LPS antagonist (39, 40, 43, 44). Data from transfected cell lines has shown that the species-specific effects of these compounds are determined not by the species of CD14, but by the genome of the host cell on which it is expressed (42). Taken together, these data suggest that the inhibitors are antagonizing LPS at a site distinct from CD14.

The first compound we tested was B287, a synthetic lipid A analogue based on the proposed structure of RSLA. B287 has activity identical to that of natural RSLA when tested in macrophage cell lines and whole human blood ex vivo (26), and has been previously shown by our laboratory to be a potent LPS mimic in CHO/CD14 cells (42). When CHO/LBP was incubated with increasing doses of B287 we found that the nuclear translocation of NF-κB was induced (Fig. 8). Similar results were found with CHO/BPI (data not shown). Thus the compound acted as an LPS mimetic in CHO/CD14, CHO/LBP, and CHO/BPI. Use of this synthetic LPS mimetic was also strong evidence that the cellular activation we observed with our ReLPS was not occurring via any contaminating bacterial proteins in the preparation.

The next compound we examined was B1287, which we recently described as a potent antagonist in the LPS responsive CHO/CD14 cell line (45).
The current understanding of LPS signaling consists of LPS binding to either membrane or soluble CD14 followed by cellular activation. The mechanism by which this occurs, at least at the molecular level, is still unclear. The central role of CD14 in the process is supported, in part, by the observation that CD14-deficient mice are highly resistant to the pro-inflammatory effects of LPS and Gram-negative bacteria (46). However, although CD14 is clearly an important LPS binding protein that participates in the initiation of LPS signaling events, one could argue that it is not truly an LPS receptor in the sense that it does not directly activate the signaling cascade. Furthermore, this ability to participate in LPS signaling events is not a characteristic that is unique to CD14. In fact, we have shown that other known surface LPS binding proteins, specifically the CD11/CD18 (β₂) integrins, are also sufficient for imparting LPS responsiveness when overexpressed in CHO-K1 cells (15, 16, 47).

As soluble LPS-binding proteins, LBP and BPI have never been shown to enable LPS signaling in the absence of CD14. In fact, BPI has been shown to inhibit LPS activity (22, 23). Thus, the ability of a soluble LPS-binding protein to participate in cell activation appears to be unique to soluble CD14. In contrast, our data demonstrates that LBP and BPI, when expressed on the surface of a CHO cell, can enable cellular activation in a manner similar to that of membrane CD14. One possible interpretation of the data is that high expression of LBP on the surface of the CHO cell enhanced signaling by efficiently transferring LPS to any contaminating soluble CD14 in our system. However, this interpretation would not explain the signaling observed in the CHO/BPI line, because BPI does not possess the same LPS-transferase activity as that of LBP (48).

Both LBP and BPI share the ability to bind LPS with high affinity, and we believe that it is their ability to bind LPS and bring it in close proximity to the cell surface that enables them to mediate activation of the LPS signaling cascade. We believe that LPS is activating the CHO/LBP and CHO/BPI lines in the same manner as CHO/CD14. This is supported by the ability of the specific LPS antagonist B287 to block signaling events. However, there appears to be at least one unique aspect of CD14-mediated signaling, at least in the transfected CHO lines—the time course of cellular activation. Unlike LBP, BPI, and CD11/CD18, CD14 is the only LPS-binding protein that turns off the signal for NF-κB translocation after 1 h; the other three lines all consistently have been shown to sustain the signal for more than 3 h. In addition, CHO cells co-transfected with both CD14 and CD11/CD18 show a time course consistent with activation via both receptors with a rapid peak and a sustained activation for up to 5 h. Thus, expression of these surface LPS binding proteins in our system has not entirely duplicated CD14-initiated signaling. It is possible that the signaling events initiated by CD14 diverge from those of the other LPS receptors at some point on the way to NF-κB translocation. In fact, this ability of CD14 to down-regulate its own signal may hold physiologic relevance.

These data are consistent with our current model of LPS signaling in which CD14 binds LPS and brings it in close proximity to the cell membrane where it can then interact with a transmembrane receptor. This second protein would be the actual LPS signal transducer/receptor. Alternate models of LPS signaling, whereby LPS uptake or intercalation into the membrane is an integral part of the downstream signaling events, are supported less well. Although LBP and CD14 can transport LPS into phospholipid bilayers (49), neither BPI nor the CD11/CD18 integrins are known to have such activity. The important observation appears to be that any protein that can bind LPS and concentrate it on the cell membrane is capable of activating the LPS-specific signaling pathway. These LPS-binding proteins thus enhance sensitivity to endotoxin, but
lack the specificity usually observed with true signal transducing receptors.

Recently two Toll-like receptors, TLR-2 and TLR-4, have been implicated in LPS signaling (50–53), and it is possible that one of the TLRs receive endotoxin from these natural and engineered surface receptors. However, any conclusions about the relevance of the TLRs in LPS signaling will have to account for the properties of the anchored LPS-binding proteins, as well as the species-specific effects of the LPS inhibitors. The ability of GPI-anchored LBP and BPI to enhance signaling in response to LPS exposure underscores the complex interactions between the multiple receptors involved in LPS recognition and cellular activation.

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