Structure and Function of Lipopolysaccharide Binding Protein

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The primary structure of lipopolysaccharide binding protein (LBP), a trace plasma protein that binds to the lipid A moiety of bacterial lipopolysaccharides (LPSs), was deduced by sequencing cloned complementary DNA. LBP shares sequence identity with another LPS binding protein found in granulocytes, bactericidal/permeability–increasing protein, and with cholesterol ester transport protein of the plasma. LBP may control the response to LPS under physiologic conditions by forming high-affinity complexes with LPS that bind to monocytes and macrophages, which then secrete tumor necrosis factor. The identification of this pathway for LPS-induced monocyte stimulation may aid in the development of treatments for diseases in which Gram-negative sepsis or endotoxemia are involved.

BOTH INVERTEBRATES AND VERTEBRATES have evolved complex mechanisms to respond to infection with Gram-negative bacteria. The response is characterized by initiation of various defense mechanisms that rely on recognition of lipopolysaccharide (LPS or endotoxin), a glycolipid present in the outer membrane of all gram-negative bacteria. The mechanisms of endotoxin action at the molecular level are being elucidated, with the identification of effector molecules, such as tumor necrosis factor (TNF), that mediate endotoxic effects (*1*). Little is known about the protein molecules that specifically recognize LPS and initiate host responses that either eliminate the pathogen or result in irreversible shock, multiorgan failure, and death.

We characterized a trace plasma protein that binds to LPS [LPS binding protein (LBP)] (*2–4*). In many species, including humans (*2, 3*), LBP is present in normal serum at <0.5 μg/ml, rising to 50 μg/ml 24 hours after induction of an acute phase response. LBP isolated from human or rabbit acute phase serum is a 60-kD glycoprotein (*2*) synthesized in hepatocytes as a 50-kD single-chain polypeptide (*5*). LBP has a binding site for lipid A, binding with high affinity to LPS from rough- (R-) and smooth- (S-) form bacteria (*4*). LBP opsonizes LPS-bearing particles and intact Gram-negative bacteria, mediating attachment of coated particles to macrophages. This binding occurs via a cellular receptor that is mobile in the plane of the membrane (*6*). Herein we describe the cloning and sequencing of LBP cDNA, together with an analysis of the function of LBP. In the accompanying report, we provide evidence that identifies the receptor for LPS-LBP complexes as the monocytic differentiation antigen, CD14 (*7*).

The NH_2-terminal sequence of rabbit LBP (*2*) is similar to the NH_2-terminal end of human bactericidal/permeability–increasing protein (BPI) (*8*). A synthetic oligonucleotide was designed that encoded the rabbit sequence; nucleotide usage was determined by those in common with the nucleotide sequence of human BPI cDNA (*9*), in order to make a consensus sequence (*10*). A human liver cDNA library in λgt10 was screened (*11, 12*), seven hybridizing clones isolated, and a clone of ~1.7 kb subcloned into pUC119. Both strands of this clone were sequenced (*13*). The deduced amino acid sequence of this cDNA is shown in Fig. 1. A hydrophobic signal sequence of 25 residues precedes the mature protein of 452

Fig. 1. Comparison of the deduced amino acid sequences of human LBP, rabbit LBP, and human BPI. The numbering is based on the rabbit LBP sequence, potential glycosylation sites are denoted by shaded areas, and identical residues are within blocks. The single-letter amino acid code is shown in (*22*). The nucleotide sequences are deposited at GenBank and are available on request: accession number M-35533 (human LBP) and M-35534 (rabbit LBP).

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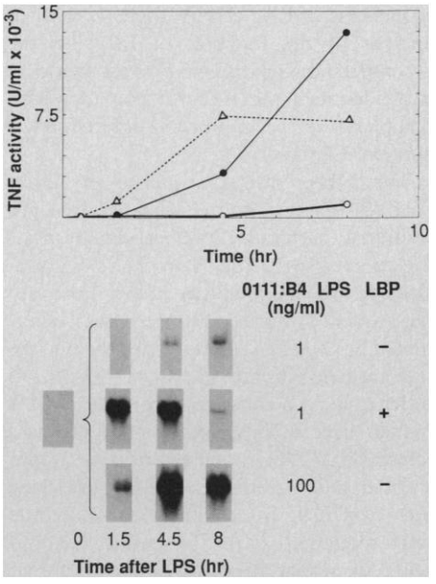

Fig. 2. The potentiating effect of rabbit LBP on LPS (0111:B4)-induced TNF production by explanted rabbit PEM (*18*). Cells were treated with 0111:B4 LPS in the presence or absence of rabbit LBP (0.1 μg/ml); conditioned medium was harvested at various times between 0 and 8 hours for the TNF assay (△, LBS-LBP, 1 ng of LPS per milliliter; ○, LPS, 1 ng of LPS per milliliter; ●, 100 ng of LPS per milliliter). Total RNA was isolated and Northern blot analysis was performed, as described (*20*). The effect of LBP shown here requires active LBP and is specific, since replacement of LBP (90°C, 30 min) that does not bind LPS or BPI (up to 10 μg/ml) did not change the response to LPS. Data shown are mean values of duplicate assays and are representative of two experiments performed with 0111:B4 LPS (mRNA and TNF activity measurements).

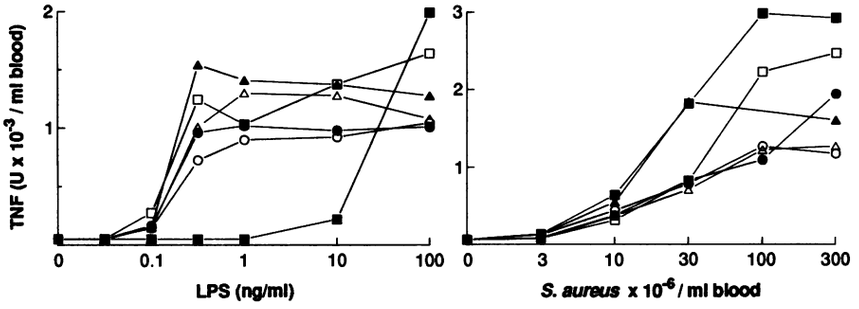

Fig. 3. The effect of immunodepletion of LBP on LPS-induced TNF production in whole rabbit blood. Heparinized (10 U/ml) rabbit blood (New Zealand White rabbits, male, 2.5 kg) was used as a source of platelet-free plasma (PFP). IgG fractions of the following goat polyclonal antibodies: (i) nonimmune IgG (△), (ii) antibody to rabbit IgG (▲), (iii) antibody to rabbit fibronectin (□), (iv) antibody to rabbit LBP (■), or (v) pyrogen-free sterile saline (●) were added to PFP as 10 mg/ml solutions to a final concentration of 2 mg/ml. Before use of the IgG, preparations were treated to remove LPS (*23*). Antibody-treated or control PFP solutions were maintained at 4°C for 18 hours and then centrifuged at 14,000*g*. On the morning of the experiment, heparinized rabbit blood was again collected from the same animal, and cells separated from plasma as follows: 200*g* followed by resuspension of cells in RPMI 1640 and recentrifugation (800*g*) and washing two times. The washed cell pellet was then gently mixed with the immunodepleted PFP (i–v) and an additional sample of rabbit blood (○) incubated with various concentrations of LPS from *S. minnesota* Re595 (*2*) or with heat-killed *S. aureus* (*2*) for 6 hours. Cell-free supernatants were collected and assayed for TNF (*24*). The initial sample of blood and all subsequent plasma and solutions contained heparin (10 U/ml) and 10 μM indomethacin. Data shown are the average of two determinations of TNF activity.

residues. The mature protein contains four cysteines and five potential glycosylation sites. This sequence was confirmed as human LBP by the purification and microsequencing of LBP from human acute phase serum (*2, 3*). The sequence of the NH_2-terminal 15 residues was identical to the encoded cDNA sequence (*14*).

Rabbit LBP cDNA clones were isolated from a λgt10 library prepared from rabbit liver RNA that was isolated 24 hours after the induction of an acute phase response (*2*). This library was screened (*11, 12*) with a 0.9-kb Eco RV–Eco RI fragment from the human LBP cDNA clone and with the synthetic oligonucleotide probes used in isolation of human LBP cDNA (*10*). The 1.8-kb insert that hybridized with both probes was subcloned and sequenced (*13*). The deduced amino acid sequence for rabbit LBP is shown in Fig. 1. The encoded sequence is similar to the human, with a 26-residue signal sequence and a 456-residue mature protein with two cysteines and three potential glycosylation sites. The first 39 residues of the mature protein deduced from the cDNA match the sequence reported for the NH_2-terminal end of natural rabbit LBP (*2*).

Sequence comparisons (Fig. 1) for LBP and BPI reveal 69% amino acid identity (78% nucleotide identity) between rabbit and human LBP, and 40% and 44% identity between rabbit and human LBP, respectively, with human BPI (*9*). These results confirm our earlier hypothesis of homology between LBP and BPI (*8*). Computer searches of protein sequences revealed that human LBP also shows sequence identity with the cholesterol ester transport protein (CETP) (*15*) from human plasma. Approximately 23% of the residues are identical, and another 23% of the residues are conservative substitutions (*16*).

Lipopolysaccharide forms high-affinity complexes with LBP in acute phase serum and in solution when purified LBP and LPS are mixed (*2, 4*). Polyclonal antibody to LBP (anti-LBP) immunoprecipitates R- and S-form LPS from mixtures of LPS and normal rabbit plasma (*17*). Because macrophages bind particles containing LBP-LPS complexes (*6*), LBP might serve as a carrier protein that brings LPS to the surface of cells. To determine if LBP participates in macrophage responses to LPS, we examined the effect of LBP on LPS-induced TNF production by peritoneal exudate macrophages (PEMs) (*18*) elicited from rabbits and also evaluated the effects of polyclonal anti-LBP on LPS-induced TNF in whole rabbit blood, ex vivo (*19*).

We determined the kinetics of TNF mRNA and protein induction in PEMs by Northern (RNA) blot analysis and by measuring TNF activity of cell-free supernatants as described (*18, 20*). Cells were stimulated with LPS (from *Escherichia coli* 0111:B4) in the presence and absence of highly purified rabbit LBP (*2*) (Fig. 2). LBP enhanced LPS-induced TNF production; TNF levels increased from slightly above base line (~40 U/ml) to 10,000 U/ml. This is accompanied by an increase in the rate and extent of TNF mRNA induction and occurs with a concentration of LBP found in normal serum (*2*). Exposure of PEMs to 0111:B4 (1 ng/ml) in the absence of LBP for up to 12 hours did not increase TNF above that shown. LBP alone did not induce detectable TNF mRNA or protein.

Lipopolysaccharide or heat-killed *Staphylococcus aureus* induces release of TNF in whole blood ex vivo, and this model system approximates in vivo conditions (*19*). Immunodepletion of LBP from normal rabbit plasma with immunoglobulin G (IgG) purified from polyclonal goat antibodies to rabbit LBP markedly inhibited *Salmonella minnesota* Re595 LPS-induced TNF production from whole blood cells without changing the dose-response characteristics for heat-killed *S. aureus*–induced TNF (Fig. 3). These data are representative of LBP immunodepletions performed in five separate experiments with a total of 12 blood donor rabbits. Addition of nonimmune IgG and antibody to rabbit IgG or antibody to rabbit fibronectin had no effect on LPS-induced TNF induction. The release of TNF by high concentrations of LPS in LBP-immunodepleted samples may occur from cell stimulation via putative LPS receptors that are

independent of LBP and CD14 (*21*). LBP immunodepletion also inhibited TNF production induced with LPS isolated from S-form organisms (*Salmonella abortus equi* or *Salmonella typhimurium*).

The LBP binds to the lipid A moiety of LPS, and thus to a diverse array of Gram-negative organisms (*4*). The sequence identity to CETP suggests that LBP and CETP may be members of a family of proteins that bind to lipids and transport these ligands that are hydrophobic or amphipathic, or both, in aqueous environments. Our data show that LBP functions as a carrier protein for LPS in plasma and controls LPS-dependent monocytic responses. These data provide support for a model of LPS-induced monocytic stimulation under physiologic conditions, in which LPS first forms high-affinity complexes with LBP (*2, 4*), and these complexes then bind to cells through a specific receptor (*7*).

Recognition of the presence of LPS is important for an efficient response to infection with Gram-negative bacteria. Thus, a principal function of LBP may be to enhance the ability of the host to detect LPS early in infection; release of cellular products would then enhance natural resistance mechanisms that combat infection. As LBP concentrations rise during the acute phase response, its concentration in extravascular sites may also rise, which would provide an additional defense mechanism through its opsonic activity (*6*). The molecular details of LPS-LBP interactions will probably provide new approaches to managing the problems of Gram-negative sepsis and endotoxin shock.

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CD14, a Receptor for Complexes of Lipopolysaccharide (LPS) and LPS Binding Protein

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Leukocytes respond to lipopolysaccharide (LPS) at nanogram per milliliter concentrations with secretion of cytokines such as tumor necrosis factor–α (TNF-α). Excess secretion of TNF-α causes endotoxic shock, an often fatal complication of infection. LPS in the bloodstream rapidly binds to the serum protein, lipopolysaccharide binding protein (LBP), and cellular responses to physiological levels of LPS are dependent on LBP. CD14, a differentiation antigen of monocytes, was found to bind complexes of LPS and LBP, and blockade of CD14 with monoclonal antibodies prevented synthesis of TNF-α by whole blood incubated with LPS. Thus, LPS may induce responses by interacting with a soluble binding protein in serum that then binds the cell surface protein CD14.

LIPOPOLYSACCHARIDE BINDING PROTEIN is a 60-kD serum glycoprotein that forms high-affinity stoichiometric complexes with bacterial endotoxin (LPS) (*1, 2*). LBP also functions as an opsonin (*3*). It binds to the surface of bacteria or to LPS-coated erythrocytes and mediates the adhesion of these coated particles to macrophages. Interaction of LPS-LBP complexes with macrophages subserves not only this adhesive function but also induces the synthesis of TNF by the macrophages (*4*). The molecule on the cell surface that mediates binding of LBP-coated particles is restricted to monocytes and macrophages, is mobile in the plane of the membrane, and is distinct from receptors for other known opsonins such as immunoglobulin and complement (FcγRI, FcγRII, FcγRIII, CR1, and CR3) (*3*). In order to determine the role of various surface structures in the binding of erythrocytes coated with LPS-LBP complexes (ELBPs), macrophages were allowed to spread on surfaces coated with monoclonal antibodies (MAbs) to macrophage surface proteins. Mobile membrane proteins diffuse to the substrate-attached portion of a spread macrophage and are trapped by interaction with the specific MAb, thus causing the apical surface to be specifically depleted of a single protein (*5, 6*). Depletion of proteins with MAbs to CD16 (FcγRIII), CD11b/CD18 (CR3), CD18, or HLA caused no diminution of the binding of ELBPs (Fig. 1). However, three separate MAbs to CD14 caused strong down-modulation of the binding of ELBP, suggesting that expression of CD14 on the apical surface of the macrophage is necessary for recognition of ELBPs.

For down-modulation to occur, antibody attached to the substrate must bind receptor, but it need not mask the ligand binding site of the receptor. Indeed, only two of the anti-CD14 MAbs tested appear to mask the binding site. Soluble MAb 3C10 and 60b both caused complete inhibition of binding of ELBPs to macrophages, with a half-maximal inhibition occuring at <1 μg/ml

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