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The Level of Lipopolysaccharide-Binding Protein Is Significantly Increased in Plasma in Patients with the Systemic Inflammatory Response Syndrome

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Currently, there is no way to predict with a high degree of sensitivity and specificity which patients are likely to develop systemic inflammatory response syndrome (SIRS) following systemic infection, trauma, organ rejection, or blood loss. The level of human lipopolysaccharide-binding protein (LBP) was determined in the plasma of 22 patients with a clinical diagnosis of early SIRS. Twenty-nine plasma samples from healthy volunteers were used as controls. The mean level of LBP in the plasma of healthy volunteers was 7.7 μg/ml (standard deviation, 6.2 μg/ml). Twenty-one of 22 patients (95%) with SIRS had an LBP level on admission at least 2 standard deviations above the mean LBP level for a healthy volunteer control group (range, 4.9 to 114.2 μg/ml; mean, 36.6 μg/ml; standard deviation, 22.2 μg/ml; $P < 0.0001$). The level of LBP in the plasma of the majority of patients with early SIRS is significantly increased compared to that in healthy controls. The sensitivity, specificity, and predictive value of elevated plasma LBP levels in patients with SIRS remain to be determined.

The systemic inflammatory response syndrome (SIRS) represents an accentuated homeostatic response to a wide variety of infectious and noninfectious stimuli resulting in progressive multiorgan dysfunction, failure, and death (5). Although infection is the most common cause of the systemic inflammatory response, other conditions such as severe trauma, burns, pancreatitis, shock due to blood loss, and organ rejection can cause an identical clinical syndrome. Patients with SIRS have a high mortality rate which is independent of the presence of a culture-documented infection. The end stage of SIRS due to infection is commonly referred to as "septic shock," a syndrome of hypotension, multiorgan failure, and disseminated intravascular coagulation, accounting for approximately 175,000 deaths annually in the United States (20).

Multiple pharmacological intervention studies have been conducted over the past 20 years in an attempt to intercede in the progression of SIRS (17, 18). These approaches have included the use of antilipopolysaccharide (anti-LPS) antibodies (4, 11, 27) and inhibitors of vasoactive proteins such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (1, 9). A predictable and significant impact on overall survival has been very difficult to demonstrate in any of these trials, and the mortality rate from established septic shock continues to be very high.

Currently, no laboratory assay is able to predict with a high degree of sensitivity and specificity which patients are likely to develop SIRS (2). If the systemic response to either infection or other causes of SIRS could be detected at an early stage, prior to the point of irreversible progression, it might be possible to reverse this trend with appropriate therapeutic intervention.

Activation of macrophages by LPS originating from the cell wall of almost all gram-negative bacteria results in the release of a spectrum of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, which are thought to play an important role in the control of systemic infection as well as being important mediators of SIRS (26). It has been recognized for some time that LPS binds with a high affinity to a variety of serum proteins, including serum high-density lipoproteins (25). Recently, a specific LPS-binding protein (LBP) has been described. This protein binds to LPS with a high affinity (10^{-9} M) and with a 1:1 stoichiometry (23, 24). LBP is a 58-kDa, heavily glycosylated, acute-phase protein whose level rapidly increases in the serum of animals within 15 to 30 minutes of exposure to LPS (19). Early studies with humans have demonstrated that the LBP concentration in human plasma ranges from 1 to 24 μg/ml and that the concentration in serum appears to increase in the presence of bacterial infection (6, 14, 16).

We describe the use of a sensitive, specific, rapid, and reproducible enzyme-linked immunosorbent assay (ELISA) that can be used to quantify the level of LBP in human plasma and the results of a pilot study designed to compare LBP levels in the plasma of healthy individuals with the plasma LBP levels in patients with early SIRS.

MATERIALS AND METHODS

Informed consent was obtained for this study from the Institutional Review Board of St. Vincents Hospital, Indianapolis, Ind., and from the Institutional Review Board of Indiana University Medical Center, Indianapolis.

Reagents. A murine monoclonal antibody, designated 17-G4, was generated from CB6F1 mice immunized with purified human LBP derived from the plasma of patients with gram-negative sepsis. Monoclonal antibody 17-G4 is an immunoglobulin G2A (IgG2A) κ antibody which reacts only with native LBP and which does not bind to completely denatured LBP.

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UPC10 (Sigma Immunochemicals, St. Louis, Mo.) is a mouse IgG2A κ monoclonal antibody used in these studies as an irrelevant isotype control.

Goat anti-LBP polyclonal antiserum developed against human recombinant LBP was kindly provided by R. Ulevitch (Department of Immunology, Scripps Research Institute, La Jolla, Calif.).

Recombinant LBP (rLBP) was expressed in CHO cells transfected with a human LBP cDNA (kindly provided by R. Ulevitch) and was purified by the chromatographic methods described below.

LBP-free serum was generated from pooled male type AB human serum which was incubated overnight at 4°C with 17-G4 immobilized on Affi-Gel (Bio-Rad, Richmond, Calif.). Serum was eluted and incubated on anti-murine IgG-coated plates to remove the 17-G4. After this two-step process, no LBP was detectable in this serum by ELISA.

Bio-Rex 70 resin was purchased from Bio-Rad. SP Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). The LPS used in all experiments was derived from *Salmonella minnesota* RE595 (Re) and was purchased from List Biological Laboratories.

Purification of LBP from plasma. LBP was purified from plasma derived from patients with gram-negative sepsis by modifications of the technique described by Tobias and Ulevitch (25). Briefly, hydrated Bio-Rex 70 resins were equilibrated with 40 mM NaCl in 50 mM phosphate buffer (pH 7.3) containing 2 mM EDTA (PBE). Pooled plasma in 5 mM EDTA was applied to the column. Elution was performed with a step gradient of NaCl in PBE.

LBP activity was tested by ELISA (as described below). LBP-active fractions were subjected to further purification by high-pressure liquid chromatography with a Mono-Q column (Pharmacia). The purity of LBP was over 90%, as assessed by mini-gel electrophoresis and silver staining.

Purification of rLBP. Human rLBP was purified from LBP-transfected CHO cells growing in serum-free medium (GIBCO-BRL) by a modification of the procedure described by Theofan et al. (22). Briefly, 10×-concentrated conditioned medium was incubated with SP Sepharose overnight at 4°C and washed with 20 mM sodium acetate (pH 4) buffer containing 0.4 M NaCl, and rLBP was eluted with 1 M NaCl. LBP-positive fractions were subject to further purification on SP Sepharose by using a fast-performance liquid chromatography column (Pharmacia). rLBP was >90% pure, as assessed by polyacrylamide gel electrophoresis and silver staining.

LBP ELISA. Ninety-six-well plates were coated with LPS (100 μl/well; concentration, 50 μg/ml) in coating buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate [pH 9.5]), sealed, and left overnight at 4°C. On the following day, LPS-coated plates were blocked with 100 μl of 1% dry milk in phosphate-buffered saline (PBS) per well for 30 min at 37°C. After blocking, the plates were dried, sealed, and stored at −20°C until they were used. On the day of assay, the plates were warmed to 37°C for 10 min. Plasma samples diluted in PBS were added, and the plates were incubated for 90 min at 37°C. The plates were washed and incubated with 100 μl of pure 17-G4 (10 μg/ml) or goat anti-human LBP polyclonal serum (1:2,000 dilution) per well for 30 min at 37°C. The plates were then washed and incubated with 100 μl of a 1:2,000 dilution of the relevant antibody conjugated to alkaline phosphatase per well for an additional 30 min at 37°C. After washing, 100 μl of chromagen (Sigma 104 phosphatase substrate) per well was added, and the reaction was allowed to develop at room temperature for 10 min. The reaction was stopped by the addition of 100 μl of a 1 N solution of NaOH per well. The absorbance at 405 nm was determined with an automated ELISA reader (Molecular Devices Inc.).

All plasma samples were assayed in triplicate at least twice. A standard consisting of human rLBP diluted in LBP-free serum was run with all assays. The levels of LBP in the plasma samples were calculated by regression analysis by reference to the levels in the standard.

Immunoprecipitation and immunoblotting. To test the binding of anti-LBP antibodies to LBP for interference by LPS, immunoprecipitation and immunoblotting (Western blotting) were performed. Aliquots of 15 μl agarose-conjugated anti-murine IgG (Sigma Immunochemicals) or anti-goat IgG were washed and incubated with the appropriate antibody: (i) 10 μg of 17-G4 or an irrelevant murine IgG (UPC 10) or (ii) 1 μl of goat anti-LBP serum or normal goat serum (Cappel Organon Teknika Corporation, Durham, N.C.).

Antibody conjugates were washed and mixed with normal human plasma or 1.5 μg of recombinant LBP with or without 100 μg of LPS per ml. After 30 min of incubation on ice, the samples were washed extensively and eluted with 25 μl of elution buffer (0.1 M glycine, 0.15 M NaCl [pH 2.4]). LBP samples were diluted with sample buffer, boiled for 5 min, and electrophoresed on a 10% acrylamide gel, followed by electrophoretic transfer to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was blocked with 1% dry milk in PBS, followed by incubation for 60 min at room temperature with goat anti-human rLBP serum, washed, and then incubated with anti-goat IgG–alkaline phosphatase conjugate. After washing, the membranes were stained with the chromagen 5-bromo-4-chloro-3-indolyphosphate toluidinium salt–nitroblue tetrazolium (Sigma Chemical Co.), and development was stopped by rinsing with PBS containing 20 mM EDTA.

Determination of levels of LBP in plasma of patients with SIRS. Plasma was obtained from 22 patients with a clinical diagnosis of SIRS. SIRS was defined by the criteria of Bone et al. (5). All patients with a diagnosis of SIRS evaluated in this study had the following clinical signs: tachycardia (heart rate, >90 beats/min), tachypnea (respiratory rate, >20/min), and fever or hypothermia (temperature, >101 or <96.1°F).

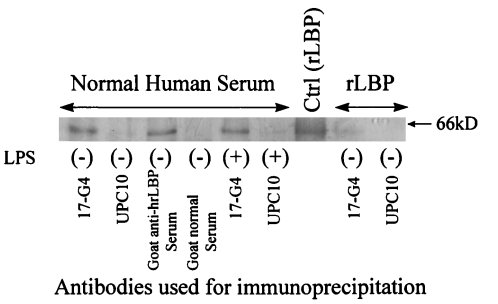

FIG. 1. Monoclonal antibody 17-G4 immunoprecipitates both human LBP and human LBP mixed with LPS. To test the specificity of 17-G4, immunoprecipitation was performed as follows. Monoclonal antibody 17-G4 or goat anti-LBP antibodies and an irrelevant isotype control antibody (UPC10) were conjugated to either anti-mouse or anti-goat IgG agarose and were then mixed either with plasma from healthy human volunteers (the plasma was preincubated with or without LPS) or human rLBP (rLBP). The samples were washed, eluted, electrophoresed, and transferred to a polyvinylidene difluoride membrane and probed with goat anti-human rLBP serum.

A single blood sample was obtained from each patient who met the criteria given above. Blood samples were collected via arterial lines, placed into heparinized tubes, and centrifuged at 400 × *g* for 5 min at 4°C, and the plasma was aliquoted and frozen at −80°C until it was assayed. Patients were excluded from the study if they had (i) major surgery or trauma in the previous 2 weeks, (ii) were on immunosuppressive therapy, (iii) had received antiendotoxin therapy, (iv) were leukopenic, (v) had known malignancy or treatment with chemotherapy in the previous 12 months, or (vi) had a known immunosuppressive disorder.

Determination of LBP Levels in a control group of healthy volunteers. Ten milliliters of blood was collected from a group of 29 healthy volunteers of both sexes. Blood was collected into a heparinized tube and centrifuged at 400 × *g* for 5 min at 4°C, and the plasma was aliquoted and frozen at −80°C until use.

RESULTS

Monoclonal antibody 17-G4 can immunoprecipitate LBP and LBP-LPS complex. As shown in Fig. 1, monoclonal antibody 17-G4 immunoprecipites both rLBP and native LBP, irrespective of whether the LBP is mixed with nonphysiological amounts of LPS. The high binding affinity of LBP for LPS and the ability of 17-G4 to recognize LBP in the presence of a high concentration of LPS was therefore used to develop an assay to quantitate LBP in human plasma.

Evaluation of 17-G4 to detect LBP in plasma samples: development of a quantitative ELISA. Plasma LBP levels were measured by reference to a series of standard curves generated with rLBP (data not shown). The optical density readings at 405 nm were linear from 0.1 through 3 μg/ml ($r > 0.9$), thereby requiring appropriate dilution of clinical plasma samples prior to assay.

To determine the reproducibility of the LBP ELISA, 50 plasma samples from healthy volunteers and study patients were assayed at least twice. As shown in Fig. 2, the correlation coefficient between LBP levels obtained in repeat assays was 0.989 ($P < 0.001$). The average difference between two LBP measurements was less than 2.5 μg/ml and never exceeded 10 μg/ml.

Determination of LBP levels in plasma of healthy humans. To determine the mean plasma LBP level in healthy individuals, plasma samples from 29 healthy volunteers of both sexes were assayed. The mean LBP level in the plasma of healthy individuals was 7.7 μg/ml (range, 0.5 to 25.4 μg/ml; standard deviation [SD], 6.2 μg/ml).

Determination of LBP levels in patients with SIRS. The LBP levels of LBP in the plasma of 22 patients with SIRS were determined. As noted in Fig. 3, 21 of 22 these patients (95%)

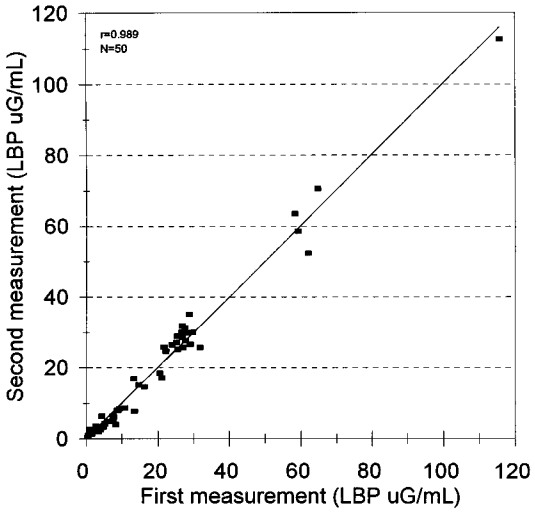

FIG. 2. Reproducibility of the LBP ELISA assessed by measuring LBP levels on two occasions by using a series of 50 plasma samples from healthy volunteers and study patients. The correlation coefficient for these two assays was 0.989.

had an LBP level ≥2 SDs above the mean LBP level for the healthy volunteer control group (range, 4.9 to 114.2 μg/ml; mean, 36.6 μg/ml; SD, 22.2 μg/ml; $P < 0.0001$).

DISCUSSION

Over the past 10 years significant progress has been made in understanding the pathogenesis of the complex multifactorial condition now referred to as SIRS (5). Profound local and systemic inflammatory responses can occur as a result of a wide variety of causes, including infection, severe trauma, burns, pancreatitis, hemorrhage, or immune-mediated tissue injury. If not successfully treated, these disparate forms of systemic injury often lead down a final common pathway, resulting in multiple-organ dysfunction, organ failure, and death. As stated by Bone (3), "SIRS represents a hierarchical continuum of an increased inflammatory response to infectious and non-infectious stimuli, since end-organ dysfunction and mortality increase with each stage of the inflammatory response." The identification of an early biochemical marker of a systemic inflammatory response to infection or other systemic stimuli would likely be of significant diagnostic utility. Early detection of SIRS might facilitate early therapeutic intervention prior to the often irreversible cascade of events leading to multiple-organ failure and death.

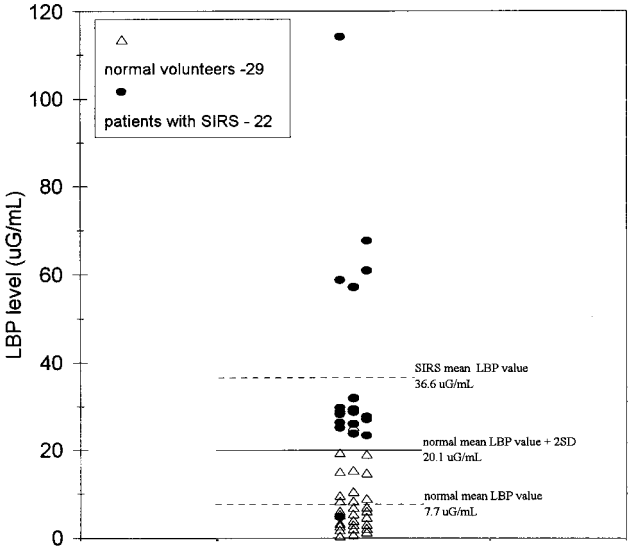

FIG. 3. LBP levels in the plasma of healthy volunteers and study patients. The difference in LBP levels between study patients and healthy volunteers was statistically significant ($P < 0.0001$).

LBP is an acute-phase reactant protein released by hepatocytes following LPS challenge (19). LBP binds with high affinity to LPS, the complex of which binds to CD14, activating these cells to produce vasoactive cytokines such TNF-α, IL-1β, and IL-6, which are recognized to have a role in the pathogenesis of SIRS. LBP expression can also be induced by cytokines, particularly IL-6. Primary hepatocyte cultures and the HEp-G2 cell line can be induced to produce LBP following exposure to IL-6 (12).

LBP release following early exposure to gram-negative organisms is mediated directly by LPS, inducing the production of IL-6 and other proinflammatory cytokines. These proteins appear to be able to independently stimulate LBP production (12). As SIRS develops over time, cytokine-induced LBP release would continue and plasma LBP levels would remain elevated, even though the initial stimuli may no longer be present, e.g., following antibiotic therapy. In support of this hypothesis is the recent report from Gallay et al. (10) that the mean concentration of LBP in serum from patients with septic shock was 40 to 60 μg/ml, which was independent of whether the patients had gram-negative sepsis. This was significantly greater than the mean concentration of LBP in the serum of healthy subjects (18.1 μg/ml) (10). With this in mind, our pilot study was specifically designed to assay LBP levels in the plasma of patients in the earliest stages of SIRS, irrespective of cause. Plasma samples were thus obtained from 22 patients with a clinical diagnosis of SIRS immediately following their hospital admission.

Our assay was specifically designed to measure the LBP capable of binding to LPS by exploiting the high binding affinity of LPS for LBP. LPS bound in the solid phase was used to "capture" LBP in plasma. Bound LBP was then detected with a highly specific LBP monoclonal antibody (17-G4) that recognizes both serum and recombinant nondenatured LBP, either alone or in the presence of a high concentration of LPS (Fig. 1). This assay detects unbound LBP, i.e., that fraction of LBP not complexed with LPS. It has been reported that LPS binds to LBP in a 1:1 molecular stoichiometry (23). It has also been reported that <1 ng of LPS per ml is sufficient to induce the symptoms of sepsis (15). The mean concentration of LBP in the plasma of healthy humans is approximately 10 μg/ml. One can thus calculate that LPB is present in at least a 500-fold molar excess for the maximal amount of LPS likely to be circulating in a patient with sepsis. There is thus a large reservoir of LBP available for binding to LPS circulating in the blood. Physiological levels of LPS are thus unlikely to seriously affect the quantitation of unbound LBP in this assay.

The range and mean levels of LBP in healthy volunteers was 0.5 to 25.4 μg/ml (mean, 7.7 μg/ml), which was similar to the data obtained by Leturcq et al. (14) (1 to 24 μg/ml; mean, 7 μg/ml) and somewhat lower than the levels reported by Heumann et al. (13) (5 to 41 μg/ml; mean, 17.8 μg/ml). LBP levels in blood from patients with clinical SIRS ranged from 4.9 to 114.2 μg/ml (mean, 36.6 μg/ml; SD, 22.2 μg/ml). This represents a 3- to 15-fold increase compared to the levels seen in healthy volunteers ($P < 0.0001$). This finding is consistent with

previous observations reported by Calvano and coworkers (6), who reported that the levels of LBP in plasma were 8 to 15-fold higher in patients with sepsis compared to those in healthy volunteers. There was little overlap between LBP levels in healthy volunteers and those in patients with SIRS in our study. Only 1 of 21 patients (4.5%) with SIRS had an admission plasma LBP level below the arbitrary cutoff point of 2 SDs above the normal mean level, i.e., 20.1 μg/ml. Only one healthy volunteer had a plasma LBP level of >20.1 μg/ml.

The specificity of an elevated plasma LBP level as an early marker for SIRS is not yet established by the data presented here. One fundamental issue that is being addressed in ongoing studies is whether there is any relationship between the levels of LBP in plasma and sepsis, defined as "the systemic response to infection in the context of a positive microbiological culture" (5). Because LBP is a protein that binds to LPS, LBP might be a particularly specific and sensitive marker for the presence of gram-negative bacteria in the early stages of SIRS due to these infections. Complete bacterial culture data for the 22 patients with SIRS enrolled in this study were not systematically collected and were not required for patient entry into this pilot study.

The sensitivity, specificity, and predictive value of the LBP level as a marker for early SIRS will be assessed in a large prospective study specifically designed to follow plasma LBP levels over time in a wide variety of patients with a clinical diagnosis of SIRS who are admitted to intensive care units at a large academic medical center.

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