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Purification and Characterization of Murine Lipopolysaccharide-Binding Protein

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The serum protein lipopolysaccharide (LPS)-binding protein (LBP) seems to play an important role in regulating host responses to LPS. Complexes of LPS and LBP form in serum and stimulate monocytes, macrophages, or polymorphonuclear leukocytes after binding to CD14. Previous reports have described the structure and properties of LBP from human and rabbit sera. Since mice are used in some experimental models of endotoxemia or gram-negative bacterial infections, information is needed about the properties of murine LBP. Murine LBP was purified by ion-exchange chromatography and high-pressure liquid chromatography; its NH_2-terminal sequence (TNPGLVTRIT) was very similar to those of human and rabbit LBPs (80 to 90% amino acid identity). Murine LBP resembled LBPs from other species in that it promoted the binding of LPS to monocytes and enhanced the sensitivity of monocytes to LPS at least 100-fold. Mouse LBP, like rabbit and human LBPs, was found to be an acute-phase protein. Further in vivo studies with mice and anti-CD14 or anti-LBP reagents should help determine the role of LBP in response to LPS challenges.

Lipopolysaccharide (LPS) is a major component of the outer envelope of gram-negative bacteria. It is believed to play a major role in the serious clinical consequences of gram-negative bacterial infections, leading to fever, disseminated intravascular coagulation, shock, and possibly death (20). The immune system of the host, particularly the cells of the monocytic lineage, has been shown to play a pivotal role in the mediation of the biological effects of LPS, namely, the overproduction of cytokines (5, 12, 15). Since the first description of the presence of LPS-binding protein (LBP) in the serum of rabbits (18), a model to explain the presentation of LPS to cells of the monocytic lineage has been proposed. LPS first forms a complex with LBP in blood; this complex binds to CD14 on the surface of monocytes, resulting in increased expression of LPS-inducible genes (14, 21). In rabbit macrophages, LBP-LPS complexes were 1,000-fold more active than LPS alone in tumor necrosis factor (TNF) induction (14).

LBPs from humans and rabbits have been cloned and sequenced (14). The high DNA sequence homology in these two species suggests that other species could share a similar mechanism of LBP-mediated recognition of LPS. Mice are often used in experimental models of LPS inoculation or of gram-negative bacterial infections. The purification of mouse LBP and the development of specific anti-LBP reagents would be helpful in determining the role played by LBP in the response to LPS.

We report here on the purification of mouse LBP and on some of the properties of the protein. As shown by experiments reported herein, mouse LBP shares features with rabbit and human LBPs in terms of NH_2-terminal amino acid sequence and biological activity.

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MATERIALS AND METHODS

Materials. *Escherichia coli* O111 LPS, either native or labeled with fluorescein isothiocyanate (FITC-LPS), was purchased from Sigma (St. Louis, Mo.). The following strains of mice were used in this study: OF1, C57BL/6J, CBA/J, DBA/2, and BALB/c (IFFA Credo); and C3H/HeN and C3H/HeJ (Harlan). Serum was collected by bleeding through the retroorbital plexus; the blood was allowed to clot at 37°C for 1 h and at 0°C overnight, centrifuged, and stored frozen. Acute-phase mouse serum (APMS) was collected 8, 16, and 24 h after induction of an acute-phase response by subcutaneous injection of 0.2 ml of 3% (wt/vol) silver nitrate in water with a protocol similar to that designed for the acute-phase response in rabbits (18). Polyclonal antiserum to mouse LBP was raised in rabbits by subcutaneous injection of 10 μg of purified LBP in complete Freund adjuvant followed by two successive booster injections of LBP in incomplete Freund adjuvant given at 3 and at 6 weeks. Rabbit antibodies were purified by protein A affinity chromatography.

Purification of LBP. APMS was collected from OF1 mice 24 h after subcutaneous injection of silver nitrate. APMS was fractionated with 20 ml of Bio-Rex 70 resin (Bio-Rad Laboratories, Basel, Switzerland) equilibrated with 40 mM NaCl in 50 mM phosphate buffer (pH 7.3) containing 2 mM EDTA (phosphate-EDTA). Then 5 mM EDTA was added to APMS; 300 ml of APMS was run over the column, and the column was washed with phosphate-EDTA until the A_{280} of the eluate was <0.02 absorbance unit. The flow-through fraction was kept for measuring residual LBP activity. Protein absorbed to the resin (Bio-Rex eluate) was eluted with a linear gradient of 40 to 1 M NaCl in phosphate-EDTA. Pools of fractions to be tested for LBP activity were dialyzed against 50 mM phosphate buffer (pH 7.3) and concentrated to a 10-ml volume by using YM10 membranes in an Amicon ultrafiltration cell (Amicon Corp., Danvers, Mass.).

Fractions containing LBP activity were adsorbed on a

Mono-Q column (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM phosphate buffer (pH 8.2). The material was eluted by high-pressure liquid chromatography (chromatograph from Beckman Instruments, Berkeley, Calif.). After injection of the sample, proteins were eluted with the following concentrations of saline in phosphate buffer: no saline for the first 20 fractions (1 ml each), 150 mM saline for fractions 20 to 60, 300 mM saline for fractions 60 to 85, and then a linear gradient up to 1 M saline. Fractions containing LBP activity were pooled, dialyzed against phosphate buffer (pH 7.3), and concentrated to a final volume of 1 ml.

Methods for sequencing. Samples were reduced with 2-mercaptoethanol and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 6% acrylamide gels (10). After electrophoresis, gels were stained with Coomassie blue, and parallel gels were transferred to nitrocellulose sheets. The blotted proteins were reversibly stained with 0.5% Ponceau S (Serva, Heidelberg, Germany) in 1% acetic acid. The strip containing the antigen (LBP) was cut apart. This material was sequenced by automated Edman degradation with an Applied Biosystems 470 A Protein Sequenator attached to a 120 A PTH analyzer in the presence of Polybrene. Concentration of purified LBP was estimated by absorbance measurement (1 A_{280} unit was normalized to 1 mg/ml). The reactivity of immune rabbit immunoglobulin G (IgG) fractions for purified mouse LBP was investigated by Western immunoblotting (19).

Test for LBP activity. Heparinized blood samples obtained from normal donors were used to prepare peripheral blood mononuclear cells (PBMC) by centrifuging blood over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMC (10^6) were incubated at 37°C with 1 μg of FITC-LPS in 1 ml (final volume) of RPMI medium enriched with the various fractions to be tested for LBP activity or with 1% albumin as a control. After 1 h of incubation, the cells were washed twice with cold phosphate-buffered saline and analyzed with a FACScan flow cytometer (Becton Dickinson). The fluorescence signal was recorded on a logarithmic scale expressed in fluorescence units (FU), and the data were analyzed with the Consort-30 software and a lysis program package from Becton Dickinson. To restrict the analysis of monocytes, side-scatter parameters were used to apply computerized gating (16). In all these experiments, the signal shown by lymphocytes was not significant compared with that shown by monocytes.

For CD14 blockade, monocytes were pretreated for 10 min with a CD14-specific monoclonal antibody, MY4 (Coulter Immunology, Luton, England) (7), used at 10 μg/ml.

TNF release of human monocytes. PBMC (0.5×10^6) were suspended in 250 μl of RPMI medium supplemented with 10% serum or with purified LBP in each well of 96-well flat bottom microtiter plates (Becton Dickinson). The cells were stimulated with nonfluoresceinated O111 LPS at the indicated concentrations. Supernatants were collected for TNF measurements after 4 h of culture at 37°C. At that time, the release of TNF was optimal. Under the conditions used in this study, TNF release by peripheral blood lymphocytes present in the PBMC fraction was negligible (8). TNF was measured with WEHI-clone 13 cells as described previously (1, 4).

RESULTS

Screening of LBP activity. We previously described a technique for detecting complexes between rabbit LBP and LPS on human monocytes (9). Fluorescence-activated cell sorter (FACS) analysis was used to measure the binding of FITC-LPS to human monocytes; the presence of rabbit LBP resulted in increased binding relative to that in controls without LBP. Similar results were observed with rabbit serum or with purified rabbit LBP. The increased binding in serum was suppressed by immunodepletion of LBP. The binding was also suppressed by CD14 blockade of monocytes, confirming previous observations that the binding of LPS-LBP complexes was restricted to the CD14 of monocytes (21).

In the present experiments, we used human monocytes, because there is no available blocking antibody to CD14 of mouse monocytes. Similar findings were observed when mouse serum was used instead of rabbit serum (Fig. 1). Indeed, although weak fluorescence was found on human

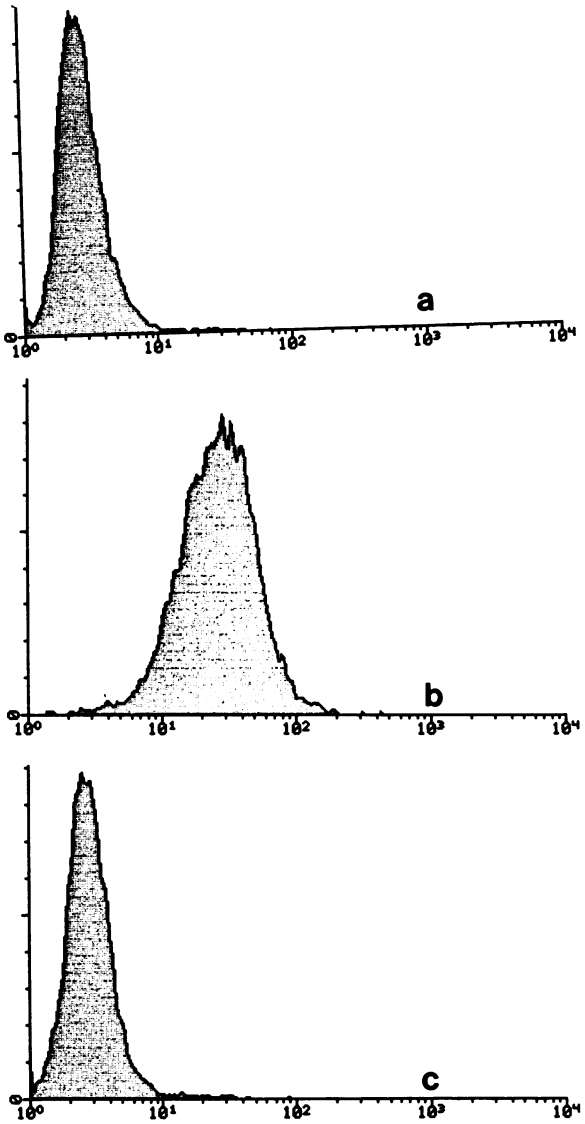

FIG. 1. Binding of FITC-LPS to human monocytes incubated in APMS. PBMC (10^6) were incubated at 37°C for 1 h with 1 μg of FITC-LPS per ml in 1% albumin (a), 10% APMS (b), or 10% APMS after preincubation of PBMC with anti-CD14 MY4 (c). *y* axis, number of monocytes; *x* axis, intensity of fluorescence.

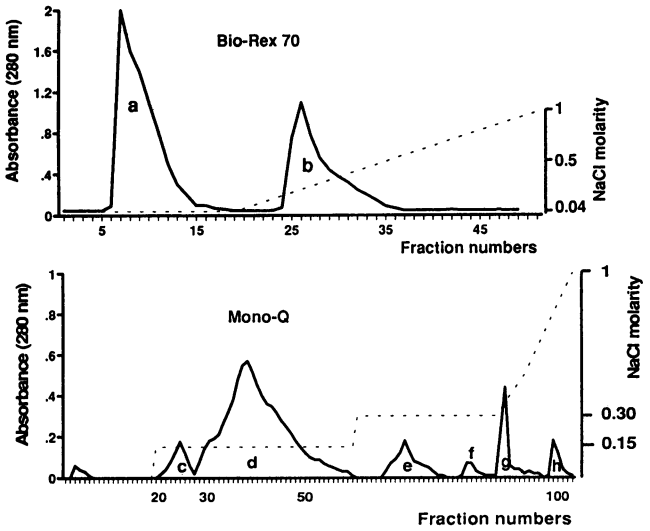

FIG. 2. Purification of mouse LBP. (a) Ion-exchange chromatography of APMS on Bio-Rex 70 resin. Pools: a, flow-through fraction; b, Bio-Rex eluate. (b) Ion-exchange chromatography of Bio-Rex eluate of APMS on Mono-Q resin. LBP activity was found only in fraction c. ——, A_{280}; – – –, molarity of NaCl.

monocytes incubated with FITC-LPS in medium containing 1% albumin (Fig. 1a), more than 90% of the monocytes incubated with FITC-LPS in the presence of 10% APMS showed a distinctly brighter fluorescence (Fig. 1b). This increased binding was suppressed when cells were preincubated with an anti-CD14 monoclonal antibody, MY4 (Fig. 1c). Thus, FITC-LPS binding permitted detection in mouse serum of an activity similar to that found with purified rabbit LBP or rabbit serum. We hypothesized that it represented mouse LBP, and this technique was adopted for the purification of mouse LBP.

Purification of mouse LBP. Two chromatographic procedures were involved in the purification of LBP essentially as described previously for rabbit LBP (18), with slight modifications. First, chromatography of APMS on Bio-Rex 70 resin effectively concentrated LBP activity. The absorbance profile of APMS passed over the column is shown in Fig. 2a. Pool a represents nonadsorbed proteins, and pool b represents proteins eluted with concentrations of saline ranging between 150 and 350 mM. When these pools were mixed with FITC-LPS and binding was determined by FACS analysis (Fig. 3), no residual LBP activity was found in nonadsorbed proteins (Fig. 3a). LBP activity was found in the whole Bio-Rex eluate after concentration (Fig. 3b).

LBP was purified further on a Mono-Q column by high-pressure liquid chromatography. Figure 2b shows the absorbance profile of the Bio-Rex eluate passed over the column after elution with increasing concentrations of NaCl. Among the various collected fractions, only fractions eluted with <150 mM (Fig. 2b, fraction c) contained LBP activity as measured by FACS analysis (Fig. 3c). All other tested fractions (Fig. 2b, fractions d, e, f, g, and h) had no LBP activity.

The LBP-containing fractions were pooled, concentrated, and dialyzed against phosphate buffer (pH 7.3). An SDS-PAGE gel of fractions from different steps of the purification of mouse LBP is shown in Fig. 4. Relative to the standards in lane a, the apparent mass of mouse LBP was 61 kDa (lane c). LBP was not detectable by Coomassie blue staining of

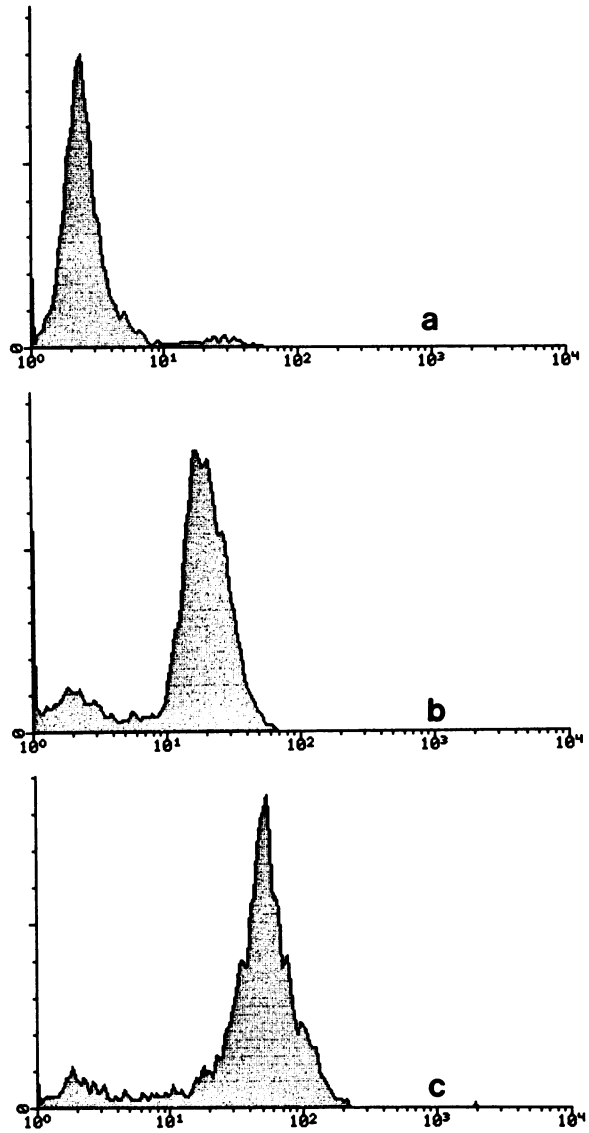

FIG. 3. Binding of FITC-LPS to human monocytes incubated with Bio-Rex eluate and HPLC-purified mouse LBP. PBMC (10^6) were incubated at 37°C for 1 h with 1 μg of FITC-LPS per ml in 1% albumin (a), 100 μl of Bio-Rex eluate (b), or 1 μg of HPLC-purified mouse LBP (c). *y* axis, number of monocytes; *x* axis, intensity of fluorescence.

total Bio-Rex eluate (lane b). Silver staining (data not shown) and Coomassie blue staining of purified LBP showed a single band.

Amino acid sequence data. The NH_2 sequence TNPGLV TRIT was obtained from purified mouse LBP (Table 1). The 10-amino-acid sequence was used to search for homologous sequences in the GenBank and EMBL data bases. This procedure resulted in matches only to human and rabbit LBPs and to human and bovine bacterial permeability-increasing proteins, which are structurally related to LBP (14).

Responses of mouse LBP to temperature and pH changes. The stability of purified mouse LBP and LBP in murine serum was evaluated at various temperatures. The proteins were heated for 1 h at various temperatures (from 37 to

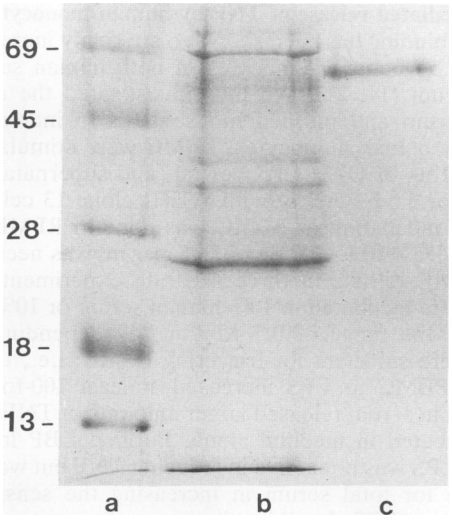

FIG. 4. SDS-PAGE analysis of chromatography fractions (Coomassie blue stain). Lanes: a, molecular mass markers (69, 45, 28, 18, and 13 kDa); b, Bio-Rex eluate of APMS; c, 10 μg of mouse LBP (pool a from Mono-Q chromatography [Fig. 2b]).

59°C), and binding of FITC-LPS to monocytes was measured by FACS analysis. Mouse LBP was stable at 50°C, but loss of biological activity occurred between 53 and 56°C. After mouse serum or purified murine LBP was heated to 59°C, all activity was lost. For comparison, human serum promoted LPS binding after heating up to 56°C, but binding was abolished after heating to 59°C.

APMS and purified mouse LBP were incubated overnight in buffers ranging from pH 2 to pH 11 and dialyzed against RPMI, and then binding of FITC-LPS to human monocytes was tested. Mouse LBP was active at all pH values, except at pHs 4 and 5. Purified mouse LBP in physiological buffers was stable for months at 4°C.

Inhibition of FITC-LPS binding with rabbit anti-LBP IgG. When examined in Western blotting experiments, rabbit IgG to mouse LBP recognized both purified LBP and LBP in Bio-Rex eluates (data not shown). These immune IgGs were then tested for their ability to inhibit the formation of LPS-LBP complexes in APMS. Immune rabbit IgG or control rabbit IgG (2 mg/ml) was preincubated for 10 min with 10% APMS, and binding of FITC-LPS in these preparations was measured on human monocytes by FACS analysis. In a typical experiment, the fluorescence peaked at 35 FU in APMS after incubation of FITC-LPS with monocytes (Fig. 5a). When APMS was incubated with FITC-LPS in the presence of immune rabbit IgG, the fluorescence decreased to 3 FU (Fig. 5b), which was the level of binding to control monocytes incubated in 1% albumin (data not shown). In contrast, preincubation of APMS with a nonimmune rabbit IgG preparation did not decrease the binding of FITC-LPS to monocytes (32 FU) (Fig. 5c). Inhibition of FITC-LPS binding with rabbit anti-LBP IgG was also effective when normal mouse serum was used instead of APMS (data not shown).

In additional experiments, anti-LBP rabbit IgG antibodies were added after LPS had been incubated with LBP. No inhibition of LPS binding to CD14 occurred (data not shown), demonstrating that preformed LPS-LBP complexes remained stable in the presence of anti-LBP antibodies. This experiment also suggested that the antibody did not recognize its epitope on LBP when LBP was complexed with LPS or that the binding of anti-LBP antibodies to LPS-LBP

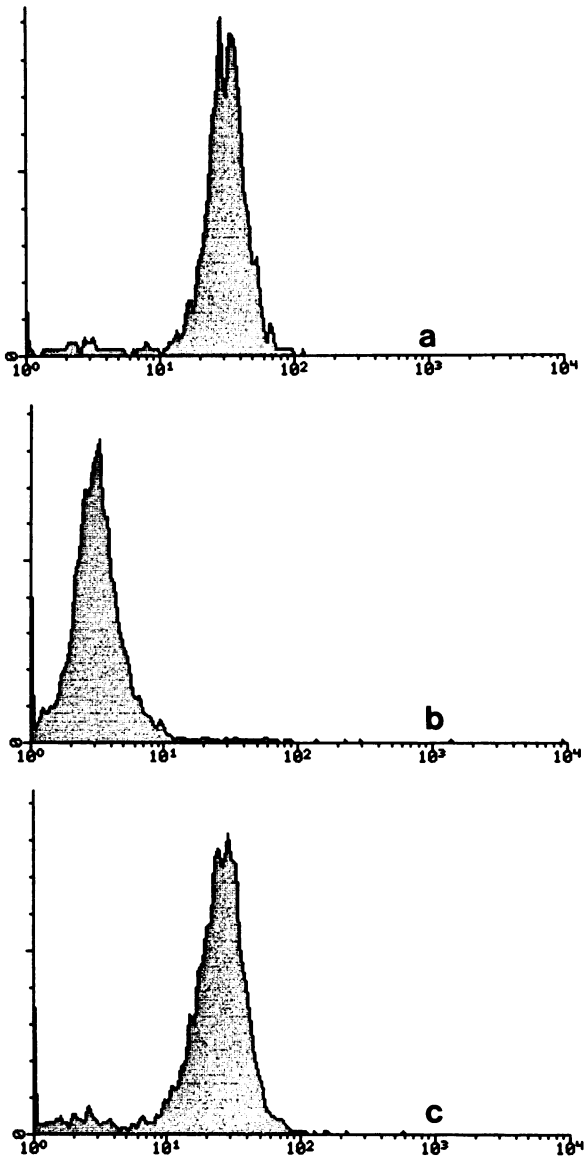

FIG. 5. Inhibition of FITC-LPS binding with rabbit anti-mouse LBP IgG. PBMC (10^6) were incubated at 37°C for 1 h with 1 μg of FITC-LPS per ml in 10% APMS (a), 10% APMS mixed with rabbit anti-mouse LBP IgG (b), or 10% APMS mixed with nonimmune rabbit IgG. *y* axis, number of monocytes; *x* axis, intensity of fluorescence.

TABLE 1. Amino acid sequence homologies among LBPs and bacterial permeability-increasing proteins (BPIs) of various species[a]

Species	Protein	NH_2-terminal amino acid sequence
Mouse	LBP	Thr-Asn-Pro-Gly-Leu-Val-Thr-Arg-Ile-Thr
Rabbit	LBP	Thr-Asn-Pro-Gly-Leu-Ile-Thr-Arg-Ile-Thr
Human	LBP	Ala-Asn-Pro-Gly-Leu-Val-Ala-Arg-Ile-Thr
Bovine	BPI	Thr-Asn-Pro-Gly-Ile-Val-Ala-Arg-Ile-Thr
Human	BPI	Val-Asn-Pro-Gly-Val-Val-Val-Arg-Ile-Ser

[a] Sequences obtained from references 6, 11, 14, and 17.

TABLE 2. Binding of FITC-LPS in normal sera and APMS from various strains of mice[a]

Mouse strain	Binding (FU) in:			
	Normal serum		APMS	
	1:10	1:100	1:10	1:100
OF1	11.3	6.7	16.8	10.6
C57BL/6J	9.6	6.1	21.1	9.6
DBA/2	12.0	7.0	16.2	10.2
CBA/J	11.1	6.8	17.6	9.0
BALB/c	10.8	6.1	18.0	8.9
C3H/HeN	11.4	6.0	24.0	11.0
C3H/HeJ	17.6	3.5	18.6	8.3

[a] Sera from five mice (normal serum or serum collected after a 24-h acute-phase response) were pooled and assessed for LBP activity. The results represent one of two experiments with very similar findings. PBMC (10^6) were incubated with 1 μg of FITC-LPS in 10 and 1% normal mouse serum or APMS. The data are expressed as FU, representing the mean fluorescence channel observed for FITC-LPS binding on monocytes. In this experiment, 2.1 FU was measured in control monocytes incubated in 1% albumin.

complexes did not prevent the complex from binding to CD14.

Presence of LBP in normal mouse serum and APMS. LBP has been described as an acute-phase reactant in rabbits (18), but it can also be detected in normal rabbit serum (9). We estimated the levels of mouse LBP in normal mouse sera and APMS by diluting sera from 10% to 0.01% and by measuring FITC-LPS binding on monocytes. In all strains of mice tested, the addition of normal serum down to 0.1% still promoted a positive fluorescence signal over that in control monocytes incubated in 1% albumin. Below 0.01%, mouse serum from all strains of mice tested behaved like the 1% albumin control (data not shown).

The kinetics of the acute-phase response in mice was measured. APMS was collected in all strains 8, 16, and 24 h after injection of silver nitrate. Very similar levels of LBP activity were found from 8 to 24 h (only data from 24 h are shown). LBP activity increased in APMS relative to that measured in normal sera (Table 2). The LPS binding measured in 1% APMS was quite similar to that of 10% normal serum, except for C3H/HeJ mice. In that strain, a high LBP activity was already observed in 10% normal serum and did not increase significantly after induction of the acute phase. However, this activity in normal serum dropped when serum was diluted to 1%, suggesting that the affinity of LBP in C3H/HeJ mice might differ from that in other mouse strains. Similar results were observed in a second experiment with another batch of C3H/HeJ mice.

LBP-mediated release of TNF by human monocytes. LBP-mediated binding has been shown to strikingly increase TNF secretion by human monocytes in both human serum and rabbit serum (14, 21). We thus investigated the ability of mouse serum and purified mouse LBP to increase TNF release by human monocytes. PBMC were stimulated with 0.1 to 100 ng of O111 LPS per ml, and supernatants were assayed for TNF activity with WEHI clone 13 cells (Table 3). When incubation of PBMC was done in RPMI without serum, at least 10 to 100 ng of LPS per ml was necessary to trigger TNF release in three separate experiments. When PBMC were incubated in 10% human serum or 10% APMS, stimuli in the range of 0.1 to 1 ng/ml (depending on the donor) were sufficient for triggering PBMC; i.e., the sensitivity of PBMC to LPS increased at least 100-fold. Cells incubated in serum released larger amounts of TNF than did cells incubated in medium alone. Purified LBP in the absence of LPS was not active in inducing TNF but was able to substitute for total serum in increasing the sensitivity of monocytes to LPS. In all incubation media, pretreatment of monocytes with anti-CD14 monoclonal antibody MY4 suppressed TNF release at LPS concentrations of 10 to 100 ng/ml.

DISCUSSION

The complex effects of LPS in mammals include activation of the immune system, which results in an overproduction of cytokines such as TNF and interleukin-1. The initial events that control the activation of monocytes are of special interest. Recent studies have assigned an important role to serum (3), and more specifically to LBP (9, 14, 21), in mediating presentation of LPS to monocytes. Complexes of LPS and LBP are recognized by cell surface CD14. This report provides further evidence supporting this model, showing that mice share with humans and rabbits a similar mechanism of recognition of LPS.

Originally, rabbit LBP was purified in acute-phase rabbit serum and detected by centrifugation in a CsCl density gradient (18). We were successful in purifying mouse LBP by detecting LBP with a different detection system, i.e., FACS analysis of the binding to monocytes of complexes of LBP and fluorescent LPS. Anti-LBP antibodies suppressed the serum-mediated binding of LPS in acute-phase serum. A similar suppression was found with CD14 blockade of human monocytes. These experiments strongly argue for the formation of an LPS-LBP complex in mouse serum, which is presented to CD14 of human monocytes, in parallel with the mechanism described previously for rabbit and human LBPs (14, 21). On mouse macrophages, an LBP-mediated activa-

TABLE 3. Influence of serum, mouse LBP, and CD14 blockade on TNF synthesis by human PBMC[a]

Incubation medium	TNF synthesis (pg/ml) with the following concn of LPS (ng/ml):			
	0.1	1	10	100
RPMI	0 (0–0)	0 (0–0)	50 (0–150)	300 (250–500)
RPMI + MY4	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
10% human serum	75 (25–75)	400 (200–950)	2,400 (1,600–3,200)	3,200 (1,600–3,200)
10% human serum + MY4	0 (0–0)	0 (0–0)	0 (0–0)	150 (0–150)
10% APMS	0 (0–0)	400 (300–400)	800 (450–1,200)	800 (400–2,400)
10% APMS + MY4	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
RPMI + 100 ng of LBP	0 (0–0)	100 (50–100)	800 (400–950)	1,440 (850–1,600)
RPMI + 100 ng of LBP + MY4	0 (0–0)	0 (0–0)	0 (0–0)	50 (0–50)

[a] PBMC (0.5×10^6) were cultivated at 37°C in various incubation media with LPS concentrations ranging from 0.1 to 100 ng/ml. The supernatants, harvested after 4 h, were measured for TNF release. The results represent the medians and ranges of three separate experiments.

tion pathway was also demonstrated; LBP enhanced binding of LPS, and triggering of mouse macrophages resulted in TNF release, NO_2 production, and killing of leishmaniae (2). By analogy with human monocytes, binding of LPS-LBP complexes to CD14 on mouse macrophages is likely, but we cannot demonstrate this because we lack blocking anti-CD14 reagents for mouse macrophages. The ability of mouse LBP to increase the sensitivity of human monocytes to minute concentrations of LPS was comparable to the activity of human or rabbit LBP (14, 21).

LBP was found to be a major mouse serum protein that increases LPS binding to monocytes. LBP was present in sufficient amounts in normal mouse serum to signal the presence of LPS to monocytes, since increased LPS binding was detected in normal sera diluted down to 0.1%. An acute-phase response further induced LBP synthesis in mice, as previously described for rabbits (18). Precise measurements of LBP levels in mouse serum will need appropriate quantification with immunoassays and anti-LBP reagents. Our binding experiments suggest a 10-fold increase of LBP after an acute-phase response. All strains of mice, except C3H/HeJ mice, shared this mechanism. Surprisingly, the LBP activity was elevated in 10% normal serum from C3H/HeJ mice but dropped sharply when the serum was diluted to 1%. This could suggest a difference in the amount of LBP and in its affinity for LPS or CD14 in C3H/HeJ mice. These findings are worthy of further investigation, because C3H/HeJ mice are highly resistant to LPS (13).

The isolation and characterization of mouse LBP have shown that the NH_2-terminal sequences in the whole family of LBP, including LBP and bacterial permeability-increasing proteins, are very similar (6, 11, 14, 17). The high degree of sequence homology of LBPs among various animal species could explain the finding that fetal calf, human, and rabbit LBPs present LPS to human monocytes in similar ways (9, 14, 21). LBPs from all these species promote the binding of LPS to human CD14 and trigger TNF production by monocytes. Similar conclusions also apply to mouse LBP, as shown in the present study, again suggesting a highly conserved manner of signaling the presence of minute amounts of LPS to immune cells in normal and acute-phase sera (9). The further development of monoclonal antibodies to mouse LBP and to mouse CD14 will, it is hoped clarify the role played in vivo by LBP in response to LPS, challenge.

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