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Monoclonal Antibodies to Murine Lipopolysaccharide (LPS)-Binding Protein (LBP) Protect Mice from Lethal Endotoxemia by Blocking Either the Binding of LPS to LBP or the Presentation of LPS/LBP Complexes to CD14[1]

Didier Le Roy,* Franco Di Padova,† Reet Tees,† Sylvain Lengacher,‡ Regine Landmann,§ Michel Pierre Glauser,* Thierry Calandra,* and Didier Heumann[2]*

Cellular responses to LPS, the major lipid component of the outer membrane of Gram-negative bacteria, are enhanced markedly by the LPS-binding protein (LBP), a plasma protein that transfers LPS to the cell surface CD14 present on cells of the myeloid lineage. LBP has been shown previously to potentiate the host response to LPS. However, experiments performed in mice with a disruption of the *LBP* gene have yielded discordant results. Whereas one study showed that *LBP* knockout mice were resistant to endotoxemia, another study did not confirm an important role for LBP in the response of mice challenged in vivo with low doses of LPS. Consequently, we generated rat mAbs to murine LBP to investigate further the contribution of LBP in experimental endotoxemia. Three classes of mAbs were obtained. Class 1 mAbs blocked the binding of LPS to LBP; class 2 mAbs blocked the binding of LPS/LBP complexes to CD14; class 3 mAbs bound LBP but did not suppress LBP activity. In vivo, class 1 and class 2 mAbs suppressed LPS-induced TNF production and protected mice from lethal endotoxemia. These results show that the neutralization of LBP accomplished by blocking either the binding of LPS to LBP or the binding of LPS/LBP complexes to CD14 protects the host from LPS-induced toxicity, confirming that LBP is a critical component of innate immunity. *The Journal of Immunology,* 1999, 162: 7454–7460.

Exposure to LPS induces a wide variety of host defense mechanisms, including macrophage/monocyte activation. The activation of circulating monocytes by low doses of LPS is mediated by two proteins, plasma LPS-binding protein (LBP)[3] and membrane CD14 (present at the surface of monocytes) (1). LBP augments the host responses to low doses of LPS levels, triggering cell responses such as cytokine synthesis (1, 2). The LPS released from Gram-negative bacteria is present as aggregates, due to the amphiphilic structure of the molecule. From aggregates, LPS is transformed in monomers through the action of LBP. LBP functions as a lipid transfer molecule, catalyzing the movement of LPS monomers either to phospholipids or to CD14 (3–7). This process results either in the neutralization of LPS, when it is transferred to lipoproteins, or in cellular activation, when it is transferred to soluble or membrane-bound CD14 (8). Thus, the predominance of one of these two pathways will determine the response of the host to LPS.

LBP is a critical component of innate immunity that is implicated in the initiation of host defense against Gram-negative bacteria. LBP serves to alarm the host to the presence of minute amounts of LPS (9). However, upon exposure to larger quantities of LPS, the amplification of LPS effects mediated by LBP may be detrimental to the host. In fact, LBP has been shown to contribute to LPS toxicity in experimental endotoxemia. Blockade of LBP activity with polyclonal Abs was found to protect mice from lethal endotoxemia (10, 11). These data suggest that LBP contributes to the toxicity of high doses of LPS, and that the transfer of LPS to high density lipoproteins is not sufficient to prevent the activation of cells via the LPS/LBP/CD14 pathway.

Experiments using mice with a disruption of the *LBP* gene have yielded conflicting results. In one study, disruption of the LBP gene was associated with a suppression of TNF production and protected mice challenged with LBP-dependent doses of LPS (12). In contrast, the results of another study showed that wild-type or *LBP*-deficient mice produced similar amounts of TNF upon exposure to LBP-dependent doses of LPS in vivo, raising doubts about the role of LBP as a mediator of LPS-induced cell activation (13). Moreover, it was recently reported that i.p. injections of high doses of recombinant LBP exerted protective rather than detrimental effects in mice challenged with LPS (14). Given these apparently discordant observations, we used a classical approach to study the role of LBP in the host responses to LPS and generated well-characterized rat mAbs to murine LBP. Using mAbs that inhibit the binding of LPS to LBP or the presentation of LPS/LBP complexes to CD14, we found that LBP is an important component of the host response to LPS, confirming the central role of LBP in innate immunity.

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; rmLBP, recombinant murine LBP; CHO, Chinese hamster ovary; D-gal, D-galactosamine; OPD, *o*-phenylenediamine; KO, knockout.

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Materials and Methods

LPS-binding protein

Recombinant murine LBP (rmLBP) was expressed in the baculovirus using the cotransfection method with the Baculo-Gold (PharMingen, San Diego, CA), expressed in SF9 insect cells, and cultivated in SF9-II medium (Life Technologies, Basel, Switzerland), as described previously (15). The concentration of rmLBP in the supernatant of LBP-expressing SF9 cells was 2 μg/ml as measured by an LBP-specific ELISA (see below). rmLBP was further purified to homogeneity (single band of 55 kDa, data not shown), as described previously (16). The LPS content of LBP preparations was <1 pg/μg of protein. Recombinant human LBP expressed in Chinese hamster ovary (CHO) cells (17) was a kind gift of P. S. Tobias (Scripps Clinics, La Jolla, CA).

Antibodies

rmLBP was electrophoresed in preparative gels (SDS-PAGE), sliced from the gel, and microsequenced for confirmation. Rabbits were immunized with 100 μg of rmLBP emulsified in 1 ml of Ribi adjuvant (Ribi Immunochem, Hamilton, MT) that was injected five times within 4 wk. Each immunization consisted of 6×50 μl administered intradermally, 2×300 μl administered i.m., and 1×100 μl administered s.c. Immune IgG was purified by HPLC using Hitrap protein G-columns (Amersham Pharmacia Biotech, Dubendorf, Switzerland). Rats were immunized seven times with 10 μg of rmLBP emulsified in 500 μl of Ribi adjuvant. Each injection was divided into two s.c. injections (2×200 μl) and one i.p. injection (100 μl).

At 14 days after the last injection, the rat with the highest titer was boosted (day -3 before fusion with 50 μg of rmLBP i.v. and 100 μg of rmLBP i.p.). On days -2 and -1, another 80 μg of rmLBP was given i.p. On the day of fusion, the rat was killed by CO_2 inhalation, and two separate fusions were made using PEG 4000. Fusion RR (rat-rat) was made by fusing 1.5×10^8 rat spleen cells with 5×10^7 cells of the 8-azaguanine-resistant rat myeloma YB 2/0 (purchased from American Type Culture Collection, Manassas, VA). Fused cells were plated out in 480 Nr 3524 wells (Costar, Cambridge, MA) in HAT medium supplemented with RPMI 1640 containing 10% FCS and 5 ng/ml human rIL-6. Fusion M (mouse) was made by fusing 1.5×10^8 rat spleen cells with 5×10^7 cells of the 8-azaguanine-resistant mouse myeloma PAI-O (a kind gift from Dr. T. Staehlin, Basel, Switzerland) (18). A total of 480 wells were plated out in HAT medium on mouse peritoneal cell feeder layers. Supernatants from hybridomas, which had reached 80% confluency, were assayed by ELISA using a coating of rmLBP at a concentration of 5 μg/ml (100 μl/well). Blocking was conducted with skimmed milk in PBS. Milk and BSA were used as controls for nonspecific binding. Screening was only conducted for hybridomas of the IgG class; therefore, alkaline phosphatase-conjugated affinity-purified $F(ab)_2$ fragments of goat anti-rat IgG (Fcγ-specific Ab) were used at a 1/25,000 dilution (112-056-071, Jackson ImmunoResearch, West Grove, PA). Hybridomas found to be positive for LBP were cloned by limiting dilution and adapted to serum-free growth; production was conducted in roller bottles.

mAbs were purified by protein G chromatography, dialyzed into PBS, and stored at -80°C. The LPS content of the anti-LBP mAbs was <5 pg/μg of protein. Anti-LBP IgG (polyclonal and monoclonal) recognized purified rmLBP and native mouse plasma LBP by ELISA or Western blotting (data not shown).

Isotyping of rat Abs

Isotyping of mAbs was performed using reagents and protocols from Immunopartners (Omega, Lyon, France). Briefly, microplates were coated with 1 μg/ml of MARG1-2 (mouse mAb against the heavy chain of rat IgG1), MARG2a-1 (against IgG2a), MARG2b-3 (against IgG2b), or MARG2C-5 (against IgG2c) in 0.1 M carbonate buffer (pH 9.5). After washing, purified mAbs were then added at a 1 μg/ml concentration; binding of mAbs was revealed with peroxidase-labeled MARK1 plus MARL15 (1 μg/ml), a mixture of Abs recognizing rat λ- and κ-chains at a 1 μg/ml concentration. Peroxidase was revealed using *o*-phenylenediamine (OPD), and OD was measured by spectrophotometry using 490/690-nm filters.

Cells

CHO cells were transfected with the murine CD14 gene as follows: Full-length mouse cDNA was cloned into the pRc/CMV2 plasmid (Invitrogen, Leek, The Netherlands) and stably transfected by electroporation into CHO cells. CD14-expressing cells were grown under G418 selection, analyzed, and sorted by FACS using hamster anti-murine CD14 mAbs (Abs to be published). CHO cells transfected with the human CD14 gene (CHO/huCD14) and control CHO cells transfected with the neomycin resistance vector alone (CHO/NEO) (19) were a kind gift of Dr. D. T. Golenbock (Boston, MA). RAW 264.7 cells and J774 cells were cultured in RPMI 1640 containing 10% FCS.

ELISA for measuring LBP

The 96-well microtiter plates (Dynatech Laboratories, Embrach, Switzerland) were coated overnight at room temperature with 1 μg/well of purified rmLBP in 100 μl of 0.1 M carbonate buffer (pH 9.5). Negative control plates included plates coated with saline. Thereafter, all incubation steps were performed for 1 h at room temperature. The plates were then incubated successively with 1% milk, followed by dilutions of purified rmLBP, SF9/LBP supernatant, or plasma and subsequently with rabbit anti-LBP IgG (10 μg/ml). LBP was detected with 1/1000 dilutions of rabbit anti-LBP IgG labeled with peroxidase (Sigma, St. Louis, MO). Washings were performed with PBS, and dilutions of reagents were made in PBS with 0.1% milk. Peroxidase was revealed using OPD, and OD was measured by spectrophotometry using 490/690-nm filters.

ELISA for measuring binding of rat mAbs to murine LBP

Plates were coated with various amounts of Re595-LPS (List Biologicals, Campbell, CA) diluted in 100 μl of 0.1 M carbonate buffer containing 20 mM of EDTA (pH 9.6) for 3 h at 37°C. The LPS solution was flicked out, and the plates were rinsed thoroughly under running water and air-dried overnight, as described previously (20). Excess binding sites were then blocked with 200 μl/well of PBS containing 1% BSA. rmLBP (usually supernatant of transfected cells) or an equivalent amount of plasma LBP was then added in 100 μl of PBS containing 0.1% BSA for 2 h at 37°C. After washing, rat mAbs were added at various concentrations in PBS containing 0.1% BSA. The binding of rat mAbs was evaluated with 1/1000 dilutions of anti-rat IgG conjugated with peroxidase (Sigma), and peroxidase was revealed using OPD. OD was measured by spectrophotometry using 490/690-nm filters.

To establish the optimal protocol for detecting the binding of rat anti-LBP mAbs to LBP, plates were coated with concentrations of ReLPS ranging from 0.3 to 30 μg/well. A total of 0.1–100 nM of purified LBP was added, and the LBP bound to LPS was detected with 0.1–100 nM of anti-LBP mAbs. The optimal conditions were 3 μg/well of ReLPS for coating, 4 nM of LBP, and 25 nM of mAbs (data not shown).

In an alternative protocol, 25 nM of mAbs were first preincubated with 4 nM of LBP or with murine plasma (dilutions containing 4 nM of LBP) before addition of the LBP/anti-LBP complexes to plates coated with rough LPS (Re595-LPS, 3 μg/well). The binding of LBP was then evaluated using rabbit IgG anti-murine LBP (10 μg/ml), followed by the appropriate peroxidase conjugate.

Evaluation of the capacity of anti-LBP mAbs to neutralize LBP activity in vitro

Two protocols were used to investigate the capacity of anti-LBP mAbs to neutralize LBP activity. The capacity of LBP to present LPS-FITC to $CD14^+$ cells was analyzed using a binding assay; the LBP-dependent TNF production of LPS-stimulated RAW 264.7 cells was measured by a bioassay.

Because anti-LBP mAbs could block either LBP directly or LBP bound to LPS (LBP/LPS complexes), two experimental protocols were performed to determine the mode of action of the mAbs. In the first series of experiments, LBP was initially preincubated with mAbs for 15 min at 37°C before the addition of LPS-FITC (binding assay) or LPS (TNF assay). In the second series of experiments, LBP was first mixed with LPS-FITC (binding assay) or LPS (TNF assay) for 15 min at 37°C before the addition of anti-LBP mAbs.

Flow cytometry assay for measuring reactivity of rat mAbs with rmLBP

CHO cells transfected with the murine or the human CD14 gene (CHO/muCD14 or CHO/huCD14) and murine macrophage J774 and RAW 264.7 cells were used to investigate the effects of rat anti-LBP mAbs on the binding of FITC-LPS to CD14. The binding of FITC-LPS to CD14 was LBP-dependent for all four cell lines. CD14 was required for the efficient binding (or uptake) of LPS-FITC through LBP, as LPS-FITC did not bind to control CHO cells transfected with the vector alone (CHO/NEO) or to CHO/huCD14 cells pretreated with a neutralizing anti-CD14 mAb (3C10) (data not shown). We could not investigate the contribution of murine CD14 in this setting, because neutralizing Abs to murine CD14 are not available.

LPS from *Serratia marcescens* (Sigma) was labeled with FITC (21) and used at concentrations of 5 nM (0.2 μg/ml), 20 nM, or 100 nM. These experiments were performed using supernatant of LBP-expressing SF9

cells. The optimal concentration of LBP in the assay was 4 nM (data not shown), and the Abs were tested from 2 nM to 64 nM. Two protocols were used: 1) preincubation of LBP with anti-LBP mAbs followed by the addition of FITC-LPS; or 2) preincubation of FITC-LPS with LBP before the addition of anti-LBP mAbs. Reagents were incubated for 1 h with 10^5 target cells in a final volume of 200 μl. Next, cells were washed, and fluorescence on $CD14^+$ cells was detected using a FACScan, as described previously (2).

Neutralization of LBP measured by the TNF bioassay

Two different experimental protocols were used. In the first protocol, rat mAbs (4 μg/ml in a 20-μl volume) were initially preincubated with rmLBP (200 ng in a 20-μl volume) for 15 min to generate LBP/anti-LBP complexes. The LBP/anti-LBP complexes were then added to RAW 264.7 cells (2×10^4 cells in a 200-μl final volume). A total of 10 ng/ml LPS (20 μl volume) was added for 4 h at 37°C. In the second protocol, LPS was first reacted with LBP to form LPS/LBP complexes. Complexes were then added to RAW 264.7 cells together with rat anti-LBP mAbs and incubated for 4 h at 37°C. All supernatants were collected for TNF determination.

Evaluation of LBP blockade in vivo

Female BALB/c mice (8 wk old) were purchased from IFFA Credo (Lyon, France) and were acclimatized 1 wk before experimentation. To investigate whether mAbs could block LBP activity in vivo, BALB/c mice were injected i.v. with 100 μg/mouse of rat anti-LBP mAbs. Plasma was collected at 1 h or 7 h postinjection and kept frozen at −80°C. LBP activity was evaluated by measuring the capacity of LBP to present LPS-FITC to $CD14^+$ cells. To do so, 2 μg/ml of LPS-FITC was added to 5% plasma, and the binding of LPS/LBP complexes to CHO/muCD14 was assessed by flow cytometry.

Endotoxemic model in mice

For all experiments, mice received 25 mg of D-galactosamine (D-gal) (Sigma) i.p. and 100 μg/mouse of rat mAbs i.v. in a volume of 250 μl of saline. After 15 min, mice were injected i.v. with *Escherichia coli* O111 LPS (30–1250 ng) from Sigma dissolved in 100 μl of saline. Plasma was obtained via the tail vein at 75 min after LPS challenge to determine peak plasma TNF concentrations.

TNF and LPS determination

TNF was measured by bioassay using WEHI clone 13 as a target, as described previously (2). The presence of LPS in reagents was determined with the *Limulus* assay (Chromogenix, Embrach, Switzerland).

Statistics

Statistical analyses were done using the χ^2 test or Fisher's exact test for comparisons of proportions and the ANOVA test to compare intergroup differences.

Results

Anti-LBP mAbs

A total of 63 clones (IgG2a, IgG2b, or IgG2c isotype) were obtained after limiting dilution that were either of mouse/rat origin (clone M) or rat/rat origin (clones RR). All clones produced Abs that reacted in ELISA with rmLBP and mouse plasma LBP but not with purified human LBP (data not shown). Three pairs of clones of the same isotype (IgG2a) (Table I) but with different inhibitory properties (see thereafter) were selected for the present studies. Cross-competition experiments showed that these clones recognized different epitopes (data not shown).

Binding of anti-LBP mAbs to LBP or to LPS/LBP complexes

As shown in Table I, all six clones reacted with LBP by ELISA. Using LPS-coated plates, we subsequently tested 1) whether the mAbs bound to preformed LPS/LBP complexes and 2) whether the binding of the mAbs to LBP inhibited the binding of LBP to LPS.

To test the first hypothesis, rmLBP or murine plasma was first incubated with LPS plates to allow the formation of LPS/LBP complexes. Under these conditions, clones 2A, 2B, 3A, and 3B bound to LPS/LBP complexes, whereas clones 1A and 1B did not (Fig. 1*A*), even in the presence of a 20-fold molar excess of mAbs (data not shown). To test the second hypothesis, rmLBP or murine plasma was first mixed with the mAbs and then added to the LPS-coated plates. Preincubation of LBP with clones 1A and 1B, but not with the other four clones, prevented the binding of LBP to LPS-coated plates (Fig. 1*B*). In both experimental settings, identical results were obtained using either recombinant or native (plasma) murine LBP. Thus, these experiments suggested that clones 1A and 1B were directed against the epitope(s) implicated in the binding of LPS to LBP, whereas the other four clones recognized other epitopes of the LBP molecule.

Table I. *Reactivity of rat anti-LBP mAbs with rmLBP*[a]

Clones	Abbreviation Code	ELISA (OD units)
M330-19	1A	0.81
M367-2	1B	1.04
M119-3	2A	1.31
RR433-8	2B	1.37
M306-5	3A	0.52
M399-44	3B	1.21

[a] For ELISA, plates were coated with 1 μg/well of rmLBP. mAbs were added at a 1 μg/ml concentration. M, mouse/rat hybridomas; RR, rat/rat hybridoma. Background was 0.05 OD units in the absence of mAbs or LBP.

Effect of anti-LBP mAbs on the binding of FITC-LPS to $CD14^+$ target cells

In the next series of experiments, we investigated whether the anti-LBP mAbs blocked the LBP-mediated binding of LPS to CD14. The experiments were performed with CHO cells transfected with either murine CD14 (CHO/muCD14) or human CD14 (CHO/huCD14) and with two murine macrophage cell lines (J774 and RAW 264.7). The binding of LPS-FITC to $CD14^+$ cells was measured by flow cytometry (2). Clones 1A, 1B, 2A, and 2B were found to inhibit the binding of LPS to CHO/muCD14; clones 3A and 3B did not (Fig. 2). Complete inhibition of LPS binding to CHO/muCD14 cells required a mAb to LBP molar ratio of ≥4:1.

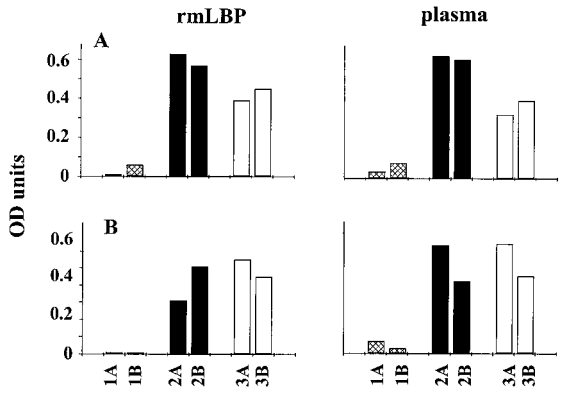

Figure 1. Binding of anti-LBP mAbs to LPS/LBP complexes. *A*, A total of 4 nM of rmLBP or 10% murine plasma (containing ~4 nM in LBP) was added to LPS-coated plates (3 μg/well), followed by 25 nM of anti-LBP mAbs and peroxidase conjugate. OD units were <0.02 in the absence of LPS, LBP, plasma, or anti-LBP mAbs. *B*, Similar LBP concentrations were first incubated with 25 nM of anti-LBP mAbs for 15 min at 37°C, and were then added to LPS-coated plates. The LBP bound to LPS was revealed with polyclonal rabbit anti-LBP IgG (10 μg/ml) followed by peroxidase conjugate. OD units were <0.01 in the absence of LPS. A total of 0.6 OD units were measured after incubation of LPS/LBP with polyclonal anti-LBP IgG in the absence of competitive mAbs.

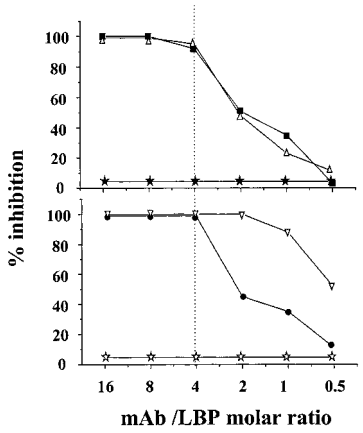

Figure 2. LBP-dependent binding of LPS-FITC to CHO/muCD14: Effect of anti-LBP mAb concentrations. A total of 4 nM of LBP was preincubated with various concentrations of mAbs. After 15 min, LBP/anti-LBP complexes were mixed with 5 nM of FITC-LPS and incubated for 1 h at 37°C with CHO/muCD14. The fluorescence of target cells was measured by FACScan. Data are expressed as percent inhibition of LPS binding when compared with a positive control consisting of 4 nM of LBP without mAbs (△, clone 1A; ■, clone 1B; ▽, clone 2A; ●, clone 2B; ⋆, clone 3A; ☆, clone 3B).

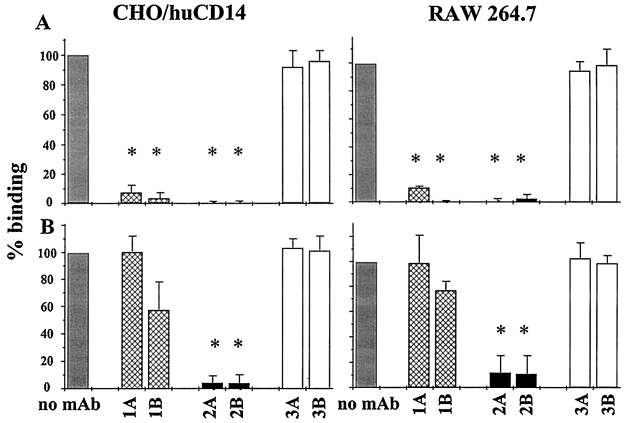

Figure 4. LBP-dependent binding of LPS-FITC to CHO/huCD14 and RAW 264.7 cells. *A*, Preincubation of anti-LBP mAbs with LBP prior to the addition of FITC-LPS. *B*, Preincubation of LBP with FITC-LPS prior to the addition of anti-LBP mAbs. Data are expressed as the percent binding of LPS to the cells. 100% refers to the binding of LPS to cells in the absence of anti-LBP mAb. Data are the mean ± SD of five different experiments. According to an ANOVA comparing all groups: $p = 0.002$ (CHO/huCD14), $p = 0.0001$ (RAW 264.7 cells). ∗, $p < 0.05$ compared with other groups and the reference control.

Decreasing the concentration of mAbs resulted in a progressive loss of the inhibition that was ~40% at a 1:1 mAb to LBP molar ratio. We subsequently examined whether LPS was able to displace the LBP/anti-LBP complexes (Fig. 3). Dissociation of LBP/anti-LBP complexes occurred at high concentrations of LPS (20 or 100 nM) with clone 1B, and also, but to a lesser extent, with clone 1A. Increasing the LPS concentrations had no effect with clone 2A.

We subsequently investigated whether the anti-LBP mAbs inhibited the binding of LPS to LBP or the binding of preformed LPS/LBP complexes to CD14. For these experiments, we used concentrations of mAbs (64 nM), LBP (4 nM) (a mAb to LBP molar ratio of 16), and LPS-FITC (20 nM) that resulted in a complete inhibition of the binding of LPS to CD14 when clones 1A, 1B, 2A, and 2B were preincubated with LBP (Figs. 2 and 3). Fig. 4 shows the results obtained with CHO/huCD14 and RAW 264.7 cells (similar observations were made with CHO/muCD14 and J774 cell lines, data not shown). Clone 2A and clone 2B inhibited the binding of FITC-LPS to $CD14^+$ targets in both experimental conditions, whereas clone 1A and clone 1B suppressed the binding of FITC-LPS to $CD14^+$ targets only when LBP was first preincubated with anti-LBP mAbs before the addition of LPS. Clone 3A and clone 3B had no effect. Moreover, preincubation of CHO/huCD14 cells with clones 2B or 1A followed by washing and subsequent addition of LBP together with LPS-FITC did not inhibit the binding of FITC-LPS to CD14 (data not shown), indicating that the effect of the anti-LBP mAbs was due to a specific inhibition of LBP activity and not to a nonspecific interaction with the target cells.

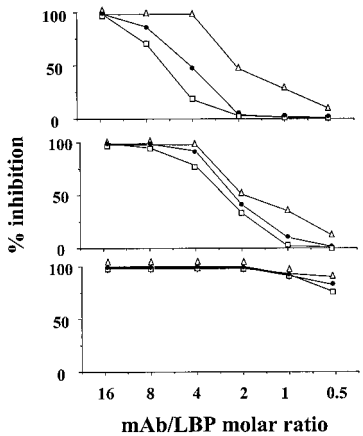

Figure 3. LBP-dependent binding of LPS-FITC to CHO/muCD14: Effect of LPS on the stability of LBP/anti-LBP complexes. A total of 4 nM of LBP was preincubated with various concentrations of mAbs. After 15 min, LBP/anti-LBP complexes were mixed with 5, 20, or 100 nM of LPS-FITC and incubated for 1 h at 37°C with CHO/muCD14. The fluorescence of target cells was measured by FACScan. Data are expressed as percent inhibition of LPS binding when compared with a positive control consisting of 4 nM of LBP without mAbs (△, 5 nM of LPS; ●, 20 nM of LPS; □, 100 nM of LPS). *Top panel*, clone 1B; *middle panel*, clone 1A; *bottom panel*, clone 2A.

Thus, the results of these binding experiments suggested that clones 1A and 1B blocked the binding of LPS to LBP, because no effect was noted once LBP was bound to LPS. The fact that clones 2A and 2B were inhibitory under both experimental conditions suggested that they interfere with the presentation of LPS/LBP complexes to CD14 and not with the binding of LPS to LBP.

Effect of anti-LBP mAbs on TNF production by RAW 264.7 cells

Binding of LPS to membrane $CD14^+$ at the surface of monocytes triggers cell activation and induces the release of cytokines. Therefore, we studied the effect of anti-LBP mAbs on the production of TNF by RAW 264.7 cells stimulated with LPS. In the presence of LBP in the cell-culture medium, RAW 264.7 cells released abundant amounts of TNF (1500 pg/ml) after stimulation with 10 ng/ml of LPS (Fig. 5*A*); no such release was observed in the absence of LBP (<20 pg/ml, data not shown). When anti-LBP mAbs were first incubated with LBP, clones 1A, 1B, 2A, and 2B almost completely suppressed TNF production in LPS-stimulated RAW 264.7 cells. Clones 3A and 3B had no effect (Fig. 5*A*). Clones 2A and 2B, but not clones 1A and 1B, also inhibited TNF production when LPS was first incubated with LBP to form LPS/LBP complexes (Fig. 5*B*). Clones 3A and 3B did not block TNF production under this experimental setting either. Similar results were obtained with

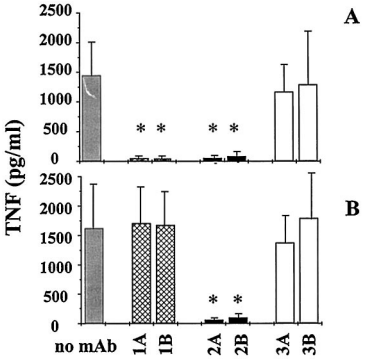

Figure 5. Effect of anti-LBP mAbs on LPS-induced TNF production by RAW 264.7 cells. Cells were stimulated in the presence of LBP with 10 ng/ml of LPS for 4 h at 37°C. Supernatants were collected, and TNF content was measured by bioassay. Data are the mean ± SD of five separate experiments. *A*, Preincubation of LBP (4 nM) with mAbs (25 nM) 15 minutes prior to the addition of LPS. *B*, Preincubation of LPS with LBP 15 minutes prior to the addition of mAbs. According to ANOVA, $p = 0.0002$ (*A*), $p = 0.001$ (*B*). *, $p < 0.05$ compared with other groups and the reference control.

J774 cells (data not shown). Therefore, these results confirm the observations made in previous experiments.

Taken together, these data show that clones 1A and 1B prevented the binding of LPS to LBP and the subsequent activation of target cells, but that they have no effect on preformed LPS/LBP complexes. In contrast, clones 2A and 2B block the biological effects of LBP regardless of whether or not it is already bound to LPS, strongly suggesting that these mAbs inhibit the binding of LPS/LBP complexes to CD14.

In vivo neutralization of LBP

We subsequently investigated whether the anti-LBP mAbs also blocked the LBP activity in vivo. This was first done by measuring the functional activity of LBP, assessed by its ability to present FITC-LPS to CHO/muCD14 cells, after the injection of anti-LBP mAbs. The concentration of LBP in the plasma of normal BALB/c mice is 20 nM as measured by ELISA. Consequently, mice were injected i.v. with 100 μg of mAb (corresponding approximately to 750 nM based on an estimated circulating blood volume of 1 ml), and plasma was collected at 1 h postinjection. Confirming the results obtained in vitro, clones 1A, 1B, 2A, and 2B completely blocked the ability of plasma LBP to present LPS to CHO/muCD14, whereas clone 3A and clone 3B had no effect (Table II).

Table II. *Blockade of LBP in vivo with anti-LBP mAbs*[a]

mAbs	LBP Activity (fluorescence units)
1A	4.1 ± 1.2*
1B	4.0 ± 1.0*
2A	2.8 ± 0.7*
2B	3.5 ± 0.6*
3A	15.0 ± 1.2
3B	16.2 ± 2.6
Saline	17.1 ± 4.4

[a] Mice were injected with 100 μg of mAbs. Plasma was collected 1 h after the injection of mAb to assess the functional activity of LBP, which was measured by its ability to present LPS-FITC (fluorescence units) to CHO/muCD14 cells in vitro. In the absence of plasma (albumin as a control), the binding of LPS-FITC to $CD14^+$ target cells was 3.6 fluorescence units. Data are the mean ± SD of eight mice per group. $p = 10^{-9}$ by ANOVA when all groups were compared.
*, $p < 0.05$ when compared with saline.

Table III. *Dose-dependent effect of LPS on survival and peak plasma TNF levels of* D*-galactosamine-sensitized mice treated with saline or anti-LBP mAbs*[a]

	LPS (ng/mouse)	LBP Activity[b] (FU)	TNF (pg/ml)[c]	Deaths/Total
Saline	50	15.7 ± 2.4	2315 ± 560	7/9 (78%)
	1250	17.0 ± 4.5	4850 ± 3250	9/9 (100%)
2A	50	3.9 ± 0.05*	155 ± 75*	1/9 (11%)**
	250	ND	2275 ± 1450	6/6 (100%)
	1250	4.0 ± 0.07*	4740 ± 2560	9/9 (100%)

[a] Mice received an i.v. injection of 100 μg of clone 2A or saline 15 min before the indicated doses of LPS were administered i.v. Data are the mean ± SD of two experiments.
[b] LBP activity was assessed by the capacity of the plasma collected 7 h after LPS challenge to enhance FITC-LPS to CHO/muCD14$^+$ cells. In the absence of plasma, the negative control without LBP was 4.1 fluorescence units (FU).
[c] Plasma TNF levels were measured 75 min after LPS challenge.
* $p < 0.05$ when compared with the other groups by ANOVA.
** $p < 0.02$ by Fisher's exact test comparing mice receiving saline with mice receiving mAbs. According to an ANOVA comparing treatment groups: 1) $p = 8 \times 10^{-9}$, 2) $p = 6 \times 10^{-9}$.

We subsequently examined the effect of anti-LBP mAbs in a model of lethal endotoxemia in mice sensitized with D-gal. Initial experiments were designed to determine the dose of LPS that induced lethality in mice pretreated with saline or with clone 2A, which neutralized the LBP activity (Table III). Injection of 50 ng or 1250 ng of LPS per mouse induced massive TNF production and killed almost all mice pretreated with saline. Pretreatment with clone 2A before a challenge with 50 ng of LPS completely suppressed the LBP activity of plasma, caused a marked reduction of the circulating levels of TNF, and improved survival. At a higher LPS dose (250 ng/mouse), clone 2A did not reduce TNF production and did not protect the mice, despite the fact that the LBP activity of plasma was inhibited for ≤7 h after LPS challenge (Table III), ruling out the possibility that the dose of mAb was insufficient to block LBP at higher LPS doses. Thus, these results suggest that death is triggered by LBP-independent events at a high dose of LPS.

Based on these preliminary experiments, a dose of 50 ng of LPS was selected to compare the protective effects of the different anti-LBP mAbs in D-gal-sensitized mice (Table IV). Mortality was 69% in mice treated with saline, whereas pretreatment with clones

Table IV. *Effect of prophylactic administration of anti-LBP mAbs on survival and on plasma TNF levels in* D*-galactosamine-sensitized mice challenged with LPS*[a]

	Deaths/Total	Plasma TNF (pg/ml)[a]
Saline	11/16 (69%)	910 ± 360
mAbs		
1A	0/16 (0%)*	55 ± 60**
1B	3/16 (18%)*	230 ± 280[b],**
2A	0/16 (0%)*	30 ± 15**
2B	1/16 (6%)*	40 ± 30**
3A	9/16 (56%)	600 ± 470
3B	10/16 (62%)	840 ± 690

[a] Mice received an i.v. injection of 100 μg of mAbs or saline 15 min prior to LPS challenge (expt. 1, 30 ng/mouse; expt. 2, 50 ng/mouse). Data are a pool of the two experiments, with eight mice per treatment group. The χ^2 test indicated a significant difference in survival between the groups ($p < 0.04$).
[b] TNF levels in mice treated with clone 1B were different from those of mice treated with clones 1A, 2A, and 2B.
* $p < 0.02$ by Fisher's exact test comparing mice receiving saline with mice receiving mAbs. According to an ANOVA comparing the seven treatment groups: 1) $p = 7 \times 10^{-9}$, 2) $p = 3 \times 10^{-8}$.
** $p < 0.05$ indicates statistical difference over other groups by ANOVA.

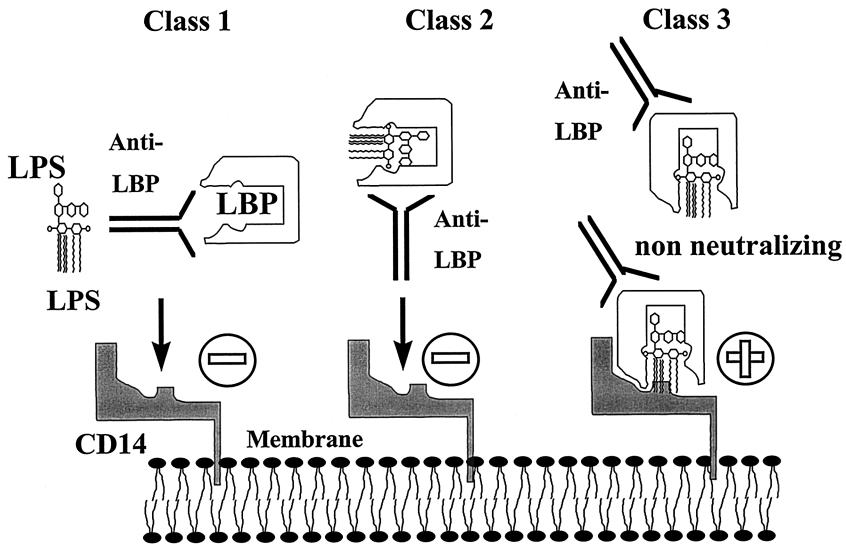

Figure 6. Mode of action of different types of anti-LBP mAbs. Class 1 mAbs block the binding of LPS to LBP. Class 2 mAbs inhibit the presentation of LPS/LBP complexes to CD14. Class 3 mAbs bind to LBP but do not neutralize LBP activity.

1A, 1B, 2A, or 2B resulted in almost complete protection (mortality: 0%, 18%, 0%, and 6%, respectively). Protection was associated with very low circulating levels of TNF in all survivors. As predicted by the results obtained in vitro, clones 3A and 3B did not reduce plasma TNF levels and did not protect mice (mortality: 56% and 62%).

Discussion

Previous in vitro studies have shown that LBP is required for optimal CD14-dependent stimulation of monocytes by LPS (2, 9, 22). Neutralization of murine LBP with polyclonal rabbit anti-LBP IgG protected mice from lethal endotoxemia (10, 11). More recently, disruption of the *LBP* gene in BALB/c/129 Sv mice was found to be associated with resistance to LPS (12), confirming previous observations made in vitro and in vivo (9–11, 22). Although this report convincingly demonstrated that the *LBP* knockout (KO) mice did not respond to low doses of LPS, differences between the KO mice and the control wild-type mice were not striking, presumably because the genetic background of the wild-type mice comprised both the BALB/c phenotype (strong LPS responder) and the 129 Sv phenotype (weak LPS responder). Recent results obtained with other *LBP* KO mice on a C57BL/6J background challenged the concept that LBP plays an important role in LPS-induced cytokine production in vivo (13). Indeed, although LBP was required for optimal production of TNF by whole blood stimulated with a low dose of LPS ex vivo, surprisingly, hemizygous and *LBP* KO mice produced equal amounts of TNF in vivo (13). These results thus suggested that the in vivo response to LPS injections was mediated by an LBP-independent pathway, and that TNF production occurred predominantly in the tissue rather than in the blood stream. No LBP was detected in the blood, but a truncated form of LBP was detected in the livers of KO mice (13). Thus, synthesis of a truncated but functional LBP might have taken place in the liver, but not in the blood, possibly because of the null mutation that suppressed part of the first intron and the signal peptide (13). The first potential initiation codon (methionine 146) of truncated LBP is beyond a region (amino acids 90–105) that has been described as a part of the LPS-binding site. Although lysines 92, 95, and 99 are involved in the recognition of LPS, other portions of the LBP molecule also may participate (reviewed in Ref. 23), so that one cannot totally rule out that this truncated form of LBP might exhibit LPS-binding activity. Alternatively, disruption of genes may lead to adaptive responses, which could have resulted in an LBP-independent LPS response in C57BL/6J KO mice, but not in BALB/c KO mice.

To further investigate the role of LBP in innate immunity, we generated well-characterized neutralizing anti-LBP mAbs and tested their effects in experimental endotoxemia. The present data provide evidence to support the concept that LBP enhances host response to low doses of LPS in vivo. Neutralization of LBP activity with anti-LBP mAbs was found to suppress TNF production by murine macrophages and to protect mice from LPS-induced lethality. Suppression of cytokine production and protection from lethal endotoxemia was achieved by two different mechanisms. A first class of anti-LBP mAbs (class 1 mAbs, Fig. 6) inhibited LBP activity by blocking the binding of LPS. According to ELISA, class 1 mAbs did not bind to LBP/LPS complexes and were unable to bind to LPS upon preincubation of mAbs with LBP. Class 1 mAbs inhibited the binding of FITC-LPS to $CD14^+$ cells when they were first preincubated with LBP but not when FITC-LPS/LBP complexes were formed before incubation with anti-LBP mAbs. Class 1 mAbs also suppressed LPS-induced TNF production by murine macrophage cells, but only when anti-LBP mAbs were first incubated with LBP. A second class of anti-LBP mAbs (class 2 mAbs, Fig. 6) recognized either LBP alone or LBP/LPS complexes. They inhibited LBP activity by blocking the binding of LPS/LBP complexes to cells expressing the CD14 receptor. In contrast to class 1 mAbs, class 2 mAbs inhibited the binding of FITC-LPS/LBP complexes to $CD14^+$ cells or LPS-induced TNF production by macrophages regardless of whether or not the mAbs had been preincubated with LBP before adding LPS.

The in vivo activity of the mAbs was very similar to that measured in vitro. Prophylactic administration of class 1 and class 2 mAbs neutralized the LBP activity in plasma, suppressed TNF production, and protected D-gal-sensitized mice from death. All class 1 and class 2 mAbs were able to neutralize the LBP activity when used in a 10-fold molar excess and with low LPS concentrations. Minor differences in activity were observed between the various mAbs used. For example, clone 1B was less potent than clone 1A, as shown by a residual TNF production noted in mice treated with clone 1B. A lower affinity for LBP or a possible dissociation of the LBP/anti-LBP complex by LPS, as noted for clone 1B (Fig. 2), could account for these slight differences.

Anti-LBP mAbs protected mice from death induced by low doses of LPS (50 ng/mouse), but not high doses of LPS (250 ng/mouse), a finding well in agreement with the results of previous in

vitro studies (9, 22). High doses of LPS trigger death by activating LBP-independent pathways, as shown by the fact that mice succumb despite a complete neutralization of LBP for ≤7 h after LPS challenge, indicating that the dose of mAbs was not a limiting factor in these experiments.

Recent reports have suggested that LBP could suppress rather than enhance LPS-induced cytokine production by activated mouse macrophages in vitro (24, 25). An i.p. injection of high doses of recombinant LBP (100 μg) was observed to protect mice from an otherwise lethal LPS challenge (14). However, in our hands, the protection afforded by the anti-LBP mAbs did not differ regardless of whether the LPS was administered i.v. or i.p (data not shown). Normal levels of LBP are low in the peritoneal cavity (usually <10 ng/ml, data not shown), and such levels are unlikely to play a protective role in endotoxemia, as observed in the present experiments. However, this does not rule out the possibility that the high levels of LBP produced during the acute phase responses might exert a protective role by a mechanism that remains to be identified (14).

In summary, we have produced anti-LBP mAbs that recognize different epitopes of the LBP molecule. Abs blocking either the binding of LPS to LBP or the binding of LPS/LBP complexes to CD14 were found to neutralize the LBP activity, to inhibit macrophage TNF production in vitro and in vivo, and to protect mice from lethal endotoxemia. Studies are now in progress to characterize the epitopes recognized by these Abs, which should give us more insight into the interactions between LPS, LBP, and CD14. Although *LBP* KO mice are more resistant than wild-type mice to LPS, they exhibit increased sensitivity to Gram-negative infections and readily succumb to *Salmonella typhimurium* infections, presumably because of impaired host defense mechanisms (12). Using the anti-LBP mAbs we have generated, we are currently investigating the role of LBP in infections caused by Gram-negative bacteria. These investigations should broaden our understanding of the pathophysiology of Gram-negative infections and conceivably may offer new treatment opportunities for the management of patients with sepsis and septic shock.

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