Lipopolysaccharide (LPS)-binding protein (LBP) is an acute phase reactant that may play a dual role in vivo, both potentiating and decreasing cell responses to bacterial LPS. Whereas low concentrations of LBP potentiates cell stimulation by transferring LPS to CD14, high LBP concentrations inhibit cell responses to LPS. One inhibitory mechanism involves the ability of LBP to neutralize LPS by transferring it to plasma lipoproteins, whereas other inhibitory mechanisms, such as the one described here, do not require exogenous lipoproteins. Here we show that LBP can inhibit monocyte responses to LPS that has already bound to membrane-bound CD14 (mCD14) on the cell surface. LBP caused rapid dissociation of LPS from mCD14 as measured by the ability of LBP to inhibit cross-linking of a radioiodinated, photoactivatable LPS derivative to mCD14. Whereas LBP removed up to 75% of the mCD14-bound LPS in 10 min, this was not accompanied by extensive release of the LPS from the cells. The cross-linking data suggest that much of the LPS that remained bound to the cells was associated with LBP. The ability of LBP to inhibit cell responses could not be explained by its effect on LPS internalization, because LBP did not significantly increase the internalization of the cell-bound LPS. In cell-free LPS cross-linking experiments, LBP inhibited the transfer of LPS from soluble CD14 to soluble MD-2. Our data support the hypothesis that LBP can inhibit cell responses to LPS by inhibiting LPS transfer from mCD14 to the Toll-like receptor 4-MD-2 signaling receptor.

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**Reagents and Cells**—Recombinant human LBP (C-terminal His tag) was expressed in a baculovirus system in SF-9 cells in SF-900 II SFM (Invitrogen). The protein was purified as described previously. Recombinant human sCD14 and soluble MD-2 (sMD-2) were produced by baculovirus expression as previously described (17, 26). The recombinant proteins did not stimulate cells when added alone at the indicated concentrations. THP-1 cells (provided by LiWu Li, Wake Forest University, Winston-Salem, NC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. 293T cells (ATCC) were covalently bound to agarose beads using Carbolink™ (BD Biosciences) in a cleavable, photoactivatable cross-linker, as previously described (1). Anti-human CD14 monoclonal antibodies, 60bca and 63D3, were purified from the culture supernatants of hybridomas obtained from the ATCC. 60bca was covalently bound to agarose beads using Carbolink™ from Pierce. Affinity-purified rabbit polyclonal anti-human LBP antibody, K1970602, was a gift of XOMA Corp. (Berkeley, CA). IgG fractions of rabbit polyclonal antibodies to recombinant human soluble CD14 (number 4-2051-5) and recombinant human LBP (number 4-1855-9) were produced in the Tobias laboratory. Peroxidase-conjugated donkey anti-goat IgG was obtained from Jackson ImmunoResearch (West Grove, PA). All other reagents were from Sigma unless otherwise stated.

**Experimental Procedures**

**LPS—[3H]LPS was biosynthetically labeled in the fatty acyl chains in* Escherichia coli* L2525 (Ra phenotype) (32).** 125I-ASD-LPS was made by derivatization of ReLPS (*Salmonella minnesota* R5) with sulfo-L-cysteine methyl ester and dithiopropionate (SASD) (Pierce), a cleavable, photoactivatable cross-linker, as previously described (33). The stimulatory activity of the 125I-ASD-LPS was tested in THP-1 (VD₃) cells and found to be similar to that of the derivatized ReLPS. Each LPS preparation stimulated cytokine production at threshold concentrations of 0.03–0.1 ng of LPS/ml in the presence of LBP (0.1 μg/ml) or as preformed LPS–sCD14 complexes (data not shown). Fluorescent LPS (Alexa Fluor488-2,4-dinitrophenyl-LPS derived from E. coli 055:B5 LPS) was obtained from Molecular Probes, Inc. (Eugene, OR) and is referred to here as Alexa-LPS. Protein contamination was not found in any of the preparations on silver-stained SDS-PAGE gels.

**LPS-Cell Binding and Stimulation**—LPS-cell binding was performed in the absence of LBP using preformed LPS–sCD14 complexes (34). For cell stimulation assays, the cells (7 × 10⁶ cells/0.5 ml) were incubated with LPS–sCD14 at 1 ng of LPS/ml for 2 min at 37 °C. The cells were then washed in cold RPMI 1640 medium to remove unbound LPS and were incubated in serum-free medium (SFM) (RPMI 1640, 20 mM Hepes, pH 7.4, 0.1 μg/ml bovine serum albumin) containing the indicated proteins for 2 or 3 h at 37 °C with brief mixing at 2-min intervals.

Cytokines were measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA). Background activity, defined by equally sized control areas of the gel, was subtracted using the software provided by the manufacturer. In experiments in which LPS was cross-linked to soluble proteins, autoradiography was performed by exposing the gels to x-ray film.

To test the specificity of the immunoprecipitations, CD14 and LBP were immunoprecipitated as described above using specific antibodies or control antibodies (an irrelevant isotype-matched (IgG1) mAb bound to beads or nonimmune rabbit IgG and protein A-Sepharose, respectively). After separating the proteins by SDS-PAGE, they were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) according to the manufacturer’s instructions. The membranes were blocked with 1% fish gelatin in PBS plus 0.05% Tween 20, and the proteins were detected with goat anti-human CD14 (7 μg/ml) or goat anti-human LBP (3 μg/ml) followed by peroxidase-conjugated donkey anti-goat IgG. The blots were developed with ECL reagents (Amersham Biosciences) and exposed to x-ray film. The CD14 and LBP bands were found at their expected molecular sizes (~55 and 60 kDa, respectively), whereas no bands were detected in control antibody immunoprecipitates (data not shown).

**Results**

To determine whether LBP can inhibit cell responses after LPS–sCD14 binding has occurred, we incubated LPS–sCD14 complexes with mCD14-expressing THP-1 cells for 2 min at 37 °C. We previously found that nearly maximal binding of LPS monomers to mCD14 occurs under these incubation conditions (34). After washing to remove unbound LPS, we found that incubating the cells with LBP inhibited cytokine production. As shown in Fig. 1, 50% inhibition of cytokine production occurred at LBP concentrations of 1–2 μg/ml. In experiments not shown, LBP also inhibited responses to cell-bound LPS in normal human monocytes in peripheral blood mononuclear cell
mixtures with similar potency. Because the LPS receptor (Tlr4) and the IL-1 receptor use many of the same intracellular signaling proteins to induce the expression of inflammatory cytokine genes, we stimulated the cells with IL-1β to test whether the inhibitory effect of LBP is LPS-specific. As shown in Table I, the cells produced cytokines in response to both LPS and IL-1β, and LBP inhibited responses only to LPS.

As shown in Fig. 2, LBP had its strongest inhibitory effect when added immediately after LPS-cell binding, and delaying the addition of LBP caused a progressive loss of its ability to inhibit the responses. Under the conditions of our 2-h incubation, LBP measurably inhibited IL-8 and tumor necrosis factor-α production when its addition was delayed for up to 30 min, and it inhibited IL-1β production when added up to 1 h after LPS-cell binding had occurred.

We next asked whether LBP could enhance the release or internalization of cell-bound LPS. LPS binding and internalization were measured by two independent methods (31, 34). In the first (Fig. 3A), cell surface-bound [3H]LPS was measured by the ability of proteinase K to release the [3H]LPS from the cells. Protection of cell-associated [3H]LPS from proteolytic release may either reflect its internalization or its transfer to a protease-resistant site on the cell surface. Therefore, we use a second method in which the binding and internalization of a fluorescent derivative of LPS were measured by flow cytometry; trypan blue was used to quench the fluorescence of the cell surface-exposed LPS (Fig. 3B).

The results showed that whereas LBP slightly increased the release of LPS from the cells, most of the LPS remained associated with the cells after they were exposed to 3 μg/ml of LBP, an LBP concentration that produced a 60–75% inhibition of the cell response (Fig. 1 and Table I). Whereas the LBP-induced reduction of cell-associated Alexa-LPS (Fig. 3B) was greater than that of [3H]LPS (Fig. 3A), it is unclear whether more Alexa-LPS was actually released from the cells or whether the binding of LBP to the cell-associated Alexa-LPS caused a slight reduction in its fluorescence intensity. In any case, LBP did not significantly increase the rate of release of cell-associated Alexa-LPS except at the earliest time point. Also shown in Fig. 3, LBP did not significantly increase the internalization of cell-bound LPS.

To determine whether the cell-associated LPS remained bound to mCD14 after the cells were incubated with LBP, we derivatized LPS with a radioiodidatable, photoactivatable cross-linker (SASD) and measured the effect of LBP on the ability of cell-bound 125I-ASD-LPS to cross-link to mCD14. The results shown in Fig. 4 suggest that LBP dramatically increased the dissociation of cell-bound 125I-ASD-LPS from mCD14 in both CD14-transfected HEK 293 cells and THP-1 cells. The ability of LBP to remove LPS from mCD14 occurred rapidly; 60% inhibition of cross-linking occurred after the cells were incubated with LBP for 5 min (data not shown), and 64–75% inhibition occurred after 10 min (Fig. 4). Immunoprecipitation of LBP from lysates of the LBP-treated cells revealed that a significant amount of cell-bound LPS was associated with LBP (Fig. 4). No labeled protein band was immunoprecipitated from cells that were not incubated with LBP (data not shown).

The specificities of the immunoprecipitations were demonstrated by Western blotting (see “Experimental Procedures”). In other experiments not shown, we confirmed that the 125I-labeled CD14 immunoprecipitates were not derived from sCD14 that may have bound to the cells. When we cross-linked 125I-ASD-LPS to sCD14 and ran this product on the gel beside the 125I-labeled immunoprecipitate from the cell lysate, the 125I-s-CD14 was smaller (~46 kDa) than the 125I-mCD14 band (~55 kDa), as expected.

We could not measure LPS binding to MD-2 and Tlr4, which are present in very low abundance on the cell surface. We therefore used soluble recombinant proteins in a cell-free assay to measure the impact of LBP on the transfer of 125I-ASD-LPS from CD14 to MD-2. As shown in Fig. 5, sCD14 (lane 1) transferred LPS to sMD-2 (lane 2). LBP inhibited LPS transfer from sCD14 to sMD-2 (lanes 3 and 4) in keeping with the previous finding that LBP can inhibit the binding of free LPS to sMD-2 (17). When LBP was added after LPS had already been transferred to sMD-2 (lanes 5 and 6), LBP removed a significant amount of the LPS from sMD-2 (lanes 7 and 8). Taken together, our data support the hypothesis that LBP may inhibit the transfer of LPS from mCD14 to the Tlr4-MD-2 signaling receptor.
LBP Inhibits Responses to Cell-bound LPS

**DISCUSSION**

Our data reveal a new mechanism by which LBP can inhibit cell responses to LPS. LBP rapidly removed up to 75% of cell-bound LPS from mCD14 (Fig. 4) and a significant amount of LPS from MD-2 (Fig. 5), suggesting that LBP attenuates signal responses by interfering with LPS interactions with the extracellular domains of mCD14 and the Tlr4-MD-2 receptor complex. Whereas our data show that LBP promoted some release of the cell-associated LPS into the medium (Fig. 3), it seems unlikely that this low percentage of LPS release could account for the strong inhibitory effect. The ability of LBP to rapidly and almost completely remove cell-bound LPS from mCD14 provides a more likely explanation for the impact of LBP on cellular responses. Our data also suggest that a significant amount of the cell-bound LPS forms a complex with LBP that remains associated with the cells. It is unclear if the LPS-LBP complexes remain bound to mCD14 or if they move to another membrane location. If the complexes remain associated with mCD14, their inability to stimulate the cells is consistent with the previous finding that most ternary LBP-LBP-mCD14 complexes do not trigger signal responses and are eventually internalized (30). If the LPS-LBP complexes become bound to another membrane structure, they may not be able to induce signaling, because LPS-LBP complexes can induce little or no signal response without the help of CD14 (10). Seydel and co-workers (36) showed that LBP may insert into monocyte membranes and enhance cell responses to LPS. In their experiments, LBP did not inhibit monocyte activation when LBP and LPS were added together, possibly because the LBP concentrations (≤0.2 μg/ml) in their experiments were below the threshold required for inhibition.

Although we used sCD14 to promote the binding of LPS to
higher concentrations (1 ng/ml) promote beneficial inflammation at local sites of infection found in human blood may help to prevent LPS-induced systemic effects. Moderate to high concentrations of LBP and sCD14 that are inhibitory mechanism of sCD14 differs from that of LBP. Taken together, these findings suggest mechanisms by which the moderate to high concentrations of LBP and sCD14 that are found in human blood may help to prevent LPS-induced systemic inflammation, whereas lower concentrations of these proteins, which presumably occur in extravascular fluids, may promote beneficial inflammation at local sites of infection (40–42).

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Patricia A. Thompson, Peter S. Tobias, Suganya Viriyakosol, Theo N. Kirkland and Richard L. Kitchens

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