Lipoplysaccharide (LPS)-binding Protein Accelerates the Binding of LPS to CD14

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Summary
CD14 is a 55-kD protein found as a glycosylphosphatidylinositol (GPI)-anchored protein on the surface of monocytes, macrophages, and polymorphonuclear leukocytes, and as a soluble protein in the blood. Both forms of CD14 participate in the serum-dependent responses of cells to bacterial lipopolysaccharide (LPS). While CD14 has been described as a receptor for complexes of LPS with LPS-binding protein (LBP), there has been no direct evidence showing whether a ternary complex of LPS, LBP, and CD14 is formed, or whether CD14 binds LPS directly. Using nondenaturing polyacrylamide gel electrophoresis (native PAGE), we show that recombinant soluble CD14 (rsCD14) binds LPS in the absence of LBP or other proteins. Binding of LPS to CD14 is stable and of low stoichiometry (one or two molecules of LPS per rsCD14). Recombinant LBP (rLBP) does not form detectable ternary complexes with rsCD14 and LPS, but it does accelerate the binding of LPS to rsCD14. rLBP facilitates the interaction of LPS with rsCD14 at substoichiometric concentrations, suggesting that LBP functions catalytically, as a lipid transfer protein. Complexes of LPS and rsCD14 formed in the absence of LBP or other serum proteins strongly stimulate integrin function on PMN and expression of E-selectin on endothelial cells, demonstrating that LBP is not necessary for CD14-dependent stimulation of cells. These results suggest that CD14 acts as a soluble and cell surface receptor for LPS, and that LBP may function primarily to accelerate the binding of LPS to CD14.

Recent work has described several serum and cell surface proteins that are necessary for responses of leukocytes to low concentrations of bacterial LPS (endotoxin) (1). LPS-binding protein (LBP), an acute phase reactant, binds LPS (2) and greatly enhances the sensitivity of cells to LPS (3). Normal serum and plasma also enhance responses to LPS, and a multicomponent factor termed septic has been proposed to serve this function (4). LBP (5) and septic (4) each bind to LPS-coated particles and promote the interaction of these particles with CD14 (6, 7), a glycosylphosphatidylinositol (GPI)-anchored protein of monocytes, macrophages, and PMN (8, 9, 10). CD14 is necessary for serum- or LBP-mediated responses of cells to LPS, such as the production of TNF by monocytes (6) and an increase in the adhesive properties of β1-integrins on PMN (7).

Cells that do not express CD14, such as endothelial cells, also respond to low concentrations of LPS in the presence of serum. We have shown that these responses require a soluble form of CD14 (sCD14) (11), which is found at significant concentrations (2–6 μg/ml) in serum (12, 13). The involvement of soluble CD14 in inducing functional responses of cells to LPS has recently been confirmed by other laboratories (14–16).

CD14 on macrophages binds erythrocytes coated with LPS and LBP, but not erythrocytes coated with LPS or LBP alone (5, 6). Moreover, CD14-dependent responses of monocytes to low concentrations of LPS occur in response to LPS and LBP together, but not to either LBP or LPS alone (3). CD14 has thus been known as a receptor for LPS–LBP complexes. Here we provide direct evidence that rsCD14 binds LPS in the absence of other proteins, and that rsCD14–LPS complexes initiate physiologic responses from both CD14-expressing and CD14-negative cells. We further show that rLBP increases the rate of LPS binding to rsCD14 but is not necessary for inducing functional responses of cells.
Materials and Methods

Reagents. LPS from Salmonella minnesota strain R595 (Re), S. minnesota strain R60 (Ra), and from Escherichia coli 011:B4 were purchased from List Biological Laboratories (Campbell, CA). 3H-labeled LPS from E. coli K12 strain LCD25 (17) was a generous gift of Dr. Robert Munford (University of Texas Southwestern Medical Center, Dallas, TX). Synthetic lipid IVa (LA-14-PP) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Anti-C14 mAbs 3C10 (18), 60b (19), and 26ic (19) were purified from ascites fluid by chromatography on Protein G. Anti-E-selectin mAb BB11 (20) was a generous gift of Dr. R. Lob6 (Biogen Inc., Cambridge, MA). Fresh frozen normal human plasma (NHP) was supplied by the New York Blood Center (New York).

Stable Expression of rsCD14. An expression plasmid containing the full-length cDNA for human CD14 (9) was used as a template in a PCR to generate a fragment that lacks codons for eight COOH-terminal amino acids (rsCD14). The oligonucleotide pair (5' GTC CCT CTA GAC CAC CAT GGA GCG CTC C + 5' TCC AGG TCG ACT TAC AGC ACC AGG GTC CCG CCA) used in the PCR was designed to introduce an XbaI site and a perfect Kozak sequence preceding the rsCD14 initiator codon, and to introduce a SalI site after the stop codon introduced after amino acid 367 in CD14. DNA sequence analysis verified the PCR-generated changes as well as the integrity of the remainder of the CD14 cDNA. The rsCD14 gene was then cloned as a XbaI–SalI fragment into the mammalian expression vector pDSRcr (European Patent Application A20398753) that was modified to include unique XbaI and SalI restriction sites. pDSRcr2 allows high-level expression of recombinant proteins in Chinese hamster ovary (CHO) transfectants. The rsCD14 expression vector was used to transfect a CHO cell line deficient in dihydrofolate reductase (CHO D) (22) by selecting for transfectants in medium lacking hypoxanthine and thymidine (23). An RNase protection assay (24) was used to screen for transfectants that had high levels of rsCD14-specific mRNA. A single clone was grown without serum as described (23) to generate conditioned medium containing rsCD14.

Purification of rsLBP. rsLBP-containing conditioned medium was concentrated 17-fold using an S10Y30 spiral wound cartridge (Amicon). Concentrated medium was diluted 1:3 with 50 mM sodium phosphate, pH 7.3, 2 mM EDTA, 40 mM NaCl, then applied to a Mono S column (Pharmacia Fine Chemicals) that had been equilibrated with the same buffer. The column was then washed with equilibration buffer to baseline absorbance, and bound proteins were eluted with a 120-ml gradient of 40–300 mM NaCl in 50 mM phosphate, pH 7.3, 2 mM EDTA. rsLBP was detected by silver staining a 10% SDS-PAGE gel loaded with eluted proteins. NH2-terminal sequencing confirmed an isolated 60-kD band as rsLBP. Purity was estimated at greater than 95% and the Limulus amebocyte lysate assay indicated that endotoxin levels were less than 0.9 ng LPS/mg rsLBP. Purified rsLBP exhibited the predicted biological activities. It enabled responses of PMN to low doses of LPS but did not by itself stimulate cells (Fig. 1). rsLBP also opsonized LPS-coated erythrocytes for recognition by CD14 on macrophages (Fig. 2).

Electrophoresis. Proteins and sonicated LPS were diluted in PD-EDTA (Dulbecco's PBS lacking Ca2+ and Mg2+, with 1 mM EDTA) and incubated at 37°C for various times, and bromophenol blue and glycerol were added before electrophoresis. Tris-glycine polyacrylamide gradient gels (continuous buffer, pH 8.6; 1.0 or 1.5 mm thick) were purchased from Novex (San Diego, CA) and electrophoresed at 100–150 V for 2 h in a running buffer containing 192 mM glycine, 24 mM Tris, pH 8.3, without detergents. Ammoniacal silver staining was performed according to published procedures (26). Gels containing 3H-LPS were fixed in 30% methanol,

Figure 1. Adhesion of PMN to fibrinogen in response to LPS and plasma or LPS and LBP. The indicated concentrations of LPS (Re, R595) alone (○), with 2% NHP (●), or with 1 μg/ml rLBP (△) were incubated with PMN and adhesion to fibrinogen-coated wells was measured as described in Materials and Methods. Mean values for triplicate determinations are shown. Bars, PMN were pretreated with 10 μg/ml of the indicated anti-C14 mAb, then washed and incubated with 100 ng/ml LPS and 2% NHP before measuring adhesion.
rate experiments. was then measured as described in Materials and Methods. Results are

Figure 2. rsCD14 enables HUVEC to express E-selectin in response to LPS. HUVEC were incubated for 4 h with the indicated concentrations of LPS in the presence of medium alone (■), 0.5% CD14-depleted human serum (○), 0.5% CD14-depleted serum reconstituted with 0.07 µg/ml rsCD14 (Δ), or 0.07 µg/ml rsCD14 (△). Expression of E-selectin was then measured as described in Materials and Methods. Results are expressed in arbitrary fluorescence units and are representative of six separate experiments.

10% acetic acid for 1 h, soaked in EN'HANCE (Dupont, Boston, MA), washed in dH2O, dried, and exposed to Kodak XAR film for 2-10 d.

Human Umbilical Vein Endothelial Cells (HUVEC). HUVEC were cultured, stimulated with LPS, and assayed for expression of E-selectin (ELAM-1) exactly as described (11). Briefly, monolayers of confluent cells were exposed to dilutions of serum or CD14-depleted serum and LPS for 4 h at 37°C. The monolayers were washed, and antigen expression was measured using the anti-E-selectin monoclonal antibody 2G12, which does not block responses of monocytes or PMN or LPS (6). A control anti-CD14 monoclonal antibody, 26ic, which does not block responses of monocytes (Van Kessel, K. P. M., and S. D. Wright, manuscript in preparation). We have previously shown that PMN bind fibrinogen-coated wells in response to LPS and LBP or LPS and plasma (Fig. 1). The stimulation of binding by LPS requires CD14 since binding is blocked by 60b and 3C10, anti-CD14 mAbs that have previously been shown to block LPS-dependent responses of CD14-bearing cells (6). A control anti-CD14 monoclonal antibody, 26ic, which does not block responses of monocytes or PMN or LPS (6), did not block responses in this assay (Fig. 1, bars). Binding of the stimulated cells to the fibrinogen-coated surface is mediated by leukocyte integrins (Van Kessel, K. P. M., and S. D. Wright, manuscript in preparation).

Results

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Addition of rsCD14 with LPS caused increased expression of E-selectin on HUVEC even in the absence of LBP or serum proteins (Fig. 2). This result confirms our previous finding that scCD14 isolated from serum enables an LPS-dependent but serum-independent response from three types of cells that do not bear CD14: HUVEC, bovine endothelial cells, and the astrocytoma cell line U373 (11). It is also consistent with the recent findings of several laboratories (14-16, Sundan, A., S. D. Wright, and T. Espevik, manuscript in preparation).

Adhesion of PMN to Fibrinogen. We have previously shown that PMN respond to LPS with a dramatic rise in the ability of the leukocyte integrin CR3 (Mac-1, αMβ2, CD11b/CD18) to bind C3bi-coated erythrocytes (7). CR3 recognizes not only C3bi but also fibrinogen (27, 28), and thus the activation of CR3 on PMN can be measured using surface-bound fibrinogen rather than surface-bound C3bi as the ligand (Van Kessel, K. P. M., and S. D. Wright, manuscript in preparation). We have measured the effect of LPS on PMN by observing adhesion of PMN to fibrinogen-coated surfaces. Briefly, PMN were purified from freshly drawn blood from normal human donors using Neutrophil Isolation Medium (Cardinal Associates, Santa Fe, NM), according to the manufacturer's instructions. Suspensions of 5 × 10⁶ PMN/ml in HAP (Dulbecco's PBS containing 0.5 U/ml of aprotinin, 0.05% (wt/vol) human serum albumin, and 3 mM D-glucose) were fluorescently labeled with 5 µM 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester for 20 min at room temperature. The PMN were then pelleted and resuspended at 2.5 × 10⁶/ml in HAP. 72-well Terasaki plates (Robbins Scientific Corp., Sunnyvale, CA) were coated with 1 mg/ml human fibrinogen in PBS for 1 h at room temperature, and washed three times with PBS. 5 µl of PMN were added to each well, followed by 5 µl of protein/LPS samples diluted in PD-EDTA. After a 20-min incubation at 37°C, fluorescence was measured in a Cytofluor apparatus (Millipore Corp.). Plates were washed vigorously in PBS to remove unbound PMN, and fluorescence was measured again. Percent adhesion was calculated as postwash fluorescence divided by prewash fluorescence for each well. Values given in figures are mean values for triplicate determinations.

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that sCD14 from serum, urine, and transfected cells can enable responses of cells to LPS in the apparent absence of serum or LBP. In our previous study (11) we speculated that this apparent lack of a need for serum opsonins could be explained if the sCD14 from serum were contaminated with serum opsonins. This explanation is unlikely for the sCD14 employed here, since rsCD14 was isolated from serum-free conditioned medium. Moreover, rsCD14 is unlikely to be physically associated with a protein of significant mass, since monomeric rsCD14 from a gel filtration column was capable of stimulating HUVEC in an LPS-dependent and serum-independent fashion (not shown). These results suggest that rsCD14 may interact with LPS directly, without the intercession of opsonic proteins such as LBP or septin.

rsCD14 Prevents Opsonization of ELPS for Recognition by Macrophages. Membrane-bound CD14 (mCD14) is known to mediate binding of LBP-opsonized ELPS (ELPS-LBP) to macrophages (6). Addition of rsCD14 with rLBP during opsonization of ELPS inhibited the subsequent binding of the erythrocytes to macrophages (Fig. 3). This finding suggests that rsCD14 can bind to LPS-LBP complexes on the surface of the erythrocyte, blocking interaction with mCD14. However, rsCD14 was also able to prevent binding of ELPS-LBP to macrophages when it was incubated with ELPS in a step preceding opsonization with rLBP (Fig. 3). This observation suggests that rsCD14 is able to interact directly with LPS in the erythrocyte membrane, preventing subsequent opsonization of ELPS by rLBP. This observation, and the ability of rsCD14 to mediate functional responses of HUVEC to LPS, led us to investigate whether rsCD14 binds LPS directly.

sCD14–LPS Complexes Can Be Detected Using Native PAGE. We used native PAGE to examine the binding of rsCD14 to LPS in solution. When rsCD14 was incubated with LPS for 30 min at 37°C before electrophoresis, the rsCD14 band exhibited increased electrophoretic mobility. Three distinct forms of rsCD14 could be observed: Form 1, seen when rsCD14 was incubated without LPS (Fig. 4, lane 1); Form 2, of intermediate mobility (Fig. 4, lane 3), resulting from incubation of rsCD14 with 50 μg/ml LPS; and Form 3, of highest mobility (Fig. 4, lane 4), seen only after incubation of rsCD14 with very high concentrations (>500 μg/ml) of LPS. At lower LPS concentrations (5 μg/ml), both Forms 1 and 2 were present (Fig. 4, lane 2).

Several observations suggest that the shift in electrophoretic mobility described above is caused by stable binding of LPS to rsCD14. (a) The shift in mobility is not an artifact caused by high concentrations of LPS in native PAGE, since several unrelated proteins showed no change in electrophoretic mobility in native PAGE upon incubation with LPS (Fig. 5). (b) The proportion of rsCD14 migrating as Form 2 increased upon longer incubation with LPS before electrophoresis (see Fig. 11), suggesting that the phenomenon under study occurs.

![Figure 4](https://example.com/f4.png)

**Figure 4.** Incubation of LPS with rsCD14 induces a shift in the electrophoretic mobility of CD14 in native PAGE. rsCD14 (30 μg/ml) was incubated for 30 min at 37°C with different concentrations of LPS (Re), and run in a 4–20% native polyacrylamide gel, which was subsequently stained with silver. Lane 1, no LPS; lane 2, 5 μg/ml LPS; lane 3, 50 μg/ml LPS; lane 4, 500 μg/ml LPS. Positions of protein standards are indicated in kilodaltons.

![Figure 5](https://example.com/f5.png)

**Figure 5.** LPS does not affect the mobility of proteins unrelated to CD14. The indicated proteins were incubated at 30 μg/ml alone or with 10 or 100 μg/ml LPS (Re) for 2 h at 37°C, and run in a 4–12% native polyacrylamide gel, which was subsequently stained with silver. Lanes a, no LPS; lanes b, 10 μg/ml LPS; lanes c, 100 μg/ml LPS.
during incubation of LPS with rsCD14, not during electrophoresis. (c) There was no change in the mobility of rsCD14 in SDS-PAGE after incubation with LPS (not shown). This result rules out the possibility that incubation with LPS caused proteolytic degradation of rsCD14, resulting in increased electrophoretic mobility. (d) Most importantly, incubation of rsCD14 with radioactively labeled LPS yielded a radioactive band in native PAGE at the same position as rsCD14–LPS complexes seen with silver staining (Fig. 6).

We used LPS labeled to high specific activity with tritium ($^3$H-LPS) (17) to confirm that CD14–LPS complexes are stable in native PAGE. $^3$H-LPS ran with a high apparent molecular weight in native PAGE, presumably in micellar form (Fig. 6, lane 2). When incubated with rsCD14 before electrophoresis, a portion of the $^3$H-LPS migrated in a complex with rsCD14, at the same position as rsCD14–LPS complexes seen in silver-stained gels (Fig. 6, lane 3). Incubation with as much as 100 μg/ml human serum albumin did not affect the mobility of $^3$H-LPS (not shown), suggesting that formation of a complex with LPS that is stable in native PAGE may be specific for CD14. While we have not observed $^3$H-LPS comigrating with rLBP in native PAGE, rLBP does increase the mobility of $^3$H-LPS (see Fig. 8, lanes 1 and 5), suggesting a rapidly reversible interaction of LBP with LPS.

To further explore the specificity of the interaction of LPS with rsCD14, binding was measured in the presence of competing doses of unlabeled endotoxins. Unlabeled LPS completely eliminated the $^3$H-LPS–rsCD14 band on native gels (Fig. 6). Competition was seen with rough and smooth forms of LPS, as well as with lipid IVα, a synthetic precursor of LPS. These forms of LPS also caused a shift in the mobility of rsCD14 in native PAGE, but the shift was less pronounced than that caused by Re LPS (data not shown).

**LBP Accelerates Binding of LPS to rsCD14 without Forming Stable Complexes with rsCD14 and LPS.** To determine whether LBP forms a stable complex with LPS and CD14, we incubated various concentrations of rLBP and rsCD14 with LPS and analyzed the samples by native PAGE. There was no decrease in staining of monomeric rsCD14 or rLBP, and no proteins were seen at positions predicted for LBP-CD14 dimers or higher order aggregates (Fig. 7). Thus, we were not able to detect complexes of rLBP with rsCD14 and LPS. However, rLBP had a profound effect on the binding of LPS to rsCD14. During 30-min incubations of $^3$H-LPS with rsCD14, the amount of rsCD14–LPS complex formed at a given concentration of rsCD14 was greatly increased in the presence of rLBP (Fig. 8). These results show that rLBP promotes the interaction of LPS and rsCD14 without forming part of the final complex, and suggests that rLBP increases the rate of binding of LPS to CD14.

To confirm the kinetic effect of rLBP on interactions of rsCD14 with LPS, we examined the time course of the binding of $^3$H-LPS to a molar excess of rsCD14. The rate of binding of $^3$H-LPS to rsCD14 was dramatically increased by rLBP at a molar concentration ∼150-fold lower than the concentration of $^3$H-LPS (Fig. 9). rLBP did not appear to affect the affinity of $^3$H-LPS for rsCD14, because a quantitative shift of $^3$H-LPS from its aggregated form to complexes with rsCD14 was seen in the presence or absence of rLBP. However, the time required for complete binding to rsCD14 was reduced from several hours to less than 30 min by the presence of rLBP. After 30 min of incubation, less than half of the $^3$H-LPS was bound to rsCD14 in the absence of rLBP (Fig. 9, lane 4), whereas in the presence of rLBP, virtually all of the LPS appeared to be bound to rsCD14 (Fig. 9, lane 8).

**Figure 6.** $^3$H-LPS comigrates with rsCD14 in native PAGE. $^3$H-LPS (1 μg/ml) was incubated with or without rsCD14 (50 μg/ml) for 4 h at 37°C, then run in an 8–16% native polyacrylamide gel. Fluorography was performed as described in Materials and Methods. Lane 1, $^{14}$C-molecular mass markers (Sigma Chemical Co.), indicated in kilodaltons; lane 2, $^3$H-LPS; lane 3, $^3$H-LPS and rsCD14. Lanes 4–15 show competitive inhibition of the binding of $^3$H-LPS to rsCD14 with unlabeled endotoxins. Unlabeled LPS (lane a, 5 μg/ml; lane b, 50 μg/ml; lane c, 500 μg/ml) was added with $^3$H-LPS to rsCD14 and incubated as in lane 3. Lanes 4–6, lipid IVα: lanes 7–9, Re LPS (R595); lanes 10–12, Ra LPS (R50); lanes 13–15, smooth LPS (O111:B4).

**Figure 7.** rLBP does not form stable complexes with rsCD14 and LPS. rsCD14 and rLBP were incubated with or without LPS (Re) at the indicated concentrations for 30 min at 37°C and run in a 4–20% native polyacrylamide gel, which was subsequently stained with silver. Lane 1, rsCD14 (30 μg/ml) and rLBP (3 μg/ml); lanes b, rsCD14 (30 μg/ml) and rLBP (5 μg/ml); lanes c, rsCD14 (30 μg/ml) and rLBP (10 μg/ml); lanes d, rsCD14 (30 μg/ml) and rLBP (30 μg/ml); lanes e, rsCD14 (10 μg/ml) and rLBP (30 μg/ml); lanes f, rsCD14 (10 μg/ml) and rLBP (30 μg/ml). The rsCD14 migrates as Form 1 in lane 1, as Form 2 in lanes 2–8, and as form 3 in lane 9.

**Figure 8.** rLBP increases the amount of LPS bound to rsCD14. $^3$H-LPS (3 μg/ml) was incubated with the indicated concentrations of rsCD14 and rLBP for 30 min at 37°C, run in a 4–20% native polyacrylamide gel, and detected by fluorography.
LBP Enables Stoichiometric Binding of LPS to CD14 at Substoichiometric Concentrations. To confirm that LBP was effective in substoichiometric concentrations, we used silver staining of native PAGE to examine the proportion of rsCD14 that binds to LPS (Re) in the presence or absence of rLBP (Fig. 10). During a 30-min incubation, the presence of rLBP increased the amount of rsCD14 exhibiting increased mobility at a given LPS concentration. When approximately equimolar concentrations of rsCD14 and LPS were used (Fig. 10, lanes 4 and 8), the presence of 20-fold less rLBP resulted in half of the rsCD14 binding to LPS, as measured by densitometric scanning (not shown). A single molecule of rLBP thus caused the transfer of at least 10 molecules of LPS to rsCD14 in 30 min, confirming that LBP is able to accelerate binding at substoichiometric concentrations. These results indicate that LBP facilitates binding of LPS to rsCD14 without being consumed, and we thus propose that LBP acts as a classical catalyst to transfer LPS to CD14. A further consequence of this model is that LBP is not directly involved in the stimulation of LPS-responsive cells (see below).

An estimate of the stoichiometry of the binding of LPS and rsCD14 can be made based on the amount of rsCD14 complexed with a given concentration of LPS. As noted above, equimolar concentrations of LPS and rsCD14 resulted in half of the rsCD14 binding to LPS, when rLBP was present to facilitate binding (Fig. 10, lane 8). This suggests a low stoichiometry of binding of LPS to rsCD14, i.e., one or two LPS molecules per molecule of CD14. The stoichiometry of binding may also be estimated by observing the amount of rsCD14 needed to completely shift the mobility of a fixed dose of $^2H$-LPS. 17 $\mu$g/ml of rsCD14 ($M_e \approx 50,000$) bound nearly all of a twofold excess of $^3H$-LPS (3 $\mu$g/ml; $M_e \approx 4,000$) (Fig. 8, lane 7), suggesting again that one or two LPS molecules bind to CD14 to cause a shift in mobility.

CD14-LPS Complexes Cause Activation of PMN. HUVEC are induced to synthesize E-selectin by mixtures of LPS and rsCD14 but not by either reagent alone (Fig. 2), suggesting that rsCD14 and LPS form complexes that activate cells. To explore this possibility, rsCD14 and LPS were incubated at 37°C for various times, and samples were tested for their ability to increase adhesion of PMN to fibrinogen (Fig. 11) and also examined by native PAGE for the extent of binding of LPS and rsCD14 (Fig. 11, inset). Our assay of PMN activation permits kinetic analysis since cells are exposed to LPS for only 20 min as opposed to the several hours used in assays of cytokine production or expression of surface molecules. We found that preincubation of rsCD14 with LPS was necessary to observe stimulation of PMN. Full activity in this assay required incubation of LPS with rsCD14 for at least 30 min before adding the samples to the PMN. The extent of binding of LPS to rsCD14, seen as the proportion of rsCD14 showing increased mobility in native PAGE, followed a similar time course (Fig. 11, inset). LPS with rLBP also caused adhesion of PMN to fibrinogen-coated surfaces (Fig. 1), but no preincubation of LPS with rLBP was required to observe full activity (not shown). These experiments strongly suggest that rsCD14 and LPS must form complexes in order to activate...
cells, and show that rLBP and other serum proteins are not necessary for stimulating cells with rsCD14–LPS complexes. They also suggest that the interaction of LPS with LBP is much faster than the binding of LPS to CD14, which is consistent with the proposed role of LBP in facilitating the transfer of LPS to CD14.

We wish to stress that rsCD14 enhances the adhesion of PMN to fibrinogen (Fig. 11) yet blocks the adhesion of macrophages to ELPS (Fig. 3) because these two adhesion assays measure entirely different adhesion events. Adhesion of ELPS to macrophages represents binding of LPS on the erythrocyte to membrane-bound CD14 on the macrophages, and competition by rsCD14 is thus easy to envision. On the other hand, the adhesion of PMN to fibrinogen represents binding of leukocyte integrins on the PMN to fibrinogen on plastic surfaces. Since CD14 does not serve as a receptor or a ligand in this adhesion, rsCD14 should not block adhesion. Rather, our assay exploits the role of CD14 as an agonist; rsCD14 stimulates the leukocyte to activate its integrins and adhesion is the readout.

Discussion

Here we show that rLBP accelerates the direct binding of LPS to rsCD14 and that LPS–rsCD14 complexes stimulate cells. These findings suggest a general mechanism for the interaction of LBP, LPS, and CD14 that is consistent with and explains a number of findings in the literature. Some studies with CD14-bearing cells have shown an apparently absolute requirement for LBP or serum proteins in mediating CD14-dependent stimulation of cells in the absence of LBP or serum proteins, can stimulate cells in a CD14-dependent fashion (29–31). CD14-dependent stimulation of cells in the absence of LBP or serum was also seen in cells that lack cell surface CD14 (11), a finding confirmed here and in additional recent reports (14–16). Finally, Kitchens et al. (31) observed low levels of CD14-dependent binding of radiolabeled LPS to cells in the absence of LBP. Our observation that LBP facilitates binding of LPS to CD14 but is not absolutely required for binding offers an explanation for these findings. Assays employing a very short incubation of cells with LPS, such as the assay measuring adhesion of PMN to fibrinogen described here (20 min), may show a strong requirement for LBP while assays that require longer incubations, such as induction of cytokine synthesis in macrophages, provide time for uncatalyzed binding of LPS to CD14. These assays show that LBP increases the rate and sensitivity of the response (3). The above interpretation is underscored by the finding that the strongly LBP-dependent activation of PMN (Fig. 1) can be made LBP-independent by preincubation of LPS with rsCD14 (Fig. 11).

The binding of LPS to CD14 is strongly enhanced by sub-stoichiometric concentrations of LBP. Fig. 10 shows that each molecule of LBP causes the movement of 10 molecules LPS into CD14. If the LBP, previously shown to have a binding site for a single LPS molecule (32), were consumed upon transfer of that molecule of LPS, it could not perform multiple rounds of this reaction. Since we see multiple rounds of transfer, the LBP must not be consumed. This point is amplified in Fig. 9. In the first 10 min of the reaction, each molecule of LBP has transferred at least 50 copies of LPS (about one third of the total) to CD14. If the LBP were consumed after these 50 cycles, the reaction would halt. However, it is clear from Fig. 9 that in the following 20 min the LBP continues to transfer LPS to CD14, not stopping until all of the LPS (a 150-fold molar excess over LBP) is transferred. Noncatalytic transfer of LPS to CD14 is negligible during this interval. While it is possible that a small fraction of the LBP is inactivated or lost upon each round of transfer, measurements of this loss would be difficult and would not materially alter the finding that LBP may transfer LPS to CD14 without being consumed. We conclude from these findings that LBP acts as a lipid transfer protein, a view consistent with the structural homology of LBP to cholesterol ester transfer protein (CETP), a protein that redistributes cholesterol esters among lipoprotein particles. We believe that binding of LBP to CD14 is necessary to achieve transfer of LPS to CD14. However, the interaction of LBP with CD14 is likely to be short-lived, and we presume that this explains our inability to observe stable complexes of LBP and rsCD14 in native PAGE. The interaction of LBP with CD14 might be more stable when surface-bound components, rather than soluble components, are used. Particles coated with LPS–LBP complexes bind to CD14 on macrophages, suggesting the formation of a relatively stable ternary complex. We have observed, however, that ELPS–LBP detach from macrophages upon incubation at 37°C (Wright, S. D., unpublished observation), suggesting that even the interaction of surface-bound CD14 and LBP is transient. The apparent catalytic role of LBP suggests that its description as a "binding protein" may be misleading and that to reflect its function faithfully, assays of LBP should measure a rate.

We have demonstrated here that rsCD14 binds LPS directly, and that complexes of LPS and rsCD14 are biologically active. Stimulation of PMN by LPS was dramatically enhanced by rsCD14, and the magnitude of the response to LPS increased with the degree of binding of LPS to rsCD14 (Fig. 11). These observations confirm the biological importance of the binding of LPS to CD14. They further suggest that the role of rLBP is limited to accelerating movement of LPS into CD14 and that it may not play a further role in interactions with cells and signal transduction.

Our finding of direct stoichiometric binding of LPS to CD14 confirms several suggestive findings in the literature. Tobias et al. observed that 125I-ASD-LPS, a photoactivatable cross-linking probe, could be cross-linked to mCD14 (33) and sCD14 (15), when added in the presence, but not in the absence, of LBP. These studies suggest a close association of LPS with CD14 in LPS-LBP–CD14 complexes, but do not establish specific binding of LPS to CD14. Very recently, Haziot et al. (14) showed binding of biotinylated LPS to plastic plates that was increased significantly if the plates were first coated with recombinant CD14, a finding consistent with direct binding of LPS to CD14. Our results represent the first direct evidence for stoichiometric binding of LPS to CD14.
using soluble components, and the first study linking this binding with functional responses of cells.

The binding of LPS to rsCD14 that we observed appears to be of high affinity inasmuch as complexes were stable during electrophoresis. The affinity of LBP for LPS has been reported as \( \sim 10^{-9} \) M (32), and the affinity of rsCD14 for LPS must be greater than this, since LBP facilitates binding of CD14 to LPS, rather than competing for binding to LPS. Strong interaction with a lipid such as LPS is consistent with the primary structure of CD14, which exhibits 13 “leucine-rich” motifs. These repeat motifs have been proposed to bind lipids by forming amphipathic beta sheets (34, 35).

It should be noted that most of our studies used concentrations of LPS much higher than the presumed binding affinity of CD14 and much higher than the concentrations needed to stimulate cells. Our studies required high concentrations of LPS in order to equal the molar concentration of rsCD14 needed for detection in our gel assays. However, high concentrations of LPS do not appear to be required for binding of LPS to rsCD14; the concentration of free, soluble LPS appears to be very low after incubation with rsCD14 (Fig. 8, lanes 7 and 8; Fig. 9, lanes 9–13). We are currently seeking methods to accurately measure binding of LPS to CD14 using lower concentrations of these reagents.

We observed a discrete enhancement of electrophoretic mobility of rsCD14 upon binding of LPS. One explanation of this finding is that binding of each LPS molecule (Re, R595) only adds \( \sim 3 \) kD of weight, a small increment in a protein of \( \sim 50 \) kD, but adds at least four negative charges (36); complexes of CD14 and LPS might therefore have a higher charge/mass ratio than monomers of CD14. Alternatively, a conformational change in CD14 may occur upon binding LPS, resulting in a more compact tertiary structure. Our studies do not distinguish between these two possibilities.

The LPS–rsCD14 complexes formed with equimolar LPS and rsCD14 (Fig. 10, lane 8) had the electrophoretic mobility of Form 2 (Fig. 4). However, there was a second discrete increase in the mobility of rsCD14, which required at least a 100-fold molar excess of LPS (Form 3, Fig. 4). Thus, rsCD14 may have an additional, lower-affinity binding site for LPS. Because the biological activity of rsCD14–LPS complexes correlated with a shift in mobility of rsCD14 to Form 2 (Fig. 11), the second shift and the proposed second binding site may not be physiologically relevant.

Smooth LPS, several forms of rough LPS, and the biosynthetic LPS precursor lipid IVa all competed for binding of \( ^3 \text{H}-\text{LPS} \) to CD14, suggesting that CD14 can bind many forms of LPS (Fig. 6). The interaction of CD14 with lipid IVa is consistent with the studies of Kitchens et al. (31), who observed that deacylated LPS, which, like lipid IVa lacks secondary fatty acids, is bound by leukocytes in a CD14-dependent fashion. Whereas rough and smooth LPS strongly promote cellular responses, lipid IVa and deacylated LPS fail to stimulate cells, acting instead as antagonists of LPS (37). Since lipid IVa is recognized by both LBP (33) and CD14 (Fig. 6), the discrimination between agonist and antagonist may occur in a step subsequent to binding CD14, as has been previously suggested (36).

LPS–rsCD14 complexes induced expression of E-selectin on HUVEC, a cell type that does not express mCD14, and induced activation of leukocyte integrins on PMN, a cell type that does express functionally active mCD14. These data suggest that sCD14 may play an important role in the responses of all cells to LPS. The importance of sCD14 is further supported by its abundance in serum: sCD14 represents at least 99% of the total amount of CD14 in blood, the remaining 1% being found on the surface of phagocytic cells. Since it is present in at least 1,000-fold molar excess of the maximal amount of LPS present during sepsis (38), we believe that sCD14 represents an enormous reservoir for binding LPS in the blood, and that partitioning of LPS into this and other reservoirs may play a crucial role in modulating the host response to gram-negative bacterial infection.

We thank Drs. Patricia A. Detmers, Leslie A. Leonard, C. Thomas Park, and Bo Yu for critical reading of the manuscript.

This work was supported by United States Public Health Service grant AI-30556 and by the Medical Scientist Training Program (E. Hailman and M. M. Wurfel).

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Received for publication 13 August 1993 and in revised form 12 October 1993.

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