Soluble CD14 Acts as a Shuttle in the Neutralization of Lipopolysaccharide (LPS) by LPS-binding Protein and Reconstituted High Density Lipoprotein

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Summary

We have recently shown that lipopolysaccharide (LPS)-binding protein (LBP) is a lipid transfer protein that catalyzes two distinct reactions: movement of bacterial LPS (endotoxin) from LPS micelles to soluble CD14 (sCD14) and movement of LPS from micelles to reconstituted high density lipoprotein (R-HDL) particles. Here we show that LBP facilitates a third lipid transfer reaction: movement of LPS from LPS–sCD14 complexes to R-HDL particles. This action of LBP is catalytic, with one molecule of LBP enabling the movement of multiple LPS molecules into R-HDL. LBP-catalyzed movement of LPS from LPS-sCD14 complexes to R-HDL neutralizes the capacity of LPS to stimulate polymorphonuclear leukocytes. Our findings show that LPS may be transferred to R-HDL either by the direct action of LBP or by a two-step reaction in which LPS is first transferred to sCD14 and subsequently to R-HDL. We have observed that the two-step pathway of LPS transfer to R-HDL is strongly favored over direct transfer. Neutralization of LPS by LBP and R-HDL was accelerated more than 30-fold by addition of sCD14. Several observations suggest that sCD14 accelerates this reaction by serving as a shuttle for LPS: addition of LBP and sCD14 to LPS micelles resulted in LPS–sCD14 complexes that could diffuse through a 100-kD cutoff filter; LPS–sCD14 complexes appeared transiently during movement of LPS to R-HDL facilitated by purified LBP; and sCD14 could facilitate transfer of LPS to R-HDL without becoming part of the final LPS–R-HDL complex. Complexes of LPS and sCD14 were formed transiently when LPS was incubated in plasma, suggesting that these complexes may play a role as intermediates in the neutralization of LPS under physiological conditions. These findings detail a new activity for sCD14 and suggest a novel mechanism for lipid transfer by LBP.

The serum proteins soluble CD14 (sCD14)[1] (1) and LPS-binding protein (LBP) (2) play a crucial role in enabling responses of cells to gram-negative LPS (endotoxin). We have recently found that CD14 binds LPS stably and with low stoichiometry (3), and that LPS–sCD14 complexes stimulate cells (3, 4). Spontaneous binding of LPS to CD14 occurs very slowly, but the rate of binding is dramatically accelerated by LBP. In this catalytic reaction, a single molecule of LBP may promote the movement of >100 molecules of LPS in 30 min (3). The acceleration of LPS binding to CD14 catalyzed by LBP may thus explain the increased sensitivity of CD14-bearing cells to LPS in the presence of LBP (2).

M. M. Wurfel and E. Hailman made equal contributions to this work.

In whole plasma, LBP may catalyze movement of LPS to destinations other than CD14. Lipoprotein particles, such as high density lipoproteins (HDL), bind LPS avidly (5, 6), presumably because of partitioning of the amphiphilic LPS molecule into the phospholipid surface of the lipoprotein. Binding of LPS to HDL effectively neutralizes its biological potency both in vitro and in vivo (5–7). Recently, reconstituted HDL particles (R-HDL) prepared from purified apolipoprotein A-I (apo A-I), phosphatidylcholine (PC), and cholesterol have been shown to protect from LPS-induced shock, fever, and death in animal models of endotoxemia (8, 9). We have found that the rate of spontaneous binding of LPS to R-HDL particles is negligible, but that LBP accelerates this rate (10). As with the LBP-mediated movement of LPS to CD14, LBP acts catalytically to promote partitioning of LPS into R-HDL (10). Thus, LBP may catalyze two distinct reactions with opposite biological effects. It may move LPS to CD14 and initiate cellular responses, or it may move LPS to R-HDL where it is biologically inert. Here we describe a third reaction catalyzed by LBP: movement of LPS from LPS–sCD14 complexes

to R-HDL. This finding suggests that R-HDL particles may be effective blockers of the response to LPS, even after formation of LPS–sCD14 complexes.

LBP catalyzes movement of LPS to sCD14 (3) and, as shown here, from sCD14 to R-HDL. These two reactions thus result in the net movement of LPS into R-HDL. Here we show that under defined conditions this two-step process may be far more efficient than direct LBP-mediated transfer of LPS to R-HDL: addition of sCD14 accelerated the LBP-mediated movement of LPS into R-HDL by as much as 100-fold. We propose that sCD14 accelerates the transfer of LPS from micelles to R-HDL by acting as a carrier or shuttle for LPS between these two sites.

Materials and Methods

Reagents. LPS from *Salmonella minnesota* strain R60 (Ra) and ³H-labeled LPS from *Escherichia coli* LCD25 (K12) were purchased from List Biological Laboratories (Campbell, CA). Human serum albumin (HSA) was purchased from Armour Pharmaceutical Co. (Kankakee, IL). Recombinant human LBP and sCD14, expressed and purified as described (3), were a generous gift of Dr. Henri Lichenstein (Amgen, Inc., Thousand Oaks, CA).

Preparation of R-HDL Particles. R-HDL was prepared by the sodium cholate dialysis method, as previously described (11). Briefly, apo A-I, purified by sequential ultracentrifugation and size exclusion chromatography (12), was mixed with egg PC, cholesterol, and cholate at a molar ratio of 80:4:1:80 (PC/cholesterol/apo A-I/cholate), and cholate was removed with extensive dialysis against PDEDTA (Dulbecco's PBS lacking Ca^{2+} and Mg^{2+}, with 1 mM EDTA), containing 0.01% sodium azide. Final preparations were stored in PDEDTA with 0.01% azide at 4°C. All concentration values for R-HDL particles are expressed as the equivalent concentration of apoA-I in micrograms per milliliter.

Formation of LPS–sCD14 Complexes. LPS and sCD14, at concentrations 500-fold higher than those stated in each figure, were incubated together for 5–18 h at 37°C in APBS (Dulbecco's PBS with Ca^{2+} and Mg^{2+}, 0.5% HSA). These high concentrations were designed to ensure complete binding of LPS to sCD14 as previously described (3).

Stimulation of PMN by LPS. To assess the biologic activity of LPS, we measured adhesion of human PMN to fibrinogen-coated surfaces as described (13). In this assay, stimulation of PMN adhesion is dependent on either the binding of LPS to cell surface CD14 in the presence of LBP, or exposure to preformed LPS–sCD14 complexes in the absence of LBP. Adhesion of the stimulated PMN to fibrinogen is mediated by the leukocyte integrin CD11b/CD18 (CR3, Mac-1) (13). Briefly, mixtures containing LPS were diluted in APBS to the concentrations indicated, yielding a final volume of 50 µl. 10 µl of freshly isolated PMN (2 × 10⁷ cells/ml in HAP [Dulbecco's PBS with 0.5 U/ml aprotinin, 0.05% HSA, 3 mM D-glucose]) fluorescently labeled with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester as described (13), were added and incubated for 10 min at 37°C to stimulate the cells. PMN were then washed into HAP and added to a 72-well Terasaki plate (Robbins Scientific, Sunnyvale, CA) precoated with fibrinogen. After 15 min at 37°C, adherence of PMN to the plate was quantitated. The fluorescence in each well was measured using a Cytofluor 2300 (Millipore Corp., Bedford, MA) as a way of quantitating the total number of cells per well. The plate was then washed and fluorescence was measured again. Binding is expressed as the percentage of cells remaining in the well after the washing step (adhe-

sion %). Donor-to-donor variation in maximal responses (25–75% adhesion) prohibited averaging results of separate experiments, but the pattern of responses was highly reproducible.

Neutralization of LPS and LPS–sCD14 Complexes. Neutralization of LPS was measured as the loss of the ability to stimulate adhesion of PMN to fibrinogen and was assayed as previously described (10). Briefly, LPS, R-HDL, and variable amounts of CD14 and LBP were diluted in APBS to a final volume of 50 µl. After incubation for the stated time at 37°C, the amount of available LPS remaining in the tube was assessed by adjusting the LBP concentration, if necessary, to 1 µg/ml with 2.5 µl of concentrated LBP, adding fluorescently labeled PMN and measuring adhesion as described above. The neutralization of LPS–sCD14 complexes by R-HDL and the effect of LBP on this neutralization was measured in a similar fashion, but with one difference. Because LPS–sCD14 complexes stimulate cells with no requirement for LBP (3), the concentration of LBP was not adjusted to 1 µg/ml before the addition of PMN.

Electrophoresis. [³H]LPS was sonicated for 1 min and incubated at 37°C with R-HDL and other proteins, as indicated in the figure legends. When [³H]LPS–sCD14 complexes were used, a preincubation step was added: [³H]LPS was sonicated and incubated with sCD14 overnight at 37°C in PDEDTA at concentrations fivefold higher than those used in each experiment, then added to samples with R-HDL and other proteins at the final concentrations indicated in the figure legends. Just before electrophoresis, 1/2 vol of a loading buffer containing 0.005% bromophenol blue and 20% glycerol in PBS or PDEDTA was added to each sample. The samples were run in continuous buffer (pH 8.6), nondenaturing Tris-glycine polyacrylamide gels (Novex, San Diego, CA) at 100–150 V for 2–3 h in a running buffer containing 192 mM glycine, 24 mM Tris, pH 8.3. After electrophoresis, the gels were fixed in 50% EtOH, 10% HAc for 1–18 h, soaked in ENHANCE™ (Dupont-NEN, Boston, MA) for 1 h, washed three times for 15 min in double-distilled H_2O, dried, and exposed to XAR film (Eastman Kodak Co., Rochester, NY) for 2 d.

Movement of [³H]LPS across 100-kD Cutoff Filters. [³H]LPS (10 ng/ml) was diluted in HAP with buffer, rLBP (1 µg/ml), rsCD14 (1 µg/ml), or rLBP and rsCD14 in a final volume of 500 µl and incubated for 60 min at 37°C. Each solution was then subjected to ultrafiltration using a Microcon unit (Amicon Corp., Beverly, MA) with a 100-kD cutoff membrane. Ultrafiltration was accomplished by centrifugation at 8,000 g, until all the solution had passed through the filter. Radioactivity in the ultrafiltrate was evaluated by scintillation counting in 3 ml of Ready Safe™ (Beckman Instruments, Palo Alto, CA). The movement of LBP and sCD14 across the ultrafiltration membrane was assessed by ELISA, as described (1, 10).

Immunoprecipitation of sCD14. Immunoprecipitation was used to examine the binding of [³H]LPS to sCD14 in plasma. 26ic, an mAb that recognizes CD14 without blocking binding of LPS (14), and IB4, an mAb directed against the β2-integrin molecule (15), were coupled to protein-G sepharose, using dimethyl pimelimidate as described (16). The resulting beads had ~1.5 mg of mAb bound per milliliter of wet beads and were stored as a 50% slurry. [³H]LPS (10 ng/ml; sp act 1,000 dpm/ng) and 40% normal human plasma (NHP) (vol:vol), diluted to a final volume of 200 µl in AHPBS (APBS with 5 U/ml heparin), were incubated for the stated interval at 37°C. 160 µl of the LPS – NHP mixture were placed on ice, split into two tubes of 80 µl, mixed with 20 µl of either 26ic or IB4-coupled beads, and incubated for 40 min at 4°C. After precipitating the beads by centrifugation at 2,000 g for 2 min, 90 µl of supernatant was recovered. 85 µl was saved for scin-
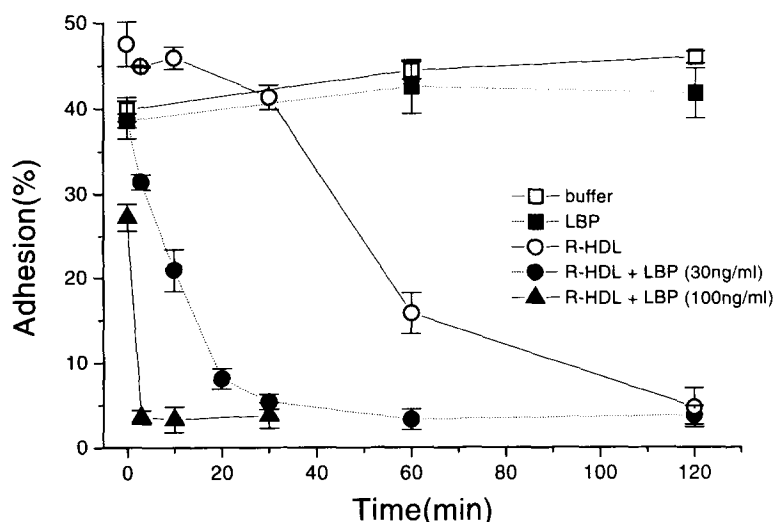
Figure 1. Spontaneous movement of LPS from LPS–sCD14 complexes to R-HDL is accelerated by LBP. LPS–sCD14 complexes (10 ng/ml and 1 μg/ml, respectively) were incubated for the stated intervals in the absence (□, O) or presence (30 ng/ml; ●), (100 ng/ml; ▲, ■), of LBP with (O, ●, ▲) or without (□, ■) R-HDL (100 μg/ml). Biologically active LPS–sCD14 complexes remaining were assessed by adding PMN and by measuring their binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.

tillation counting, and the remaining 5 μl were assayed for sCD14 by ELISA (1) to confirm that sCD14 had been successfully precipitated. The pelleted beads were then washed once with 20 vol of AHPBS, eluted with 300 μl of 0.1 M glycine pH 3.0, and the radioactivity in the eluate and the supernatant was evaluated by scintillation counting in 3 ml of Ready-Safe™. To assess the biologic activity of the LPS–NHP mixtures, CFSE-labeled PMN were added to the remaining 40 μl from each time point, incubated for 10 min at 37°C, washed, and evaluated for binding to fibrinogen-coated surfaces as described above.

Results

LBP Accelerates the Transfer of LPS from LPS–CD14 Complexes to R-HDL. We previously showed that LBP catalyzes transfer of LPS from micelles to CD14 (3) and transfer of

LPS from micelles to R-HDL (10). To determine if LBP also catalyzes movement of LPS from LPS–sCD14 complexes to R-HDL, complexes of LPS with sCD14 were produced by incubating LPS with a stoichiometric excess of sCD14 for at least 5 h at 37°C. As demonstrated in previous work from our laboratory (3), the resulting LPS–sCD14 complexes strongly stimulated integrin-mediated adhesion of PMN (Fig. 1), and stimulation of PMN was not observed unless the LPS and sCD14 were precincubated together (not shown). LPS–sCD14 complexes retained the capacity to stimulate PMN during incubation at 37°C for 120 min (Fig. 1) or longer (not shown). However, addition of R-HDL caused gradual loss of the stimulatory capacity of LPS–sCD14 complexes. R-HDL thus "neutralized" LPS–sCD14 complexes.
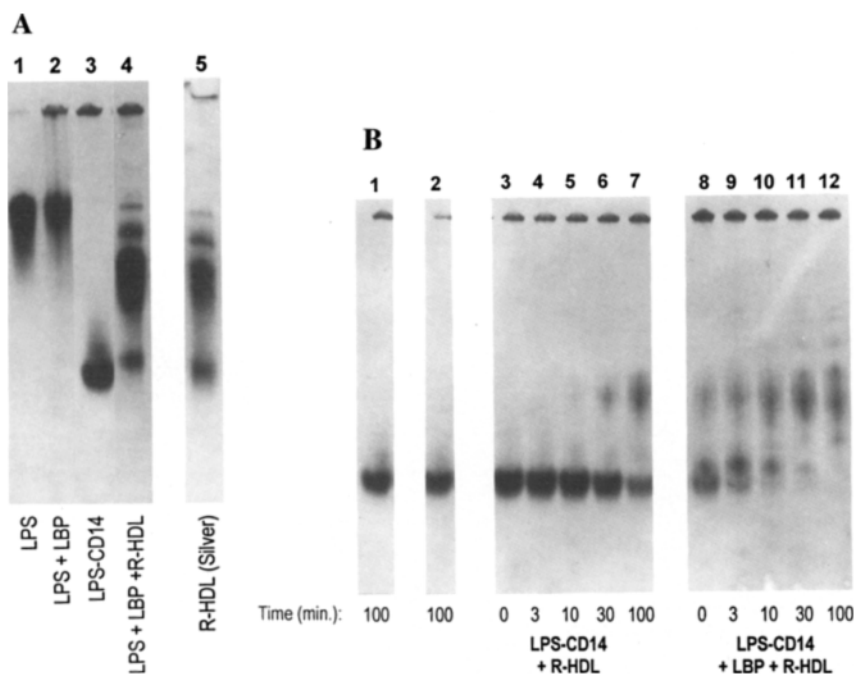
Addition of low concentrations of LBP dramatically ac-

Figure 2. Transfer of [³H]LPS to R-HDL from sCD14 is accelerated by LBP. *(A)* [³H]LPS was incubated for 4 h with buffer (lane *1*); LBP (1 μg/ml; lane *2*); or LBP and R-HDL (100 μg/ml; lane *4*). [³H]LPS–sCD14 complexes, formed as described in Materials and Methods, were run in lane *3*. A silver stain of R-HDL is shown for comparison (lane *5*). *(B)* [³H]LPS–sCD14 complexes were prepared as described in Materials and Methods, diluted to a final concentration of 1 μg/ml [³H]LPS and 50 μg/ml sCD14, and incubated for the stated interval with buffer (lane *1*); LBP (1 μg/ml; lane *2*); R-HDL (100 μg/ml; lanes *3–7*); or LBP and R-HDL (lanes *8–12*), before separation by native PAGE.
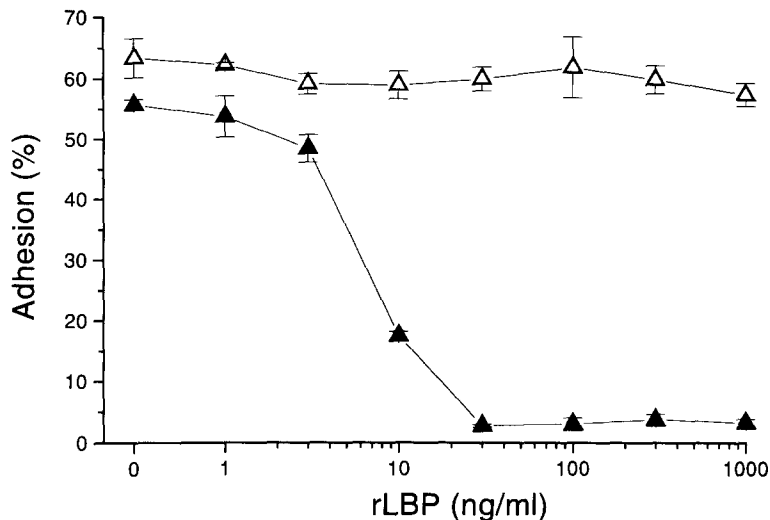
Figure 3. Low doses of LBP enable neutralization of LPS–sCD14 complexes by R-HDL. LPS–sCD14 complexes, prepared as described in Materials and Methods and diluted to an equivalent of 10 ng/ml of LPS and 1 μg/ml of sCD14, were incubated for 30 min with increasing concentrations of LBP in the absence (Δ) or presence (▲) of R-HDL (100 μg/ml). Biologically active LPS–sCD14 complexes remaining were assessed by adding PMN and by measuring their binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.

celerated neutralization of LPS–sCD14 complexes. 100 ng/ml of LBP caused at least a 20-fold enhancement of the neutralization rate (Fig. 1). By analogy with the finding that LBP catalyzes binding and neutralization of micellar LPS, these results suggest that LBP catalyzes neutralization of LPS–sCD14 complexes by catalyzing the movement of LPS from LPS–sCD14 complexes to R-HDL.

We used ^{3}H-labeled LPS ([^{3}H]LPS) and native PAGE to directly examine the transfer of LPS to R-HDL. Preliminary studies established the feasibility of this approach. [^{3}H]LPS, alone, ran as a high molecular weight aggregate (Fig. 2 *A*, lane *1*), while R-HDL, alone, ran in a lower molecular weight range, reflecting particles composed of 2, 3, or 4 copies of apo A-I (Fig. 2 *A*, lane *5*). Neither LBP (Fig. 2 *A*, lane *2*) nor R-HDL (Fig. 7, lanes *1–4*) caused a significant change in mobility of [^{3}H]LPS. However, incubation of [^{3}H]LPS with LBP and R-HDL caused the radioactivity to comigrate precisely with R-HDL (Fig. 2 *A*, lane *4*). This result demonstrates LBP-dependent binding of [^{3}H]LPS to R-HDL and

confirms our finding that LBP causes the transfer of LPS to R-HDL, as shown previously by ultracentrifugal separation (10). [^{3}H]LPS–sCD14 complexes exhibit high electrophoretic mobility and are well separated from R-HDL (Fig. 2 *A*, lane *3*). Thus, native PAGE resolves micellar LPS aggregates, LPS bound to R-HDL, and LPS bound to sCD14.

[^{3}H]LPS–sCD14 complexes were incubated for increasing intervals in the presence or absence of LBP and R-HDL and were then analyzed on native gels. [^{3}H]LPS–sCD14 complexes were very stable, with no change in the amount of radioactivity or the mobility of [^{3}H]LPS occurring after 100 min of incubation at 37°C in the presence or absence of LBP (Fig. 2 *B*, lanes *1* and *2*). Incubation of [^{3}H]LPS–sCD14 complexes with R-HDL, however, resulted in a time-dependent shift of [^{3}H]LPS to a mobility consistent with R-HDL (Fig. 2 *B*, lanes *3–7*). This finding demonstrates that [^{3}H]LPS can move spontaneously from sCD14 to R-HDL and is consistent with the spontaneous neutralization of LPS–sCD14 complexes observed in Fig. 1. Addition of LBP
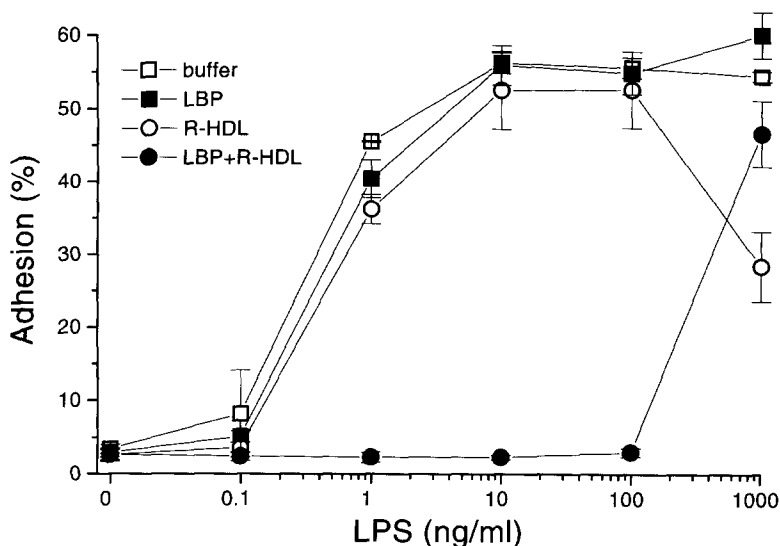

Figure 4. LBP enables neutralization of large amounts of LPS–sCD14 complexes. LPS–sCD14 complexes were formed as described in Materials and Methods with a fixed concentration of sCD14 and increasing concentrations of LPS. Complexes were diluted to a final sCD14 concentration of 1 μg/ml and the final LPS concentrations shown in the figure. The LPS–sCD14 complexes were incubated for 30 min in the presence of buffer (□), LBP (1 μg/ml; ■), R-HDL (100 μg/ml; ○), or LBP and R-HDL (●), and biologically active LPS–sCD14 complexes remaining were assessed by adding PMN and by measuring their binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated twice.

during the incubation accelerated the movement of [³H]LPS from [³H]LPS–sCD14 complexes to R-HDL 10–30-fold (Fig. 2 B, lanes 8–12). These results confirm that LBP accelerates the movement of LPS from LPS–sCD14 complexes to R-HDL and suggest that this movement causes neutralization of the biological activity of LPS–sCD14 complexes by R-HDL (Fig. 1). Quantitative aspects of the neutralization of LPS–sCD14 complexes by R-HDL were explored in several assays.

LBP Acts Catalytically in the Transfer of LPS from LPS–sCD14 Complexes to R-HDL. LPS–sCD14 complexes were incubated for 30 min with increasing concentrations of LBP in the presence or absence of R-HDL. 30 min was insufficient time for significant neutralization of LPS–sCD14 complexes by R-HDL alone (Fig. 3). However, low concentrations of LBP (10 ng/ml) enabled strong neutralization of the stimulatory capacity of LPS–sCD14 complexes. The amount of LPS–sCD14 complexes neutralized by LBP and R-HDL was calculated from a standard curve relating LPS concentration to cell adhesion (data not shown). At a concentration of 10 ng/ml, each molecule of LBP enabled the neutralization of at least three molecules of LPS by R-HDL. Similarly, substoichiometric amounts of LBP caused the complete transfer of [³H]LPS from sCD14 to R-HDL within 10–30 min (Fig. 2, lanes 8–12). Taken together, these data show that LBP can catalytically accelerate the transfer of LPS out of sCD14 into R-HDL.

LBP Enables Neutralization of Large Amounts LPS–sCD14 Complexes by Low Concentrations of R-HDL. LPS–sCD14 complexes were formed with increasing concentrations of LPS and a fixed concentration of sCD14. Complexes containing as little as 1 ng/ml of LPS strongly stimulated adhesion of PMN (Fig. 4). The stimulatory capacity of these complexes was not significantly reduced by incubation with R-HDL for 30 min. However, addition of LBP enabled R-HDL to neutralize LPS–sCD14 complexes formed with up to 100 ng/ml of LPS. Thus, LBP caused at least a 100-fold increase in the amount of LPS–sCD14 complexes neutralized by R-HDL within 30 min.

LBP also dramatically reduced the concentration of R-HDL necessary for rapid and complete neutralization of LPS–sCD14 complexes. In the absence of LBP, at least 30 μg/ml of R-HDL was necessary to observe complete neutralization of LPS–sCD14 complexes within 60 min (Fig. 5). However, addition of LBP enabled as little as 1 μg/ml of R-HDL to completely neutralize the LPS–sCD14 complexes. Thus, LBP reduced the concentration of R-HDL necessary to observe complete neutralization by ∼30-fold. The data presented above provide compelling evidence that LPS moves spontaneously from LPS–sCD14 complexes to R-HDL, and that movement of LPS from sCD14 to R-HDL is catalyzed by LBP.

sCD14 Acts Cooperatively with LBP in the Transfer of LPS to R-HDL. We have previously demonstrated that LBP can directly catalyze the movement of LPS to R-HDL and thereby promote neutralization of LPS (10). The above experiments suggest that LBP could enable neutralization of LPS through another pathway in which LBP first catalyzes binding of LPS to sCD14 and then catalyzes the transfer of LPS from sCD14

to R-HDL. To examine whether such a pathway is favored over direct transfer of LPS to R-HDL, we compared the rate of neutralization of LPS caused by sCD14 and LBP individually or in combination. sCD14 alone was ineffective in enabling neutralization of LPS by R-HDL (Fig. 6 B). This implies that, for sCD14 to promote neutralization of LPS by R-HDL, LPS must first be allowed to bind sCD14. As we have shown previously, LBP was effective in enabling the neutralization of LPS by R-HDL, with more than half of the stimulatory capacity of LPS neutralized within 60 min (Fig. 6 A). However, addition of sCD14 with LBP caused very rapid neutralization by R-HDL, with more than half of the LPS activity neutralized within 2 min. This represents a 30-fold increase over the rate of LPS neutralization by R-HDL made possible by LBP alone. Since sCD14 did not demonstrate any ability to enable neutralization of LPS by R-HDL over the time tested (Fig. 6 B), LBP and sCD14 clearly act synergistically: the rate of neutralization observed when LBP and sCD14 are combined is much greater than the sum of the rates observed with either protein alone. This synergism implies that LBP interacts with sCD14 in the rapid neutralization of LPS and supports the conclusion that LPS can be transferred to R-HDL, via sCD14, in two sequential half-reactions catalyzed by LBP: transfer of LPS into sCD14 and transfer of LPS out of sCD14 into R-HDL.

To confirm that LBP and sCD14 function together in transfer of LPS to R-HDL, we used [³H]LPS and native PAGE. No spontaneous transfer of [³H]LPS to R-HDL was observed at any time point (Fig. 7, lanes 1–4), nor was sCD14 able to cause any transfer of [³H]LPS to R-HDL (Fig. 7, lanes 5–8). LBP alone did promote transfer of most of the
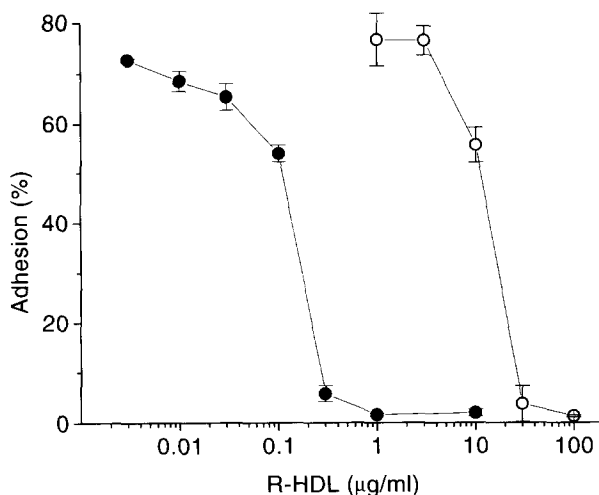

Figure 5. LBP enables neutralization of LPS–sCD14 complexes by low concentrations of R-HDL. LPS–sCD14 complexes, prepared as described in Materials and Methods and diluted to a final concentration of 0.5 μg/ml sCD14 and 10 ng/ml LPS, were incubated for 60 min with increasing concentrations of R-HDL in the absence (O) or presence (●) of LBP (1 μg/ml). Biologically active LPS–sCD14 complexes remaining were assessed by their ability to stimulate PMN binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.
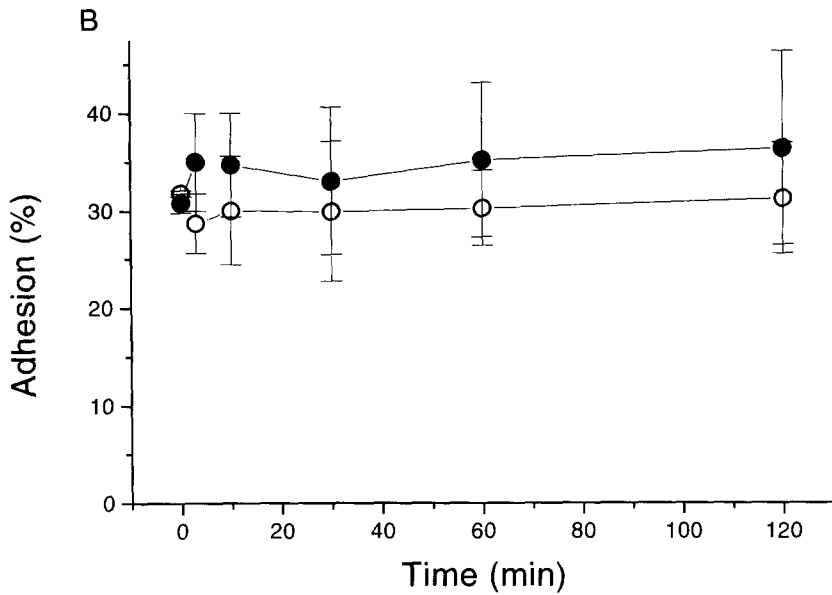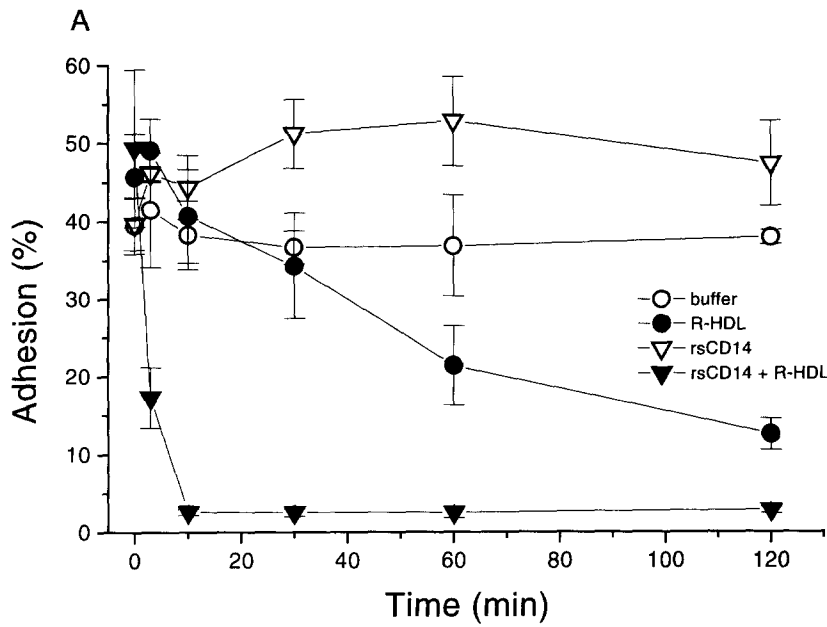
A

B

Figure 6. sCD14 and LBP act cooperatively to enable neutralization of LPS by R-HDL. (*A*) LPS (10 ng/ml) and rLBP (1 μg/ml) were incubated for the stated intervals with buffer (O), rsCD14 (1 μg/ml; ▽), RHDL (100 μg/ml; ●), or RHDL and sCD14 (▼). Biologically active LPS remaining was assessed by addition of PMN and by measuring binding to fibrinogen. sCD14 alone is ineffective in enabling neutralization of micellar LPS by R-HDL. (*B*) LPS (10 ng/ml) was incubated for the stated intervals with sCD14 (1 μg/ml) in the presence (●) or absence (□) of R-HDL (100 μg/ml). Biologically active LPS remaining was assessed by addition of LBP (1 μg/ml) and PMN and by measuring binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times (*A*), or twice (*B*).
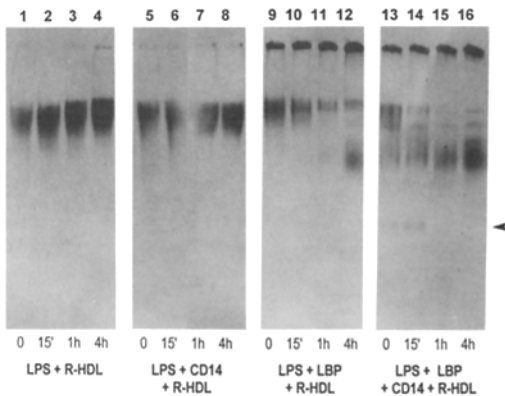

Figure 7. LBP and sCD14 cooperate in the transfer of LPS to R-HDL. [³H]LPS (1 μg/ml) was incubated for the stated intervals with R-HDL (100 μg/ml; lane *1–4*); sCD14 (1 μg/ml) and R-HDL (lane *5–8*); rLBP (1 μg/ml) and R-HDL (lane *9–12*), or LBP, sCD14, and R-HDL (before separation by native PAGE; lane *13–16*). The mobility of LPS–sCD14 complexes is indicated with an arrowhead.
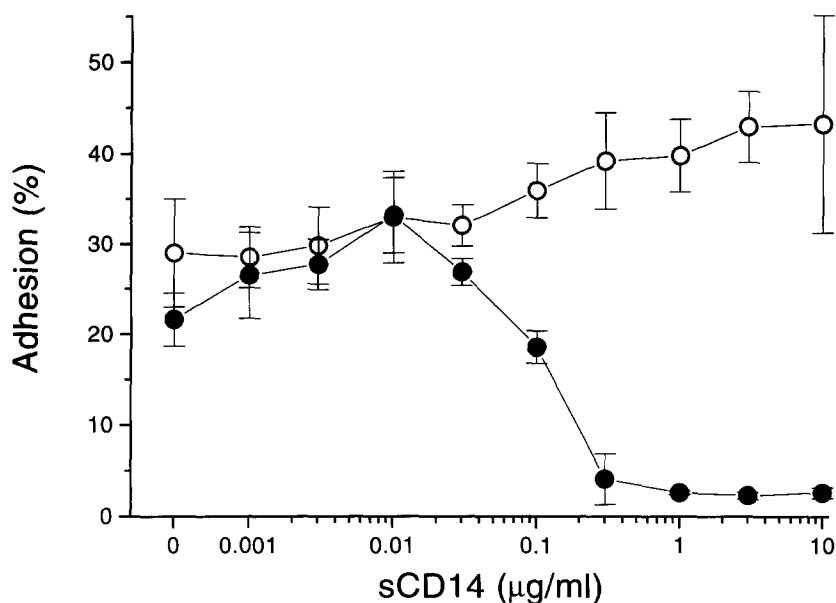
Figure 8. Concentration-dependent enhancement of LPS neutralization by sCD14. LPS (10 ng/ml) and LBP (1 μg/ml) were incubated for 30 min with increasing concentrations of rsCD14 in the absence (O) or presence (●) of R-HDL (100 μg/ml). Biologically active LPS remaining was assessed by addition of PMN and by measuring binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.

[³H]LPS from high molecular weight micelles to R-HDL within 4 h of incubation (Fig. 7, lanes 9–12). However, combining LBP and sCD14 caused very rapid transfer of [³H]LPS from LPS micelles to R-HDL. Partial transfer occurred within 10 min, and complete transfer occurred by 1 h of incubation (Fig. 7, lanes 13–16). This rate represents at least a fourfold increase over the rate at which LPS is transferred to R-HDL by LBP alone. LPS–sCD14 complexes appear to serve as a reaction intermediate in the transfer of LPS from micelles to R-HDL in the presence of LBP and sCD14. A band corresponding to [³H]LPS–sCD14 complexes was observed at the two earliest time points (Fig. 7, lanes 13 and 14, arrowhead), but disappeared at later time points (Fig. 7, lanes 15 and 16) after complete transfer of LPS to R-HDL. The disappearance of [³H]LPS from the band corresponding to LPS–sCD14 represents transfer of LPS to R-HDL and not a shift in the mobility of LPS–sCD14 complexes. Western blot analysis demonstrated that the bands staining with anti-CD14 migrated with a high mobility consistent with the mobility of sCD14, both before and after movement of [³H]LPS to R-HDL. No sCD14 comigrated with R-HDL under any conditions (data not shown). These binding studies thus confirm that LBP and sCD14 synergize in the transfer of LPS to R-HDL, with LBP first transferring LPS to sCD14 and subsequently from sCD14 to R-HDL. Several aspects of this LPS transfer mechanism were studied in subsequent experiments.

Rapid Neutralization of LPS by R-HDL and LBP Is Dependent on the Concentration of sCD14. LPS was incubated for 30 min with increasing concentrations of sCD14 in the presence or absence of R-HDL. 30 min was insufficient time for LBP to enable significant neutralization of LPS by R-HDL (Fig. 8). However, addition of sCD14 enhanced the neutralization of LPS caused by LBP, with complete neutralization occurring in the presence of 0.3 μg/ml sCD14.

LBP and sCD14 Act Synergistically to Enable the Neutralization of High Concentrations of LPS by R-HDL. Increasing concentrations of LPS were incubated for 30 min with LBP in the presence or absence of R-HDL and sCD14. Addition of R-HDL caused only partial neutralization of 1 ng/ml of LPS (Fig. 9). In contrast, combining sCD14 and R-HDL enabled complete neutralization of as much as 100 ng/ml of LPS. Thus, the addition of sCD14 caused at least a 100-fold increase in the amount of LPS that can be neutralized by R-HDL in the presence of LBP.

Combining LBP and sCD14 reduced the concentration of R-HDL necessary to accomplish complete neutralization of LPS activity. LBP incubated with high concentrations of R-HDL (100 μg/ml) caused only partial neutralization of LPS after 30 min of incubation (Fig. 10). However, combining LBP and sCD14 enabled complete neutralization of LPS by as little as 10 μg/ml of R-HDL. This is further evidence that LBP and sCD14 work synergistically to enhance the neutralization of LPS by R-HDL.

sCD14 Carries LPS in a Low Molecular Weight Form. The above results suggest that sCD14 may accelerate the transfer of LPS between LPS micelles and R-HDL by acting as a carrier, shuttling LPS between these two sites. To provide evidence that sCD14 can function as a shuttle for LPS, we measured the movement of LPS through 100-kD cutoff filters. LPS in aqueous solutions forms large aggregates, which migrate in native PAGE with an apparent mol wt of at least 500 kD (3). We reasoned that, while LPS in this form could not pass through a 100-kD cutoff ultrafiltration membrane, LPS in the monomer form (mol wt ≈5 kD) or bound with low stoichiometry to LBP (mol wt ≈60 kD) or sCD14 (mol wt ≈55 kD), should pass through the membrane. [³H]LPS incubated with buffer alone for 1 h did not exhibit significant movement through the membrane (Fig. 11), implying that LPS remained in the aggregated form. Incubation of a stoichio-
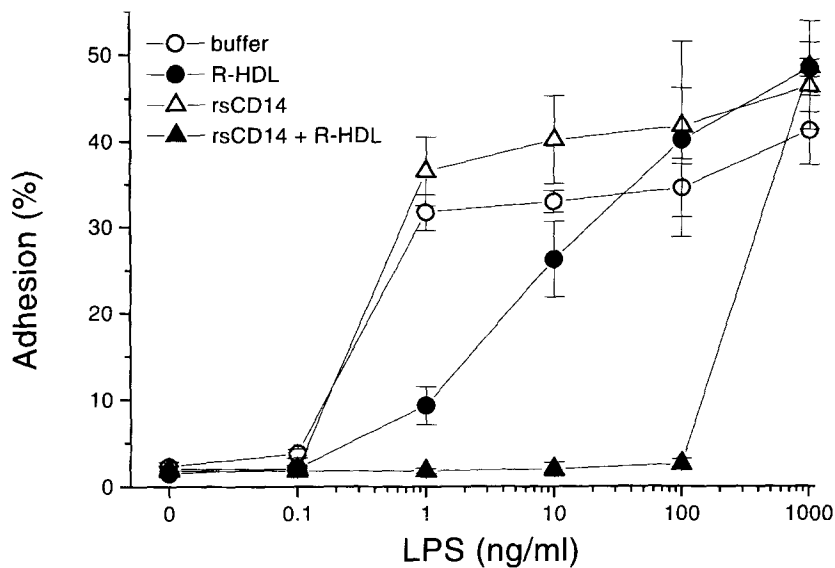
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Figure 9. LBP and sCD14 act cooperatively to enable neutralization of high concentrations of LPS. Increasing concentrations of LPS were incubated for 30 min with LBP (1 μg/ml) in the presence of buffer (O), sCD14 (1 μg/ml; △), R-HDL (100 μg/ml; ●), or sCD14 and R-HDL (▲). Biologically active LPS remaining was assessed by adding PMN and measuring their binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.

metric excess of LBP or sCD14 with [³H]LPS did not significantly increase the movement of LPS through the filter. However, combining LBP and sCD14 caused a 10-fold increase in the amount of LPS which moved across the filter. The LPS appears to be carried across the membrane in association with sCD14, rather than LBP, since ELISAs on the ultrafiltrate showed that sCD14 passed readily through the 100-kD cutoff membrane, both in the presence and absence of LPS and LBP, but in no case was LBP, itself, able to cross the membrane (data not shown). LBP may, thus, self-associate or bind to LPS micelles to form an aggregate with a total mol wt >100 kD. These findings demonstrate a shuttle activity for sCD14 that may underlie its role in the neutralization of LPS by R-HDL and LBP.

LPS Binds Transiently to sCD14 in Plasma. LPS binds to lipoproteins in plasma and is thereby neutralized (7, 9, 17). To determine if LPS-sCD14 complexes are an intermediate

in this process, [³H]LPS was incubated at 37°C for increasing intervals in plasma, then sCD14 was isolated by immunoprecipitation with an anti-CD14 mAb. Binding of [³H]LPS to sCD14 in plasma occurred rapidly, attaining a maximum within 10 min (Fig. 12 *A*). Upon further incubation at 37°C, however, the amount of [³H]LPS bound to sCD14 declined markedly. LPS thus binds to and then dissociates from sCD14 in plasma, and this transient interaction mirrors that observed in mixtures of purified proteins (Fig. 7). It should be noted that maximal specific binding of LPS to sCD14 (10 min; Fig. 12 *A*) represented only 32% of the total LPS recovered (data not shown). The relatively low proportion of LPS associated with sCD14 at any given time point may reflect the dynamic process of LPS moving into and out of sCD14. These results suggest that sCD14 may be an intermediate in the transfer of LPS to lipoproteins in plasma. These results do not, however, indicate whether
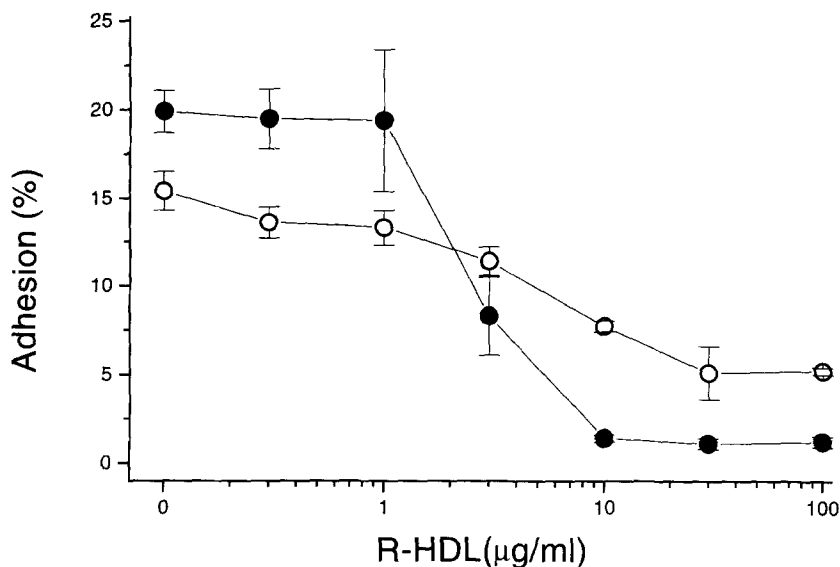

Figure 10. LBP and sCD14 act cooperatively to enable neutralization of LPS by low concentrations of R-HDL. LPS (10 ng/ml) and LBP (1 μg/ml) were incubated for 30 min with increasing concentrations of R-HDL in the absence (O) or presence (●) of sCD14 (1 μg/ml). Biologically active LPS remaining was assessed by adding PMN and measuring binding to fibrinogen. Each point represents the mean of three wells, ± SD of a representative experiment repeated three times.
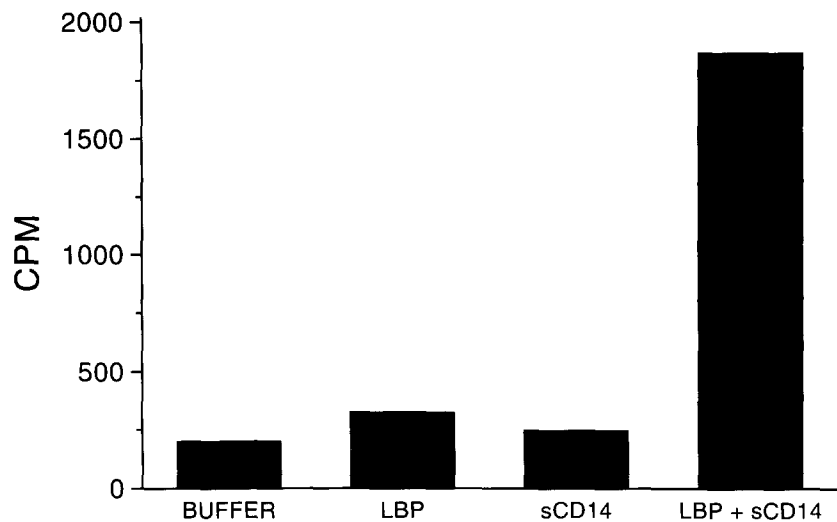
Figure 11. sCD14 carries LPS across a 100-kD cutoff ultrafiltration membrane. [^{3}H]LPS (10 ng/ml) was incubated for 60 min at 37°C with buffer alone, LBP (1 μg/ml), sCD14 (1 μg/ml), or LBP and sCD14, and subjected to ultrafiltration through a 100-kD cutoff membrane. The radioactivity in the ultrafiltrate was measured by scintillation counting. Under these conditions, in the presence of LBP and sCD14, ~25% the total cpm added to the filter were recovered in the ultrafiltrate. Each bar represents the mean of two counts of a representative experiment repeated twice.
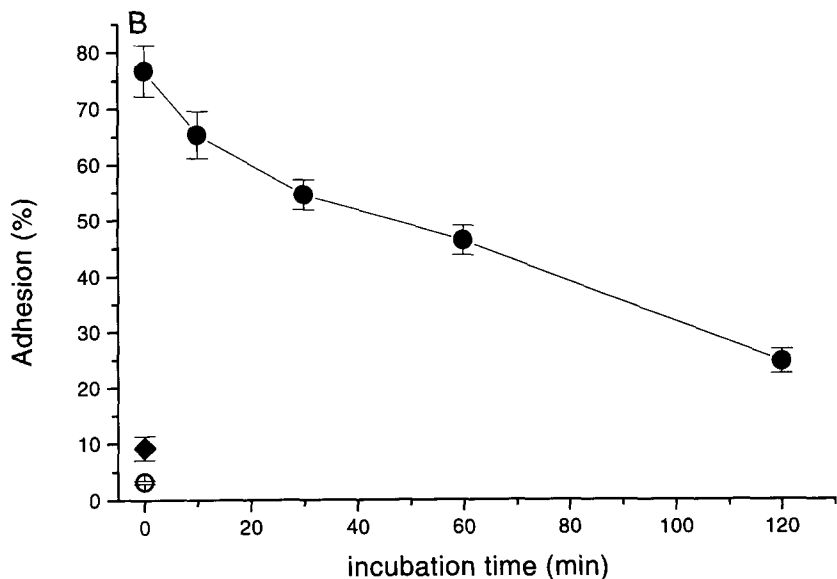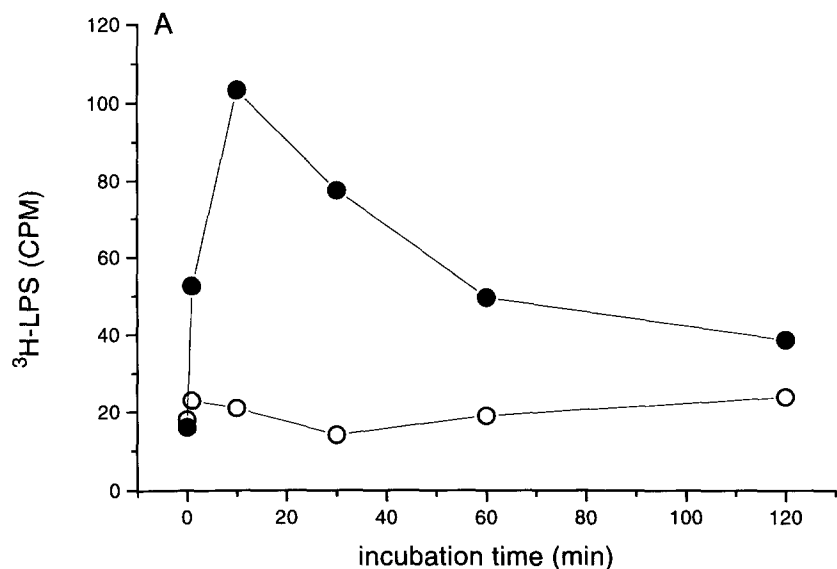

Figure 12. [^{3}H]LPS binds transiently to plasma sCD14. [^{3}H]LPS (10 ng/ml) was incubated with NHP (40%) for the stated intervals at 37°C: (A) The amount of [^{3}H]LPS specifically bound to sCD14 was determined by immunoprecipitation of the mixture with either an anti-CD14 (●; 26ic), or a control (○; IB4) mAb. The values represent the number of cpm found in the immunoprecipitate from a representative experiment repeated three times. (B) Biologic activity of the LPS-NHP mixtures was assessed by addition of PMN for 10 min, washing, and measuring binding of cells to fibrinogen-coated surfaces (●). Neither LPS alone (○), nor NHP alone (◆), stimulated cell adhesion over background. Values represent the mean of three wells, ± SD of a representative experiment repeated three times.
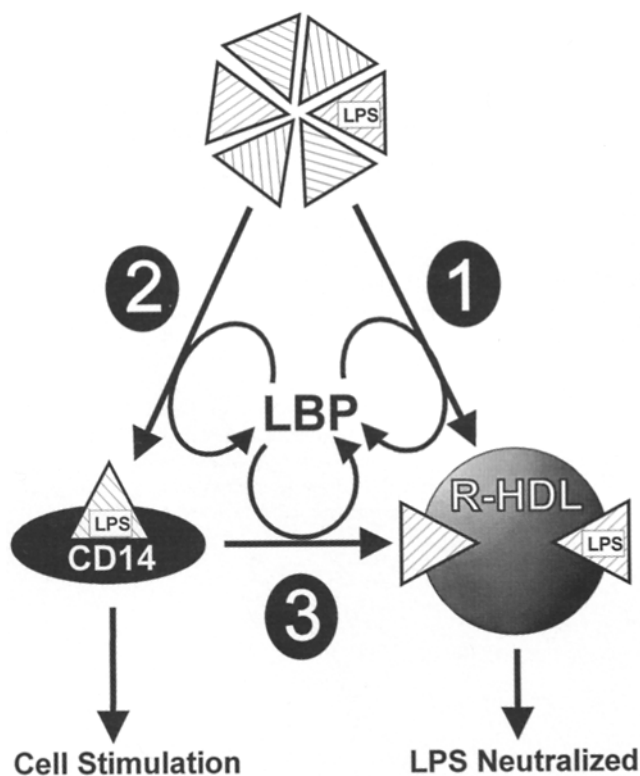
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Figure 13. LPS transfer reactions catalyzed by LBP. See text for details.

all the LPS transferred to lipoproteins in plasma passes through sCD14. Alternative pathways for transfer of LPS to lipoproteins may also exist.

Parallel studies measured the capacity of LPS–NHP mixtures to stimulate PMN. In keeping with our previous work (10), plasma rapidly enabled LPS to stimulate cells, and further incubation resulted in a loss of the stimulatory capacity (Fig. 12 B). The rapid rise and subsequent fall in the biologic potency of LPS–NHP mixtures corresponded closely with the rapid binding and gradual dissociation of LPS and sCD14. These results suggest that LPS–sCD14 complexes represent a key intermediate in cellular responses to LPS and that dissociation of LPS from sCD14 is required for neutralization of the LPS.

Discussion

Previous work from our laboratory has shown that LBP catalyzes two distinct reactions: transfer of LPS from micelles to CD14 (3) and transfer of LPS from micelles to R-HDL particles (10). Here we demonstrate that LBP facilitates a third reaction: transfer of LPS from LPS–CD14 complexes to R-HDL (Fig. 13). As in the previously characterized LPS transfer reactions, LBP acts catalytically to enable the movement of LPS from CD14 to R-HDL, with each LBP molecule enabling the movement of at least three molecules of LPS within 30 min (Fig. 3). The catalytic action of LBP is consistent with its structural similarity to cholesterol ester transfer protein (CETP) (2) and phospholipid transfer protein (PLTP) (18), plasma proteins with well-characterized ca-

pacity to facilitate the movement of lipids between lipoprotein particles (19, 20).

An inspection of the three reactions catalyzed by LBP reveals two possible paths by which micellar LPS may be transferred to R-HDL: direct transfer (reaction 1, Fig. 13) and indirect transfer via sCD14 (reactions 2 and 3, Fig. 13). Here we show that, under our experimental conditions, the indirect pathway is kinetically favored. Addition of sCD14 to LBP enhanced the rate of transfer of LPS to R-HDL (Figs. 6 A and 7), increased the amount of LPS that could be neutralized by a fixed dose of R-HDL in a fixed time (Fig. 9), and decreased the amount of R-HDL needed to neutralize a fixed dose of LPS in a fixed time (Fig. 10). Since sCD14 demonstrated no ability to transfer micellar LPS on its own (Fig. 6 B), these results suggest that LBP and sCD14 work synergistically to transfer LPS to R-HDL.

sCD14 may enhance the LBP-mediated transfer of LPS to R-HDL by shuttling LPS between LPS micelles and R-HDL. Our previous work showed that LBP transfers a single molecule of LPS to sCD14 (3). Here we have shown that LPS–sCD14 complexes formed from this reaction are diffusible across a 100-kD cutoff filter (Fig. 11). We further showed that LPS–sCD14 complexes donate their LPS to R-HDL in a transfer reaction that is catalyzed by LBP (Fig. 1). A role for LPS–sCD14 complexes as intermediate carriers of LPS was confirmed by the observation that these complexes are transiently formed in the movement from LPS micelles to R-HDL facilitated by LBP and sCD14 (Fig. 7). A requirement for an intermediate carrier is suggested by the fact that LBP does not exhibit the properties of an effective shuttle. It binds tightly to bacteria or LPS-coated surfaces (21, 22), it fails to move LPS across a 100-kD cutoff filter (Fig. 11), and it is found in plasma associated with HDL particles (10). The use of sCD14 as a mobile carrier protein supplants the need for diffusion of LPS-LBP or LBP-HDL complexes and accelerates the transfer of LPS between these two complexes. The finding that LBP can use a soluble carrier protein (sCD14) to effect LPS transport raises the possibility that CETP or PLTP may use sCD14 or a related shuttle protein to equilibrate lipids among lipoprotein particles.

The capacity of LPS–sCD14 complexes to donate LPS to R-HDL suggests a role for sCD14 in the trafficking of LPS to lipoproteins in plasma. We have previously shown that incubation of LPS with plasma for brief intervals enables strong stimulation of PMN (10). This stimulation of PMN is likely to represent the action of LPS–sCD14 complexes, since these complexes form rapidly in the presence of LBP (3) and upon incubation of LPS with plasma (Fig. 12), and are known to stimulate endothelial cells (1, 3), PMN (3, 4), and astrocytes (1, 23). Further incubation of LPS with plasma, however, results in neutralization of LPS, with nearly complete loss of biological activity after 2 h. This neutralization is known to represent the movement of LPS to lipoprotein particles (5–7, 24), and, here, we show that neutralization is temporally correlated with loss of LPS from sCD14 (Fig. 12). These results suggest that neutralization of LPS in plasma may occur through transfer of LPS from LPS–sCD14 complexes to lipoprotein particles.

We have observed that LBP-catalyzed movement of LPS to sCD14 (Fig. 13, reaction 2) occurs much more rapidly than LBP-catalyzed movement of LPS into R-HDL (Fig. 13, reaction 1) (see Fig. 7 and reference 3). Under conditions in which sCD14 and HDL are in excess, such as those in plasma (25), movement of LPS to sCD14, and, thus, formation of LPS–sCD14 complexes, should be favored. Furthermore, the prevalence of LPS–sCD14 complexes will be dictated by the relative rates of LPS movement into sCD14 (reaction 2) and LPS movement out of sCD14 to lipoproteins (reaction 3). If reaction 2 is faster than 3, LPS–sCD14 complexes will accumulate and cell stimulation will be observed. Conversely, if reaction 3 is faster than 2, few LPS–sCD14 complexes will accumulate, and the LPS will be rapidly neutralized. Control of these two rates may thus yield a sensitive means of regulating responsiveness of mammals to LPS.

We also wish to emphasize that the effectiveness of sCD14 as a shuttle for LPS depends on the relative rates of reactions 1 and 3 in Fig. 13. If reaction 3 is faster than 1, then sCD14 may hasten neutralization of LPS by shuttling LPS to HDL, as we have found here with R-HDL particles. On the other hand, if reaction 1 is faster than 3, then sCD14 could impede movement of LPS to HDL by diverting the LPS from reaction 1. Reactions 1 and 3 involve not only LPS, LBP, and sCD14, but also a lipoprotein particle, and the nature of that lipoprotein particle may affect transfer rates. We have observed that the R-HDL particles used here rapidly neutralize LPS in the presence of LBP and sCD14 (Fig. 6), but that plasma (Fig. 12 B) and native HDL particles (M. M. Wurfel and S. D. Wright, unpublished observations) neutralize LPS much more slowly under similar conditions. Since plasma contains a wide spectrum of lipoprotein particles, our experiments do not predict whether sCD14 will hasten or slow the neutralization of LPS in plasma. The kinetics of LPS transfer reactions in whole plasma is now under study.

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