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B Yu, … , E Hailman, S D Wright

Research Article

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Bo Yu, Eric Hailman, and Samuel D. Wright

Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

Abstract

Lipopolysaccharide binding protein (LBP) is a plasma protein known to facilitate the diffusion of bacterial LPS (endotoxin). LBP catalyzes movement of LPS monomers from LPS aggregates to HDL particles, to phospholipid bilayers, and to a binding site on a second plasma protein, soluble CD14 (sCD14). sCD14 can hasten transfer by receiving an LPS monomer from an LPS aggregate, and then surrendering it to an HDL particle, thus acting as a soluble "shuttle" for an insoluble lipid. Here we show that LBP and sCD14 shuttle not only LPS, but also phospholipids. Phosphatidylinositol (PI), phosphatidylcholine, and a fluorescently labeled derivative of phosphatidylethanolamine (R-PE) are each transferred by LBP from membranes to HDL particles. The transfer could be observed using recombinant LBP and sCD14 or whole human plasma, and the plasma-mediated transfer of PI could be blocked by anti–LBP and partially inhibited by anti–CD14. sCD14 appears to act as a soluble shuttle for phospholipids since direct binding of PI and R-PE to sCD14 was observed and because addition of sCD14 accelerated transfer of these lipids. These studies define a new function for LBP and sCD14 and describe a novel mechanism for the transfer of phospholipids in blood. In further studies, we show evidence suggesting that LBP transfers LPS and phospholipids by reciprocal exchange: LBP-catalyzed binding of R-PE to LPS•sCD14 complexes was accompanied by the exit of LPS from sCD14, and LBP-catalyzed binding of R-PE to sCD14 was accelerated by prior binding of LPS to sCD14. Binding of one lipid is thus functionally coupled with the release of a second. These results suggest that LBP acts as a lipid exchange protein. (*J. Clin. Invest.* 1997. 99:315–324.) Key words: endotoxin • lipoprotein • phospholipid • phospholipid transfer protein • sepsis

Introduction

Bacterial LPS provokes dramatic responses in macrophages, polymorphonuclear leukocytes, and endothelial cells. LPS is a membrane-forming amphiphile that, like phospholipids, diffuses very slowly from aggregates, micelles, or membranes. As a consequence, very little LPS associates with cells in protein-free buffers, and responses generally require very high concentration of LPS (micrograms per milliliter to milligrams per milliliter). The amount of LPS needed to produce a cellular response is reduced by up to three logs by the addition of plasma, serum or serum proteins (1, 2). Two serum proteins, LPS binding protein (LBP)[1] and soluble CD14 (sCD14), have been shown to play a role in enhancing responses to LPS (3, 4), and work from our laboratory has established that these two proteins act to transport individual LPS molecules (5–7). LBP first catalytically transfers an LPS monomer to a binding site on sCD14. The resulting LPS•sCD14 complexes diffuse readily and may surrender LPS to lipoprotein particles (7), phospholipid vesicles (Wurfel, M.M., and S.D. Wright, manuscript submitted for publication), or to cells (7a, and Yu, B., E. Hailman, and S.D. Wright, unpublished observations) to complete the lipid transfer. The LPS transport function of LBP and sCD14 may explain their well established role in enhancing responsiveness of cells to bacterial LPS (8).

LBP catalyzes not only transfer of LPS to and from sCD14, but also direct transfer of LPS from LPS micelles to HDL particles (6) and phospholipid vesicles (8a). Here we analyze the kinetics of this process using a quantitative, real-time assay developed for measuring LPS transfer to sCD14 (9), and quantitative aspects of the transfer are described. In the course of these studies, we made the surprising finding that LBP transfers not only LPS, but also several phospholipids. Transfer of phospholipids by LBP occurs concurrently with transfer of LPS, suggesting a reciprocal transfer of lipids. Further studies showed that sCD14 can bind phospholipids, that LBP can transfer phospholipids to sCD14, and that transfer of phospholipids to sCD14 by LBP is accompanied by a reciprocal transfer of LPS out of the sCD14. These studies strongly suggest that LBP and sCD14 are phospholipid transfer proteins and that they have physiological functions in addition to transfer of bacterial LPS.

Methods

Reagents. LPS from *Salmonella minnesota* strain R595 (Re) was purchased from List Biological Labs, Inc., (Campbell, CA). Boron dipyrromethene difluoride (BODIPY)-labeled LPS (BODIPY-LPS) was prepared as previously described (9). Fresh normal human plasma was prepared from blood donated by healthy volunteers,

Address correspondence to Samuel D. Wright, Merck Research Laboratories, P.O. Box 2000, RY80W-250, Rahway, NJ 07065. Phone: 908-594-3086; FAX: 908-594-1169; E-mail: samuel_wright@merck.com

1. *Abbreviations used in this paper:* BODIPY, boron dipyrromethene difluoride; BR-HDL, biotinylated reconstituted high density lipoprotein; CETP, cholesteryl ester transfer protein; LBP, LPS binding protein; PC, phosphatidylcholine; PD, Dulbecco's phosphate buffered saline lacking Ca^{2+} and Mg^{2+}; PI, phosphatidylinositol; PLTP, phospholipid transfer protine; R-HDL, reconstituted high density lipoprotein; R-PE, *N*-(6-tetramethylrhodamineethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt.

drawn into EDTA. Anti–CD14 monoclonal antibody 3C10 (10), was purified from ascites fluid by chromatography on protein G Sepharose. Function-blocking monoclonal anti–LBP antibodies 1-23 and 1-137 (11) were kind gifts from Dr. Alexander H. Taylor (Centocor, Inc., Malvern, PA). Monoclonal anti–ICAM 84H10 was as described (12). Rabbit IgG was purified from nonimmune rabbit serum by chromatography on protein G Sepharose. Polyclonal antirecombinant LBP, recombinant human LBP, and sCD14 were the generous gifts of Dr. Henri Lichenstein (Amgen, Inc., Thousand Oaks, CA). *N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (R-PE) was from Molecular Probes, Inc. (Eugene, OR). L-α-(myoinositol-2-^{3}H(*N*))-phosphatidylinositol (5–20 Ci/mmol), [^{3}H]phosphatidylinositol (PI) and L-α-dipalmitoyl-phosphatidylcholine, (choline-methyl-^{3}H) (30–60 Ci/mmol) [^{3}H]phosphatidylcholine (PC) were from DuPont-NEN (Boston, MA) except where noted (see below). Phosphatidylinositol was from Avanti Polar Lipids (Alabaster, AL). Phosphatidylcholine and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest quality available.

Preparation of reconstituted HDL particles and biotinylated reconstituted HDL. Reconstituted HDL (R-HDL) was prepared by the sodium cholate dialysis method, as previously described (6, 13). Briefly, apo A-1, egg PC, cholesterol, and cholate were mixed at a molar ratio of 1:80:4:80 and dialyzed against PD-EDTA (Dulbecco's PBS lacking Ca^{2+} and Mg^{2+}, but containing 1 mM EDTA, 0.01% sodium azide.) (Whittaker M.A. Bioproducts, Inc., Walkersville, MD). The concentration of R-HDL is expressed as the equivalent concentration of apo A-1 in micrograms per milliliter. Biotinylated reconstituted (BR-HDL) was prepared by incubating R-HDL particles with ImmunoPure® NHS-Biotin from Pierce (Rockford, IL), according to the manufacturer's recommendation.

Measurement of fluorescence. Fluorescence was measured with an SLM-SPF500c (SLM-AMINCO, Urbana, IL) spectrofluorimeter as previously described (9). For BODIPY-LPS, the maximum excitation was at 485 nm, and emission was at 518 nm. The R-PE was excited at 535 nm and emission was measured at 570 nm. To measure time-dependent changes in fluorescence that occur upon binding of BODIPY-LPS to R-HDL, BODIPY-LPS, BODIPY-LPS•sCD14 complexes, LBP, sCD14, and antibodies were diluted in PBS (Dulbecco's phosphate buffered saline with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) as described in the figure legends and quickly mixed in a submicrocuvette (Starna Cells Inc., Atascadero, CA). The initial rate was calculated for the first 20-s period. Progress curves for fluorescence change over time were analyzed with integrated rate equations using a nonlinear, least-squares fitting software package (STATISTICA, StatSoft, Tulsa, OK). Data shown in Fig. 3 used Eq. 1 (14):

$$t = a(f - f_0) + b \ln[(f_\infty - f_0) / (f_\infty - f)] \qquad (1)$$

Where t is time, f_0 is the background fluorescence of BODIPY-LPS, f_∞ is the fluorescence at the end of transfer, f is the fluorescence of the reaction mixture at any given time, a and b are the coefficients, and b^{-1} is the first order rate constant k_{exp} (see reference 14 for details of the derivation).

Determination of fluorescence quenching in phospholipid bilayers. The fluorescence of BODIPY-LPS and R-PE in aqueous buffer is highly quenched, but quenching is relieved by disbursement of the aggregates in an excess of SDS, and we assume here that the fluorescence efficiency in SDS is 100%. We previously showed that the fluorescence efficiency of BODIPY-LPS in sCD14 is about ∼ 40% of maximal, since the fluorescence was more than doubled by addition of SDS to BODIPY-LPS•sCD14 complexes (9). To determine the fluorescence efficiency of BODIPY-LPS in a phospholipid bilayer, BODIPY-LPS and PC (1:100 molar ratio) were dissolved in a small volume of toluene, and a 40-fold excess of PBS was added. After thorough mixing, the toluene was evaporated. The fluorescence of the resulting BODIPY-LPS•PC vesicles was measured in the presence or absence of 2% SDS. Addition of SDS caused no rise in fluo-

rescence (not shown), indicating that dequenching was complete in PC vesicles. This determination suggests that dequenching is complete upon transfer of BODIPY-LPS to phopholipid vesicles and, by inference, upon transfer to R-HDL particles. The fluorescence efficiency of R-PE in PC vesicles was determined similarly, and the fluorescence ratio with and without SDS was 1:0.65.

Formation of LPS•sCD14 complexes. sCD14 (0.2 mg/ml) and LPS (5 μg/ml) or BODIPY-LPS were incubated together for 16 h at 37°C in PD (Dulbecco's PBS lacking Ca^{2+} and Mg^{2+}) or PBS (Dulbecco's PBS, with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) with 10% glycerol. Under these conditions, the molar ratio of LPS to sCD14 is 1:2. The formation of complexes was complete since addition of LBP did not cause a further increase in fluorescence.

Ultrafiltration of R-PE•sCD14 complexes. sCD14 was separated from PE membranes (diluted and sonicated in PBS) as follows: LPS•sCD14 complexes (14 μg/ml sCD14 and 380 ng/ml LPS), 1 μg/ml LBP and 1,600 ng/ml R-PE were mixed in different combinations at 37°C for 30 min, and 70 μl of the mixtures were loaded on Microcon ultrafiltration units (Amicon, Inc., Beverly, MA) equipped with 100-kD cutoff membranes. R-PE•sCD14 and LPS•sCD14 complexes appeared in the ultrafiltrate while R-PE membranes and LBP were retained (reference 7, Table I, and our unpublished observations). The fluorescence of the filtrate was then measured with or without 2% SDS.
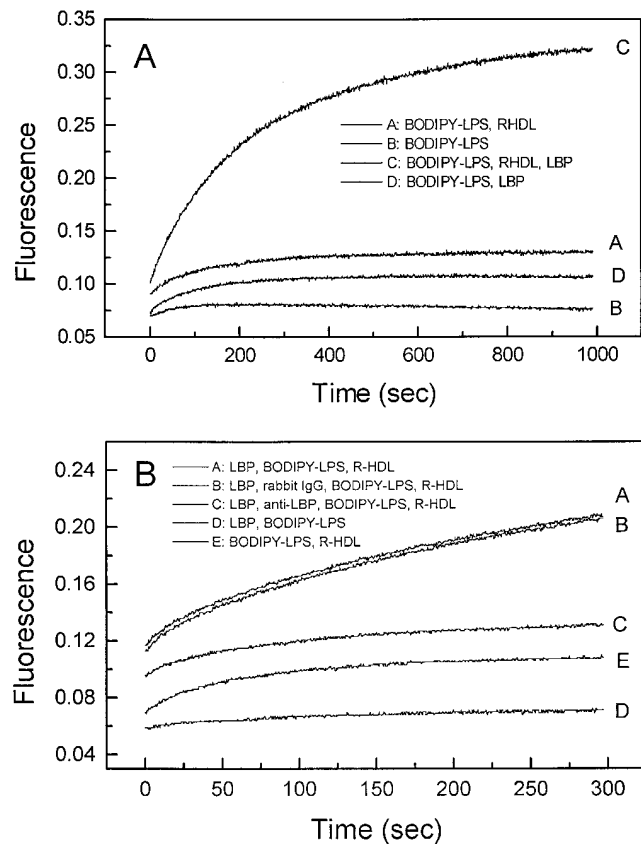
The formation of PI•sCD14 complexes and nondenaturing polyacrylamide gel electrophoresis (native PAGE). [^{3}H]PI (a 1:44 (wt/wt) mixture of [^{3}H]PI from ox brain microsomes (Amersham Corp., Arlington Heights, IL) and unlabeled PI from bovine brain (Sigma Chemical Co.) was dried under a steam of nitrogen, resuspended in PD-EDTA, and incubated alone or with LBP and sCD14 for 30 min at 37°C. Just before electrophoresis, 1⁄2 vol PD-EDTA with 20% (vol/vol) glycerol and 0.005% (wt/vol) bromophenol blue was added, and the samples were electrophoresed on an 8–16% (step-gradient) continuous buffer (pH 8.6), nondenaturing Tris–glycine polyacrylamide gel (Novex, San Diego, CA). The gel was then soaked in ENHANCE (DuPont NEN) for 45 min, washed three times for 15 min in distilled water, dried, and exposed to XAR film (Eastman Kodak Co., Rochester, NH) for 42 d (bands were visible within 7 d).

Transfer of labeled lipids to BR-HDL. Solutions of fluorescently or radioactively labeled lipids in ethanol were diluted at least 100-fold in PBS and sonicated to form membranes. Lipids were then mixed with BR-HDL in the presence or absence of LBP, plasma, or antibodies as described in the legends for Figs. 5 and 10, and incubated at 37°C in PBS, in a final volume of 50 μl. BR-HDL was then separated from the other reactants with streptavidin beads and the associated phospholipids were measured with either scintillation counting or fluorimetry. Briefly, 20 μl 50% streptavidin beads (Pierce) were added to the reaction mixtures and incubated at 4°C for 1 h on a shaker. The streptavidin beads were then washed five times with PBS, and label was eluted with 100 μl 2% SDS in PBS. The specificity of the precipitation was demonstrated in control experiments similar to those in Fig. 5, but in which BR-HDL was replaced by unbiotinylated R-HDL. Under these conditions, no [^{3}H]PI could be precipitated (data not shown).
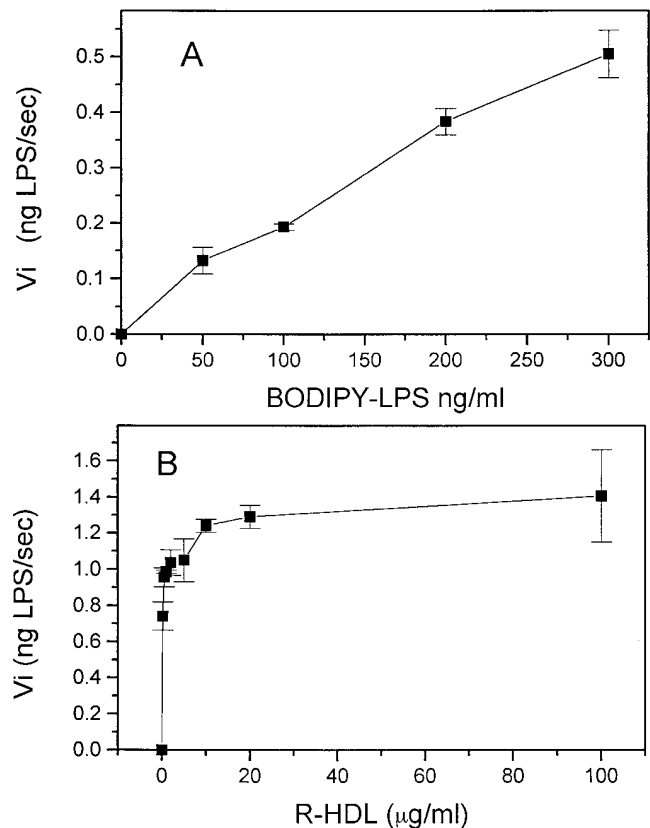
Results

LBP catalyzes transfer of BODIPY-LPS to R-HDL: kinetic analysis. Previous work from this lab has shown that LBP catalyzes transfer of LPS from LPS micelles or aggregates to R-HDL particles, and this transfer causes the toxicity of LPS to be neutralized (6). R-HDL used in these studies are disk-shaped pieces of phospholipid bilayers bounded by apo A-1. The phospholipid component of R-HDL is both necessary and sufficient for receiving LPS (8*a*). To further characterize the mechanism by which LPS is transferred to a phospholipid bilayer, we used a fluorescent LPS derivative (BODIPY-LPS) that we had previously used to study the movement of LPS

from micelles to sCD14 (9). The fluorescence of BODIPY-LPS in micelles is quenched, but quenching is relieved and fluorescence increases upon movement of BODIPY-LPS monomers out of the aggregates. The increase in fluorescence allows real-time quantitation of lipid transfer. A typical study (Fig. 1 *A*) shows that a large increase in fluorescence was observed only when both LBP and R-HDL were present with BODIPY-LPS in the reaction mixture, (curve *C*). This study confirms previous findings of increased BODIPY-LPS fluorescence upon incubation with LBP and R-HDL (15), or LBP and phospholipid vesicles (8*a*). Based on previous results showing increased BODIPY-LPS fluorescence upon movement of BODIPY-LPS out of aggregates (9) and showing LBP-dependent transfer of LPS to R-HDL (6), we interpret the increase in fluorescence as LBP-mediated transfer of BODIPY-LPS to R-HDL. To further confirm the LBP dependence of this process, polyclonal anti–LBP was added to the reaction mixture. As shown in Fig. 1 *B*, the increase in fluorescence was blocked with polyclonal anti–LBP (curve *C*), but was unaffected by control IgG (curve *B*).

To analyze the kinetics of this process, we measured the initial rate of LPS transfer to R-HDL by a fixed amount of LBP in the presence of various concentrations of LPS or R-HDL. When the concentration of R-HDL (acceptor) was kept constant, the initial rate of BODIPY-LPS transfer increased linearly with increasing of BODIPY-LPS concentration (donor; Fig. 2 *A*). The linear relationship in Fig. 2 *A* indicates that even at the highest concentration (300 ng/ml), the donor particles were well below their apparent K_m, and the K_m for LPS could not be determined. Conversely, when the concentration of BODIPY-LPS (donor) was kept constant, the initial rate of BODIPY-LPS transfer reached maximum at a very low R-HDL concentration (Fig. 2 *B*). Under these conditions, the half-maximal initial transfer rate was observed with LBP (1.7×10^{-8} M) and apo A-1 (1.7×10^{-8} M) in near stoichiometric equivalence. This finding suggests a high affinity interaction between LBP and R-HDL and is consistent with our previous

Figure 1. (*A*) LBP-mediated transfer of BODIPY-LPS to R-HDL. 100 ng/ml BODIPY-LPS, 1 μg/ml LBP, and 104 μg/ml R-HDL in different combinations were mixed at 37°C as indicated in the figure, and the fluorescence at 518 nm was recorded over time. The experiment shown is representative of three separate studies. (*B*) Anti–LBP antibody blocks transfer activity of LBP. Polyclonal anti–LBP (curve *C*) or nonimmune rabbit IgG (curve *B*) (100 (μg/ml) was incubated with LBP (1 μg/ml) for 5 min on ice. BODIPY-LPS 500 ng/ml and R-HDL 26 μg/ml were then added, and the fluorescence of the mixtures was recorded over time at 37°C. The other curves are mixtures as indicated in the graph. The experiment shown is representative of three separate studies.

Figure 2. Kinetic analysis of LBP-mediated transfer of BODIPY-LPS to R-HDL. (*A*) The relationship of the initial rate of BODIPY-LPS transfer to BODIPY-LPS concentration. 25 μg/ml R-HDL and 1 μg/ml LBP were mixed with various amounts of BODIPY-LPS as indicated in the figure and the fluorescence change was recorded over time. The initial rate was calculated for the first 20-s period. Similar results were obtained in studies using R-HDL concentrations from 5 to 200 μg/ml. (*B*) The relationship of initial rate of BODIPY-LPS transfer to R-HDL concentration. 500 ng/ml BODIPY-LPS and 1 μg/ml LBP were mixed with various amounts of R-HDL as indicated in the figure and the fluorescence change was recorded over time. The initial rate was calculated for the first 20-s period, and the background (no R-HDL) was subtracted. Each point represents the mean of three measurements±SD (*n* = 3) of a representative experiment repeated more than three times.

studies showing that LBP is found associated with apo A-1 in plasma (6, 16). A valid analysis of the K_m for R-HDL requires data obtained at varying R-HDL concentrations. Since most R-HDL in the sensitive portion of the curve in Fig. 2 *B* is apparently not free but complexed with LBP, a K_m for R-HDL could not be calculated from our data.

Progress curves for transfer of LPS to R-HDL fit a model for lipid exchange. Since the characteristics of LBP-mediated transfer of LPS to R-HDL (Fig. 2) were not suitable for classical kinetic analysis by double reciprocal plots, we sought to gain mechanistic understanding of the reaction by close analysis of reaction progress at fixed concentrations of reactants. Curve *C* in Fig. 1 was first fitted to an integrated rate equation derived from a "ping-pong" reaction mechanism (17). This model assumes that LBP first receives an LPS in a bimolecular reaction with an LPS aggregate, then transfers LPS in a second bimolecular reaction with R-HDL. A satisfactory fit to this equation could not be achieved (data not shown). We next fitted the data to a model recently described by Epps et al. (14) for cholesteryl ester transfer protein (CETP), a protein with 23% sequence identity to LBP (4). CETP is known to exchange one molecule of cholesteryl ester for one molecule of triglyceride in a reciprocal transfer (14, 18). The model features a desorption-diffusion–adsorption-exchange mechanism, and a simplified integrated rate equation based on this model (see Eq. 19 in reference 14) is shown as Eq. 1 in Meth-

ods. The data in Fig. 1 *A*, curve *C* were analyzed with the first order model described in Eq. 1 using nonlinear least squares-fitting software (Fig. 3). The agreement of the theoretical curve with the experimental data points throughout the time course and the randomness of the residuals indicate that the LPS transfer can be accurately modeled with the assumptions of Eq. 1. Under our conditions (100 ng LPS/ml, 1 μg LBP/ml, and 100 μg/ml R-HDL), the pseudo–first order rate constant for transfer of BODIPY-LPS was $4.72 \pm 0.64 \times 10^{-3}$ S^{-1} ($n = 2$).

LBP transfers phospholipid to LPS micelles. The close fit of the LBP-mediated transfer data to a model of lipid exchange and the structural similarity of LBP to CETP raised the possibility that LBP may exchange LPS for phospholipid in a reciprocal fashion. To test this, we first asked if LBP can transfer phospholipid into LPS micelles. We chose to study a derivative of phosphatidylethanolamine, which bears a rhodamine on its amino group (R-PE). As with BODIPY-LPS, the fluorescence of R-PE was strongly quenched, the quenching may be relieved by transfer of monomers into SDS micelles or phospholipid bilayers (not shown), and transfer of R-PE could be measured in real time. As expected, the fluorescence of R-PE membranes did not change upon mixing with LBP or LPS micelles (Fig. 4). However, upon the addition of LBP to the R-PE and unlabeled LPS, an enhancement of the fluorescence was observed. We interpret this finding as a reflection of LBP-mediated transfer of R-PE to LPS micelles, and we conclude that LBP can transfer not only LPS but also the phospholipid, R-PE.

We wish to point out that the LBP-mediated transfer of R-PE to LPS micelles shown in Fig. 4 represents the precise inverse of the LBP-mediated transfer of BODIPY-LPS into R-HDL particles pictured in Fig. 1 *A*. In both of these experiments, the fluorescence enhancement could derive either from movement of a fluorescent lipid monomer into a micelle of unlabeled lipid or from gradual "dilution" of fluorescent lipid by transfer of unlabeled lipid into micelles composed of labeled
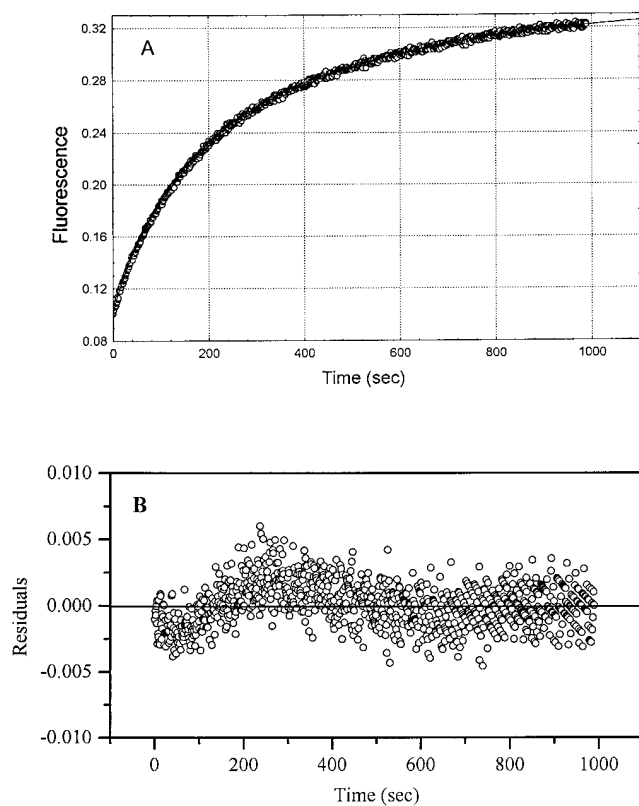
Figure 3. Progress curve analysis of LBP-catalyzed BODIPY-LPS transfer from BODIPY-LPS micelles to R-HDL. Curve *C* in Fig. 1 *A* was analyzed with Eq. 1 using a nonlinear least-squares fitting method. The solid line represents the theoretical fit to the data points. Residuals (the difference between experimental and predicted data) are plotted in *B*.
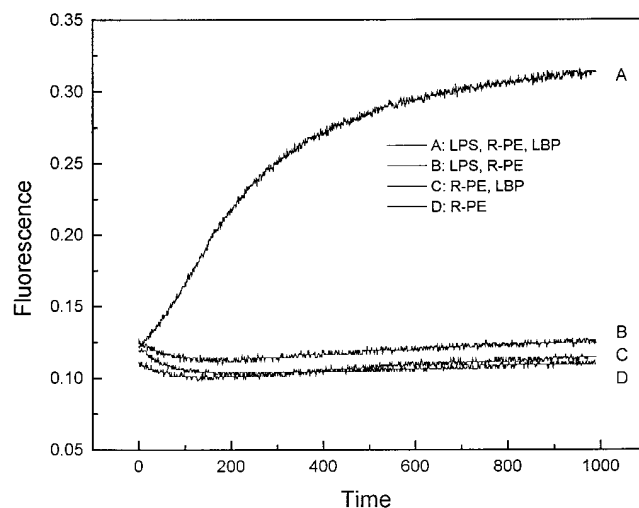
Figure 4. LBP-mediated transfer of R-PE from R-PE membranes to LPS micelles. 2,000 ng/ml unlabeled LPS, 6 μg/ml LBP, 1,000 ng/ml Rhodamine-PE in different combinations were mixed at 37°C as indicated in the graph, and the fluorescence at 570 nm was recorded over time with excitation at 535 nm. The figure is representative of three separate studies.

lipid. In both transfer of BODIPY-LPS (Fig. 1) and R-PE (Fig. 4), the former appears to be the case, since enhancement of fluorescence commences without a lag, and a lag would be expected if unlabeled lipid were acting to dilute fluorescent lipid in a fluorescent lipid micelle. While we cannot rule out a contribution from "backflow" of unlabeled lipid in our measurements, such a contribution is likely to be numerically slight. We conclude that LBP transfers not only LPS to phospholipid vesicles, but also R-PE to LPS micelles.

LBP transfers PI, PC, and R-PE to BR-HDL. To confirm the ability of LBP to transfer phospholipids and to determine if LPS is necessary for LBP to perform its lipid transfer function, we measured transfer of R-PE not into LPS micelles, but into R-HDL particles. BR-HDL particles were incubated with R-PE in the presence or absence of LBP, and then the BR-HDL was separated from donor lipid using streptavidin beads. R-PE did not spontaneously associate with BR-HDL, but LBP strongly promoted binding of R-PE to BR-HDL (Fig. 5 *A*). This experiment indicates that LPS is not necessary for LBP to act as a lipid transfer protein. This experiment also confirms by independent means that LBP causes transfer of R-PE from one bilayer to another.

To determine if LBP can transfer phospholipids in addition to R-PE, parallel experiments were performed to measure transfer of radiolabeled phosphatidylinositol and phosphatidylcholine to BR-HDL. For both of these lipids, LBP enhanced association with BR-HDL (Fig. 5, *B* and *C*). These data indicate that LBP is a lipid transfer protein capable of facilitating the movement of several lipid species. Taken together the above data indicate that LBP can transfer lipids to and from lipid bilayers. To determine if LBP-catalyzed movement of a lipid into a membrane is coupled to movement of another lipid out of that membrane, we turned to an LBP-medi-

ated transfer event in which stoichiometry can be better measured.

LBP catalyzes the transfer of BODIPY-LPS from BODIPY-LPS•CD14 complexes into R-HDL. Previous studies done in this laboratory demonstrate that LBP facilitates not only LPS transfer to sCD14 (5, 9) and to R-HDL (6), but also LPS transfer from LPS•sCD14 complexes to R-HDL (7). To measure this reaction, preformed BODIPY-LPS•sCD14 complexes were mixed with LBP and R-HDL, and the fluorescence changes over time were recorded. Although BODIPY-LPS·sCD14 complexes exhibited strong fluorescence, a twofold LBP-dependent fluorescence increase could still be seen (reference 11 and data not shown). This is due to the fact that the fluorescence of BODIPY-LPS·sCD14 complexes is only 40% of the fluorescence in SDS solution (9) or in phospholipid bilayers (see Methods). Our observation of LBP-catalyzed movement of BODIPY-LPS from LPS•sCD14 complexes to R-HDL confirms previous findings (7) and allows kinetic analysis of this process. As above, we fitted data for movement of BODIPY-LPS from BODIPY-LPS•CD14 complexes into R-HDL to Eq. 1. The data fit the theoretical curve as closely as that of Fig. 3 (data not shown), suggesting that Eq. 1 can be used to describe transfer of BODIPY-LPS from sCD14 to R-HDL and that this reaction may also involve lipid exchange.

LBP catalyzes binding of PI and R-PE to sCD14. To determine if LBP catalyzes reciprocal exchange of lipids into and out of sCD14, we first determined if sCD14 can bind phospholipids. We addressed this question using native PAGE, a technique we have used to demonstrate binding of LPS to sCD14 (5). When [³H]PI was incubated alone, a small amount of radioactivity was visible near the top of the gel (Fig. 6, lane *1*), consistent with the formation of micelles or vesicles in aqueous buffer. When [³H]PI was incubated with increasing concentra-
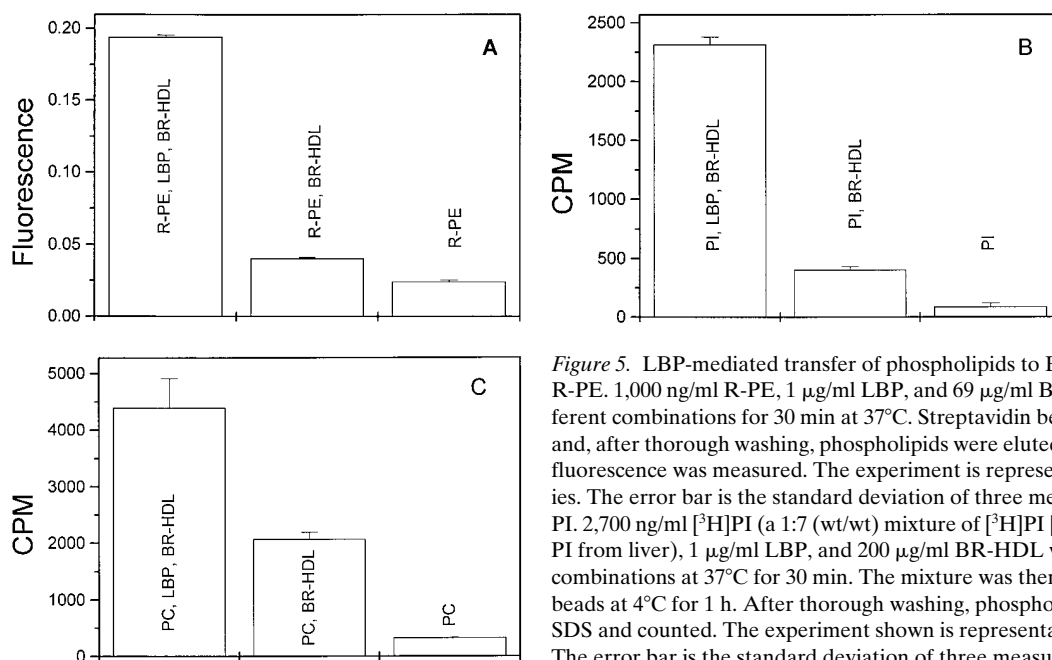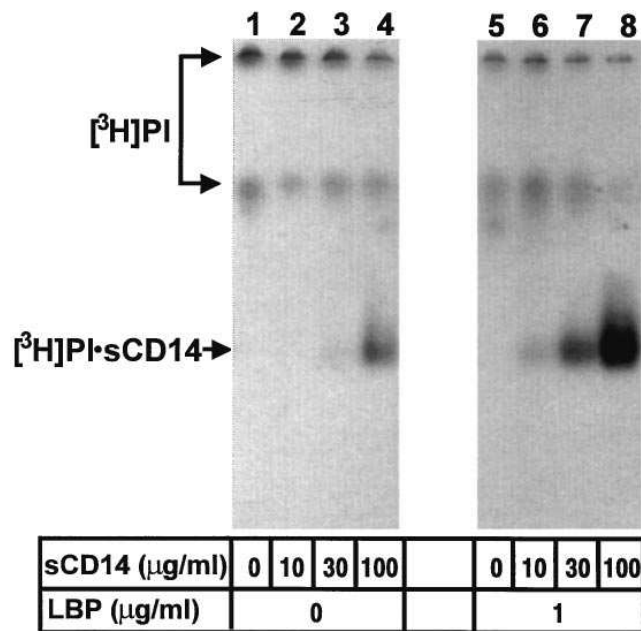

Figure 5. LBP-mediated transfer of phospholipids to BR-HDL. (*A*) Transfer of R-PE. 1,000 ng/ml R-PE, 1 µg/ml LBP, and 69 µg/ml BR-HDL were incubated in different combinations for 30 min at 37°C. Streptavidin beads were added for 1 h at 4°C and, after thorough washing, phospholipids were eluted with 2% SDS and associated fluorescence was measured. The experiment is representative of three separate studies. The error bar is the standard deviation of three measurements. (*B*) Transfer of PI. 2,700 ng/ml [³H]PI (a 1:7 (wt/wt) mixture of [³H]PI [DuPont-NEN] and unlabeled PI from liver), 1 µg/ml LBP, and 200 µg/ml BR-HDL were incubated in different combinations at 37°C for 30 min. The mixture was then incubated with streptavidin beads at 4°C for 1 h. After thorough washing, phospholipids were eluted with 2% SDS and counted. The experiment shown is representative of three separate studies. The error bar is the standard deviation of three measurements. (*C*) Transfer of PC. 2,700 ng/ml [³H]PC (a 1:33 [wt/wt] mixture of [³H]PC and unlabeled egg PC, 1 µg/ml LBP, and 69 µg/ml BR-HDL were incubated in different combinations at 37°C for 70 min, and then streptavidin beads were added for 1 h at 4°C. After a thorough washing, phospholipids were eluted from beads with 2% SDS and counted. The experiment shown is representative of two separate studies. The error bar is the standard deviation of three measurements.

tions of sCD14, a radioactive band at the position of sCD14 appeared (Fig. 6, lanes *2–4*), indicating that [³H]PI and sCD14 formed stable complexes.

To determine if LBP can accelerate the binding of PI to sCD14, the incubation of [³H]PI with sCD14 was done in the presence of 1 µg/ml LBP (Fig. 6, lanes *5–8*). The presence of LBP increased the amount of [³H]PI bound to sCD14 without affecting the mobility of the complexes. This result is very similar to our previous results with [³H]LPS (5), suggesting that LBP acts as a transfer protein, accelerating the binding of [³H]PI to sCD14 without forming part of the final complex.

To determine if R-PE can also bind sCD14, sCD14 was incubated with R-PE membranes in the presence or absence of LBP. R-PE exhibited a strong fluorescence rise that was dependent on both sCD14 and LBP (Fig. 7, curve *C*). As in previous studies with BODIPY-LPS (9), we interpret this data as indicating LBP-dependent transfer of R-PE into sCD14. To confirm this interpretation, sCD14 and R-PE micelles were incubated with or without LBP, and then passed through an ultrafiltration device with a 100-kD cutoff filter to separate sCD14 from R-PE. R-PE alone forms membranes that do not pass through the filter (not shown), and addition of LBP alone did not enable passage (Table I). sCD14 passes through these filters (7, 9), and we observed that when sCD14 was added together with LBP, R-PE appeared in the filtrate. This observation confirms the binding of R-PE to sCD14. Importantly, this binding only occurred if LBP was added to catalyze the reaction, indicating that LBP acts to facilitate the binding of R-PE to sCD14. In addition, we observed that the fluorescence of R-PE•sCD14 complexes passing through the 100-kD filter was increased approximately twofold by the addition of SDS. Identical findings were obtained with LPS•CD14 complexes (9),

Table I. LBP Facilitates Binding of R-PE to sCD14

Incubation conditions	LPS-sCD14, LBP, R-PE	LPS-sCD14, R-PE	R-PE, LBP	sCD14, R-PE, LPB
−2% SDS	0.252±0.006	0.051±0.003	0.051±0.002	0.127±0.018
+2% SDS	0.485±0.026	0.075±0.004	0.065±0.000	0.213±0.039

sCD14 (14 µg/ml), preformed LPS•sCD14 complexes (14 µg/ml sCD14 and 380 ng/ml LPS), 1 µg/ml LBP, and 1,600 ng/ml Rhodamine-PE were incubated at 37°C for 30 min in the indicated combinations. The mixtures were loaded on Microcon units with a 100-kD cutoff membrane for ultrafiltration. The fluorescence (570 nm) of the ultrafiltrate was then measured with or without 2% SDS. Data represent the average results of two samples±SD of a representative experiment repeated three times.

and this finding suggests that the interaction of the lipid with sCD14 may affect fluorescence yield.

LBP facilitates exchange of phospholipids into and out of sCD14. To determine if LBP-mediated transfer of R-PE to sCD14 is associated with a reciprocal exit of LPS from the sCD14, complexes of BODIPY-LPS and sCD14 were formed and incubated with LBP and R-PE for increasing intervals before testing for passage through 100-kD filters. The content of BODIPY and Rhodamine in the ultrafiltrate was then determined by measurement of fluorescence at appropriate wavelengths. As expected from Table I, LBP caused a time-dependent increase in the amount of R-PE complexed with sCD14 (Fig. 8). We observed a parallel decrease in the amount of BODIPY-LPS associated with the sCD14. This finding suggests that binding of R-PE is associated with a loss of BODIPY-LPS. From standard curves relating fluorescence to BODIPY-LPS or R-PE concentration, we calculated that one BODIPY-LPS molecule is lost from sCD14 for each 2.2±0.6 R-PE bound in this experiment. Since LPS bears six acyl chains and R-PE has only two acyl chains, these observa-

Figure 6. LBP enhances binding of [³H]PI to sCD14. [³H]PI (2 µg/ml) was incubated at 37°C for 30 min in PD-EDTA alone (lane *1*) or with the indicated concentrations of LBP and sCD14 (lanes *2–8*). The resulting complexes were subjected to native PAGE and ³H was detected by fluorography. The result is representative of two separate studies.
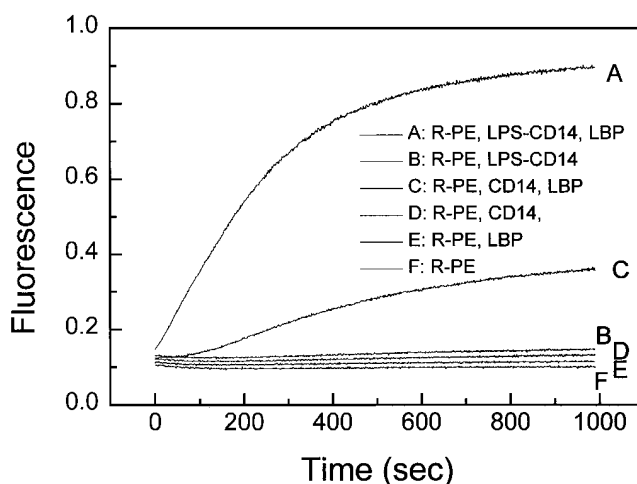

Figure 7. LBP catalyzes the exchange of R-PE from R-PE particles with LPS in LPS•sCD14 complexes. sCD14 (14 µg /ml) or LPS•sCD14 complexes (14 µg/ml sCD14 and 380 ng/ml LPS), 1 µg/ml LBP, and 1,000 ng/ml R-PE in different combinations were mixed at 37°C as indicated in the graph, and the fluorescence at 570 nm was recorded over time. The experiment shown is representative of three separate studies.
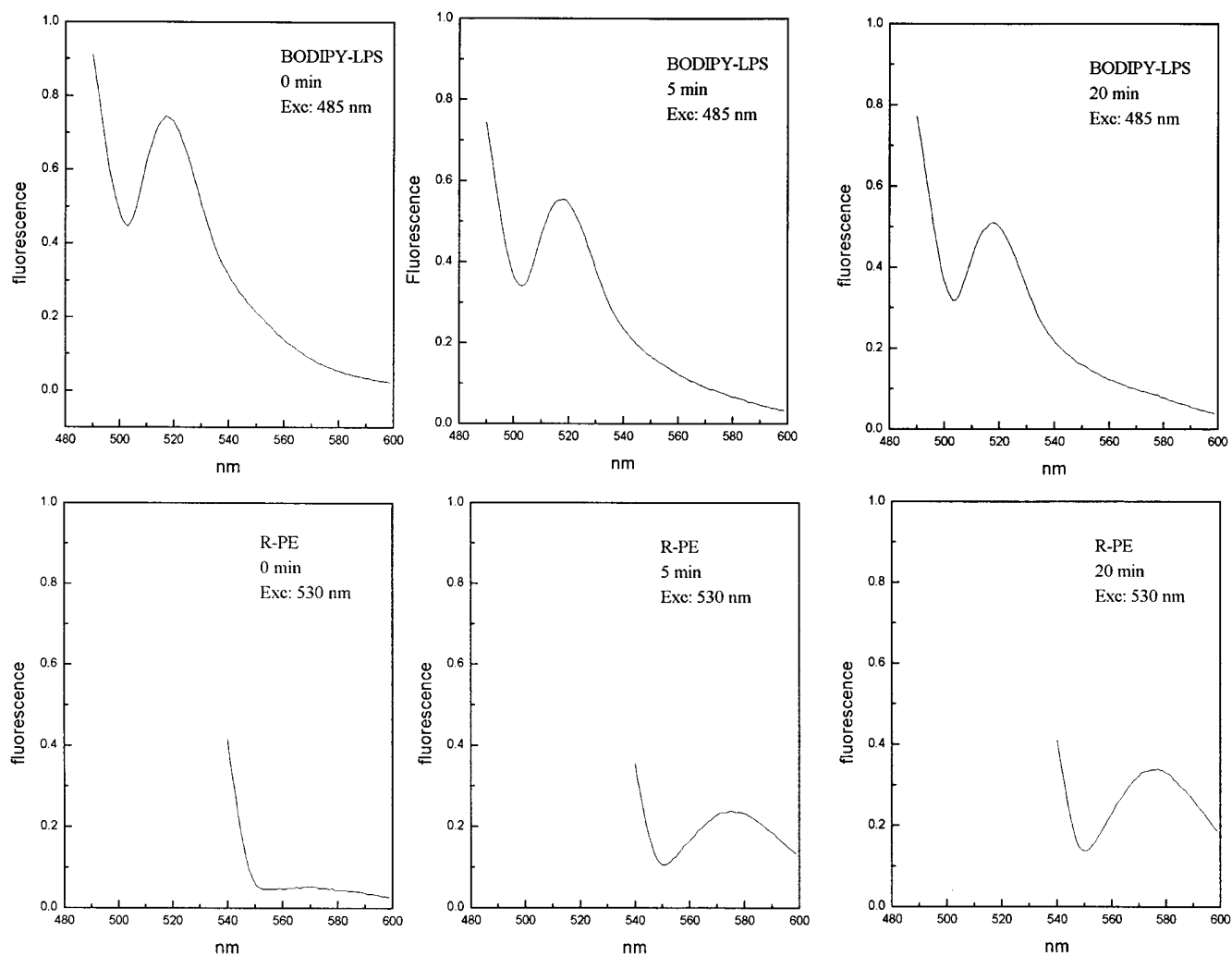
Figure 8. BODIPY-LPS exits from sCD14 upon LBP-catalyzed binding of R-PE. BODIPY-LPS•sCD14 complexes (29 μg/ml sCD14, 770 ng/ml LPS) were incubated with 710 ng/ml R-PE and 2 μg/ml LBP for the periods as indicated. The incubation mixture was then centrifuged in an ultrafiltration device with a 100-kD cutoff filter, and fluorescence emission spectra of the ultrafiltrate were recorded in the presence of 2% SDS at the excitation wavelengths indicated. The experiment shown is representative of two separate studies.

tions are consistent with sCD14 containing a single site sufficient to bind one LPS molecule or two R-PE molecules. Our results do not, however, rule out the possibility that sCD14 has two distinct binding sites and that LBP enforces binding of lipid to only one of them.

Exit of LPS from sCD14 is coupled to uptake of R-PE. The simultaneous transfer of R-PE to sCD14 and LPS from sCD14 suggested that these events are coupled and that transfer of one lipid to sCD14 may be facilitated by the transfer of another lipid from sCD14. To test this idea, we asked whether the transfer of R-PE to sCD14 by LBP was enhanced when LPS•sCD14 complexes were used. We found that the fluorescence increase of R-PE was dramatically greater when incubated with LBP and LPS•sCD14 complexes than with LBP and sCD14 alone. (Fig. 7, curves *A* and *C*). The fluorescence increase with LPS•sCD14 complexes could be caused either by binding of R-PE to sCD14 or by the formation of mixed LPS/R-PE micelles. The latter possibility is unlikely, however, because R-PE is present in a sevenfold molar excess over LPS, and this small amount of LPS is unlikely to cause significant dequenching of R-PE. As confirmation that the transfer of

R-PE to sCD14 is enhanced by the exit of LPS from sCD14, we showed that the ability of R-PE to bind sCD14 and pass through a 100-kD filter was also greater in the presence of LPS•sCD14 complexes than with sCD14 alone (Table I). These observations very strongly suggest that movement of LPS out of sCD14 and movement of R-PE into sCD14 are not simultaneous, independent reactions. Rather, exit of LPS from sCD14 facilitates movement of R-PE into sCD14, and LBP mediates this coupled lipid exchange.

LBP and sCD14 work together to shuttle phospholipids. We have previously shown that LBP-mediated transfer of LPS from micelles to R-HDL can be dramatically accelerated by addition of sCD14 and that the role of sCD14 in this process is to act as a shuttle, first binding an LPS molecule and then surrendering it to the R-HDL particle (7). We found a similar pattern for transfer of R-PE to R-HDL (Fig. 9). Addition of sCD14 (Fig. 9, curve *A*) to LBP and R-HDL (Fig. 9, curve *B*) caused dramatic acceleration of lipid transfer. It is thus evident that LBP can perform the two transfer steps involving sCD14 (transfer of R-PE first to sCD14 and then from sCD14 to R-HDL) more rapidly than it can perform direct transfer of
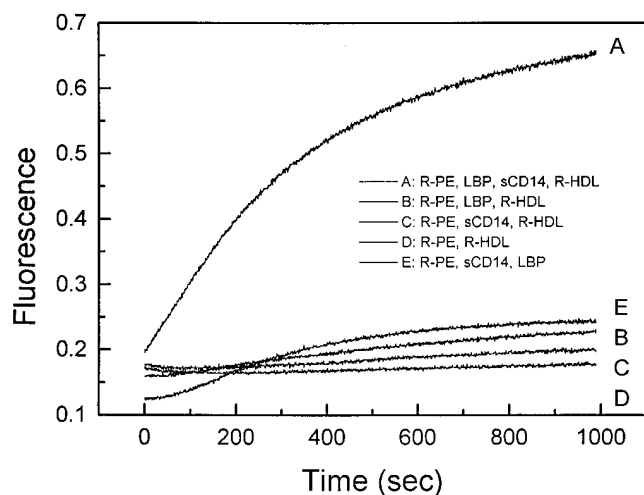
Figure 9. LBP and sCD14 transfer R-PE into R-HDL. 500 ng/ml R-PE, 1 μg/ml LBP, 2 μg/ml sCD14, and 20 μg/ml R-HDL were mixed in different combinations as indicated on the graph, and fluorescence at 570 nm was recorded over time. The experiment shown is representative of three separate studies.

R-PE to R-HDL. As a consequence, sCD14 acts as a shuttle to carry phospholipids from an R-PE membrane to an R-HDL particle.

Quantitative consideration of the transfer rates support the above conclusion. The number of lipid molecules transferred in 400 s was measured under defined conditions of 200 nM R-PE or BODIPY-LPS and 20 μg/ml R-HDL and was calculated as molecules transferred per LBP per hour (Table II). LBP directly transferred 1.2 molecules of R-PE to R-HDL per hour while the addition of 18 nM sCD14 raised the transfer rate to 5.2/h (Table II). Thus, the transfer of R-PE first to sCD14 and then to R-HDL was about fourfold faster than direct transfer under these conditions. To determine which step in sCD14-dependent shuttling of R-PE was rate limiting, we also measured the rate of R-PE transfer to sCD14 under similar conditions. This rate (7.5 R-PE/LBP per h) was slightly faster than the shuttling rate. Transfer of R-PE from R-PE•sCD14 complexes to R-HDL is thus likely to be at least as rapid as transfer to sCD14. We conclude that LBP-catalyzed movement of R-PE into sCD14 and from R-PE•sCD14 complexes into R-HDL occurs at similar rates during shuttling, and that shuttling of R-PE may occur without the buildup of a large concentration of R-PE•sCD14 complexes.

Parallel measurements for transfer of BODIPY-LPS revealed that transfer of this lipid was substantially faster than transfer of R-PE (Table II) and had a different rate-limiting step. As with R-PE, shuttling of BODIPY-LPS via sCD14 was almost threefold faster than direct transfer to R-HDL. However, transfer of LPS to sCD14 was ∼ 16-fold faster than the shuttling rate. It is thus evident that a two-step transfer process involving sCD14 may be faster than direct transfer and that transfer of BODIPY-LPS out of sCD14 into R-HDL is the rate-limiting step in the transfer process. This finding suggests that LPS•sCD14 complexes may build up during shuttling, a phenomenon we have observed in whole plasma (7).

LBP and sCD14 in whole plasma transfer phospholipid. In plasma, LBP is carried on a specialized lipoprotein particle that may affect its ability to function in lipid transfer (16). To determine if the phospholipid transfer activity we have observed with purified, recombinant LBP also obtains in plasma, we measured the transfer of [³H]PI to BR-HDL particles using whole plasma as a source of transfer proteins (Fig. 10 *A*). Significant transfer was observed with 2% plasma. The transfer appeared to be mediated by LBP since it was completely blocked by polyclonal or monoclonal anti–LBP antibodies while irrelevant IgG had no effect. sCD14 present in plasma also appears to accelerate transfer, since addition of the anti–CD14 mAb 3C10 caused a 39% depression in PI transfer (Fig. 10 *B*). These results suggest that LBP and sCD14 play a dominant role in mediating transfer of at least some species of phospholipid in plasma.

Discussion

LBP and sCD14 were originally discovered as proteins necessary for cellular responses to LPS, but their abundance has suggested additional functions. In plasma, both sCD14 (∼ 5 μg/ml) and LBP (∼ 10 μg/ml) (6) are present at ∼ 1,000-fold molar excess of the LPS levels seen in patients with fatal septic shock (19). The principal finding of this work is that LBP and sCD14 can function in transport of mammalian phospholipids. Transport of PI, PC, and a fluorescent derivative of PE were observed from membranes bearing high concentrations of these lipids to R-HDL particles. This finding suggests that LBP and sCD14 catalyze an equilibration of amphiphilic, membrane-forming lipids between membranes. Transport of lipids was observed both with purified recombinant proteins and in whole plasma, suggesting that the transport activity is operative under in vivo conditions. These studies define a novel function for LBP and sCD14.

We have previously demonstrated that LBP and sCD14 transport LPS in a two-step process. An LPS monomer is first transferred to sCD14, the sCD14 diffuses to its destination, and then LBP catalyzes release of the lipid from sCD14. Data described here suggest a similar mechanism operates for transport of phospholipids. LBP was shown to catalyze the binding of PI and R-PE to sCD14, and addition of sCD14 was shown to strongly accelerate the transport of these lipids between membranes. These results suggest that sCD14 facilitates transport of phospholipids by acting as a carrier or shuttle in the same way that serum albumin shuttles fatty acids. Our findings thus

Table II. Rates of LBP-mediated Transfer of Labeled Lipids (Molecules Lipid Transferred per LBP per Hour)

Lipid	Direct transfer to R-HDL	Transfer to R-HDL with sCD14	Transfer to sCD14
R-PE	1.19±0.058	5.23±0.52	7.54±1.23
BODIPY-LPS	13.99±0.22	35.95±1.5	561±22

For transfer to R-HDL and transfer to R-HDL with sCD14, 200 nM R-PE or 200 nM BODIPY-LPS were mixed with 1 μg/ml LBP, 2 μg/ml sCD14, and 20 μg/ml R-HDL as indicated in the table. Fluorescence was recorded for 400 s and transfer during this interval was calculated. Transfer was < 30% complete in this time frame. For R-PE transfer to sCD14, 0.3 μg/ml LBP was used. Transfer of BODIPY-LPS to sCD14 was so rapid that data was taken from studies using a much lower LBP concentration (30 ng/ml). The results are the average of three experiments±SD.
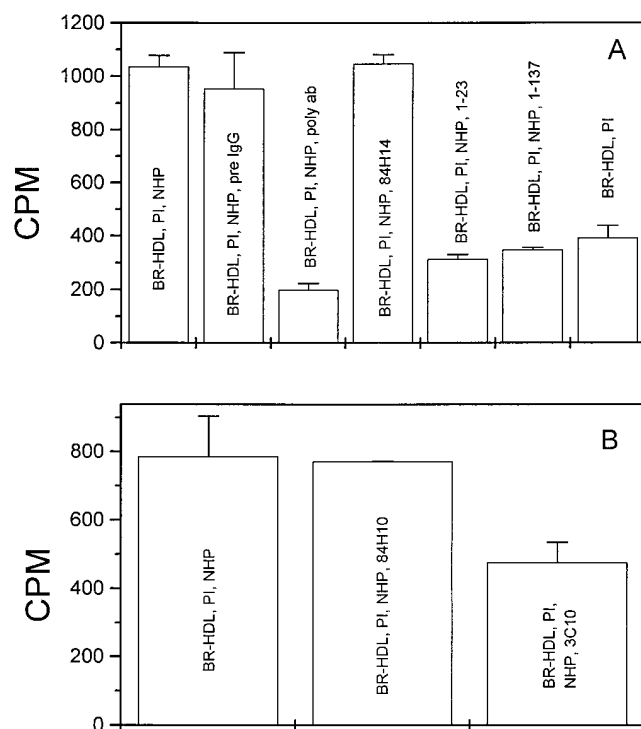
Figure 10. LBP and sCD14 mediate the plasma-catalyzed transfer of PI to BR-HDL. (*A*) Role of LBP. 2,700 ng/ml [^{3}H]PI, 2% plasma, 69 μg/ml BR-HDL, and polyclonal (100 μg/ml) or monoclonal (20 μg/ml) anti–LBP antibodies were incubated in different combinations for 30 min at 37°C in a volume of 50 μl. The mixtures were then incubated with streptavidin beads at 4°C for 1 h. After a thorough wash, phospholipid was eluted with 2% SDS and counted. The experiment is representative of two separate studies. The error bar is the standard deviation of two measurements. 84H10 is a control mAb against ICAM-1, and 1-23 and 1-137 are function-blocking anti–LBP mAbs. (*B*) Role of CD14. 2,700 ng/ml [^{3}H]PI, 2% plasma, 69 μg/ml BR-HDL, and monoclonal anti-CD14 antibody (3C10) (20 μg/ml) or control antibody (84H10) (20 μg/ml) were incubated in different combinations for 30 min. The mixtures were then incubated with streptavidin beads at 4°C for 1 h and processed as above. The result is the average of two separate experiments. The background counts (BR-HDL, PI but no normal human plasma) were subtracted from the data.

describe a novel mechanism of phospholipid transport in plasma.

The requirement for a soluble carrier protein (sCD14) distinguishes LBP from other plasma lipid transfer proteins. LBP shows ∼ 23% sequence identity to CETP and 24% to phospholipid transfer protein (PLTP) (4, 20). LBP, PLTP, and CETP are all found in plasma in association with apo A1 and all transfer plasma lipids. PLTP has been shown to transfer phospholipids between lipoprotein particles (21) with no requirement for an additional factor or protein. We have recently shown that PLTP transfers not only phospholipids between lipoproteins, but also transfers LPS from LPS aggregates to R-HDL particles (15). This transfer was not accelerated by addition of sCD14, and PLTP could not transfer LPS to sCD14. In a similar fashion, CETP has been shown to mediate reciprocal exchange of cholesteryl esters and triglyceride between lipoprotein particles with no requirement for a cofactor or carrier protein (18). While work to date on phospholipid

transfer in blood has failed to describe LBP as a participant (18), it is possible that the phospholipid transfer activity of LBP was overlooked because of its dependence on sCD14 for optimal activity. It is also possible that the contribution of any particular phospholipid transfer protein may be dictated by the identity of the donor and acceptor particles, as well as the head group and acyl chain composition of the molecule being transferred. The precise contribution of PLTP and LBP and sCD14 to phospholipid transfer in plasma now needs to be determined.

Several findings suggest that, as is the case with CETP, LBP may catalyze a reciprocal exchange of phospholipids. Progress curves for LBP-catalyzed movement of LPS from LPS micelles (Fig. 3) or from LPS•sCD14 complexes (data not shown) to R-HDL were very faithfully predicted by a model of lipid exchange. Under the conditions of LPS transfer from LPS micelles to lipid vesicle, transfer of R-PE was observed from membranes to LPS micelles (Fig. 4). LBP-catalyzed binding of R-PE to sCD14 caused simultaneous release of LPS from the sCD14 (Fig. 8). Most importantly, LBP-catalyzed transfer of R-PE into sCD14 was accelerated if sCD14 was preloaded with LPS (Fig. 7 and Table I). This finding suggests that not only is there simultaneous movement of lipids into and out of sCD14, but also that there is a coupling of those two movements, with exit of LPS speeding entry of R-PE.

While our data are consistent with LBP catalyzing reciprocal exchange of lipids into and out of sCD14, it is not clear if exchange is obligate under all conditions. For example, it is possible that LBP may catalyze either exchange or net transfer, depending on the availability of lipids. It is also not clear from our data which lipids are most readily transferred by LBP and sCD14. Since binding of a lipid to sCD14 may be coupled with exit of a lipid from the sCD14, the nature of both lipid species may affect the reaction rate. We have recently surveyed a large range of phospholipids for their ability to accept LPS from LPS•sCD14 complexes and neutralize their biological activity (8*a*), and these studies revealed strong differences in the speed of transfer. PI showed the greatest activity and lactosylceramide the least. The avidity of sCD14 for individual phospholipids and the role of this avidity in determining the rate of exchange is currently under study.

Quantitative analysis of lipid transfer suggests that although LBP can directly transfer phospholipids to lipoprotein particles, most transfer of lipids may pass through sCD14. LBP-mediated transfer of LPS to R-HDL is threefold faster in the presence of sCD14, and transfer of R-PE is fourfold faster in the presence of sCD14. Moreover, movement of LPS out of LPS•sCD14 complexes is the rate limiting step during shuttling of LPS, with movement into sCD14 being nearly 16-fold faster. These results suggest that sCD14 may be the favored conduit of certain lipids in plasma.

Once bound to sCD14, lipids may be transferred to lipoproteins (7), to phospholipid vesicles (8*a*), and to the plasma membranes of cells (7*a*). LBP can clearly mediate transfer to lipoproteins (7) and phospholipid vesicles (8*a*), but recent studies suggest that LPS may be transferred out of sCD14 to the membranes of leukocytes by a protein distinct from LBP. Cells readily bind LPS from LPS-sCD14 complexes and are strongly stimulated (references 7*a* and 8). The LPS appears to exit from CD14 during binding since the amount of cell-bound LPS far exceeds the amount of cell-surface CD14 (7*a*). Under these conditions, LBP is not necessary for either the uptake of

LPS into cells or the cellular response to the LPS (references 7*a* and 8). Uptake of LPS does appear to require a protein component since pretreatment of the cells with trypsin blocks both uptake of LPS and cellular responses to LPS without affecting cell-surface CD14 (7*a*). These results suggest the existence of an additional protein that works to load/unload CD14 at the cell surface. Such a protein may have important effects on the selection of lipids transported by sCD14 and their destination.

CD14 has been shown to bind not only LPS but also lipoteichoic acid from gram-positive bacteria (22), and additional work implicates CD14 in responses to other bacterial products such as lipoarabinomannan (23). For this reason, it has been suggested that CD14 is a "pattern recognition receptor" that specifically binds foreign molecules and initiates innate immune responses (24). Work described here makes it clear that the binding specificity of CD14 is not restricted to bacterial or foreign or injurious molecules. CD14 binds mammalian phospholipids that have no documented capacity to initiate innate immune responses. Our results thus suggest that CD14 participates in the transport but not the "recognition" of LPS. This conclusion is consistent with recent studies on the ability of certain LPS variants to act as agonists in one mammalian species but antagonists in another. Using mouse, human, or hamster cell lines transfected with mouse or human CD14, Delude et al. (25) showed that recognition of LPS variants as agonist or antagonist was dependent on the species of the cell, not on the species of the transfected CD14. We therefore propose that bacterial LPS is transferred to target cells via an existing mammalian lipid transport system (LBP and CD14), and recognition of LPS as a foreign molecule occurs thereafter in the membranes of cells.

Acknowledgments

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