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Lipopolysaccharide (LPS)-binding Protein Is Carried on Lipoproteins and Acts as a Cofactor in the Neutralization of LPS

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Summary

Lipoproteins isolated from normal human plasma can bind and neutralize bacterial lipopolysaccharide (LPS) and may represent an important mechanism in host defense against gram-negative septic shock. Recent studies have shown that experimentally elevating the levels of circulating high-density lipoproteins (HDL) provides protection against death in animal models of endotoxic shock. We sought to define the components of HDL that are required for neutralization of LPS. To accomplish this we have studied the functional neutralization of LPS by native and reconstituted HDL using a rapid assay that measures the CD14-dependent activation of leukocyte integrins on human neutrophils. We report here that reconstituted HDL particles (R-HDL), prepared from purified apolipoprotein A-I (apoA-I) combined with phospholipid and free cholesterol, are not sufficient to neutralize the biologic activity of LPS. However, addition of recombinant LPS binding protein (LBP), a protein known to transfer LPS to CD14 and enhance responses of cells to LPS, enabled prompt binding and neutralization of LPS by R-HDL. Thus, LBP appears capable of transferring LPS not only to CD14 but also to lipoprotein particles. In contrast with R-HDL, apoA-I containing lipoproteins (LpA-I) isolated from plasma by selected affinity immunosorption (SAIS) on an anti-apoA-I column, neutralized LPS without addition of exogenous LBP. Several lines of evidence demonstrated that LBP is a constituent of LpA-I in plasma. Passage of plasma over an anti-apoA-I column removed more than 99% of the LBP detectable by ELISA, whereas 31% of the LBP was recovered by elution of the column. Similarly, the ability of plasma to enable activation of neutrophils by LPS (LBP/Septin activity) was depleted and recovered by the same process. Furthermore, an immobilized anti-LBP monoclonal antibody coprecipitated apoA-I. The results described here suggest that in addition to its ability to transfer LPS to CD14, LBP may also transfer LPS to lipoproteins. Since LBP appears to be physically associated with lipoproteins in plasma, it is positioned to play an important role in the neutralization of LPS.

Studies over the past few years have shown that plasma proteins play an important role in mediating responses of cells to low concentrations of bacterial lipopolysaccharide (LPS, endotoxin). LPS-binding protein (LBP)[1] (1) and Septin (2) interact with LPS and facilitate the binding of the LPS to CD14 (3). CD14, a protein found on the surface of monocytes, macrophages, and neutrophils (4) then initiates responses of these cells (5, 6). CD14 is also found as a soluble protein in the plasma, and complexes of LPS with soluble CD14 participate in responses of endothelial cells (7), epithelial cells (8), and probably other cell types that do not express membrane CD14. These studies have emphasized the ability of plasma to potentiate responses to LPS.

In contrast, a number of older studies have focused on the ability of plasma to inactivate endotoxin (9–11). Incubation of LPS with plasma has been shown to block the ability of the LPS to cause fever and death in experimental animals (10, 11) and to block the ability of LPS to give a positive signal in the *Limulus* amebocyte lysate assay (12, 13). This "detoxification" or neutralization of LPS is thought to occur without covalent modification of the LPS, because the LPS detoxified

[1] *Abbreviations used in this paper:* AHPBS, APBS with heparin; APBS, PBS with 0.5% human serum albumin; apoA-I, apolipoprotein A-I; ELPS, LPS-coated sheep erythrocytes; HAP, Dulbecco's PBS with 0.5 U/ml aprotinin, 0.05% human serum albumin, 3 mM D-glucose; LBP, LPS-binding protein; LNF, LPS-neutralizing factor; Lp(A-I), apoA-I containing lipoproteins; NHP, normal human plasma; PC, phosphatidylcholine; PDEDTA, Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} with 1 mM EDTA; R-HDL, reconstituted high-density lipoproteins; rLBP, recombinant human LBP; SAIS, selected affinity immunosorption.

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by plasma can be extracted with organic solvents and shows full activity (14). Here we reconcile the findings of both potentiating and neutralizing activity in plasma. By using a very rapid assay of cell stimulation, we show that plasma first potentiates responses to LPS, with maximal activity observed in about 10 min. Thereafter, plasma neutralizes the LPS. These studies suggest that the relative kinetics of potentiation and neutralization may dictate the responses of mammalian cells to endotoxin.

Work from several laboratories has shown that plasma lipoproteins, particularly high-density lipoproteins (HDL), bind and neutralize LPS (15–18), and that these particles may constitute the LPS-neutralizing activity in plasma. Here we show that HDL-like particles reconstituted from purified components cannot by themselves neutralize LPS, but the LPS neutralizing capacity of these particles can be realized by the addition of purified LBP. These studies suggest that LBP is an LPS transfer protein that facilitates binding of LPS not only to CD14 but also to lipoproteins. We further found that LBP in plasma is associated with lipoproteins, suggesting an important role for LBP in the neutralization of LPS by plasma.

Materials and Methods

Reagents. LPS from *Salmonella minnesota* strain R595 (Re) and ^{3}H-labeled LPS from *Escherichia coli* K12 strain LCD25 (K12) was purchased from List Biological Laboratories (Campbell, CA). Human serum albumin was purchased from Armour Pharmaceutical Co. (Kankakee, IL). Recombinant human (r)LBP was expressed and purified as described (3). Polyclonal anti-rLBP was raised in rabbits and purified by chromatography on protein G–Sepharose. 17G4 is a murine mAb (IgG2a) directed against LBP (Myc, A., and D. Emanuel, manuscript in preparation). It was purified from ascites fluid by chromatography on protein G–Sepharose. Purified egg phosphatidylcholine, cholesterol, and sodium cholate were purchased from Sigma Chemical Co. (St. Louis, MO). Normal human plasma (NHP) was prepared from the blood of healthy donors anticoagulated with 5 U/ml sodium heparin and stored at 4°C.

Depletion of Apolipoprotein A-I from NHP. NHP was depleted of apolipoprotein A-I (apoA-I) using selected affinity immunosorption (SAIS) as previously described (19, 20). NHP passed through a column of Sepharose coupled to a goat anti–human apoA-I IgG was shown to be >95% depleted of apoA-I by ELISA (Kunitake, S. T., and J. P. Kane, manuscript in preparation). Recovery of apoA-I from the column by elution with 0.2 M acetic acid, 150 mM NaCl (pH 3.0) was >90%. The resulting preparation of apoA-I containing lipoproteins (Lp(A-I)) has been characterized previously (19). As a control, NHP was passed over a preimmune IgG column and showed no detectable loss of apoA-I from the flow through and no recovery of apoA-I in the acid eluate. The goat anti–human apoA-I antibody used to prepare the immunoaffinity column was shown not to recognize LBP by ELISA (see Fig. 10) or Western blot (data not shown).

Preparation of Reconstituted HDL (R-HDL) Particles. R-HDL were prepared by the sodium cholate dialysis method as previously described (21). Briefly, apoA-I, purified by sequential ultracentrifugation and size exclusion chromatography as previously described (22) was mixed with egg phosphatidylcholine (PC), cholesterol, and cholate at a ratio of 80:4:1:80 (PC/cholesterol/apoA-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. Another mixture was prepared in parallel in which PC and cholesterol were omitted (apoA-I alone). Incorporation of apoA-I protein into lipid-containing particles of ~200 kD was confirmed by gel filtration using a Superose 6 column (60 × 1.8 cm; Pharmacia LKB, Piscataway, NJ) and by nondenaturing polyacrylamide gel electrophoresis using an 8–25% gel run on the Pharmacia Phast System (Pharmacia LKB). Final preparations were stored in PDEDTA with 0.01% azide at 4°C. Immediately before use in assays of the bioactivity of LPS, the apoA-I preparations were exchanged into APBS (PBS with 0.5% human serum albumin) by ultrafiltration in a Centricon 10 (Amicon Corp., Beverly, MA).

ELISA for Quantitation of LBP. A 72-well Terasaki plate (Robbins Scientific Corp., Sunnyvale, CA) was coated with anti-LBP mAb 17G4 (5 μg/ml) then blocked with PD containing 10% nonfat milk. The plate was then washed with PD containing Tween 20 (0.05%) and samples, diluted in PD containing 0.1% nonfat milk, were added to the plate for 1 h at room temperature. The plate was then washed and rabbit anti–human rLBP (5 μg/ml in PD with 0.1% nonfat milk) was added for 1 h at room temperature. The plate was washed again and alkaline phosphatase-conjugated goat anti–rabbit IgG (Bio-Rad Laboratories Inc., Richmond, CA) (1:1,500 in PD with 0.1% nonfat milk) was added to the wells. After a further 35-min incubation at room temperature, the plate was washed and bound alkaline phosphatase was measured using the fluorogenic substrate Attophos (JBL Scientific, San Luis Obispo, CA) and a Cytofluor 2300 (Millipore Corp., Bedford, MA) fluorescence plate reader. Each plate included a standard curve of known concentrations of rLBP diluted in PD with 0.1% milk.

To observe the interaction of LBP with apoA-I, Terasaki wells were coated with 5 μg/ml of mAb 17G4, a mAb directed against human apoA-I, type II (Calbiochem-Novabiochem, San Diego, CA), or control mAb 26ic directed against CD14 (23) for 2 h at 21°C. Plates were blocked with 0.5% gelatin as described (24), and dilutions of rLBP, purified apoA-I, or partially purified lipoproteins were added for 30 min at 21°C. Plates were then washed and incubated with either goat anti-apoA-I (10 μg/ml) or with a rabbit anti-LBP (10 μg/ml). Bound secondary antibody was detected with alkaline phosphatase-conjugated anti–goat (Bio-Rad Laboratories Inc.) or anti–rabbit IgG as described above.

Partially purified lipoproteins were obtained by passing NHP over a column loaded with HiPak™ aldehyde (ChromatoChem, Inc., Missoula, MT). This procedure quantitatively removed LBP/Septin activity from NHP. The column was eluted with 0.5 M ammonium acetate, pH 3.0, and the eluate was immediately applied to a G-25 Sephadex column to change the buffer to PDEDTA. This eluate contained 90% of the original LBP/Septin activity of plasma but <1% of the protein of plasma (Park, C. T., and S. D. Wright, manuscript in preparation).

Stimulation of PMN by LPS. To assess the biologic activity of LPS we measured adhesion of human PMN to fibrinogen-coated surfaces as described (25). In this assay, stimulation of PMN adhesion depends on the binding of LPS to cell surface CD14 and requires LBP or Septin (LBP/Septin activity) to facilitate this binding. Adhesion of the stimulated PMN to fibrinogen is mediated by the leukocyte integrin CD11b/CD18 (CR3, Mac1). Briefly, mixtures containing LPS and a source of LBP or Septin were diluted in APBS or AHPBS (APBS with 4 U/ml sodium heparin) to the concentrations indicated, yielding a final volume of 50 μl. 10 μl of freshly isolated PMN (2 × 10^7 cells/ml in HAP [Dulbecco's PBS with 0.5 U/ml aprotinin, 0.05% human serum albumin, 3 mM D-glu-

cose]) fluorescently labeled with 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester as described (3, 25) 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 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 as a way of quantitating the total number of cells/well. The plate was then washed and the plate was read again. Binding is expressed as the percentage of cells remaining in the well after the washing step (percent adhesion). Since stimulation of the PMN requires both LPS and LBP/Septin (Fig. 1), assays performed in the presence of excess LBP measure the available concentration of LPS. In a similar fashion, assays performed in the presence of excess LPS measure the available concentration of LBP/Septin. Donor to donor variation in maximal responses (20–75% adhesion) prohibited averaging results of separate experiments, but the pattern of responses was very reproducible.

Neutralization of LPS. LPS was incubated with lipoprotein or NHP diluted in AHPBS for 0–2 h at 37°C. The amount of available LPS remaining in the tube was assessed by adjusting the LBP concentration to 0.5–1.0 μg/ml, adding PMN, and measuring adhesion as described above.

Binding of LPS-coated Erythrocytes to Macrophages. The binding of LPS-coated sheep erythrocytes (ELPS) opsonized with plasma, by CD14 on cultured human macrophages was performed as described (26). Briefly, ELPS were prepared by incubating 10 μg of sonicated LPS (R595) with 10^8 sheep erythrocytes for 1 h at 37°C. ELPS were washed extensively and resuspended to a concentration of 10^8 cells/ml in EDTA-GVB^{2-} (5 mM veronal buffer, pH 7.5, with 150 mM NaCl, 0.1% gelatin, 1 mM EDTA). To determine the ability of NHP to promote the binding of ELPS to macrophages, equal volumes of ELPS and a 1:50 dilution of NHP in PDEDTA were incubated for the stated time at 37°C. The resulting opsonized ELPS were washed, resuspended in EDTA-GVB^{2-}, and 5×10^5 red cells were added to a monolayer of macrophages in a Terasaki well. After a 15-min incubation at room temperature, unbound ELPS were removed by inverting the plate for 10 min at room temperature and gently washing the monolayer. Binding of ELPS was evaluated by phase contrast microscopy and expressed as the attachment index, the number of erythrocytes bound per 100 macrophages.

Binding of ^{3}H-labeled LPS to R-HDL. To measure binding of LPS to R-HDL, ^{3}H-LPS (100 ng/ml, specific activity ≈ 1,000 dpm/10ng), R-HDL (100 μg/ml), and rLBP (0–10 μg/ml) were diluted in 1 ml of APBS and incubated at 37°C for 1 h. The solution was then cooled to 0°C, brought to a density of 1.12 g/ml with a saturated potassium bromide solution, placed in a 5-ml Quick-Seal™ tube (Beckman Instruments, Palo Alto, CA) and spun in a Vti 65.2 rotor (Beckman Instruments) at a temperature of 10°C for 9 h at 60,000 rpm. The contents of each tube were separated into equal top and bottom fractions and the amount of radioactivity in 1 ml of each fraction was evaluated by scintillation counting in 3 ml of Ready Safe™ (Beckman Instruments). Preliminary studies demonstrated that under similar conditions, >90% of [^{3}H]phosphatidylcholine-labeled R-HDL was found in the top half of the tube. apoA-I protein detected by Western blot, also migrated to the top of the tube under these conditions.

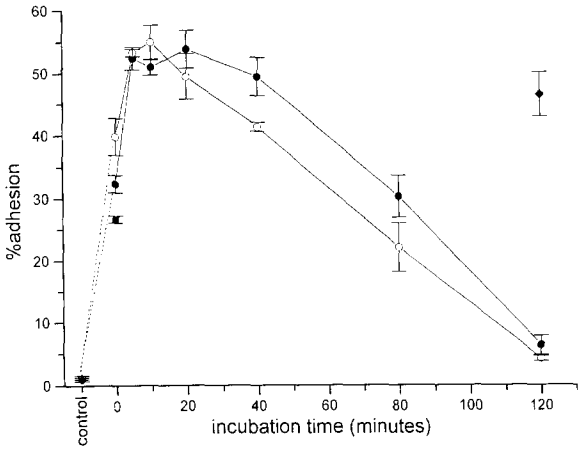

Figure 1. NHP first potentiates then neutralizes the capacity of LPS to stimulate PMN. *E. coli* K12 LPS (1 ng/ml) was incubated for the stated intervals at 37°C with NHP (10%) (●, ○). At the end of this period, buffer (●) or rLBP (1 μg/ml) (○) and fluorescently labeled PMN were added. After 10 min at 37°C the binding of PMN to fibrinogen was measured as described in Materials and Methods. Fresh LPS (1 ng/ml) added after the 120-min incubation with plasma (◆) restored cellular adhesion. rLBP (1 μg/ml) was sufficient to enable a response when mixed with LPS (1 ng/ml) just before addition of PMN (■). "Control" represents PMN treated with LPS alone (▽) or NHP alone (◆). Each point represents the mean of 3 wells ± SD of a representative experiment repeated three times.

Results

The Ability of LPS to Stimulate PMN Is Rapidly Enhanced and then Slowly Neutralized upon Incubation in NHP. LPS (1 ng/ml) was incubated with NHP (10%) at 37°C for the intervals shown in Fig. 1. The ability of the resulting mixture to stimulate cells was quantitated by adding PMN for 10 min and measuring the attachment of the cells to immobilized fibrinogen. LPS alone did not stimulate adhesion of PMN to fibrinogen, but upon incubation of LPS with NHP, the ability of LPS to stimulate adhesion was very rapidly enabled (Fig. 1). Stimulation was evident even when LPS and NHP were combined immediately before the 10-min incubation with PMN (0-min incubation) and was maximal with a 10-min incubation. We attribute this rapid enabling effect to LBP and/or Septin in the plasma and henceforth we will refer to this activity, seen at the early time of incubation, as LBP/Septin activity. Longer periods of incubation with NHP resulted in a gradual loss of the ability of LPS to stimulate PMN. After 120 min, >80% of the stimulatory capacity of the mixture was lost. The loss in activity over time was due to the loss of available LPS rather than the loss or degradation of LBP/Septin because addition of LPS at the end of the incubation restored responses while addition of rLBP did not (Fig. 1). Thus, the loss of the ability of LPS to stimulate cells after incubation in NHP results from neutralization of the LPS, and the factor(s) responsible for this neutralization we will refer to as LPS-neutralizing factor or LNF. Because incubation of LPS in NHP for times longer than 120 min resulted in only minor additional loss of activity we chose 120 min as the standard time of incubation to demonstrate LNF activity.

The ability of plasma to transiently enable recognition of

LPS by cells was confirmed in studies that measure the binding of ELPS to CD14 on macrophages. ELPS were incubated with 2% NHP for intervals, washed, and binding to macrophages was assessed (Fig. 2). Incubation of ELPS with plasma (5 min) enabled strong binding to macrophages, but further incubation caused complete loss of this binding capacity. This loss of binding was not due to the instability of LBP/Septin deposited on the ELPS surface because ELPS incubated with NHP for 5 min, washed to remove unbound protein, and incubated another 35 min at 37°C retained the ability to bind macrophages. Furthermore, the fall in binding observed after long incubation with NHP was not caused by loss of LBP/-Septin activity because NHP recovered after the incubation with the ELPS retained the ability to promote binding of fresh ELPS to macrophages, and addition of fresh NHP or LBP to the treated ELPS did not restore binding to macrophages (data not shown). Finally, it is unlikely that the fall in binding was caused by loss of LPS from the erythrocyte since ELPS formed with ^{3}H-labeled LPS demonstrated only a slow loss of <9% of the LPS between the 5- and 40-min time points (data not shown). These results confirm that plasma contains activities that first enable recognition of LPS by cells and then cause neutralization of the LPS.

R-HDL Does Not Bind or Neutralize LPS. Several laboratories have demonstrated that LPS added to blood or plasma binds to lipoproteins such as HDL resulting in a complex with reduced biological activity (15–18). Therefore we examined the role of HDL particles in the neutralization of LPS. All HDL particles contain apoA-I, but these particles also contain substoichiometric amounts of at least 17 other proteins (24, 27–31). To obtain a homogeneous preparation, we reconstituted HDL particles from purified apoA-I, phosphatidylcholine, and cholesterol.

Binding of LPS to lipoprotein was measured by incubating ^{3}H-LPS with R-HDL for 1 h at 37°C, followed by ultracentrifugation at a density of 1.12 g/ml to separate R-HDL (density <1.12 g/ml) from the relatively higher density ^{3}H-LPS (Fig. 3). Addition of R-HDL caused no increase in the amount of ^{3}H-LPS found at density <1.12 g/ml, indicating that no binding to R-HDL had occurred. In similar studies, LPS (1 ng/ml) was incubated with an amount of R-HDL approximating that found in 10% NHP (100 μg/ml), for times up to 2 h, but no neutralization of biological activity was observed (Fig. 4). Thus, R-HDL by itself cannot bind or neutralize LPS.

rLBP Enables R-HDL to Rapidly Bind and Neutralize LPS. We have previously shown that LBP acts as an LPS transfer protein, catalyzing the binding of LPS to CD14 (3). We reasoned, therefore, that LBP may be able to transfer LPS into R-HDL and cause neutralization of the LPS. Indeed, we observed that LBP enabled a dose-dependent increase in the amount of ^{3}H-LPS fractionating with R-HDL (Fig. 3). Association of ^{3}H-LPS with R-HDL appeared maximal at an LBP concentration of 1 μg/ml. Under these conditions, ~30% of the ^{3}H-LPS was shifted to the upper, HDL-containing fraction by the addition of LBP.

Parallel studies showed that LBP also enabled R-HDL to neutralize the biologic activity of LPS (Fig. 4). Neutralization of LPS by R-HDL and LBP was rapid and complete, with >50% neutralization occurring in 40 min and >88% by 120 min. We confirmed the requirement for LBP in the neutralization of LPS by R-HDL using several concentrations of LPS (Fig. 5). Addition of 1 μg/ml rLBP enabled R-HDL to strongly neutralize >30 ng/ml LPS, but R-HDL in the absence of LBP caused no neutralization of LPS at any

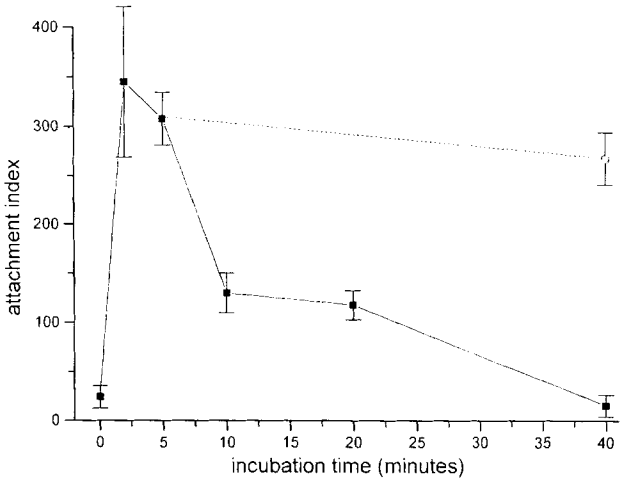

Figure 2. NHP enables then inhibits the binding of ELPS to macrophages. ELPS were incubated for the stated period with NHP (2%) (■), washed into EDTAGVB^{2-}, added to a monolayer of macrophages, and binding of erythrocytes was evaluated as described in Materials and Methods. Data are expressed as attachment index, the number of erythrocytes bound per 100 macrophages, and are the mean values ± SD for three wells of a representative experiment repeated three times. ELPS incubated with NHP 5 min, washed, and incubated another 35 min at 37°C showed no decrease in binding to macrophages (○). In this experiment, ELPS in the absence of LBP or NHP gave an attachment index of 5.

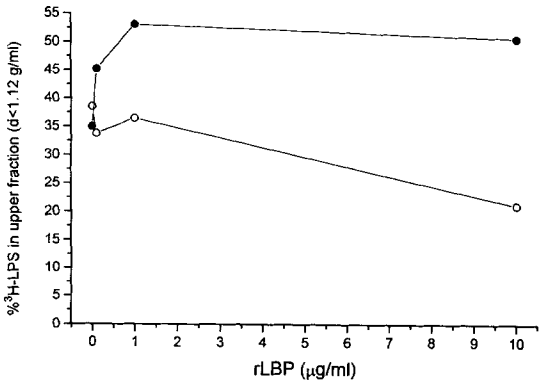

Figure 3. Binding of LPS to R-HDL depends on the concentration of rLBP. ^{3}H-LPS (100 ng/ml) was incubated with increasing concentrations of rLBP in the presence (●) or absence (○) of R-HDL (100 μg/ml) for 1 h at 37°C. After ultracentrifugation at density = 1.12 g/ml, the top and bottom half of each tube were separated and the amount of ^{3}H-LPS assessed by scintillation counting. The data is presented as the cpm measured in the top half of each tube (density <1.12 g/ml) as a percentage of the total recovered in each tube. Results are representative of two identical studies and three similar studies using a fixed dose of LBP.

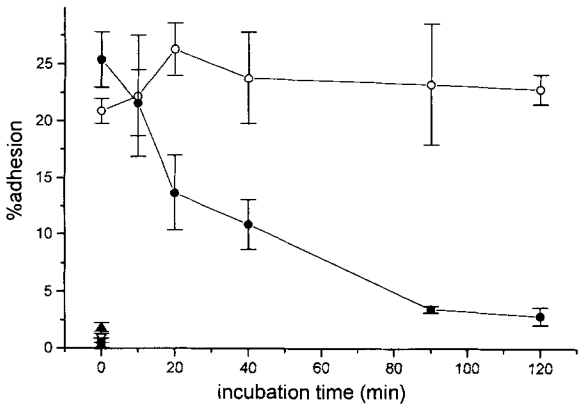

Figure 4. Time dependence of the neutralization of LPS by R-HDL and rLBP. *E. coli* K12 LPS (1 ng/ml) was incubated with R-HDL (100 μg/ml apoA-I) for the stated times in the presence (●) or absence (○) of rLBP (0.5 μg/ml). The amount of biologically active LPS remaining was assessed by adjusting the LBP concentration of all samples to 0.5 μg/ml and adhesion of PMN to fibrinogen was measured. No stimulation of adhesion was seen with buffer alone (▽), LPS alone (▲), or LPS with rHDL (■). Each point represents the mean of 3 wells ± SD of a representative experiment repeated three times.

dose. When the dose of LPS was adjusted to 100 ng/ml, LBP caused neutralization of ~30% of the biologic activity (Fig. 5). This fraction corresponds well to the 30% of ^{3}H-LPS transferred to R-HDL under similar conditions (Fig. 3).

Substoichiometric amounts of rLBP were sufficient to enable neutralization of LPS by R-HDL. Half-maximal neutralization of 10 ng/ml of LPS by R-HDL was seen with 0.01 μg/ml of rLBP (Fig. 6). Under these conditions, each molecule of LBP ($M_r \approx 60{,}000$) (1) neutralized at least seven molecules of LPS ($M_r \approx 4{,}000$) (32) in the presence of R-HDL. This result further confirms that LBP enables neutralization of LPS by R-HDL and suggests that LBP functions catalytically in this role. In contrast to R-HDL, apoA-I alone did not neutralize LPS, and the addition of even high concentrations of LBP did not enable neutralization. This result shows that neither LBP nor apoA-I has any LPS neutralizing activity and suggests an obligate role for lipids in LPS neutralization by rHDL.

Lp(A-I) Particles Represent Only Part of the LNF Activity in Plasma. To assess the contribution of Lp(A-I) particles to LNF activity in plasma, we used SAIS to remove Lp(A-I) particles from whole plasma and to recover purified Lp(A-I) particles. Chromatography on anti-apoA-I removed >95% of the apoA-I from plasma, but removed only small amounts of the LNF activity (Fig. 7). The amount of LNF activity removed varied from preparation to preparation, but removal of apoA-I from NHP never resulted in more than a 66% reduction in the amount of LPS neutralized. This observation suggests that Lp(A-I) particles are not the only lipoprotein particles involved in neutralization of LPS. This finding is consistent with studies demonstrating a role for very low density lipoprotein (VLDL) in the neutralization of LPS (33).

We also tested the purified Lp(A-I) particles for LNF activity. Lp(A-I) particles demonstrated very strong LNF activity, almost completely neutralizing 10 ng/ml of R595 LPS (Fig. 7). No LNF activity was eluted from the control preimmune column (data not shown). The efficient neutralization of LPS by Lp(A-I) particles demonstrates that like R-HDL,

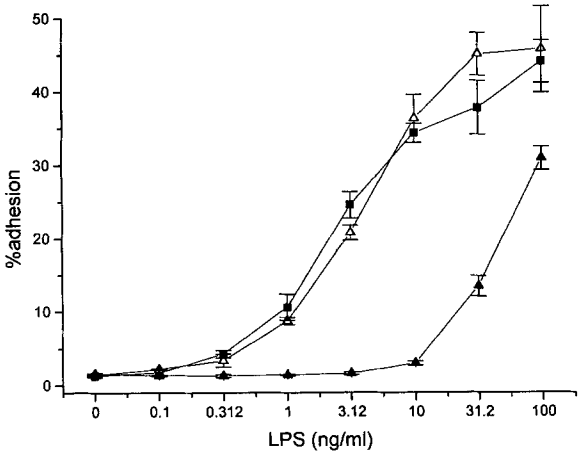

Figure 5. LBP enables neutralization of high concentrations of LPS by R-HDL. Increasing concentrations of *E. coli* K12 LPS were incubated with buffer (■) or R-HDL (100 μg/ml apoA-I) (▲, △) in the presence (■, ▲) or absence (△) of rLBP (1 μg/ml) for 2 h at 37°C. The amount of biologically active LPS remaining was assessed by adjusting the final rLBP concentration in all samples to 1 μg/ml, adding PMN and measuring binding to fibrinogen. Each point represents the mean of three wells ± SD of a representative experiment repeated three times.

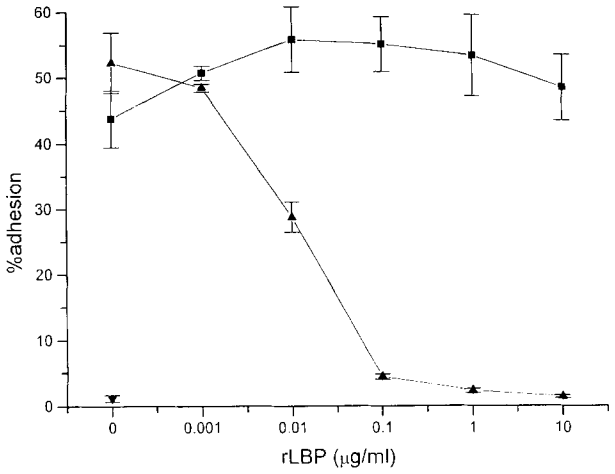

Figure 6. Neutralization of LPS by R-HDL depends on the concentration of rLBP. Increasing concentrations of rLBP were incubated with K12 LPS (10 ng/ml) for 2 h at 37°C with apoA-I (■), or R-HDL (▲), both at a concentration of 100 μg/ml apoA-I. The biologically active LPS remaining was assessed by raising the rLBP concentration by 1 μg/ml in all tubes, adding PMN and measuring adhesion to fibrinogen. No stimulation of adhesion was seen with LPS alone (▼). Each point represents the mean of three wells ± SD of a representative experiment repeated three times.

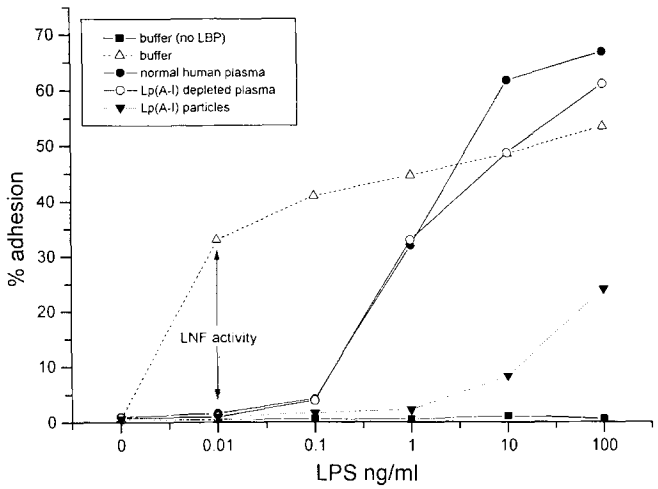

Figure 7. Lp(A-I) particles exhibit LNF activity. Increasing concentrations of R595 LPS were incubated for 2 h at 37°C with buffer (AHPBS) alone (■, △), 10% NHP (●), 10% apoA-I depleted plasma (○), or 10% purified Lp(A-I) particles (▼) (dilutions normalized to the starting volume of NHP). At the end of this incubation, buffer (■) or rLBP (△, ●, ○, ▼) was added to 0.5 μg/ml, PMN were added and adhesion to fibrinogen coated surfaces was evaluated. LPS alone caused no stimulation of PMN (■). LNF activity is represented here by the difference between LBP-stimulated adhesion (△) and the loss of that stimulatory capacity (*arrow*). Each point represents the mean of three wells of an experiment selected from a set of three.

native HDL particles are also able to neutralize LPS. However, the neutralization of LPS by purified Lp(A-I) particles showed no dependence on exogenously added LBP. This finding suggested that native Lp(A-I) particles possess a lipid transfer protein with the same function as LBP. We therefore asked whether LBP itself was present in these particles.

LBP/Septin Activity Copurifies with Lp(A-I) Particles. Lp(A-I) particles were assayed for LBP/Septin by measuring their ability to enable responses of PMN to LPS after a 10-min incubation. We observed strong LBP/Septin activity in Lp(A-I) particles as shown by the large increase in PMN adhesion over LPS alone (Fig. 8 *A*). In contrast, plasma depleted of apoA-I showed a marked reduction in LBP/Septin activity such that ~1,000 times more LPS was required to observe the level of adhesion seen in the presence of Lp(A-I). The copurification of LBP/Septin activity with Lp(A-I) was confirmed by measuring the ability of fractions to enable binding of ELPS to macrophages (Fig. 8 *B*). NHP demonstrated strong LBP/Septin activity in this assay, enabling avid binding of ELPS to macrophages. In contrast, plasma depleted of Lp(A-I) by passage over the anti-apoA-I column was devoid of this activity, promoting no binding of ELPS. Greater than 62% of the LBP/Septin activity observed in this assay, was recovered in the eluate from the anti-apoA-I column (purified Lp(A-I) particles). A nonspecific loss of activity from NHP of ~35% was seen after passage over a preimmune IgG column, but no activity was recovered in the eluate from this column, confirming that LBP/Septin activity specifically copurifies with Lp(A-I) particles. These results indicate that Lp(A-I) particles bear not only LNF activity but also bear LBP/Septin activity, and that nearly all the LBP/Septin activity in plasma resides on Lp(A-I) particles.

We confirmed the association of both LNF and LBP/Septin activity with apoA-I containing particles by following the kinetics of PMN stimulation by LPS and purified Lp(A-I) particles. LPS was incubated for various times with either purified Lp(A-I) particles or with the eluate from a control, preimmune column (Fig. 9). The capacity of LPS to stimu-

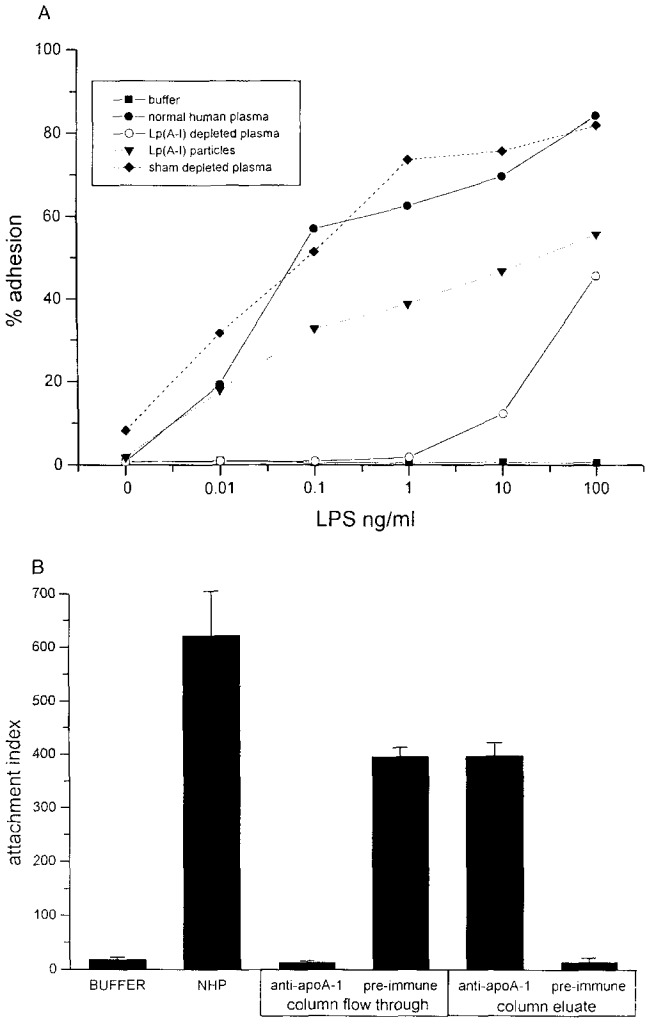

Figure 8. LBP/Septin activity copurifies with Lp(A-I) particles. (*A*) The indicated concentrations of R595 LPS were incubated for 10 min at 37°C in the presence of buffer (AHPBS) alone (■), 10% NHP (●), 10% apoA-I depleted plasma (○), 10% purified Lp(A-I) particles (▼), or 10% preimmune control (sham) depleted plasma (◆). The ability of these mixtures to stimulate adhesion of PMN to fibrinogen was measured. Each point represents the mean of three wells of a representative experiment repeated three times. (*B*) ELPS were incubated in the presence of a 1:80 dilution of the indicated samples for 10 min at 37°C. Treated ELPS were washed, added to a monolayer of macrophages, and binding was evaluated as described in Materials and Methods. Each bar represents the mean of three wells ± SD of a representative experiment repeated three times.

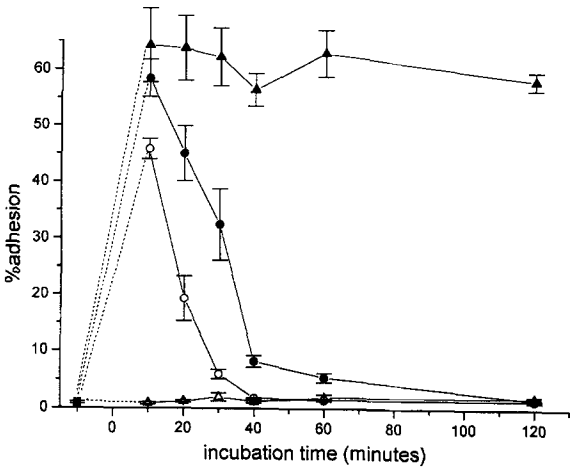

Figure 9. Lp(A-I) particles contain both LBP/Septin and LNF activities. R595 LPS (10 ng/ml) was incubated for the stated times at 37°C in the presence of 10% eluate from a preimmune IgG column (Δ, ▲) or 10% purified Lp(A-I) particles (O, ●). At the end of this incubation, buffer (AHPBS) (Δ, O) or rLBP (0.5 μg/ml) (▲, ●) was added, and the mixture was incubated an additional 10 min, PMN were then added and binding of PMN to fibrinogen-coated wells was measured. LPS alone (■) did not stimulate cells. Each point represents the mean of three wells ± SD of a representative experiment repeated three times.

late PMN was rapidly enabled by Lp(A-I) particles, thus demonstrating LBP/Septin activity. Upon further incubation, the mixture lost the ability to stimulate PMN. This loss of activity was due to neutralization of the LPS since addition of rLBP at the end of the incubation was not able to restore activity. The eluate from the control preimmune column showed no LBP/Septin or LNF activity, demonstrating that these activities are specifically associated with Lp(A-I) particles. These studies also show that the sequential enabling and neutralization of LPS-mediated responses observed in whole plasma (Fig. 1) may be recapitulated with purified Lp(A-I) particles.

LBP Is Physically Associated with Lp(A-I) Particles. Because the above functional data suggested that LBP may be present in the Lp(A-I) particles, we measured LBP associated with these particles by ELISA (Table 1). NHP was found to contain 2.8 μg/ml of LBP. Depletion of Lp(A-I) particles reduced LBP to undetectable levels (>99% depletion). Lp(A-I) particles eluted from the column contained 0.88 μg/ml of LBP whereas LBP was undetectable in the eluate from the control preimmune column. Thus, LBP detected by ELISA is specifically retained by the anti-apoA-I column suggesting that LBP is physically associated with Lp(A-I) particles.

We performed the reciprocal experiment (capture of apoA-I with anti-LBP) using a modified ELISA (Fig. 10). Solutions of lipoprotein particles were incubated in plates coated with anti-LBP mAb 17G4. This step results in capture of both LBP and any associated apolipoproteins. ApoA-I associated with the captured LBP was then detected using polyclonal anti-apoA-I. Since NHP contains a high concentration of apoA-I (~1 mg/ml), nonspecific binding of this protein from plasma precluded detection of a signal above background. We therefore performed the analysis using partially purified lipoprotein particles isolated by chromatography on a column of HiPak™ Aldehyde. This procedure results in quantitative recovery of LBP/septin activity from plasma but a 100-fold reduction in protein concentration (Park, C. T., and S. D. Wright, manuscript in preparation). Anti-LBP mAb 17G4 captured not only LBP but also apoA-I from this solution (Fig. 10 *A*). This observation confirms physical association of LBP with apoA-I containing lipoproteins. Under identical conditions, surface-bound monoclonal anti-apoA-I captured a small amount of LBP (Fig. 10 *B*). We believe this is due to the fact that the immobilized anti-apoA-I mAb is saturated and capture of the apoA-I is thus incomplete. Experiments with lower concentrations of partially purified lipoproteins showed capture of up to 79% of the LBP with anti-apoA-I (data not shown).

Parallel studies confirmed the efficacy and specificity of the antibodies used above. Surface-bound anti-LBP mAb 17G4

Table 1. *LBP Content (μg/ml) in Immunoaffinity Column Fractions*

Expt. no.	NHP	Flow through anti-apoA-I column	Eluate anti-apoA-I column	Flow through preimmune column	Eluate preimmune column
1	3.75	<0.025	1.00	1.50	<0.025
2	1.50	<0.025	1.03	1.13	<0.025
3	3.25	<0.025	0.63	2.50	<0.025
Average	2.80	<0.025	0.88	1.71	<0.025

NHP from three separate individuals was subjected to immunoaffinity adsorption on a column coupled with goat anti-human apoA-I IgG or goat preimmune IgG. Fractions were equalized to the starting volume of plasma, and the concentration of LBP levels was determined by ELISA as described in Materials and Methods. Values given are the LBP concentration in μg/ml. Each value is the mean of triplicate wells. The limit of detection of the ELISA is 0.025 μg/ml LBP.

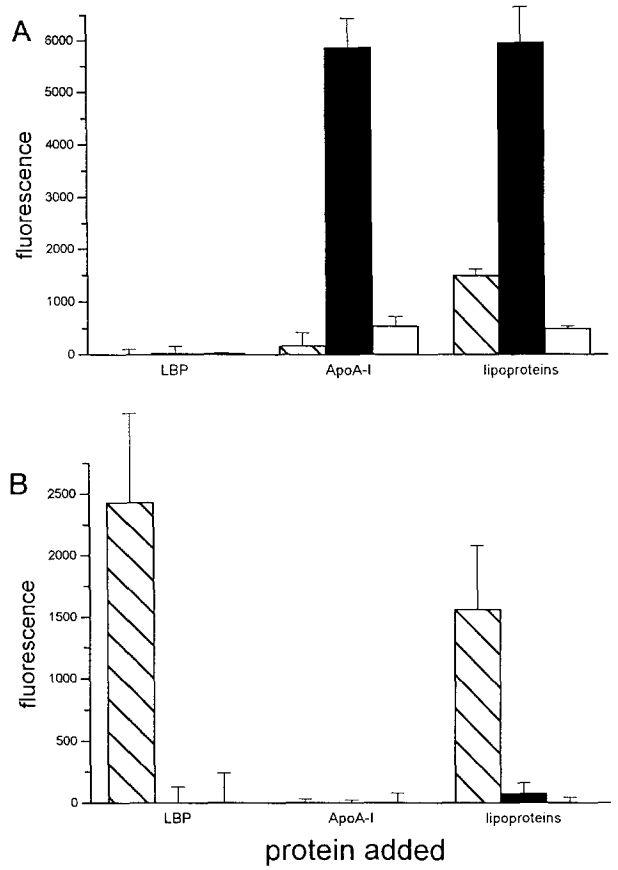

Figure 10. Anti-LBP captures apoA-I. Terasaki plates were coated with mAbs against LBP (*hatched bars*), apoA-I (*solid bars*), or CD14 (*open bars*) followed by addition of rLBP (0.1 μg/ml), apoA-I (0.1 μg/ml), or partially purified lipoproteins (5 μg/ml) for 30 min. Captured apoA-I was detected with rabbit anti-apoA-I (*A*) and captured LBP was detected with rabbit anti-rLBP (*B*). Background signal (no LBP or lipoprotein added) was determined for each combination of immobilized antibody and secondary antibody and was subtracted from the data shown. Each bar represents the mean ± SD of a representative experiment repeated three times.

captured purified rLBP as detected with a rabbit anti-LBP antibody (Fig. 10). No rLBP was captured if the 17G4 was replaced with an irrelevant monoclonal, confirming the specificity of this mAb, and the rabbit anti-LBP did not detect apoA-I captured by another monoclonal antibody, confirming the specificity of this polyclonal antibody. In a similar fashion, surface-bound anti-apoA-I captured purified apoA-I as detected with the goat anti-apoA-I second antibody. No apoA-I was captured if the monoclonal anti-apoA-I was replaced with an irrelevant monoclonal. It is important to note that the polyclonal anti-apoA-I did not detect LBP captured by 17G4 indicating that it does not recognize LBP (Fig. 10 A). Since this polyclonal anti-apoA-I antibody was used in the affinity isolation of Lp(A-I) particles described above, the coisolation of LBP with apoA-I observed above is unlikely to be an artifact caused by crossreactivity of the goat anti-apoA-I with LBP.

Discussion

We have developed an assay for the biologic activity of LPS that requires only a 10-min exposure of LPS-containing samples to cells, and we have used this assay to measure the kinetics of LPS neutralization in NHP. This assay contrasts with other assays used in the study of in vitro neutralization of LPS (e.g., production of cytokines by monocytes) which require at least 4 h incubation of cells with samples and are therefore blind to the rapid changes in the availability of LPS described here. Using our assay we found that plasma first enabled LPS to stimulate cells, with activity reaching a peak within 10 min. Subsequently, the ability of LPS to stimulate cells slowly decreased over the next 2 h. This decline was not due to a loss of LBP/Septin activity in the plasma but to sequestration of the LPS in a form which was no longer available for LBP-mediated transfer to cell surface CD14. Thus, plasma contains factors which sequentially enable then neutralize the biological effects of LPS.

Despite the well-documented ability of HDL to bind and neutralize LPS in plasma (15–18), and studies showing that intravenous R-HDL protects against death in animal models of endotoxic shock (34, 35) we showed that R-HDL particles alone, reconstituted from highly purified components (apoA-I, PC, and cholesterol), were unable to bind or neutralize purified LPS. The inability of LPS to spontaneously interact with R-HDL is consistent with the biophysical properties of LPS and phospholipids. These membrane-forming amphiphiles diffuse very slowly between bilayers, and appreciable movement of phospholipids is only observed in the presence of proteins such as phospholipid exchange proteins (36) which catalyze interbilayer movement. We observed, however, that addition of rLBP to R-HDL enabled rapid, potent, dose-dependent binding and neutralization of LPS by R-HDL. By analogy with other lipid exchange proteins, we believe that LBP enables neutralization of LPS by facilitating its diffusion from LPS vesicles or micelles into HDL. This hypothesis is consistent with previous studies that have shown that binding of LPS by lipoproteins is a necessary step in the neutralization of LPS (16, 18).

rLBP enabled effective neutralization of LPS at doses substoichiometric to the dose of LPS neutralized. Each molecule of LBP caused neutralization of at least seven molecules of LPS under the conditions shown in Fig. 6. This result implies that LBP acts catalytically to transfer LPS to R-HDL. Thus, LBP appears to catalytically transfer LPS to at least two destinations, CD14 as we have recently described (3) and now HDL.

We find that LBP circulates in plasma associated with apoA-I. Over 99% of LBP detected by ELISA and virtually all of the LBP/Septin activity was removed from plasma by passage over an anti-apoA-I column, and both LBP/Septin activity and LBP protein was recovered in the eluate from the anti-apoA-I column. Reciprocal studies showed that an immobilized mAb against LBP captured not only LBP but also apoA-I (Fig. 10). LBP thus joins a group of over 17 apolipoproteins that associate with apoA-I–containing lipoproteins (24, 27–31). Because of the relative abundance of LBP (~5

μg/ml) (37) and apoA-I (~1 mg/ml) in plasma, it is likely that fewer than one in 100 HDL particles bear an LBP. ApoA-I-containing lipoproteins are tremendously heterogeneous in composition, and our studies do not identify other constituents in Lp(A-I) particles associated with LBP.

Our studies show that neutralization of LPS requires both lipoprotein and an enzyme to catalyze movement of LPS into the lipoprotein. ApoA-I–containing lipoproteins bear both of these components and consequently are strong neutralizers of LPS (Fig. 7). Removal of apoA-I from plasma, however, caused only partial loss of LPS neutralizing activity (Fig. 7). Since apoA-I–depleted plasma contains abundant lipoproteins (LDL, VLDL), only a transfer enzyme is needed for neutralization to be observed. Fig. 6 shows that as little as 0.01 μg/ml of LBP can catalyze significant neutralization of LPS in the presence of excess lipoprotein. It is possible, therefore, that the small amount of LBP remaining in the apoA-I–depleted plasma is sufficient to enable neutralization of LPS. We cannot rule out the possibility, however, that an additional transfer activity exists in plasma.

Our finding that lipoproteins in plasma carry all the necessary cofactors for neutralization of LPS appears to differ from previous work. Studies with ultracentrifugally isolated lipoproteins demonstrated that neutralization of LPS required factors found in the nonlipoprotein fraction (16, 17). We believe this difference results from the methods used to prepare lipoproteins. The SAIS procedure used here is a far gentler procedure than ultracentrifugation and is known to preserve the association of proteins with HDL. For example, the association of transferrin (31) with HDL is disturbed by ultracentrifugal isolation but preserved by SAIS. Therefore, we believe that lipoproteins isolated by SAIS do not require exogenous cofactors such as LBP to neutralize LPS because LBP remains bound to Lp(A-I) particles.

LBP shows strong sequence homology with cholesterol ester transfer protein (CETP) (1) and phospholipid transfer protein (PLTP) (38) (23 and 24% identity, respectively). CETP is associated with HDL and transfers cholesterol esters and triglycerides between lipoproteins (39). PLTP is also associated with HDL and transfers phospholipids into HDL (40). LBP thus appears to be part of an emerging family of proteins characterized by sequence homology, association with HDL, and the capacity to transfer lipid species between lipoproteins. An additional homologous protein, bactericidal permeability increasing factor (BPI) (1), resides not on lipoproteins but on the membranes of the primary granules of PMN (41). Lipid transfer activity has not yet been demonstrated for BPI.

The dual role of LBP in both enabling and inhibiting responses to LPS makes it difficult to predict whether its effects are predominantly to enhance or to blunt responses to LPS. A dominant role for LBP in the neutralization of LPS might be inferred from the observation that LBP is an acute phase reactant, rising from <5 μg/ml to >60 μg/ml after challenge (37), and plasma from patients in the acute phase exhibits a reduced ability to enable cytokine production by monocytes in response to LPS (42). Alternatively, LBP may also have an important role in enabling responses to LPS, and factors such as the affinity of LBP for lipoproteins may modulate which activity predominates.

ApoA-I–containing lipoproteins have all the necessary components for both enabling and neutralizing the bioactivity of LPS. In fact, these purified particles recapitulated the sequential enabling and neutralizing of LPS-mediated responses (Fig. 9) observed with whole plasma (Fig. 1). Thus, HDL sits at a crossroads in LPS trafficking. Transfer of LPS to CD14 results in a biologically active complex, while retention by lipoproteins neutralizes LPS. It is possible that yet other factors in plasma influence the direction of trafficking of LPS and thereby modulate biological responses to LPS after it enters the bloodstream. We are currently searching for such factors.

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