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Reconstituted High-Density Lipoprotein Neutralizes Gram-Negative Bacterial Lipopolysaccharides in Human Whole Blood

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We have tested hypotheses relating lipoprotein structure to function as measured by the relative ability to neutralize endotoxin by comparing natural human lipoproteins, a chemically defined form of reconstituted high-density lipoprotein (R-HDL), and a lipid emulsion (Intralipid). The human whole-blood system was used as an in vitro model of lipopolysaccharide (LPS) binding protein and CD14-dependent activation of cytokine production. When lipoproteins were compared on the basis of protein content, R-HDL was most effective in reducing tumor necrosis factor alpha (TNF-α) production followed in order by very low density lipoprotein, low-density lipoprotein, Intralipid, and natural HDL. However, when these particles were compared by protein, phospholipid, cholesterol, or triglyceride content by stepwise linear regression analysis, only phospholipid was correlated to effectiveness ($r^2 = 0.873$; $P < 0.0001$). Anti-CD14 monoclonal antibodies MY4 and 3C10 inhibited LPS binding protein and CD14-dependent activation of TNF-α production by LPS at LPS concentrations up to ~1.0 ng/ml. R-HDL (2 mg of protein per ml) blocked TNF-α production by LPS from both smooth- and rough-type gram-negative bacteria at concentrations up to 100 ng of LPS per ml but had little effect on heat-killed gram-positive *Staphylococcus aureus* and no effect on other LPS-independent stimuli tested. These results support our hypothesis that LPS is neutralized by binding to phospholipid on the surface of R-HDL and demonstrate that R-HDL is a potent inhibitor of the induction of TNF-α by LPS from both rough- and smooth-form gram-negative bacteria in whole human blood.

Endotoxin is a lipopolysaccharide (LPS) that is anchored by a phospholipid-like lipid A domain in the outer monolayer of the outer membrane of gram-negative bacteria (28). When bacteria are exposed to blood or serum, LPS is released from the bacterial surface as membrane fragments, membrane blebs, or mixed vesicles of bacterial phospholipid and LPS (4, 32). In vivo, these extended molecular complexes of LPS are rapidly cleared from the circulation by the phagocytic system (22). In vitro and in vivo, they are only weakly toxic in the absence of a 60-kDa plasma glycoprotein named LPS binding protein (LBP) (13, 33). LPS is transferred from a single high-affinity binding site on LBP (34) to the CD14 antigen expressed by monocytes (42) and activated neutrophils (41). By "solubilizing" vesicular LPS and transferring it to CD14, LBP lowers the threshold for triggering production and release of a cascade of cytokines, including tumor necrosis factor alpha (TNF-α), interleukin 1, and interleukin 6 (31), from ~100 to <0.1 ng of LPS per ml.

LPS can also bind to plasma lipoproteins: high-density lipoprotein (HDL) (35), low-density lipoprotein (LDL), very low density lipoprotein (VLDL) (11, 12, 16, 36), and chylomicron remnants (17). Binding to any of these lipoproteins greatly reduces the ability of LPS to induce production and release of TNF-α, and interleukins 1 and 6 (1, 6, 24). Although normal plasma lipoprotein concentrations provide a vast excess (>1,000-fold) of LPS binding sites, we and others have recently shown that resistance to endotoxemia is increased in vivo by relatively modest increases in plasma HDL or chylomicron remnant concentrations (8, 16, 18, 21). Further in vivo studies of a chemically defined form of HDL, made by reconstituting apo-HDL protein (R-HDL) or a synthetic peptide with pure phosphatidylcholine (PC), led us to propose a model of the LPS-HDL complex (21). In this leaflet insertion model, the fatty acyl chains of lipid A are anchored in the monolayer of phospholipid that covers the surface of lipoproteins in the same orientation that LPS assumes on the outer membrane of gram-negative bacteria, phospholipid monolayers, liposomes (29, 40), and LDL (38). Binding in this orientation would effectively mask the lipid A domain which carries the biological activity of LPS (28).

The leaflet insertion model predicts that the different capacities of VLDL, LDL, and HDL to neutralize LPS should be proportional to the amount of phospholipid surface available for insertion of the lipid A domain rather than the amount of protein, cholesterol, or triglyceride. The model also predicts that HDL or R-HDL will neutralize endotoxins from both rough (*Escherichia coli* O111:B4) and smooth (*Salmonella minnesota* Re595) gram-negative bacteria that contain complete lipid A domains and either truncated or fully developed polysaccharide domains, respectively. These hypotheses were tested in an in vitro human whole-blood system that preserves the integrity of interactions between plasma proteins and the cellular elements of blood, including those between LBP and CD14.

MATERIALS AND METHODS

LPS. Smooth-strain LPS from *E. coli* O111:B4, *Salmonella typhimurium*, and *Serratia marcescens* and rough-strain LPS from *S. minnesota* R595 (Re) were purchased from List Biological Laboratories, Inc. (Cambell, Calif.). An additional rough-strain LPS, Re595, extracted from *S. minnesota* according to a method described previously (24), was kindly provided by J. C. Mathison and R. J. Ulevitch (Scripps Clinic, La Jolla, Calif.). Stock solutions of LPS (5 mg/ml) were prepared by sonication in 200 mM EDTA, pH 7.0.

Reagents. Heat-killed *Staphylococcus aureus* (HKSA), at 10^{11} cells per ml in saline and monoclonal mouse anti-human antibodies 3C10 (anti-CD14 [37])

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hybridoma line from the American Type Culture Collection (TIB 228) and IB4 (anti-CD18 [23, 43]) were kindly provided by J. C. Mathison and R. J. Ulevitch. Monoclonal anti-CD14 antibody MY4 (14) was obtained from Coulter Cytometry (Hialeah, Fla.); monoclonal anti-prolactin was obtained from Zymed Laboratories (South San Francisco, Calif.). All antibodies were immunoglobulins and were purified on a protein A column.

Natural lipoproteins. Fresh plasma was drawn from healthy, fasting human volunteers. Lipoproteins were purified by sequential density flotation in a Beckman Ti60 rotor (30), with EDTA (1 mg/ml) used as an anticoagulant and an antioxidant.

R-HDL. HDL was purified from human plasma. Beta lipoproteins were removed, and an HDL-rich concentrate was prepared by the two-stage phosphotungstic acid precipitation method of Burstein and Scolnick (5) as modified by Koizumi et al. (19). HDL was isolated by isopycnic focusing in a Beckman V Ti50 vertical rotor by a modification of the procedure described by Chung et al. (7). The purified HDL was washed by density flotation through a layer of 1.21 g of sodium bromide per ml in a Beckman Ti60 rotor (30). The final HDL preparation contained ~85% apolipoprotein A-I, 15% apolipoprotein A-II, and apolipoprotein CI-III and less than 0.5% albumin when estimated by scanning of sodium dodecyl sulfate gradient polyacrylamide (3 to 30%) gels or by direct measurement of protein in fractions separated by chromatography through columns of Superose 6 and 12 in series.

Purified HDL (5-g lots) was dialyzed against 1 mg of EDTA per ml, lyophilized, and extracted three times with 2 liters of cold chloroform-methanol (2:1, vol/vol) and twice with 500 ml of anhydrous diethyl ether. Apo-HDL was collected by filtration through Whatman no. 4 paper, dried under vacuum, and stored at −70°C.

Apo-HDL was reconstituted (R-HDL) with 99% pure egg phosphatidylcholine (PC) (2:1, wt/wt; Avanti Polar Lipids, Alabaster, Ala.) as described by Matz and Jonas (25), except that the last traces of detergent (cholic acid) were removed by adsorption to Bio-Rad SM2 beads as described by Bonomo and Swaney (3). The molar ratio of protein (as A-I) to PC in the final R-HDL preparation was 1:100, with <0.02 mol% cholic acid and ≤0.1 endotoxin unit per mg of protein (~20 pg of LPS per mg of protein).

Whole-blood TNF-α induction assays. Blood was collected in a heparinized tube, diluted with Hank's balanced salt solution (HBSS) or R-HDL, and transferred to Sarstedt tubes (250 μl per tube) (Hayward, Calif.). LPS or HKSA was added (2.5 μl) at various concentrations in pyrogen-free saline containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). After incubation for 4 h at 37°C, the tubes were chilled to 4°C and centrifuged at 10,000 × *g* for 5 min. The supernatant was collected for determination of TNF-α.

A two-way dose-response experiment was carried out in the whole-blood system by mixing a 4-mg/ml solution of R-HDL with whole blood at 6.25, 12.5, 25, 33, 50, and 75% (vol/vol) to obtain 0.25, 0.5, 1.0, 1.25, 2.0, and 3.0 mg of R-HDL (as protein) per ml, respectively. Controls contained HBSS in the place of R-HDL. HBSS controls and R-HDL were incubated with *E. coli* O111:B4 LPS at concentrations of 0, 0.1, 0.3, 1, 10, and 100 ng/ml. A total of 72 (2 × 6 × 6) incubations were run in three batches, using blood from a single donor. A 50% HBSS control was included in each run. TNF-α concentrations from incubations at various dilutions (6.25%, 12.5%, etc.) were adjusted by an appropriate factor to normalize them to 50% whole blood. Adjustment for dilution was unnecessary in all subsequent experiments, which were carried out in 50% mixtures.

Comparison of R-HDL to natural lipoproteins and Intralipid. Fresh whole human blood was supplemented with lipoprotein-free human serum or lipoproteins (VLDL, LDL, and HDL), Intralipid, or R-HDL and incubated with 10 ng of *E. coli* O111:B4 LPS per ml for 4 h. Cell-free supernatants were collected for measurement of TNF-α.

TNF-α production in PBM. Blood mononuclear cells were isolated from fresh human plasma or after preincubation with R-HDL by Ficoll-Paque density gradient centrifugation, as previously described (20). Washed human peripheral blood mononuclear cells (PBM) were suspended in RPMI containing 5% fetal calf serum (10^6 cells per ml), and 200,000 cells were added to each well of a 96-well round-bottom plate. The cells were incubated with phytohemagglutinin (2 μg/ml), anti-CD3 (100 ng/ml), *Staphylococcus aureus* enterotoxin B (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.), or LPS (*E. coli* O111:B4; 10 ng/ml) for 64 h (last 16 h in the presence of [^{3}H]thymidine, 1 μCi per well). The cells were harvested and [^{3}H]thymidine uptake was measured as described previously (20). Incubations of PBM with LPS were supplemented with 10% autologous plasma as a source of LBP. The media were collected after a 48-h incubation. TNF-α was measured by immunoassay (Genzyme, Cambridge, Mass.).

Cytolytic assay for TNF-α. TNF-α was measured by using a cytolytic assay with actinomycin D-treated WEHI-13 cells (10). WEHI cells were maintained in tissue culture medium containing RPMI 1640 supplemented with 10% fetal bovine serum (not heated), 10 mM HEPES, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. WEHI cells were plated in 96-well, flat-bottomed microtiter plates at 20,000 cells per well in a 100-μl volume. The cells were allowed to adhere for 2 to 3 h. Samples for measurement were added at 50 μl per well, and serial threefold dilutions were then made. Plates were incubated for 18 to 20 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added (10 μl of 5 mg/ml in 0.01 M phosphate-buffered saline, pH 7.5), and the plates were incubated for an additional 4 h. Isopropanol containing 40 mM HCl was added to each well (100 μl) to dissolve crystals formed as a product of the metabolism of MTT by the live cells. The degree of cytolysis was directly proportional to the amount of formazan produced and was determined by the optical density at 570 nm corrected for that at 650 nm due to cell debris.

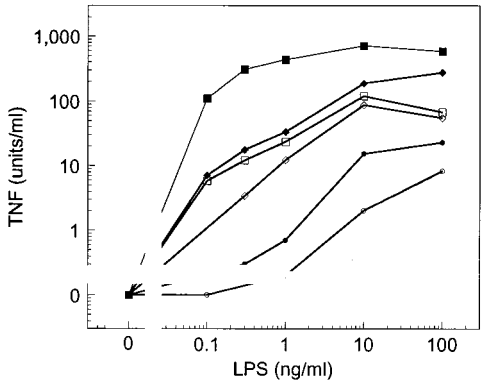

FIG. 1. LPS-dependent TNF-α production in the whole human blood system. Shown is the amount of TNF-α produced by whole human blood in response to *E. coli* O111:B4 LPS. Symbols: control (■) versus R-HDL at 0.5 (◆), 1 (□), 1.3 (◇), 2 (●), and 3 (○) mg/ml.

Each assay contained WEHI cells incubated with no TNF-α (minimum lysis) and cells incubated with a standard (conditioned medium from LPS-treated RAW 264.7 cells), which gave rise to maximum lysis. One unit of TNF-α was defined as the amount of TNF-α resulting in lysis of 50% of the WEHI cells. Samples measured to be below the sensitivity of the assay were assigned a value equal to the lowest standard.

Lipid, lipoprotein, and LPS measurements. Lipid and lipoprotein measurements were carried out using a Roche COBAS FARA II (Roche Diagnostic Systems, Branchburg, N.J.). Total cholesterol and triglycerides were measured by enzymatic methods (Boehringer Mannheim Diagnostics, Indianapolis, Ind.), as described previously (9). Cholic acid was measured with an enzymatic test kit (Sigma). Phospholipid was measured by using an enzymatic method based on measurement of the choline content of PC, sphingomyelin, and lysophosphatidylcholine (Wako Chemical USA, Dallas, Tex.); these phospholipids make up approximately 95% of total serum phospholipids (39). LPS was measured in lipoprotein preparations after dilution and heating at 75°C (15) by the *Limulus* amebocyte lysate method, using the E-Toxate kit (Sigma).

Data analysis and statistics. Results are expressed as mean TNF-α units; the standard deviation was always less than 10% of the mean and is not shown. Lipoprotein concentrations are expressed as milligrams of protein per milliliter unless otherwise stated. Regardless of the units, lipoprotein concentrations in whole-blood incubations refer to the concentration of added lipoprotein. We estimate that endogenous VLDL, LDL, and HDL concentrations, respectively, were less than 0.1, 0.2, and 0.1 mg of protein per ml in incubations containing 50% (vol/vol) whole blood. Stepwise linear regression of TNF-α against lipoprotein protein, PC, TC, and triglycerides was carried out after transformation of the independent variables to $\log_{10}$: $\log(\text{TNF}) = K_{\text{PC}}\log(\text{PC}) + K_{\text{protein}}\log(\text{protein}) + K_{\text{TC}}\log(\text{TC}) + K_{\text{TG}}\log(\text{TG}) + K_{\text{intercept}}$, where K_{PC}, K_{protein}, K_{TC}, K_{TG}, and $K_{\text{intercept}}$ are constants derived from the stepwise regression analysis, TC is total cholesterol, and TG is triglycerides. Mathematical models that included the independent variables protein, cholesterol, and/or triglyceride concentration did not "fit" the data better than the simple model that uses only PC: $\log(\text{TNF}) = K_{\text{PC}}\log(\text{PC}) + K_{\text{intercept}}$.

RESULTS

Dose-response of R-HDL on TNF-α induction by LPS in whole blood. A two-way dose-response experiment was carried out to determine the relationship between TNF-α production in whole blood and the concentrations of *E. coli* O111:B4 LPS and R-HDL. Substantial dose-response relationships, extending over more than 3 $\log_{10}$ units of TNF-α production, were observed between TNF-α production and both LPS and R-HDL concentrations (Fig. 1 and 2). Production of TNF-α decreased steadily with increasing concentrations of R-HDL at LPS concentrations ranging from 0.01 to 100 ng/ml (Fig. 2). The effect of R-HDL was clearly apparent at 0.5 mg/ml and optimal at 2 mg/ml or from approximately half- to twice the concentration of HDL protein in normal human plasma (~1

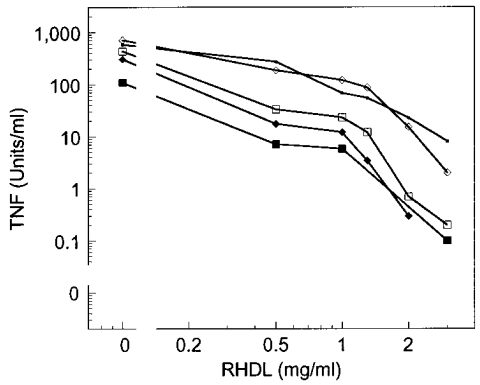

FIG. 2. Effect of R-HDL on LPS-dependent TNF-α production in whole human blood. Shown is the amount of TNF-α produced by whole human blood in response to *E. coli* O111:B4 LPS at 0.1 (■), 0.3 (◆), 1.0 (□), 10 (◇), and 100 (●) mg/ml as affected by increasing R-HDL concentrations.

mg/ml). Optimal concentrations of R-HDL (2 mg/ml) reduced TNF-α production to <5% of the control at LPS concentrations up to 100 ng/ml. All subsequent experiments were carried out with R-HDL at 2 mg/ml.

Comparison of R-HDL with natural lipoproteins and Intralipid. The effect of natural lipoproteins, R-HDL, or 20% Intralipid on LPS-mediated TNF-α production is shown in Fig. 3. Lipoprotein concentrations are expressed as milligrams of lipoprotein protein per milliliter. The compositions of the purified lipoproteins are shown in Table 1. As previously reported by others (11, 12, 16), VLDL and LDL more effectively reduced TNF-α production per unit of lipoprotein protein than did natural HDL (Fig. 3, left panel). In contrast, R-HDL was more effective than either VLDL or LDL and substantially more effective than natural HDL, confirming previous findings (9a). Stepwise regression analysis of the data identified a strong inverse correlation between TNF-α production and the concentration of added phospholipid (Fig. 3, right panel; $r^2 = 0.873$; $P < 0.0001$). The correlation was not improved significantly by including terms for protein, cholesterol, and/or triglyceride concentration in the linear regression analysis.

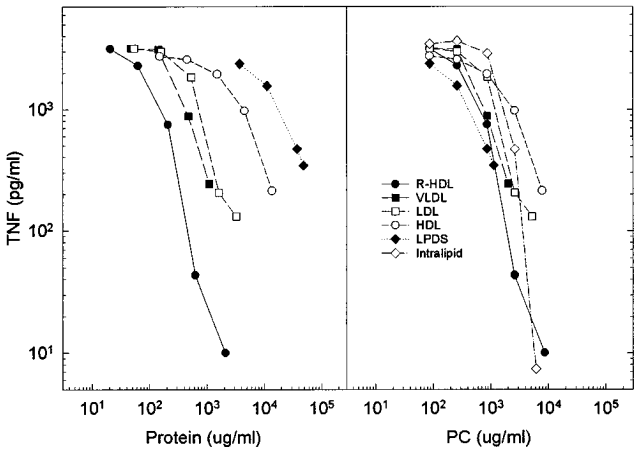

FIG. 3. Inhibition of LPS-dependent TNF-α production is inversely correlated to lipoprotein protein and phospholipid concentration. TNF-α concentration is plotted against protein (left) and phospholipid (right) concentration in whole-blood incubations containing 10 ng of *E. coli* O111:B4 LPS per ml supplemented with R-HDL, VLDL, LDL, HDL, lipoprotein-deficient serum (LPDS), and Intralipid.

TABLE 1. Lipoprotein composition

Lipoprotein[a]	% of total lipoprotein mass			
	Protein	PC	TC[b]	TG[c]
VLDL	8.7	14.2	10.2	66.9
LDL	13.8	20.2	32.7	33.3
HDL	52.9	27.4	17.2	2.5
R-HDL	21.1	78.9	ND[d]	ND
LPDS	97.9	2.1	ND	ND
Intralipid	ND	5.6	0.7	93.7

[a] The phospholipid concentrations (milligrams per milliliter) in these stock solutions of purified lipoproteins were as follows: VLDL, 3.62; LDL, 9.51; HDL, 15.17; R-HDL, 38.51; lipoprotein-deficient serum (LPDS), 2.17; and Intralipid, 12.03.
[b] TC, total cholesterol.
[c] TG, triglycerides.
[d] ND, not detectable.

R-HDL inhibits induction of TNF-α production by LPS from rough and smooth gram-negative bacteria. Two rough types (Re595 and *S. minnesota*) and three smooth types (*E. coli*, *S. typhimurium*, and *Serratia marcescens*) of LPS were used to stimulate TNF-α production in the presence or absence of R-HDL (Fig. 4). Virtually complete inhibition of TNF-α production was observed in incubations of whole blood with LPS from smooth types of gram-negative bacteria. LPS from rough-type gram-negative bacteria induced comparatively more TNF-α production in control incubations when compared on an LPS weight basis (nanograms per milliliter) with smooth-type LPS, but R-HDL inhibited TNF-α production by 90% at LPS concentrations of 100 ng/ml.

Anti-CD14 antibodies inhibit TNF-α production by LPS stimulation. Blocking the LBP-CD14 membrane receptor pathway with anti-CD14 monoclonal antibodies (MY4 and 3C10) essentially abolished TNF-α production at low concentrations of LPS up to 0.3 ng/ml (Fig. 5). Antibodies against prolactin (APA) and CD18 (IB4), used as negative controls, had no effect on TNF-α production. R-HDL so completely inhibited TNF-α production in these studies that it was not possible to draw conclusions regarding complementary or competitive interactions between R-HDL and the anti-CD14 monoclonal antibodies.

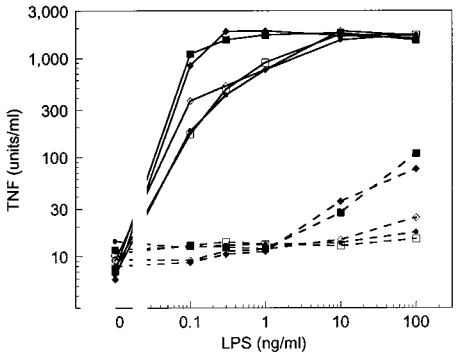

FIG. 4. TNF-α production in response to LPS from rough- and smooth-type gram-negative bacteria: rough types, Re595 (■) and *S. minnesota* (◆); smooth types, *E. coli* (□), *S. typhimurium* (◇), and *Serratia marcescens* (●). Solid lines are incubations without R-HDL; dashed lines are incubations with LPS from the same source in the presence of 2 mg of R-HDL per ml.

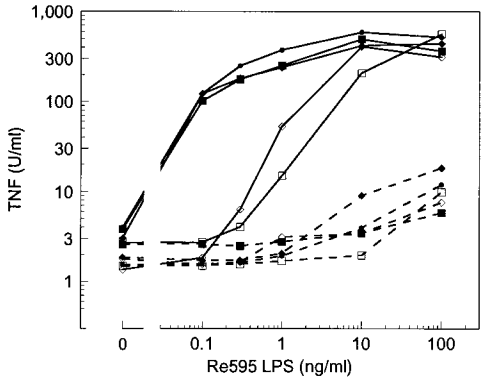

FIG. 5. Comparative inhibition of LPS-stimulated TNF-α production by R-HDL and selected monoclonal antibodies (10 μg/ml). Solid lines are incubations with HBSS; dashed lines are incubations with 2 mg of R-HDL per ml. Symbols: HBSS or R-HDL alone (■); APA (◆); MY4 (□); 3C10 (◇); IB4 (●).

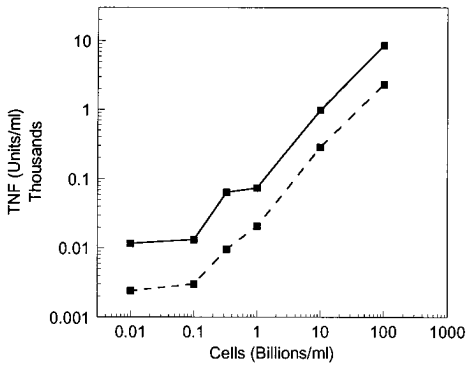

FIG. 6. Induction of TNF-α production by HKSA alone (solid line) and in the presence of 2 mg of R-HDL (dashed line) per ml.

Effect of R-HDL on non-LPS induction of TNF-α. To demonstrate that the effect of R-HDL on cells was not caused by a nonspecific cytotoxicity or immune suppression, we studied the effect of R-HDL on LPS-independent signaling pathways. HKSA activates cells by several pathways, all of which are independent of the LPS signaling pathway. Although R-HDL was not expected to block TNF-α production stimulated by HKSA, we observed a shift in the dose-response curve to the right toward higher concentrations of HKSA (Fig. 6). This could be due to a nonspecific effect of R-HDL on TNF-α production or to neutralization of unidentified gram-positive toxins that meet the structural requirements for binding to R-HDL. To rule out nonspecific effects, whole human blood was incubated with R-HDL (0.5, 1.0, and 2.0 mg/ml) for 4 h as described in Materials and Methods. R-HDL was washed out during preparation of PBM from whole blood. Preincubation with R-HDL had no effect either on the yield of PBM or the percent viable cells as determined by exclusion of trypan blue. The data shown in Table 2 indicate that washed PBM responded normally to LPS and the three non-LPS mitogens (phytohemagglutinin, anti-CD3, and staphylococcal enterotoxin B) tested.

DISCUSSION

Our working hypotheses are that R-HDL and other lipoproteins block the activation of cytokine production by competing successfully with cellular receptors for LPS, that LPS binds to lipoproteins by insertion of the lipid A domain into the phospholipid leaflet that covers the surface of lipoproteins, and that insertion masks the lipid A domain, preventing interactions between LPS and LPS receptors that are necessary for activation of cytokine production. Because LBP and CD14 recognize the lipid A domain of LPS and play an essential role in the cellular response to low (<1 ng/ml) concentrations of LPS in vitro and in vivo, it was important to study lipoprotein-mediated neutralization of LPS in a system that models these interactions.

The studies with anti-CD14 monoclonal antibodies MY4 and 3C10 demonstrated that activation of TNF-α production by LPS was dependent on LBP and CD14 in the human whole-blood system that was used in these experiments. Our finding that R-HDL blocked TNF-α production at low concentrations of LPS supports the hypothesis that R-HDL competed successfully with LBP and CD14 for binding LPS. Activation by higher concentrations of LPS, which can occur through CD14-independent pathways, was also blocked. This suggests that virtually all of the LPS was bound to R-HDL. If nearly all of the added LPS in this system was indeed bound, it would amount to ~18 ng of LPS per mg of phospholipid or <1 molecule of LPS per 3,000 R-HDL disks. (The molar ratio of LPS to PC was estimated by assuming average molecular sizes of LPS [*E. coli* O111:B4] and egg PC molecules to be 12,000 and 786 g/mol, respectively, and the weight ratio of PC to protein measured in the R-HDL to be 2.8 g/g.) Others have pointed to this apparent excess of binding capacity and argued that further increases should be without effect, but neutralization of LPS remains clearly dependent on HDL concentration both in vivo and in vitro (21; this paper). The continued relationship between plasma HDL concentration and inhibition of TNF-α production in response to LPS may be explained by data that indicate that LPS does not dissociate from preformed LPS-lipoprotein complexes while they remain circulating in the vascular compartment (12, 22, 26, 27).

In the whole-blood system used in these experiments, LPS is present initially as free vesicles and LPS-LBP complexes that can interact with cell membranes directly or through receptors, but the concentration of "exchangeable" LPS decreases rapidly with time as the fraction bound to lipoproteins increases. (Wurfel et al. [44] have recently reported the transient presence of exchangeable LPS on LBP and LBP-dependent transfer to R-HDL.) In our system LPS was still present as LPS-lipoprotein complexes, but TNF-α production was 5% or less of that in control incubations when R-HDL and other lipoproteins were added. This would not be the case if LPS readily dissociated from LPS-lipoprotein complexes. Under these conditions, competition among LBP, CD14, and lipoproteins occurs primarily during the first encounter between LPS (or LPS bound to LBP) and lipoproteins or cellular LPS receptors, and the probability of encountering a lipoprotein would be determined by lipoprotein concentration.

Previous studies (11, 12, 16) have compared HDL with the other lipoproteins and concluded that chylomicron remnants and VLDL are as good as or better than HDL at neutralizing LPS. These studies compared lipoproteins by protein content and therefore made the implicit assumption that the protein portion of the lipoprotein particle is the active component that governs binding of LPS. Our model of the LPS-HDL complex predicts that the phospholipid surface of the lipoproteins is the active component. Stepwise regression analysis of the data comparing natural lipoproteins and R-HDL in whole blood showed a strong inverse correlation between TNF-α production and phospholipid concentration. After allowing for the

TABLE 2. Response of PBM to LPS and non-LPS mitogens after R-HDL washout[a]

R-HDL (mg/ml)	TNF-α with LPS (pg/ml)	[^{3}H]thymidine uptake (dpm/10^6 cells)			
		Control	PHA	Anti-CD3	SEB
0	888 ± 211	263 ± 69	32,699 ± 1,401	21,470 ± 1,520	17,462 ± 174
0.5	606 ± 164	301 ± 182	34,476 ± 1,852	16,747 ± 2,317	18,611 ± 2,246
1	872 ± 292	368 ± 163	29,832 ± 4,513	16,242 ± 3,902	17,611 ± 1,343
2	752 ± 66	178 ± 54	30,608 ± 3,938	23,718 ± 654	20,038 ± 3,260

[a] R-HDL was present at the indicated concentrations during a 4-h preincubation in whole blood. R-HDL was removed during the isolation of PBM. Subsequent incubations with LPS and mitogens were carried out in the absence of R-HDL as described in Materials and Methods. TNF-α concentrations in control incubations without LPS averaged 14 pg/ml. Values are given as means ± standard deviations. PHA, phytohemagglutinin; SEB, staphylococcal enterotoxin B.

contribution of phospholipid, no other component, lipoprotein protein, cholesterol, or triglyceride, was significantly correlated with TNF production. Phospholipid content accounted for 94% of the activity of lipoproteins as measured by change in TNF-α production.

Additional support for the role of phospholipid comes from the observations that protein-free Intralipid and cholesterol- and triglyceride-free R-HDL were active. The weak inhibition of TNF-α production by lipoprotein-deficient serum appears to be due to very high density lipoprotein in this fraction as indicated by the presence of phospholipid and by the good correlation of inhibition with phospholipid concentration (Fig. 3, left panel). We conclude that R-HDL was more effective than the natural lipoproteins because it delivered more phospholipid per unit of protein. The small differences in effectiveness between preparations that remained after adjustment in phospholipid could be explained by the presence on the surface of components other than phospholipid that were not measured in this study. Thus, we speculate that the ability of R-HDL to present a clean, unhindered phospholipid surface favors insertion and inactivation of LPS.

The comparative study of LPS from both smooth and rough gram-negative bacteria also supports the hypothesis that the phospholipid leaflet covering lipoproteins provides a surface for insertion and neutralization of LPS. R-HDL neutralized LPS from both groups of bacteria independently of the size and structure of their polysaccharide domains. However, the difference in TNF-α production curves of LPS from both smooth and rough gram-negative bacteria was not expected. LPS isolated from the rough-form bacteria activated TNF-α production at lower concentrations (nanograms per milliliter) than did high-molecular-weight LPS from smooth-form bacteria (Fig. 4). We speculate that this is explained by the difference in molecular weight (the higher molar concentration of the low-molecular-weight forms at the same nanogram-per-milliliter level). Tobias et al. (34) found similar differences in the mass of LPS required to inhibit LBP binding to an LPS-coated surface: 13 versus 50 ng/ml for LPS from *S. minnesota* Re595 compared with *E. coli* O111:B4. Nevertheless, it remains possible that the polysaccharide domains affect association of LPS with LBP or transfer of LPS in LPS-LBP complexes to CD14 and/or R-HDL.

Several lines of evidence argue that inhibition of TNF-α production was not due to a nonspecific inactivation of the cellular immune response. Preincubation with R-HDL had no effect of recovery of viable PBM from whole blood. Moreover, washed PBM responded normally to LPS and mitogens in the absence of R-HDL. However, the modest inhibition of TNF-α production in response to HKSA was unexpected. Gram-positive bacteria do not contain LPS but may contain other membrane-anchored toxins which could in theory be neutralized by R-HDL. Examples include lipoteichoic acid from *Staphylococcus aureus* and the lipoarabinomannans of mycobacteria. At least one pore-forming alpha-toxin of *Staphylococcus aureus* is known to be neutralized by LDL (2). Although natural HDL does not inactivate this alpha-toxin, the effect of R-HDL remains to be determined.

Overall, these results from the human whole-blood system extend our previous data showing increased survival in the mouse endotoxemia model and demonstrate that R-HDL is a potent inhibitor of the induction by LPS of TNF-α production in human whole blood.

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