New Function for High Density Lipoproteins

ISOLATION AND CHARACTERIZATION OF A BACTERIAL LIPOPOLYSACCHARIDE-HIGH DENSITY LIPOPROTEIN COMPLEX FORMED IN RABBIT PLASMA

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ABSTRACT The addition of bacterial lipopolysaccharide (LPS) from *Salmonella minnesota* R595 to rabbit plasma results in a marked reduction of the hydrated buoyant density of the parent R595 LPS, from 1.38 to <1.2 g/cm^3. Using immunopurified anti-R595 LPS antibody covalently linked to Sepharose 4B, we were able to separate the altered R595 LPS ($d < 1.2$ g/cm^3) from the remainder of the plasma proteins by elution of the bound material with 2.5 M KSCN. The KSCN eluate was shown to have a $d < 1.2$ g/cm^3 and to contain both R595 LPS as well as protein and lipid characteristic of high density lipoprotein (HDL). The major protein in the KSCN eluate is a single polypeptide chain with an apparent molecular weight of 26,000 in sodium dodecyl sulfate and an amino acid composition essentially identical to that of apoprotein AI, the major protein of rabbit HDL. The lipid composition of the KSCN eluate is similar to that of HDL, although marked differences in the cholesterol ester/cholesterol ratio and the phosphatidyl choline/phosphatidyl ethanolamine ratio were observed when the KSCN eluate and rabbit HDL were compared. The formation of this R595 LPS-protein-lipid complex in plasma accounts for the marked reduction in buoyant density found when LPS is added to plasma.

INTRODUCTION

Bacterial lipopolysaccharides (LPS)[1] produce multiple pathophysiological changes in man and experimental animals, including fever, hypotensive shock, disseminated intravascular coagulation, diminished oxygen delivery to the tissues, and disruption of normal carbohydrate metabolism. These changes often result in tissue necrosis and most likely contribute to the lethal effects of LPS. Although these pathophysiologic changes have been well characterized, the sequence of events following LPS injection has not been defined in molecular terms.

Recent studies from this laboratory have demonstrated that an intravenous injection of LPS is rapidly partitioned between the vascular compartment and the tissues (1). The major site of tissue localization was shown to be in the fixed macrophages of the liver and spleen. In contrast, LPS remaining in the blood was not bound to formed blood elements, but was present in the plasma. Using LPS isolated from a smooth strain of *Escherichia coli*, 0111:B4, or from the rough mutant *Salmonella minnesota* R595, we showed that the LPS remaining in the plasma after injection had undergone a marked decrease in both particle size as well as buoyant density (1–3).

The interaction of LPS with plasma resulting in the marked decrease in buoyant density was studied in vitro, and data were obtained to support a mechanism whereby the particle size of the parent LPS is first reduced by disaggregation, followed by interaction of disaggregated LPS with lipid containing plasma constituents (2, 3). The reduction in buoyant density was shown to be dependent upon the presence of high density lipoprotein (HDL), and evidence was presented that suggested that enzymatic degradation of LPS could not account for the decrease in buoyant density (2, 3).

Because the low density LPS retains the capacity to produce shock, disseminated intravascular coagulation, and death in rabbits (1, 4) despite the marked abrogation of other endotoxic activities such as complement

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[1] *Abbreviations used in this paper*: apo, apoprotein; HDL, high density lipoprotein; LPS, lipopolysaccharide; NH_2, diaminodipropylamine-substituted; NRP, normal rabbit plasma; NRS, normal rabbit serum; PAGE, polyacrylamide gel electrophoresis; Pi, phosphate; Seph, Sepharose; TLC, thin-layer chromatography.

activation and pyrogenicity, it is important to characterize thoroughly this altered LPS. Thus, the experiments described in this report were undertaken to isolate the altered LPS from LPS/plasma mixtures, and to identify and quantitate the plasma lipid and protein components associated with the low density LPS.

Studies performed here with LPS isolated from *S. minnesota* R595 show that the low density LPS may be separated from the rest of the plasma constituents by affinity chromatography with insoluble immunopurified anti-R595 LPS antibody, and that the reduction in density of LPS by plasma results from a complex between disaggregated R595 LPS and plasma protein and lipid constituents that are characteristic of HDL.

METHODS

LPS. LPS was purified from *S. minnesota* R595 essentially as described by Galanos et al. (5), except that after the final ether wash, the dried R595 LPS was redissolved in 20 mM EDTA (pH 7.5) and subjected to sonic oscillation (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.; model W-375 Sonicator) to dissolve the LPS. This solution was dialyzed against 3 liters of sterile water for 72 h, with a change of the dialysis bath every 12 h, and then lyophilized. Fresh stock solutions of 5 mg/ml LPS were prepared by addition of the LPS to the appropriate buffer, followed by sonic oscillation at 25°C. Radioiodinated R595 LPS was prepared as described by Ulevitch (6) with $Na^{125}I$ and the Enzymo-Bead Reagent (Bio Rad Laboratories, Richmond, Calif.).

The ^{125}I-R595 LPS was shown to coband with the parent R595 LPS in a CsCl gradient at $d = 1.38$ g/cm^3, to coelectrophorese with the parent R595 LPS in sodium dodecyl sulfate/urea polyacrylamide gel electrophoresis (SDS-PAGE) (1), and to react with anti-R595 LPS antibody when examined by immunodiffusion analysis combining protein staining and autoradiographic analysis (data not shown).

Preparation of anti-R595 LPS antiserum. Anti-R595 LPS serum was prepared by immunization of New Zealand white rabbits with acetone-washed *S. minnesota* R595 bacteria as described by Johns et al. (7), except that injections were continued for up to 2 mo with 1.6 ml of a 4 mg/ml suspension of bacteria twice weekly. When the antiserum from individual rabbits was titered by hemagglutination assay with R595-sheep erythrocytes as described by Johns et al. (7), titers of at least 1:600 were obtained. The presence of anti-R595 LPS antibody was routinely assessed by Ouchterlony analysis with a 5 mg/ml solution of purified R595 LPS as the antigen.

Sera from at least six immunized rabbits were pooled, and to prepare an immunoglobulin (Ig)G fraction, solid ammonium sulfate was added to 50% saturation at 4°C, and the resulting precipitate was recovered by centrifugation for 30 min at 5,000 *g*. The precipitate was washed once with 50% saturated ammonium sulfate and redissolved in 0.01 M sodium phosphate buffer, pH 7.7. The redissolved precipitate was dialyzed against 10 liters of the same buffer with two changes during a 48-h period. After dialysis, the small amount of precipitate was removed by centrifugation at 5,000 *g* for 30 min.

This solution (350 ml containing 38 mg/ml of protein) was added to a column containing 80 g of DEAE-cellulose equilibrated with 0.01 M sodium phosphate, pH 7.7, and 20-ml fractions were collected while the column was washed with the initial buffer. The protein that did not bind to the DEAE-cellulose under these conditions contained the anti-R595 IgG fraction, and this fraction was pooled, the protein precipitated by the addition of solid ammonium sulfate to 50% saturation, and the resulting precipitate recovered by centrifugation at 5,000 *g* for 30 min. The precipitate was redissolved in saline and dialyzed against saline for 48 h with three changes of the dialysate. Approximately 2.9 g of protein was recovered by this procedure, and the protein was employed to prepare the immunopurified anti-R595 IgG. An IgG fraction of nonimmune rabbit serum to be used in control experiments was prepared by identical methods.

Preparation of R595-Sepharose 4B. Sepharose 4B/CL (Seph 4B) (Pharmacia Fine Chemicals, Piscataway, N. J.) was substituted with diaminodipropylamine, using CNBr as described by Nishikawa and Bailon (8). The diaminodipropylamine-substituted Seph 4B (NH_2-Seph 4B) was then activated with 6% glutaraldehyde as described by Ternyck and Avrameas (9).

To couple R595 LPS to the activated NH_2-Seph 4B, the LPS was added from a stock solution of 5 mg/ml to a 1:1 slurry of the Seph 4B; and a concentrated sodium borate buffer, pH 8.0, was added so that the final buffer concentration was 0.05 M. This mixture was maintained overnight at 25°C with gentle mechanical agitation, the mixture was then washed three times with 10 vol of saline, and the beads were resuspended in a 1:1 slurry with saline.

The uptake of R595 LPS was determined by measurement of 2-keto-3-deoxyoctulosonante remaining in the washes of the beads (10) or by determining the phosphate (Pi) content of the activated NH_2-Seph 4B exposed to R595 LPS, using a modification of the Pi assay described by Chen et al. (11). In control experiments, we showed that the presence of glutaraldehyde-activated NH_2-Seph 4B did not interfere with the measurement of either the Pi content of R595 LPS or the Pi standards. Good agreement was obtained between the loss of R595 from solution, and the uptake of R595 onto the activated NH_2-Seph 4B was determined by the Pi assay.

Up to 2.5 mg of R595 LPS could be bound to 1 ml packed volume of activated NH_2-Seph 4B, and it was determined that maintaining the R595-Seph 4B at 100°C for 15 min or treatment with 2.5 M KSCN did not cause the loss of >5% of the bound LPS. We also showed that the binding of LPS did not occur when either untreated Seph 4B, glutaraldehyde-treated Seph 4B, or NH_2-Seph 4B were tested, but required glutaraldehyde treatment of NH_2-Seph 4B for LPS binding.

Preparation of immunopurified anti-R595 IgG-Seph 4B. NH_2-Seph 4B (5 ml), to which R595 LPS was coupled to a final concentration of ~0.9 mg/ml, was placed in a 6-ml plastic column and equilibrated with 0.01 M Tris, 2.5 mM $CaCl_2$, pH 7.5. The IgG fraction of anti-R595 LPS was brought to pH 7.5 by the addition of Tris buffer to a final concentration of 0.01 M, and $CaCl_2$ was added to a final concentration of 2.5 mM.

To prepare the immunopurified anti-R595 antibody, 50 ml of the IgG fraction, containing ~1 g of protein, was added to the column, and the column was washed with 0.01 M Tris, 2.5 mM $CaCl_2$, 0.05 M NaCl, pH 7.5, until the absorbance at 280 nm was <0.05, at which time the eluting buffer was replaced by 0.01 M Tris, 2.5 mM $CaCl_2$, 2.5 M KSCN, pH 7.5. A peak of protein was eluted with this buffer that contained anti-R595 antibody activity, as determined by Ouchterlony analysis using a 5 mg/ml solution of R595 LPS as the antigen. The elution profile of a typical preparation is shown in Fig. 1. The tubes containing the anti-R595 LPS were pooled and dialyzed against 1 liter of saline for 72 h with six changes of buffer. Approximately 50 mg of protein was recovered in the peak eluted with 2.5 M KSCN.

The identity of the protein in the KSCN eluate as IgG was made from SDS-PAGE in 9.5% gels prepared according to the

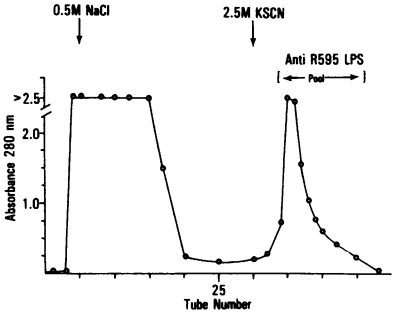

FIGURE 1 The elution pattern obtained from the addition of the an IgG fraction of anti-R595 LPS antiserum to R595-Seph 4B. Approximately 5 ml of R595-Seph 4B containing 0.9 mg/ml R595 LPS was placed in a 6-ml plastic column, and the column was run as described in the text. Each tube contained 4 ml of column eluate.

method of Laemmli (12). As shown in Fig. 2, treatment of the protein in the KSCN eluate with β-mercaptoethanol at 100°C for 5 min before the electrophoresis demonstrates two polypeptide chains (panel B) with molecular weights equivalent to the heavy and light chain of IgG, whereas in the absence of reducing agent, the protein electrophoresed as a single polypeptide chain (panel D). After dialysis the IgG was coupled to CNBr-activated Seph 4B (Pharmacia Fine Chemicals) at a concentration of 1 mg protein/ml Seph 4B according to instructions provided by Pharmacia. After the specified washing procedures an additional wash was performed with 10 columns of 2.5 M KSCN, and the anti-R595 Seph 4B was equilibrated with saline as a 1:1 slurry.

Analytical procedures. Protein was determined by a colorimetric procedure (13) or by the Bradford method (14) using bovine serum albumin as a standard. Isopycnic equilibrium density ultracentrifugation was performed in CsCl as previously described (2, 3). PAGE was performed with 9% gels containing 8 M urea and SDS as described by Downer et al. (15) in a 10 or 20 place, 3 mM thick, vertical slab gel apparatus. Standard proteins used for molecular weight determination were ovalbumin, carboxypeptidase A, chymotrypsinogen, cytochrome *c*, and trasylol. Unless otherwise noted, all samples were pretreated with 40 mM dithiothreitol for 1 min at 56°C before electrophoresis.

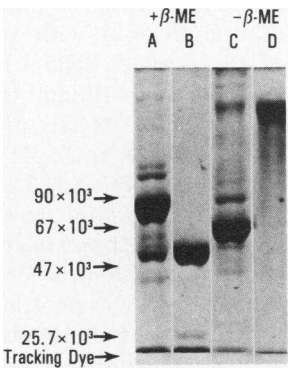

FIGURE 2 SDS-PAGE of rabbit anti-R595 LPS serum and immunopurified anti-R595 LPS antibody performed in 9.5% gels and stained with Coomassie blue according to the method of Laemmli (12). A: 50 μg anti-R595 LPS serum, 1.25% β-mercaptoethanol, 100°C, 2 min. B: 50 μg immunopurified anti-R595 LPS antibody, 1.25% β-mercaptoethanol, 100°C, 2 min. C: 50 μg anti-R595 LPS serum, 100°C, 2 min. D: 50 μg immunopurified anti-R595 LPS antibody, 100°C, 2 min.

Amino acid analysis of protein bands from SDS slab gels was performed according to the method of Houston (16). Corrections for background were made by analyzing equivalent areas of blank gels.

Samples for lipid analysis were extracted with 10 vol of chloroform-methanol (1:1, vol/vol) for 24 h at 4°C in 16 × 125-mm tubes with Teflon-lined screw caps. All glassware was cleaned with dichromate-sulfuric acid before use. Distilled water (1.5 ml/10 ml of organic solvent) was added to each tube and the samples were mixed by inversion and vigorous shaking for three 1-min periods. The samples were kept at room temperature until the upper (aqueous) phase and lower (chloroform) phase were completely separated. The chloroform phase was removed and evaporated to dryness under a flow of nitrogen at 30°C. The dried total lipid extracts were solubilized in 200 μl of chloroform-methanol (1:1) and kept at 4°C for further processing.

Aliquots of the lipid extracts were separated into individual lipid classes by the double-development thin-layer chromatographic (TLC) system of Freeman and West (16). The TLC plates were developed for a third time using hexane as the solvent to remove hydrocarbons and solvent and/or TLC plate contaminants which comigrated with the cholesterol-ester bands. Lipid classes were identified by iodine staining of purified lipids run in standard lanes on each TLC plate. Iodine was removed from the TLC plates by sublimation in a vacuum oven at 15°C. The lipid-containing bands were removed from the plates with a razor blade and were transferred to 12 × 100-mm Pyrex tubes. Cholesterol and cholesterol esters were analyzed directly on the silica gel using the ferric acetate-uranyl acetate-sulfuric acid reagent method of Jung et al. (18). Triglycerides were determined using the sulfuric acid-charring assay of Marsh and Weinstein (19) as modified by Kabara and Chen (20). Phospholipids were determined by a micromodification of the method of Bartlett (21). Samples from blank TLC plates were processed in the same manner as the experimental samples, to serve as controls.

Less than 20% of the R595 LPS present in the KSCN eluate was recovered in the washed lipid extracts, based upon measurements of ^{125}I-R595 LPS. Since >70% of this material had a mobility equivalent to that of free cholesterol or fatty acids in our TLC system, it is not likely that LPS contributes significantly to the phosphorus content of the phospholipid fraction.

Phospholipids were separated into individual species by the two-dimensional TLC method of Parsons and Patton (22). Lipids were identified by comparison of the mobility of standard polar lipid mixtures (Supelco, Inc., Bellefonte, Pa.) on reference TLC plates. Phospholipid spots were degraded and analyzed as described above. Ceramide monohexoside was analyzed by the charring method (19) using bovine cerebrosides (Applied Science Labs, Inc., State College, Pa.) as a standard.

Assays for lipids were performed in duplicate or triplicate, and the data shown are the average values of these determinations.

RESULTS

Characterization of the immunopurified anti-R595 antibody

To prepare immunopurified anti-R595 LPS antibody, we used R595 LPS covalently linked to NH_2-

Seph 4B as an affinity column, prepared as described in Methods. The specificity of the antibody eluted from R595-Seph 4B was examined by Ouchterlony analysis using a 10,000-*g* supernate of a saline-10 mM EDTA extract of whole *S. minnesota* R595 bacteria and purified R595 LPS. This extract was prepared by sonication of a 50 mg/ml suspension of acetone-washed bacteria. The results of the Ouchterlony analysis are shown in Fig. 3, where the precipitin lines obtained with the whole serum, the IgG fraction, and the immunopurified antibody are shown. These results show that the immunopurified anti-R595 recognizes both purified R595 LPS as well as that released from whole bacteria by sonication, but does not contain the antibodies against other bacterial antigens present in the whole serum and the IgG fraction.

We next used Ouchterlony analysis to examine the ability of immunopurified anti-R595 LPS antibody to recognize the serum-altered form ($d < 1.2$ g/cm^3) of R595 LPS. R595 LPS was added to either rabbit or human plasma, and the LPS with a $d < 0.5$ g/cm^3 was prepared by isopycnic density ultracentrifugation as previously described (2). The low density LPS ($d < 1.2$ g/cm^3), along with the parent R595 LPS, was used for Ouchterlony analysis, and the results are shown in Fig. 4. The immunopurified anti-R595 LPS antibody reacts with the altered R595 LPS prepared from both human as well as rabbit serum, although spurring is seen when the parent and altered form of R595 LPS ($d < 0.5$ g/cm^3) are compared.

All of the following experiments have been performed with rabbit plasma to characterize first the low density LPS in a species in which we have performed detailed studies of LPS interactions with host targets

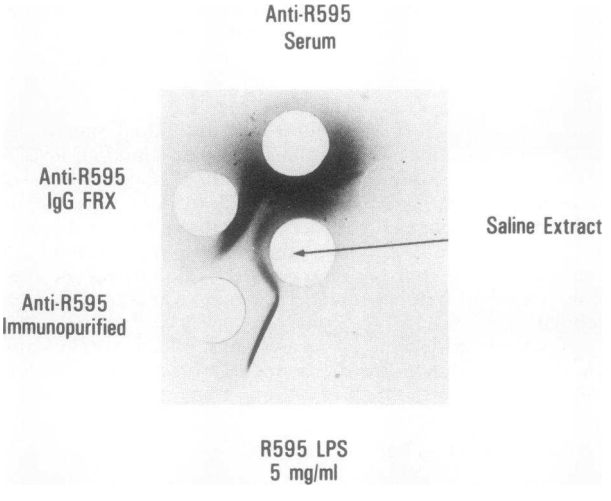

FIGURE 3 Immunodiffusion analysis of the anti-R595 LPS antiserum using a sonicated saline/EDTA extract of whole *S. minnesota* R595 bacteria and purified R595 LPS as antigen. See the text for experimental details.

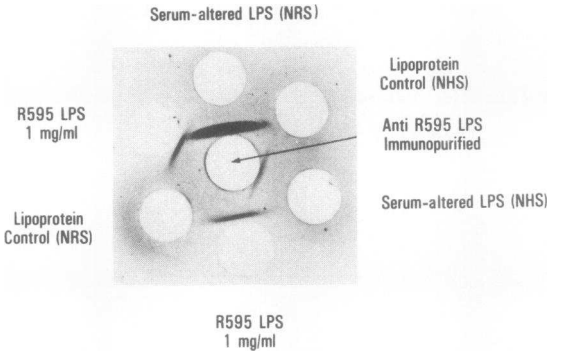

FIGURE 4 Immunodiffusion analysis of immunopurified anti-R595-LPS antibody, comparing the reactivity toward parent R595 LPS and low density ($d < 1.2$ g/cm^3) LPS prepared in rabbit or human serum. The low density LPS was prepared by preparative ultracentrifugation as previously described (2).

(1–4). Studies performed with R595 LPS and human plasma are in progress and will be reported elsewhere.

Isolation of altered R595 LPS

The insoluble anti-R595 LPS was employed as an immunoadsorbant to separate the altered R595 LPS from the remainder of the plasma proteins. Typically, the following method was used to prepare the low density LPS for the experiments described below. A 30-ml plastic syringe was filled with ~17 ml of anti-R595 Seph 4B, and the column was equilibrated with 0.01 M Tris, pH 7.5. Plasma anticoagulated with 20 mM EDTA was pooled from at least six New Zealand white rabbits, and R595 LPS (containing tracer ^{125}I-R595 LPS) was added to the pooled rabbit plasma so that the LPS concentration was 250 μg/ml. The resulting solution was maintained at 37°C for 60 min, and then the solution was diluted fivefold with sterile distilled water. 20 ml of the diluted R595 LPS/plasma was added to the anti-R595 Seph 4B, and the column was washed first with 50 ml of 0.01 M Tris, pH 7.5, next with 100 ml of 0.01 M Tris, 0.05 M NaCl, pH 7.5, and finally with 2.5 M KSCN in 0.01 M Tris, pH 7.5. The results of a typical column run where 4-ml fractions were collected are shown in Fig. 5. Approximately 50% of the applied radioactivity (equivalent to ~500 μg of R595 LPS) was recovered in the KSCN pool, and >95% of the total ^{125}I-LPS applied was recovered from the column in the various fractions. Ouchterlony analysis of the column fractions with anti-R595 LPS demonstrated precipitin formation only in the KSCN eluate (data not shown).

A number of controls were performed to examine the specificity of the anti-R595 Seph 4B. First, when R595 LPS was not added to the pooled normal rabbit plasma (NRP) there was no protein eluted from the anti-R595

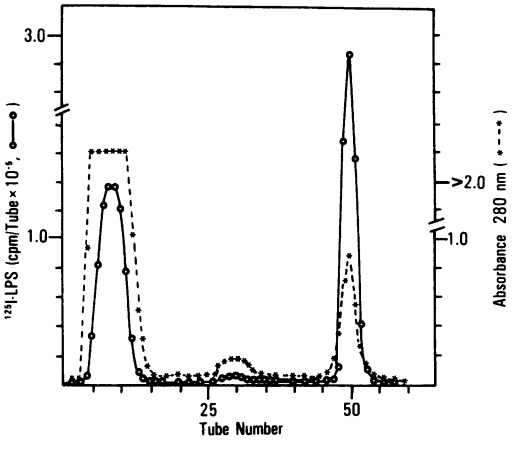

FIGURE 5 The use of insolubilized, immunopurified, anti-R595 LPS antibody to separate low density R595 LPS ($d < 1.2$ g/cm^3) from a mixture of R595 LPS and NRP (containing 20 mM EDTA). To accomplish this, 5 ml of NRP, containing 250 μg R595 LPS/ml and tracer ^{125}I-R595 LPS, was maintained at 37°C for 60 min and diluted five-fold with distilled H_2O. 20 ml of this solution were then passed over a column containing ~17 ml of the anti-R595 LPS-Seph 4B, and the column was washed sequentially with 0.01 M Tris, containing 0.05 M NaCl, and then 2.5 M KSCN. The protein content of the 4-ml column fractions is shown by the dashed line while the presence of ^{125}I-R595 LPS is shown by the solid line.

Seph 4B by KSCN. Next, we used nonimmune IgG that was covalently bound to Seph 4B so that the final protein concentration was ~1 mg/ml Seph 4B. A solution of R595 LPS containing tracer ^{125}I-R595 was added to NRP as above, and the solution was passed over the control column. In this experiment, no protein was eluted from the column with KSCN and >95% of the tracer ^{125}I-R595 LPS included in the incubation mixture was recovered in the initial column fractions and before the KSCN wash.

It was also determined that the anti-R595 Seph-4B could be regenerated by sequential washes with 10 vol of saline and 0.01 M Tris, pH 7.5, and that the columns could be reutilized at least 10 times without any alteration in the elution patterns obtained or in the properties of the KSCN-eluted material (see below).

Characterization of the KSCN eluate from anti-R595-Seph 4B

Because the KSCN eluate contained ^{125}I-LPS and reacted with anti-R595 LPS in immunodiffusion analysis, we next sought to characterize the properties of this material. To do this we performed isopycnic equilibrium density ultracentrifugation in CsCl, SDS-PAGE, amino acid analysis, molecular exclusion chromatography, and lipid analysis.

Equilibrium density ultracentrifugation. The material in the peak eluted from the anti-R595 Seph 4B with 2.5 M KSCN was dialyzed against 1 liter of saline for 72 h with six changes of the dialysis medium. An aliquot of the dialyzed KSCN eluate was subjected to isopycnic equilibrium density ultracentrifugation, and the gradient fractions were analyzed for ^{125}I-LPS as well as protein content. The results of these studies are shown in Fig. 6, where it is demonstrated that >75% of the radioactivity in the gradient was present at $d < 1.2$ g/cm^3. The major peak of the protein in the gradient was present at $d < 1.2$ g/cm^3, and cocentrifuged with the ^{125}I-LPS. A minor protein peak was also detected at $d = 1.36$ g/cm^3. The material containing ^{125}I-LPS that did not bind to the anti-R595-LPS Seph 4B (see Fig. 5) was also subjected to CsCl gradient ultracentrifugation, and no radioactivity banding at $d < 1.2$ g/cm^3 was detected in this fraction (data not shown).

SDS-PAGE and amino acid analysis. Aliquots of the individual CsCl gradient fractions were applied directly to an SDS/urea slab gel, and after completion of the electrophoresis the gels were stained for protein with coomassie blue. As shown in Fig. 7, the major protein present in the same gradient portion as the ^{125}I-LPS (fractions 9–12) has an electrophoretic mobility between the standards with molecular weights 34.6×10^3 and 25.7×10^3. The presence of high concentrations of CsCl interfered with the stacking of the proteins in the gel, so that accurate molecular weight could not be determined in this experiment. However, as described below, the major protein component in the $d < 1.2$ g/cm^3 was shown to have a molecular weight of ~26,000. In contrast, the protein present at $d = 1.36$ g/cm^3 just entered the gel and thus had a molecular weight > 47,000.

To determine accurately the molecular weight of the major protein component of the KSCN eluate, dialyzed samples were electrophoresed in SDS/urea slab gels, and gels were stained for either protein or carbohydrate. The major protein in the KSCN eluate was a single polypeptide chain with an apparent molecular

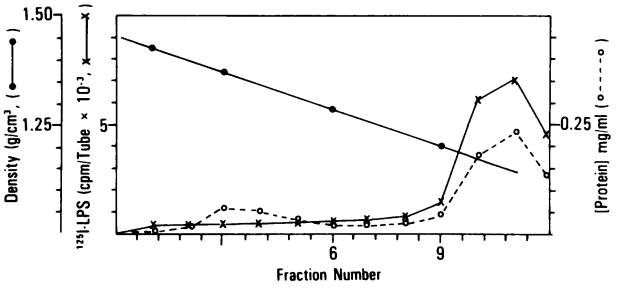

FIGURE 6 Equilibrium density ultracentrifugation in CsCl of the KSCN eluate from anti-R595-Seph 4B. Centrifugation was performed in a Ti 60 rotor at 40,000 rpm for 60 h and gradient fractions were collected and analyzed. The protein content of the fractions is shown by the dashed line, that of the ^{125}I-R595 LPS by the solid line, and the density of the fractions by the closed circles and solid line.

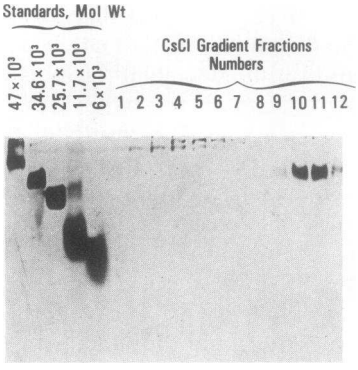

FIGURE 7 SDS-PAGE in a slab gel system of the fractions collected from the CsCl gradient shown in Fig. 6. The samples were treated with 1 mM dithiothreitol for 1 min, 56°C prior to electrophoresis and after completion were stained with coomassie blue. Standard proteins with molecular weights between 47-6 × 10^3 are shown in the first five lanes, and the individual gradient fractions are shown in the remaining lanes.

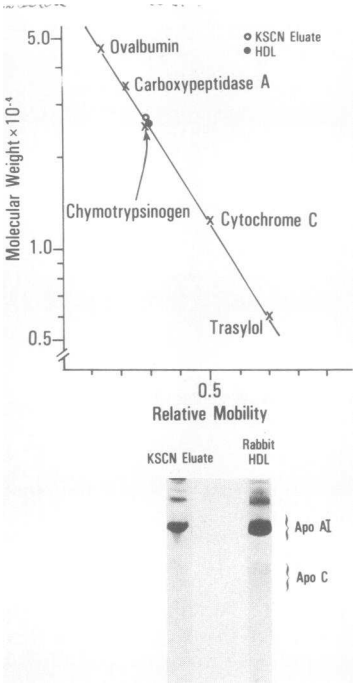

FIGURE 8 Molecular weight determination of the major polypeptide in the KSCN eluate (○) and the rabbit HDL (●) by SDS-PAGE. The upper graph shows the relative mobility of the major protein in these materials compared with the standard proteins used in this analysis. The lower panel shows the coomassie blue staining patterns of the proteins in the KSCN eluate and rabbit HDL. Each gel lane had ~30–40 μg of protein applied. The major protein in each material behaved with an apparent molecular weight of 25.7 × 10^3.

mass of ~26,000 daltons, as shown in Fig. 8. This protein component had approximately the same electrophoretic mobility regardless of whether or not the sample was treated with dithiothreitol before electrophoresis. When the gels were scanned with a soft laser densitometer equipped with a peak integrater, >70% of the protein was present in the 26,000-mol wt peak. Staining of the gels with Schiff's reagent after periodate oxidation indicated that the 26,000-mol wt protein did not contain detectable carbohydrate.

HDL, purified from pooled normal rabbit serum (NRS) (3), was also electrophoresed in SDS/urea slab gels. As shown in Fig. 8, the major protein of rabbit HDL also has an apparent molecular weight ~26,000, and this band is apoprotein (apo)AI, the major apoprotein of rabbit HDL (23). The 26,000-mol wt band from rabbit HDL, as well as from the KSCN eluate, was cut out of the slab gel and subjected to amino acid analysis. The amino acid composition of rabbit apo AI and the 26,000 mol wt protein of the KSCN eluate are shown in Table I. The amino acid composition of rabbit apo AI reported by Børresen and Kindt (23) has been included in this table for comparative purposes.

Molecular exclusion chromatography. To determine the apparent molecular weight of the KSCN eluate, we used molecular exclusion chromatography with Seph 6B. The column was calibrated with proteins of molecular weight between 68,000 (bovine serum albumin) and 450,000 (fibronectin), and a linear relationship between elution volume and log molecular weight (see Fig. 9) was observed. A 5-ml aliquot of the KSCN eluate was applied to the column, and the elution position was determined by measuring the ^{125}I-R595 LPS radioactivity in the column fractions. Additional immunodiffusion analysis of the column fractions for both protein as well as R595 LPS demonstrated that the ^{125}I-R595 LPS, protein, and immunoreactive R595 LPS coeluted from the Seph 4B column as a single homogeneous peak (data not shown). The elution position of the KSCN eluate corresponds to a molecular weight of 310,000, as shown in Fig. 9.

Lipid analysis. The KSCN eluate from anti-R595 Seph 4B was prepared from two separate pools of NRP as described above. One sample was further purified by equilibrium density ultracentrifugation in CsCl (2), and the gradient fractions at $d < 1.2$ g/cm^3 were pooled and dialyzed to remove CsCl before the lipid analysis. Neutral lipid and phospholipid analysis was performed using the procedures described in Methods. The lipid composition of the KSCN eluate, as well as that of rabbit HDL, is shown in Table II. The KSCN eluate, like HDL, was found to be ~ 50% protein and 50% lipid, with a general content of lipids that is characteristic of HDL. However, the KSCN eluate and rabbit HDL were found to differ in the relative amounts triglyceride and the cholesterol ester/cholesterol ratios.

TABLE I

Amino Acid Composition of Major Polypeptide in the KSCN Eluate from Anti-R595 LPS Seph 4B: Comparison with Rabbit APO AI

Amino acid	KSCN eluate*	Apo AI‡	Apo AI§
	Residues/100 residues		
Lysine	8.99±0.24	8.52±0.14	9.0
Histidine	0.99±0.11	1.0±0.21	1.2
Arginine	6.35±0.32	6.48±0.17	7.2
Aspartic acid	8.91±0.21	8.64±0.06	9.4
Threonine	5.1±0.06	4.65±0.03	4.2
Serine	7.18±0.35	7.13±0.19	5.6
Glutamic acid	21.7±0.29	21.1±0.14	24.5
Proline	3.3±0.25	3.9±0.1	3.6
Glycine	3.85±0.31	3.1±0.35	2.4
Alanine	8.32±0.28	7.75±0.06	5.0
Half cystine	ND‖	ND	ND
Valine	6.58±0.48	6.95±0.25	5.9
Methionine	<0.2	0.41±0.04	1.06
Isoleucine	1.08±0.06	1.34±0.01	1.47
Leucine	14.2±0.29	13.6±0.07	13.6
Tyrosine	1.48±0.29	1.92±0.21	3.4
Phenylalanine	2.24±0.11	2.43±0.21	2.8

* Data expressed as mean±SEM for four separate KSCN eluates.

‡ Data expressed as mean±SEM for three separate preparations of rabbit HDL.

§ Data from Børresen and Kindt (24).

‖ Not determined.

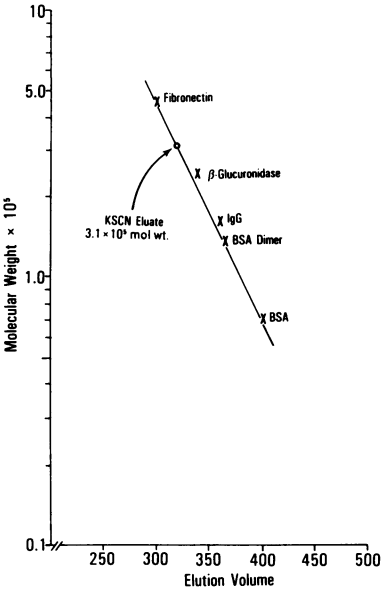

FIGURE 9 Determination of the apparent molecular weight of the KSCN eluate by molecular exclusion chromatography with Seph 6B. A 2.5 ×80-cm column of Seph 6B was equilibrated at room temperature with 0.01 M Tris, 0.1 M NaCl, pH 7.5; and a sample of the KSCN eluate was chromatographed. Calibration of the column was performed before and after chromatography with the standards shown in the graph. The KSCN eluate (○) eluted from the column in a position equivalent to an apparent molecular weight of 3.1×10^5.

In contrast, the relative amounts of total phospholipid in the KSCN eluate and HDL were quite similar.

However, when the phospholipid compositions of the KSCN eluate and HDL were compared, major differences were observed, as shown in Table III. The KSCN eluate contains somewhat more lysophosphatidyl choline and sphingomyelin than HDL, and a marked difference between the phosphatidyl choline/phosphatidyl ethanolamine ratio in the KSCN eluate and HDL was noted. In HDL there is approximately 10-fold more phosphatidyl choline than phosphatidyl ethanolamine, while in the KSCN eluate the phosphatidyl choline/phosphatidyl ethanolamine ratio was between 0.5 and 0.85.

When pooled rabbit serum was used to prepare the KSCN eluate from anti-R595-Seph 4B, similar lipid compositions to that shown in Tables II and III were obtained (data not shown), indicating that the formation of the low density R595 LPS proceeds by the same mechanism in plasma and serum.

DISCUSSION

The experiments described here demonstrate that the addition of LPS from *S. minnesota* R595 to rabbit plasma results in the formation of a complex between R595 LPS and both protein and lipids characteristic HDL. The formation of this complex accounts for the reduction in hydrated buoyant density of LPS that occurs after the addition of LPS to plasma, as we have described in previous reports (1–3).

The experiments described here were performed with R595 LPS, which consists of the lipid A and 2-keto-3-deoxyoctulosonate trisaccharide components, common to many gram-negative organisms. The LPS from many pathogenic gram-negative bacteria may also contain longer carbohydrate side chains in addition to the lipid A and 2-keto-3-deoxyoctulosonate regions. However, we have previously shown that the carbohydrate-containing LPS isolated from *E. coli* 0111: B4 undergoes a marked decrease in buoyant density when added to plasma or serum that requires the presence of HDL (2, 3). Thus, it is likely that the studies reported here with R595 LPS provide insight into a general mechanism for most, if not all, LPS isolates.

Isolation of altered R595 LPS ($d < 0.5$ g/cm³). The method employed to separate the low density R595 LPS from the remainder of the plasma proteins used affinity chromatography with an insolubilized immuno-

TABLE II
Protein and Lipid Composition of KSCN Eluate from Anti-R595 Sepharose 4B

	Composition		
	KSCN eluate-1*	KSCN-2‡	HDL§
	% by wt		
Protein	53	53	54
Total lipid	47	47	46
Lipid class‖			
Triglyceride	20	31	9
Cholesterol ester	15	14	36
Cholesterol	16	9	6
Phospholipid	46	42	46
Ceramide monohexoside	3	3	trace

* Prepared from a mixture of R595 LPS and pooled NRP (containing 20 mM EDTA); the lipid analysis was performed as described in Methods.
‡ Prepared from a mixture of R595 LPS and pooled NRP (containing 20 mM EDTA), further purified by isopycnic equilibrium density ultracentrifugation in CsCl; the lipid analysis was performed as described in Methods.
§ Rabbit HDL were prepared from pooled NRS (3), and the lipid analysis was performed as described in Methods.
‖ These data are given as the percentage of the total lipid content.

purified anti-R595 LPS antibody. Although we have not examined the specificity of the immunopurified antibody with respect to the major determinants in R595 LPS, namely the lipid A and 2-keto-3-deoxy-octulosonate trisaccharide, studies by Johns et al. (7) suggest that the anti-R595 LPS produced in rabbits by immunization with whole *S. minnesota* R595 bacteria does not cross-react with lipid A, despite the fact that it recognizes purified R595 LPS. Studies are currently in progress to elucidate further the specificity of the immunopurified anti-R595 LPS described in this report.

TABLE III
Phospholipid Composition of KSCN Eluate from Anti-R595 Seph 4B

	Composition		
	KSCN eluate-1	KSCN-2	HDL
	% by wt		
Phospholipid class			
Lysophosphatidyl choline	6	4	2
Sphingomyelin	9	15	6
Phosphatidyl choline	34	26	76
Phosphatidyl ethanolamine	40	49	8
Phosphatidyl serine Phosphatidyl inositol	11	6	7.2

See legend, Table II.

Immunodiffusion analysis with the altered R595 LPS demonstrated good reactivity with the immunopurified anti-R595 LPS antibody, although the low density LPS had a markedly increased diffusion rate in agar when compared with the parent R595 LPS (see Fig. 3). This is consistent with data previously reported by Ulevitch et al. (3), showing that the altered LPS ($d < 1.2$ g/cm^3) had a much smaller particle size than the parent LPS, and, with the data presented here, showing that the R595 LPS in the KSCN eluated from Seph 6B with an apparent molecular weight of 310,000. An apparent loss of antigenic determinants was also observed when the altered LPS ($d < 1.2$ g/cm^3) was compared with the parent R595 LPS. Whether this is due to masking or actual loss of determinants is not known, and this question is currently under study.

Approximately 50% of the LPS applied to the anti-R595 LPS column was recovered in the KSCN eluate, and this was shown by isopycnic equilibrium density ultracentrifugation in CsCl to be LPS with $d < 1.2$ g/cm^3. In a previous report we showed that 50–70% of the R595 LPS was converted to $d < 1.2$ g/cm^3 by rabbit plasma (or serum). Thus the method used here to isolate the altered LPS from the initial R595 LPS/NRP mixture yielded the expected amount of low density LPS.

In the preliminary studies with the R595 LPS that did not bind to the column, we have shown that this material does not bind to anti-R595-Seph 4B upon rechromatography, that it does not react with anti-R595 antiserum in immunodiffusion analysis, and that it does not band at $d < 1.2$ g/cm^3. Ultracentrifugation in CsCl demonstrated that this material banded as a broad peak around $d = 1.36$ g/cm^3 and may represent disaggregated LPS bound to plasma proteins (Ulevitch, R., unpublished data). Alternatively, this material may represent a minor fraction or contaminant of the original R595 LPS, which does not react with the anti-R595 antibody, but does have a high capacity to take up radioactive iodine during the labeling procedure. Studies are currently in progress to characterize further the chemical nature of the radioactive material in the column flow-through.

Properties of the low density LPS ($d < 1.2$ g/cm^3). The major protein, associated with the low density LPS representing >70% of the total protein in the KSCN eluate, has been tentatively identified as apo-AI. This is based upon results obtained by two different experimental approaches comparing authentic apo AI and the major protein in the KSCN eluate. First,

the behavior of this protein in SDS-PAGE is consistent with a single polypeptide of apparent molecular weight of 26,000, which is in agreement with the published data for purified apo AI from rabbit HDL (23) as well as other species (24). Second, the amino acid composition of the 26,000-mol wt protein from the KSCN eluate is very similar to the apo AI of rabbit HDL (see Table I).

The relative amount of total protein and total lipid in the KSCN eluate is very similar to that determined for rabbit HDL (see Table II). However, examination of the lipid composition of the KSCN eluate revealed some significant differences between rabbit HDL and the KSCN eluate. First, the KSCN eluate is relatively rich in triglyceride when compared with HDL. Second, the cholesterol ester/cholesterol ratios in the two KSCN eluates were 0.94 and 1.55, respectively, whereas this ratio was 6 in rabbit HDL. The cholesterol ester/cholesterol ratios for human HDL_2 and HDL_3 have been reported to be 2.8 and 4.4, respectively (24, 25). Whereas the total phospholipid content of the KSCN eluate and of rabbit HDL are very similar (see Table II), the relative amounts of the various phospholipids classes are markedly different in the KSCN eluate and rabbit HDL. A marked difference was noted in the phosphatidyl choline/phosphatidyl ethanolamine ratios when the KSCN eluates and rabbit HDL were compared; namely, ratios of 0.85 and 0.53 for the former and 9.5 for the latter. The phosphatidylcholine/phosphatidyl ethanolamine ratio for human HDL has been reported to be at least 10 (25–27); and Skipski et al. (26) reported phosphatidyl choline/phosphatidyl ethanolamine ratios of 13 and 25 for human LDL and VLDL, respectively.

The phosphatidyl choline/phosphatidyl ethanolamine ratio of the KSCN eluate is most similar to that observed in cell membranes. For example, the phosphatidyl choline/phosphatidyl ethanolamine ratios for human erythrocytes (28), human myelin (28), and beef heart mitochondria (28) are 1.05, 0.5, and 1.45, respectively.

Preliminary studies with the KSCN eluate have shown that, upon reinjection into rabbits, this material is cleared with a $t_{1/2}$ of ~14 h, which is in good agreement with data previously reported for the disappearance of the low density R595 LPS in rabbits (1). Furthermore, the amount of the KSCN eluate present in the blood at 1 min after injection is >90% of the theoretical amount calculated from the injected dose and the blood volume of the rabbits, suggesting that this material is fully mixed in the vascular compartment. Thus, the KSCN eluate appears to have properties similar, with respect to in vivo clearance kinetics, to the low density LPS formed in serum (1–3).

Mechanism of the density reduction of LPS. In previous reports (2, 3), we proposed the following mechanism to account for the density reduction of LPS observed after the addition of LPS to plasma or serum:

$$\text{Step 1: } (\text{LPS})_{\text{parent}} + \text{plasma factor(s)} \rightleftharpoons (\text{LPS})_{\text{disaggregated}}$$

$$\text{Step 2: } (\text{LPS})_{\text{disaggregated}} + \text{HDL} \rightarrow (\text{LPS-HDL})_{d<1.2\,\text{g/cm}^3}$$

The data presented here provide additional evidence to support this mechanism. First, the apparent molecular weight of the KSCN eluate was determined to be 310,000, and thus the parent R595 LPS, which has been shown to be at least 40S (29), must have undergone disaggregation before interaction with the plasma lipid and protein components found in the KSCN eluate. At the present time the mechanism of disaggregation is unknown, although data from a previous report by Ulevitch et al. (3) suggest that plasma lipid is not required for this disaggregation.

Evidence for the formation of an LPS-HDL complex is derived from several different experimental observations. The major protein present in the KSCN eluate from the anti-R595-Seph 4B column has biochemical characteristics that are similar if not identical to apo AI, the major apoprotein of rabbit HDL. Furthermore, when the KSCN eluate was subjected to isopycnic equilibrium density ultracentrifugation in CsCl, all of the 26,000-mol wt protein co-centrifuged with the ^{125}I-R595 LPS, suggesting that there is a firm association between the LPS, the protein, and the lipids present in the KSCN eluate. Studies with human plasma indicate that at least 90% of the apo AI is present in the density range between 1.063–1.121 g/cm^3 and not in HDL free plasma (or serum). Thus, the presence of apo AI in the KSCN eluate suggests that the disaggregated R595 LPS interacts with apo AI-containing HDL particles. In addition, Freudenberg et al. (30) have also recently presented data that have confirmed our identification of HDL as a major plasma component which interacts with R595 LPS, although no quantitative data on the composition of the complexes were presented.

Although the KSCN eluate and HDL have similar relative amounts of protein and total lipid, the lipid and phospholipid compositions of the KSCN eluate and rabbit HDL differ substantially. At least two mechanisms should be considered to explain these differences. One possible mechanism is that the disaggregated LPS may interact with a minor subgroup of HDL that does not have the same lipid composition as bulk rabbit HDL. In this regard it is now well appreciated that marked heterogeneity may occur both in HDL protein and lipid (27, 28).

Alternatively, structural studies of isolated apo AI have suggested that this protein contains helical seg-

ments with predominantly hydrophilic amino acids on one face and primarily hydrophobic amino acids on the other face. This structural feature, termed "amphipathic helix" has been suggested to bind phospholipids with the fatty acyl chains binding to the hydrophobic face and the zwitterionic phosphorylcholine of lecithin binding to the hydrophilic face. If R595 LPS binds to apo AI, the LPS could displace phosphatidyl choline bound to apo AI, and in addition, the particle could acquire phosphatidyl ethanolamine from other lipoproteins. This latter event is required because the total amount of phospholipid in the KSCN eluate is similar to that found in rabbit HDL. Studies by Tall and his collaborators (31, 32) suggest that apo AI can indeed effect transfer of phospholipids from synthetic phospholipid membranes. A possible role for plasma phospholipid exchange proteins must also be considered in the formation of the LPS-HDL complex.

Chobanian et al. (33) have shown that addition of lecithin vesicles to plasma causes incorporation of phospholipid into HDL particles. These data suggest that HDL may have the capacity to accept phospholipid, and this capacity may involve interaction with apoprotein. Kim and Nishida (34) have reported data that demonstrated that human apo AI can interact with dextran sulfate (average molecular weight, 150,000) in the presence of Ca^{2+} and phospholipid. These data suggest that apo AI may have binding sites for polyanions, and thus the disaggregated LPS could bind directly to these sites. The role of these mechanisms in the interaction of LPS with HDL in plasma is being evaluated in this laboratory.

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