New Function for High Density Lipoproteins

THEIR PARTICIPATION IN INTRAVASCULAR REACTIONS OF BACTERIAL LIPOPOLYSACCHARIDES

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ABSTRACT The addition of bacterial lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 or *Salmonella minnesota* R595 to plasma (or serum) resulted in a marked reduction of the hydrated buoyant density of the parent LPS (0111:B4 [d = 1.44 g/cm^3] and R595 [d = 1.38 g/cm^3]), to $d < 1.2$ g/cm^3. This reduction in buoyant density to <1.2 g/cm^3 of the LPS required plasma (or serum) lipid. Delipidation of plasma (or serum) by extraction with *n*-butanol/diisopropyl ether (40/60, vol:vol) prevented the conversion of the parent LPS to a form with $d < 1.2$ g/cm^3. Reversal of the effect of delipidation was accomplished by the addition of physiologic concentrations of high density lipoprotein (HDL). In contrast, as much as two times normal serum concentration of low density or very low density lipoprotein were ineffective. The ability of normal plasma (or serum) to inhibit the pyrogenic activity of LPS, lost after delipidation, was also restored after the addition of HDL. Preliminary results suggested that prior modifications of the LPS, probably disaggregation, may be required before interaction with HDL.

INTRODUCTION

Gram-negative bacterial septicemia in man may be complicated by hypotensive shock and disseminated intravascular coagulation. The major evidence to support a role for endotoxin or bacterial lipopolysaccharide (LPS)[1] in the induction of these pathophysiological changes is twofold. It has been shown that injection of purified LPS into experimental animals produces most, if not all, of the pathophysiologic changes observed in gram-negative septicemia. Most recently, the studies of McCabe and his collaborators (1, 2) and Braude et al. (3) provided additional evidence to support a role for LPS. They showed that antiserum to the core glycolipid of LPS is protective in experimental sepsis induced with heterologous bacteria.

It is clear that LPS must first interact with both the formed, as well as the soluble elements of blood. The main focus in previous studies of intravascular reactions of LPS has been on the distribution of LPS among the formed elements of blood, although it was established over 25 yr ago that >80% of the LPS remaining in the blood after injection was found in the plasma (4). This observation, by Braude and his co-workers, was later confirmed by Herring et al. (5) although the properties of the LPS remaining in the plasma were not studied. Subsequently, Skarnes and Chedid (6) examined the immunologic properties of LPS remaining in the plasma of mice after intravenous injection. They demonstrated that a change in the immunodiffusion patterns of LPS was detected for the LPS remaining in the plasma and postulated that the LPS was converted by a degradative process from a large, slow diffusing molecule to a much smaller, rapidly diffusing form. A striking finding reported by Skarnes and Chedid was the persistence in the plasma of an LPS molecule which was still toxic for adrenalectomized mice. The molecular basis for both the altered immunodiffusion patterns as well as the residual toxicity as yet remains unexplained.

Most recently, using ^{125}I-LPS (7) as a tracer molecule, we have described a reaction of LPS in plasma or serum which results in a marked reduction in the buoyant

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[1] *Abbreviations used in this paper*: DIPE, diisopropyl ether; DLHS, delipidated human serum; DLRP, delipidated rabbit plasma; HDL, high density lipoprotein; LDL, low density lipoprotein; LPS, lipopolysaccharide.

density of the parent LPS to a value of <1.2 g/cm^3 (8). We showed that the reduction in LPS buoyant density did not occur as a result of enzymatic degradation of the glycolipid backbone of the LPS and that the reduction in buoyant density of LPS resulted in the abrogation of a number of the immediate biologic responses to LPS; namely, the pyrogenic response, the rapid neutropenia, and the anticomplementary activity.

Although the reduction in buoyant density accompanied a loss of these activities of the LPS, the mechanism associated with the physical change of the LPS remains unknown. We have therefore undertaken further experiments designed to gain understanding of the mechanism of the density reduction of LPS. The studies reported here, performed with LPS prepared from *Escherichia coli* 0111:B4 and from *Salmonella minnesota* R595, will provide evidence to support a critical role for plasma high density lipoprotein (HDL) in the conversion of the parent LPS to a form with a buoyant density of <1.2 g/cm^3. A preliminary report of these findings has been presented in abstract form (9).

METHODS

Rabbits

Male New Zealand white rabbits (2–2.5 kg) from a closed colony were obtained from Rancho de Conejo, Vista, Calif.

Lipopolysaccharide

LPS from *S. minnesota* and the lipid A-rich fraction of LPS from *E. coli* 0111:B4 were prepared by Dr. D. C. Morrison (Scripps Clinic and Research Foundation, La Jolla, Calif.) The methods for preparation of the LPS have been described in detail (10, 11). Radioiodination of 0111:B4 or R595 LPS was accomplished as described by Ulevitch (7). The radioiodinated LPS has been shown to have the following properties: (*a*) the radioactivity is covalently bound and does not dissociate after treatment with either 10% sodium dodecyl sulfate, 8 M urea, or 5 M guanidine HCl; (*b*) the biophysical and immunologic properties of the radioiodinated LPS are identical with those of the parent LPS; and (*c*) the endotoxic properties are equivalent to those of the parent LPS. Radioiodinated LPS was stored at −20°C in the presence of 0.01% sodium azide.

Serum and plasma

Human venous blood was collected from the antecubital fossae of normal donors, allowed to clot in a glass tube for 60 min, 37°C, and the serum separated by centrifugation at 3,000 *g* for 30 min, 4°C. The serum was aliquoted, and stored at −70°C, thawed at 37°C and used immediately. Whole blood was also collected from the medial ear artery of rabbits, allowed to clot at 37°C in a glass tube and processed as above. Platelet-free plasma was prepared from rabbit blood (anticoagulated with 5 mM EDTA) and delipidated as described below.

Delipidation of serum or plasma was accomplished by extraction with *n*-butanol/diisopropyl ether (DIPE) (40:60, vol/vol) essentially as described by Cham and Knowles (12). Serum or plasma was extracted at 25°C twice with 2 vol of butanol/DIPE for 60 min and once with 2 vol of DIPE for 2 min. To reduce the residual butanol after delipidation, samples, in a container covered with surgical gauze, were placed in a chemical hood overnight. A final concentration of 0.01% sodium azide was added to these solutions as an antibacterial agent.

Lipoproteins

Lipoproteins were prepared from normal human serum as previously described (13, 14). Human lipoproteins were prepared from pooled human serum (usually four to six donors) so that the 1-wk preparation was initiated within 1 d of the serum collection. For the experiments described here the very low density lipoprotein (VLDL) was utilized within 3 d of isolation whereas the low density lipoproteins (LDL) and HDL were always used within 1 wk after isolation. Briefly, VLDL was isolated by centrifugation at plasma density (d = 1.006 g/cm^3) for 24 h at 50,000 rpm in a Beckman-Spinco L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using a 60Ti rotor. LDL was isolated as the d = 1.02–1.063-g/cm^3 fraction after adjustment of density by addition of solid NaBr and HDL was isolated as the d = 1.090–1.21-g/cm^3 fraction. Lipoprotein fractions were washed by floatation through NaBr solutions at the appropriate densities in an SW-41 rotor at 38,000 rpm for 24–48 h. Lipoproteins were dialyzed exhaustively against 50 mM phosphate-saline buffer (pH 7.4) containing 0.3 mM EDTA and were stored at 4°C after sterilization by passage through 0.45-μm sterile filters. Lipoprotein fractions were shown to be free of cross-contamination with other density fractions by immunodiffusion against specific rabbit antisera to human LDL and HDL. Reconstitution of delipidated serum or plasma with purified lipoproteins used the following concentrations of lipoproteins, expressed as milligrams of lipoprotein protein per (milliliters of serum) to approximate normal human serum concentrations: HDL = 1.35 mg/ml; LDL = 0.8 mg/ml; and VLDL = 0.1 mg/ml (15).

Analytical techniques

Ultracentrifugation. CsCl isopycnic density gradient ultracentrifugation was performed and data analyzed as previously described (10). Although the CsCl ultracentrifugation experiments were usually run for 60 h, 48- and 72-h runs were initially performed to establish that equilibrium had been reached by 48 h. In no case were differences noted in the distribution of ^{125}I-LPS in the 48-, 60-, or 72-h runs. Sucrose gradient ultracentrifugation was performed with 5–20% sucrose gradients at 45,000 rpm for 3 h in a SW 50.1 rotor. The distribution of radioactivity in CsCl gradients is plotted as total radioactivity per fraction vs. normalized gradient position where the normalized gradient position is calculated from the ratio of the (individual fraction number) /(total number of fractions).

LPS-induced physiologic changes in rabbits. The methods employed to determine the effect of LPS of ^{51}Cr platelet levels and core temperature are described in detail in a previous publication (16).

Measurement of radioactivity. The radioactivity in samples containing ^{125}I-LPS was quantitated with a Searle model 1195 automatic gamma counter equipped with a Teletype and paper tape punch (Searle Radiographics Inc., Des Plaines, Ill.). These data were analyzed and plotted with a Hewlett-Packard 9862 A calculator equipped with a paper tape reader and 9862 A data plotter (Hewlett-Packard Co., Palo Alto,

Calif.). The determination of integrated areas under peaks of radioactivity was performed with the Hewlett-Packard 9862 A calculator.

Molecular exclusion chromatography. Sepharose 2B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) chromatography was performed at room temperature. The sample (0.5 ml) was added to a 10-ml column of Sepharose 2B in 0.15 M NaCl (0.9 × 24 cm, prepared in a 10-ml plastic pipette) and 0.2-ml fractions were collected. The column was calibrated with blue dextran, ^{125}I-immunoglobulin (Ig)G and cytochrome *c* for determination of Vo, mol wt = 160,000 marker and V_t, respectively.

Experimental protocols

The experiments described in this paper were performed with normal or delipidated plasma or serum from human and rabbit sources. The source of the plasma or serum used for individual experiments will be specified in the text. The effect of serum or plasma on the bouyant density of either 00111:B4 or R595 LPS was examined as follows. The final volume of each reaction to be studied was 0.4 ml and consisted of the following reagents added together sequentially: (*a*) 0.3 ml normal or delipidated serum or plasma; (*b*) 0.09 ml saline or purified lipoprotein; (*c*) 0.01 ml ^{125}I-LPS.

These solutions were mixed and maintained at 37°C for varying lengths of time. The reaction between LPS and serum or plasma was terminated by the addition of 5.0 ml of cold CsCl as previously reported (8). For experiments with 0111:B4 LPS each reaction mixture contained 20 μg of LPS. Experiments with R595 LPS utilized 10 μg of LPS and the reaction mixture always contained 20 mM EDTA, pH 7.5. We have previously shown that the addition of EDTA to normal serum markedly increased the amount of R595 LPS which is converted to a form with $d < 1.2$ g/cm^3 (8).

Preparation of serum altered ($d < 1.2$ g/cm^3) LPS. LPS with a $d < 1.2$ g/cm^3 was isolated from solutions of delipidated rabbit plasma (DLRP) and HDL by the preparative methods as previously described (8). The LPS with a $d < 1.2$ g/cm^3 was dialyzed exhaustively against 0.15 M saline at 4°C. The concentration of the LPS at $d < 1.2$ g/cm^3 was calculated from the specific activity of the starting ^{125}I-LPS. In control experiments we established that CsCl ultracentrifugation of LPS/saline solutions did not alter the biologic activity of the parent 0111:B4 or R595 LPS (7, 8).[2]

RESULTS

Serum from human, lapine, or murine sources produces a marked decrease in the hydrated buoyant density of LPS (8) and this change can be quantitated by CsCl isopycnic equilibrium density ultracentrifugation of LPS/serum mixtures. CsCl gradients of ^{125}I-LPS/serum mixtures showing the effect of normal human serum on the buoyant density of 0111:B4 and R595 LPS are shown in Fig. 1*a* and *b*. Experimental protocols for this and all subsequent experiments are described in Methods. Under these experimental conditions, ≅50% of the 0111:B4 and R595 LPS was found banded at $d < 1.2$ g/cm^3 with the remaining LPS distributed in the rest of the gradient ($d > 1.2$ g/cm^3).

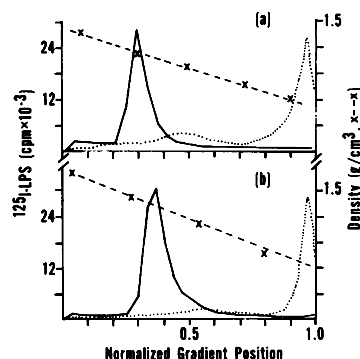

FIGURE 1 The distribution of parent and serum-altered LPS in CsCl gradients after centrifugation at 40,000 rpm, 60 h in an SW 50.1 rotor. (*a*) 0111:B4 LPS; parent LPS is shown by the solid line and 0111:B4 after exposure to normal human serum is shown by the dotted line. (*b*) R595 LPS; parent LPS is shown by the solid line and R595 LPS after exposure to normal human serum is shown by the dotted line. In both panels the density (grams per cubic centimeter) of gradient fractions is shown as a dashed line. The experimental protocol is given in Methods. Each reaction was performed in a final volume of 0.4 ml consisting of 0.3 ml of serum, 0.1 ml containing ^{125}I-LPS and saline with a total amount of 20 μg of 0111:B4 LPS or 10 μg of R595 LPS.

Role of serum lipid in the decrease in buoyant density of LPS. To investigate the role of lipids in the serum-induced density reduction we prepared delipidated human serum (DLHS) by extraction of normal human serum with butanol/DIPE (12). The effect of DLHS on the buoyant density of 0111:B4 and R595 LPS was then examined in the following experiment; ^{125}I-LPS was added to DLHS or saline, maintained at 37°C for 60 min, and then equilibrium density gradient ultracentrifugation was performed. The analysis of the distribution of radioactivity is shown in Fig. 2. As shown, the addition of R595 LPS to DLHS (containing 20 mM EDTA) produced a decrease in density from 1.38 (Fig. 2*a*) to 1.34 g/cm^3 (Fig. 2*b*). The addition of 0111:B4 LPS to DLHS (Fig. 2*d*) resulted in a partitioning of LPS between that with a $d = 1.42$ g/cm^3 and that with a $d = 1.34$ g/cm^3. In marked contrast to results obtained with normal plasma (or serum) no LPS was observed to band with a $d < 1.2$ g/cm^3. Experiments were also performed with delipidated rabbit serum and delipidated plasma from human and rabbit sources and in no case was LPS found at a $d < 1.2$ g/cm^3 under these conditions.

It is of interest that the band width of R595 LPS (Fig. 2*b*) and 0111:B4 LPS (Fig. 2*d*) is broadened by DLHS. Thus, the small changes in density of the parent LPS, induced by DLHS, might also be associated with disaggregation of the parent macromolecular aggregate. The band width of a macromolecule banded by isopycnic equilibrium density gradient ultracentrifugation is related to molecular size with larger

[2] Ulevitch, R. Unpublished data.

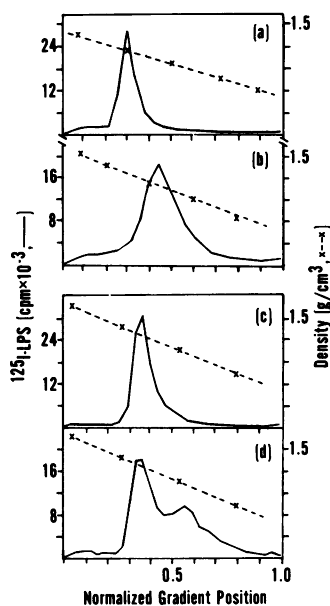

FIGURE 2 The effect of delipidated serum on the distribution of LPS in CsCl gradients after centrifugation at 40,000 rpm, 60 h in an SW 50.1 rotor. (*a*) R595 LPS after exposure to 0.15 M NaCl, 20 mM EDTA is shown by the solid line. (*b*) R595 LPS after exposure to delipidated human serum containing 20 mM EDTA is shown by the solid line. (*c*) 0111:B4 LPS after exposure to 0.15 M NaCl is shown by the solid line. (*d*) 0111:B4 LPS after exposure to delipitated human serum is shown by the solid line. In all panels the density grams per cubic centimeters of gradient fractions is shown as a dashed line. The experimental protocol is given in Methods and in the legend of Fig. 1.

molecules having narrower band widths. More direct evidence suggesting that delipidated serum can indeed disaggregate R595 LPS will be presented in a subsequent section of this report.

Reconstitution of delipidated serum with purified lipoproteins. The previous experiment demonstrated that the removal of lipid from serum or plasma prevented the reduction in buoyant density of LPS to <1.2 g/cm^3. We next sought to determine if the activity of delipidated serum could be restored with purified serum lipoproteins because we could not exclude the possibility that the delipidation procedure denatured a component of serum required for the density reduction to <1.2 g/cm^3.

^{125}I-LPS was added to DLHS containing increasing amounts of VLDL, LDL, or HDL so that the lipoprotein concentrations were reconstituted to between 20 and 200% of normal human serum levels. The reaction mixtures were maintained at 37°C for 60 min and, after the addition of cold CsCl, centrifuged to equilibrium. The distribution of the radioactivity in these gradients was determined and the percentage of LPS banding at a $d < 1.2$ g/cm^3 was calculated.

The effect of adding increasing concentrations of purified lipoproteins to DLHS on the buoyant density of R595 as well as 0111:B4 LPS are summarized in Fig. 3. Results obtained with 0111:B4 LPS shown in Fig. 3*a*) indicate that the activity of DLHS can be reconstituted with HDL but not VLDL or LDL. The concentration of HDL required to reconstitute DLHS fully was approximately equal to the normal serum level of HDL. Likewise, in the case of R595 LPS, HDL was found to be the most effective at reconstitution of DLHS (Fig. 3*b*). LDL partially reconstituted the activity of DLHS but was significantly less effective than HDL. The same results have been obtained when purified human lipoproteins were utilized to reconstitute delipidated rabbit serum or plasma. The maximum amount of ^{125}I-LPS at a $d < 1.2$ g/cm^3 when either 0111:B4 or R595 LPS is added to DLHS and HDL is similar to the value obtained in control experiments shown in Fig. 1.

The actual ^{125}I-LPS distribution in CsCl gradients of solutions of either R595 or 0111:B4 LPS and DLHS reconstituted with normal serum levels of HDL are similar to, if not indistinguishable from, those obtained when ^{125}I-LPS (0111:B4 or R595) was mixed with normal human serum and centrifuged in CsCl as shown in Fig. 1.

Participation of lipid-free serum protein in the decrease in buoyant density of LPS. The previous experiments indicate that HDL participates in the serum-induced reduction of the hydrated buoyant density of LPS. We next sought to determine if components of delipidated serum were required for HDL to participate in the reduction of the LPS density. To examine this question we mixed ^{125}I-0111:B4 or R595 with purified lipoproteins without DLHS and incubated these solutions for 60 min at 37°C before CsCl ultracentrifugation. For these experiments the lipoprotein concentration utilized was approximately that of normal human serum concentration (15). Data for the

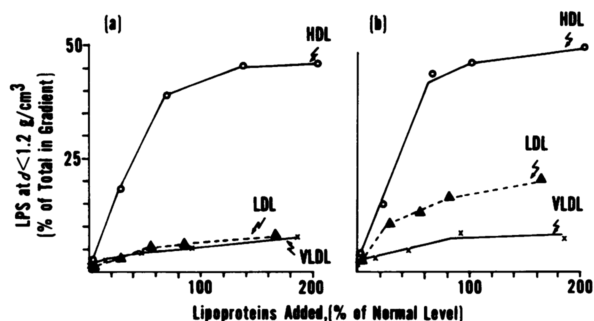

FIGURE 3 Reconstitution of delipidated human serum with purified human lipoproteins. (*a*) The effect of delipidated serum/lipoprotein mixtures on the buoyant density of 0111:B4 LPS. (*b*) The effect of delipidated serum/lipoprotein mixtures on the buoyant density of R595 LPS. The experimental protocol is given in Methods and in the legend of Fig. 1.

amounts of 0111:B4 and R595 LPS found to band at $d < 1.2$ g/cm³ are shown in Table I. The results obtained when 0111:B4 LPS was mixed with purified lipoproteins indicate that VLDL or HDL interacted poorly with 0111:B4 LPS in the absence of delipidated serum so that no more than 12% of the 0111:B4 LPS banded at a $d < 1.2$ g/cm³. In contrast, LDL did interact more effectively so that 20% of the LPS in the gradient was found at a $d < 1.2$ g/cm³. These results suggest then that components of delipidated serum participate in the density reduction of 0111:B4 LPS, although 0111:B4 LPS can interact with LDL in the absence of serum.

The effect of addition of purified lipoproteins in the absence of serum, on the density of R595 LPS, was somewhat greater. Both VLDL and HDL caused 25% of the R595 LPS to shift to a $d < 1.2$ g/cm³ whereas LDL altered 40% of the LPS in this way.

The amount of either 0111:B4 or R595 LPS shifted to a $d < 1.2$ g/cm³ by lipoproteins in the absence of serum, was identical to that shown in Table I regardless of whether the lipoprotein concentration was 20 or 200% of normal serum levels. The LPS which was not converted by the purified lipoproteins to a $d < 1.2$ g/cm³ remained at the same density and displayed an identical band width as the parent LPS (data not shown). Studies are in progress to elucidate the mechanism of this direct effect of lipoproteins on the density of LPS.

TABLE I
Modification of the Buoyant Density of LPS by Purified Lipoproteins

LPS	Lipoprotein*	LPS at $d < 1.2$ g/cm³
		% of total in gradients mean±SEM
0111:B4‡	—	<3
	VLDL	8±2
	LDL	20±3
	HDL	12±1.5
R595§	—	<3
	VLDL	25±4
	LDL	42±2
	HDL	25±3

* The final concentration of lipoproteins was equal to the approximate normal serum concentration; VLDL = 0.1 mg/l; LDL = 0.08 mg/ml; HDL = 1.35 mg/ml.

‡ 20 μg of 0111:B4 LPS was added in a volume of 0.1–0.3 ml of saline containing the appropriate lipoprotein and the mixture was maintained at 37°C for 60 min. The data shown are the mean±1 SEM for three separate experiments.

§ 10 μg of R595 LPS was added in a volume of 0.1–0.3 ml of saline containing the appropriate lipoprotein and the mixture was maintained at 37°C for 60 min. The data shown are the mean±1 SEM for three separate experiments.

Mechanism of the density reduction. The previous experiments established a role for HDL as well as for plasma (or serum) components not extracted by butanol/DIPE in the reaction of LPS with plasma resulting in a reduction in LPS buoyant density to <1.2 g/cm³. To further characterize the mechanism of the density reduction the following experiments have been performed. We first sought to determine the molecular weight of the serum altered form ($d < 1.2$ g/cm³) of 0111:B4 and R595 LPS by chromatography on a Sepharose 2B column calibrated with blue dextran, human IgG and cytochrome *c*. Results shown in Fig. 4 demonstrate that the serum-altered forms of both LPS preparations eluted slightly ahead of IgG and after the parent LPS. Greater than 80% of the radioactivity applied to the Sepharose 2B column was recovered in the peaks shown in Fig. 4. The parent R595 LPS elutes largely in the void volume (V_0) and the elution position of the parent 0111:B4 LPS is shown in Fig. 4. Therefore, these findings suggest that size reduction of the parent LPS has occurred before or at the same time as the interaction with HDL. In experiments not shown here we have determined that the addition of HDL to the parent 0111:B4 LPS in the absence of other serum components, did not produce a disaggregation of the LPS.[2] Thus, it seemed possible that a component not extracted by butanol/DIPE might produce the disaggregation.

To determine if delipidated serum had the capacity to alter the particle size of the parent LPS we performed the following experiment with R595 LPS. Peak tubes from CsCl gradients of the parent R595 LPS (Fig. 2*a*) or R595 LPS plus delipidated serum (Fig. 2*b*) were pooled and dialyzed vs. 0.15 M NaCl to remove the CsCl. The dialyzed samples were then applied to the

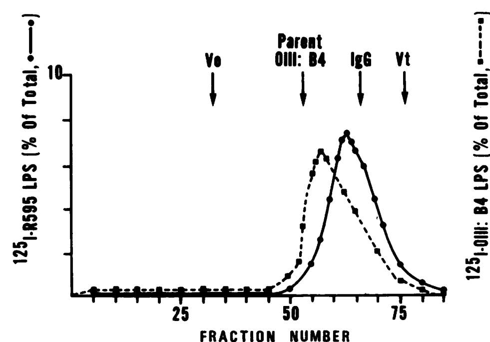

FIGURE 4 Sepharose 2B molecular exclusion chromatography of serum-altered R595 and 0111:B4 LPS. The column was 0.9 × 24 cm in 0.15 M NaCl and 0.2-ml fractions were collected. Elution position of Vo, parent 0111:B4 LPS, IgG, and V_t are shown in the figure. The elution profile of the R595 LPS is shown by the closed circles whereas that of 0111:B4 LPS is shown by the closed boxes. These data are plotted as the percent of total radioactivity vs. column fraction number.

top of a 5–20% sucrose gradient and centrifuged at 45,000 rpm for 3 h in an SW 50.1 rotor. The amount of radioactivity in both individual gradient fractions as well as that pelleted was determined and these data are shown in Fig. 5. The parent R595 LPS pelleted with 77% of the ^{125}I-LPS found at the bottom of the tube (Fig. 5*a*). In contrast, only 5% of the ^{125}I-LPS from the delipidated serum/LPS mixture was found in the pellet and ≅50% of the LPS was observed to sediment in the upper fourth of the gradient as a relatively homogeneous peak. These data suggest that a marked disaggregation of R595 LPS occurs in delipidated serum and does not require the presence of lipid. Alternatively these findings could be explained by a conformational change in the LPS which would result in a markedly slower sedimentation rate.

Endotoxic activity of LPS with $d < 1.2$ g/cm^3. We next sought to examine the endotoxic properties of LPS ($d < 1.2$ g/cm^3) isolated from delipidated serum or plasma reconstituted with HDL. The assays chosen for the present study are those which reflect the immediate biologic response to LPS; namely, the pyrogenic response, neutropenia induction, and anticomplementary activity.

We first examined the ability of serum-altered 0111:B4 LPS ($d < 1.2$ g/cm^3) to produce a pyrogenic response in rabbits. In this experiment the pyrogenic response produced by the injection of 50 ng of 0111:B4 LPS preincubated with 1 ml of DLRP or 1 ml of HDL (1.35 mg/ml) and the pyrogenic response of 5 μg of 0111:B4 LPS ($d < 1.2$ g/cm^3) isolated from an DLRP + HDL mixture was determined. The injection into rabbits of 50 ng of native 0111:B4 LPS is the minimal dose of LPS which produced a 1°–1.5°C increase in core temperature during a 300-min period (8). The results of this experiment are shown in Fig. 6, where we have plotted the change from base-line temperature vs. time after LPS injection. These data, the mean value for three animals in each group, show that neither DLRP nor HDL alone inhibit the pyrogenic activity of 0111:B4 LPS, but that when DLRP and HDL are combined the LPS ($d < 1.2$ g/cm^3) isolated from this mixture is devoid of pyrogenic activity. These data demonstrate that reconstitution of delipidated serum or plasma with HDL restores the ability of this mixture to inactivate LPS. Identical findings have been obtained for R595 LPS.

In experiments to be reported elsewhere we have determined that the LPS isolated from mixtures of LPS + DLRP or LPS + HDL retained the ability to induce a pyrogenic response and neutropenia (see below) which was indistinguishable from that produced by the parent LPS. Furthermore, separate injections of DLRP or HDL alone did not produce a pyrogenic response or neutropenia (see below).

LPS injection into rabbits results in a rapid and profound neutropenia (8, 16) and this phenomenon can be utilized as a sensitive assay for the presence of LPS. We next examined this activity using either serum-altered LPS isolated from mixtures of 0111:B4 + DLRP + HDL or 0111:B4 LPS mixed with DLRP. The serum-altered 0111:B4 LPS isolated from DLRP + HDL mixtures was unable to induce an immediate neutropenia after the injection of up to 5 μg of this material. In contrast, the injection of 0111:B4 LPS incubated with DLRP resulted in an immediate neutropenia. In

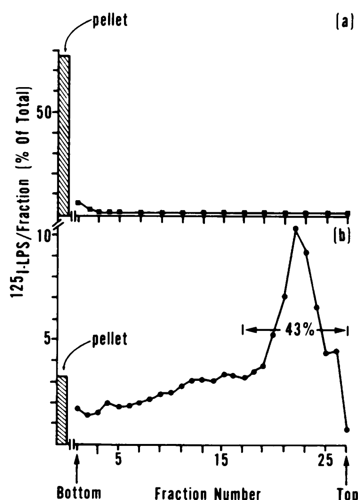

FIGURE 5 Sucrose density gradient ultracentrifugation of the parent R595 LPS (*a*) and R595 LPS after exposure to delipidated serum (*b*). The radioactivity which was pelleted at the bottom of the tube is shown by the shaded area. The experimental details are described in the text.

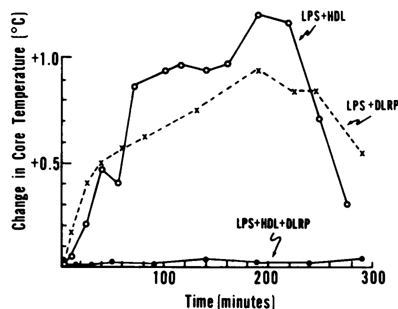

FIGURE 6 Pyrogenic response in rabbits over a 300-min period after the intravenous injection of 0111:B4 LPS. The data is expressed as change in base-line core temperature at different times after injection. The pyrogenic response of 5 μg of serum-altered 0111:B4 LPS ($d < 1.2$ g/cm^3) is shown by the closed circles. The pyrogenic response of 50 ng of 0111:B4 LPS exposed to 1 ml of DLRP for 60 min before injection is shown with the "x" symbols. The pyrogenic response of 50 ng of 0111:B4 LPS exposed to 1 ml of HDL (1.35 mg) for 60 min before injection is shown as the open circles. The experimental data are the mean obtained with three rabbits in each experimental group. At each measurement the SEM never exceeded ±0.04°C change in core temperature.

these experiments the neutrophil levels remained at preinjection levels for 60 min after the injection of 5 μg of serum-altered 0111:B4 LPS ($d < 1.2$ g/cm³), whereas the injection of as little of 50 ng of 0111:B4 LPS in 1 ml of DLRP reduced the circulating neutrophil levels to <10% of the preinjection value. Thus, DLRP was unable to inhibit this activity of LPS.

One sensitive measure of the anticomplementary activity of R595 LPS is the rapid, transient thrombocytopenia following intravenous injection into rabbits (16, 17). This effect is dependent upon intravascular complement activation and as little as 50 μg of R595 LPS produces a 30–50% decrease in circulating platelets 5 min after injection (16). Experiments were performed in which we compared the ability of the parent R595 ($d = 1.38$ g/cm³) and serum-altered R595 ($d < 1.2$ g/cm³) LPS isolated from LPS + DLHS + HDL mixtures to induce an immediate thrombocytopenia in rabbits. A 250-μg dose of parent R595 LPS and a 350-μg dose of serum-altered R595 was injected intravenously in a 2.5-ml volume as a bolus. The results shown in Fig. 7 indicate that the serum-altered LPS does not produce the immediate thrombocytopenia observed with the parent LPS. The minor changes in levels of ^{51}Cr-platelets produced by the serum-altered LPS are indistinguishable from those observed with a saline injection. These studies suggest that the anticomplementary activity of the serum-altered R595 is markedly reduced and are consistent with our previously published findings that the serum-altered form of R595 LPS ($d < 1.2$ g/cm³) no longer retains the ability to activate complement (8).

DISCUSSION

These experiments demonstrate that HDL participates in the reaction of LPS with plasma (or serum) components which results in the reduction of the bouyant density of LPS to <1.2 g/cm³. The present studies also showed that nonlipid components of plasma (or serum) modify LPS, possibly by disaggregation and thus facilitating the interaction with HDL. The delipidated plasma (or serum) failed to inhibit several different LPS-dependent biologic responses, namely, the pyrogenic response and the immediate neutropenia in rabbits. Importantly, the addition of purified HDL to delipidated plasma (or serum) restored both the ability to reduce the buoyant density of LPS to <1.2 g/cm³ as well as the capacity to abrogate the activities of LPS responsible for the pyrogenic response, immediate thrombocytopenia and anticomplementary activity.

Two different LPS preparations have been utilized for these experiments; namely that isolated from a smooth strain of *E. coli*, 0111:B4 and from a rough mutant of *S. minnesota*, R595. The former LPS is highly water soluble, has a mol wt of ≅1 × 10⁶, consists of ≅50% by weight of lipid A, and has been shown to be an activator of the alternative complement pathway (18). In contrast R595 LPS is a large macromolecular aggregate with a sedimentation coefficient of >50S, consists of >90% by weight of lipid A and has been shown to be an activator of the classical complement pathway (18). Despite these differences both LPS preparations have been previously shown to produce similar pathophysiologic changes in rabbits (16). Furthermore as shown in the present paper these LPS preparations interact with serum components, apparently by the same mechanism, so that the buoyant density of the parent LPS is reduced to <1.2 g/cm³.

The evidence to support the participation of HDL is derived from experiments in which we compared the ability of purified lipoproteins to reconstitute delipidated serum (or plasma). These experiments clearly showed that HDL, at physiologic concentrations, restored the ability of delipidated serum to reduce the density of both 0111:B4 as well as R595 LPS to a value of <1.2 g/cm³. In contrast, VLDL and LDL, added to twice normal serum levels were ineffective although LDL partially restored the activity of delipidated serum when R595 was examined.

Our experimental findings also suggest that LPS undergoes modification before interaction with HDL. In the case of 0111:B4 LPS the addition of HDL to the parent LPS did not result in a significant amount of LPS being altered to a $d < 1.2$ g/cm³ (Table I). Furthermore, HDL did not inactivate the pyrogenic activity of this LPS in the *absence* of delipidated serum. The requirement for modification of R595 LPS before interaction with HDL is less certain (Table I), since all three lipoprotein classes tested appeared to produce a reduction in $d < 1.2$ g/cm³ in the absence of delipidated serum. However, the results of the reconstitution experiments (Fig. 3) indicated that the presence of components of delipidated serum markedly reduced

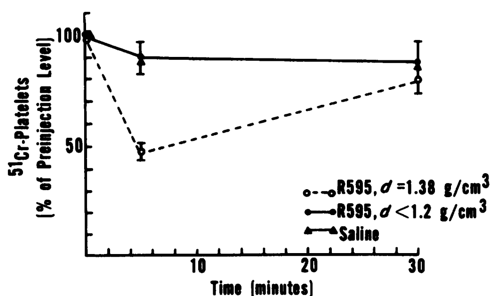

FIGURE 7 The effect of parent and serum-altered R595 LPS injection on levels of circulating ^{51}Cr-platelets. The effect of injection of a 350-μg dose of serum-altered LPS is shown by the closed circles. The effect of injection of 250 μg of parent R595 LPS is shown as the open circles. The effect of injection of saline is shown by the open triangles. The data are shown as the mean±1 SEM for three rabbits in each experimental group.

the effect of VLDL and LDL shown in Table I and appeared to confer a specificity for HDL. Furthermore, the changes in LPS density produced by lipoproteins in the absence of delipidated serum would be of lesser importance because in vivo it is unlikely that LPS would be exposed to any of the lipoproteins in the absence of other plasma components.

We performed a number of experiments to characterize the modification of LPS by delipidated plasma (or serum). Delipidated serum reduced the buoyant density of the parent R595 LPS (d = 1.38 g/cm^3) so that R595 LPS was found to band at a d = 1.34 g/cm^3 after incubation with delipidated serum. A similar small reduction in the buoyant density of 0111:B4 LPS was also observed after exposure to delipidated serum. The broadening of the band width in CsCl of both 0111:B4 and R595 LPS after addition to delipidated serum suggested that disaggregation of the LPS may have accompanied the small change in buoyant density. More direct evidence to suport this is found in the results of sucrose gradient analysis of parent R595 LPS and R595 LPS + delipidated serum mixtures (Fig. 5). This experiment showed that delipidated serum produced a marked reduction in the sedimentation rate of the parent LPS. The possibility that lipid-free serum components produced a conformational change in the LPS, resulting in a markedly decreased sedimentation rate cannot be excluded. However, the modifications of LPS, produced after exposure to delipidated plasma (or serum), may reflect changes which occur in normal plasma and are of critical importance in the conversion of the parent LPS to a form with $d < 1.2$ g/cm^3. This possibility is currently under investigation.

Finally, the molecular weight of the serum-altered form of 0111:B4 or R595 LPS ($d < 1.2$ g/cm^3) was markedly less than that of the parent LPS. These data also suggest that a disaggregation of the LPS occurs before or in concert with the reduction in $d < 1.2$ g/cm^3. In an earlier publication we suggested that the interaction of LPS with plasma (or serum) resulting in a reduction of the buoyant density of LPS to <1.2 g/cm^3 could be explained by a two-step mechanism. The experimental results reported in the present paper are consistent with this scheme shown below:

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

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

The loss of endotoxic activity requires the interaction of the LPS product of step I with HDL to produce the LPS with a $d < 1.2$ g/cm^3. It is likely that the conversion of LPS to a form with a $d < 1.2$ g/cm^3 results from complex formation between the disaggregated LPS and HDL so that the LPS acquires the density characteristics of the HDL. This possibility is currently under investigation.

We have previously shown that conversion of LPS to a form with $d < 1.2$ g/cm^3 results in the abrogation of several biologic activities of LPS, most notably those expressed by the immediate host responses to LPS. However, it was demonstrated that the serum-altered LPS ($d < 1.2$ g/cm^3) was still toxic when assayed in a mouse lethality assay (8). These findings suggested that the serum-altered LPS may still retain the ability to interact with host defense systems and produce mediators responsible for LPS-induced injury.

To further explore the toxic properties of the serum-altered LPS ($d < 1.2$ g/cm^3) we have examined the ability of this LPS to produce shock and disseminated intravascular coagulation in rabbits. These studies, to be reported elsewhere, indicated that the capacity of the serum-altered form ($d < 1.2$ g/cm^3) to induce shock and disseminated intravascular coagulation in rabbits is not inhibited.[3]

The mechanism of this toxicity as yet remains unexplained. However, we have shown that in contrast to the parent LPS, the serum-altered form of R595 LPS was specifically concentrated in the adrenals reaching concentrations of LPS per gram tissue up to fivefold greater than that appearing in any other organs including the liver, lungs, and spleen. The mechanism and biologic effect of the adrenal uptake is of particular interest because this tissue contains cells with high affinity receptors for lipoproteins (19–21). Our finding of accumulation of the serum-altered LPS in the adrenal suggests that the LPS may have acquired apolipoproteins responsible for interaction at these sites.

Experiments are currently in progress to examine the following: (*a*) An evaluation of the importance of step I of the proposed mechanism for conversion of the parent LPS to the low-density form. (*b*) The contribution which lipoprotein lipids and apolipoproteins make to the conversion of LPS to a form with $d < 1.2$ g/cm^3.

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REFERENCES

1. McCabe, W. R. 1972. Immunization with R mutants of *S. minnesota*. I. Protection against challenge with heterologous gram-negative bacilli. *J. Immunol.* **108:** 601–610.
2. Bruins, S. C., R. Stumacher, M. A. Johns, and W. R. McCabe. 1977. Immunization with R mutants of *Salmonella minessota*. III. Comparison of the protective effect of

[3] Mathison, J., and R. Ulevitch. Unpublished data.

immunization with lipid A and the Re mutant. *Infect. Immun.* **17**: 16–20.

3. Braude, A. I., E. T. Ziegler, H. Douglas, and J. A. McCutchan. 1977. Antibody to cell wall glycolipid of gram-negative bacteria: induction of immunity to bacteremia and endotoxemia. *J. Infect. Dis.* **136**(Suppl.): S167–S173.
4. Braude, A. I., F. J. Carey, and M. Zalesky. 1955. Studies with radioactive endotoxin. II. Correlation of physiologic effects with distribution of radioactivity in rabbits injected with lethal doses of *E. coli* endotoxin labeled with radioactive sodium chromate. *J. Clin. Invest.* **34**: 858–866.
5. Herring, W. B., J. C. Herion, R. I. Walker, and J. G. Palmer. 1963. Distribution and clearance of circulating endotoxin. *J. Clin. Invest.* **42**: 79–86.
6. Skarnes, R. C., and L. C. Chedid. 1964. Biological degradation and inactivation of endotoxin. *In* Bacterial Endotoxins. M. Landy and W. Brown, editors. Rutgers University Press, New Brunswick. 575–587.
7. Ulevitch, R.J. 1978. The preparation and characterization of radioiodinated bacterial lipopolysaccharide. *Immunochemistry.* **15**: 157–164.
8. Ulevitch, R. J., and A. R. Johnston. 1978. The modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. *J. Clin. Invest.* **62**: 1313–1324.
9. Weinstein, D. B., and R. J. Ulevitch. 1979. A new protective role for high density lipoprotein (HDL): detoxification of endotoxins. *Circulation.* **58**(Suppl. II): II-90. (Abstr.)
10. Morrison, D. C., and L. Leive. 1975. Fractions of lipopolysaccharide from *Escherichia coli* 0111:B4 prepared by two extraction procedures. *J. Biol. Chem.* **250**: 2911–2919.
11. Galanos, C., O. Luderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**: 245–249.
12. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17**: 176–181.
13. Weinstein, D. B., T. E. Carew, and D. Steinberg. 1976. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* **424**: 404–421.
14. Miller, N. E., D. B. Weinstein, T. E. Carew, T. Koschinsky, and D. Steinberg. 1977. Interactions between high density and low density lipoproteins during uptake and degradation by cultured human fibroblasts. *J. Clin. Invest.* **60**: 78–88.
15. Patch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holaseck, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate zonal ultracentrifugation. *J. Lipid. Res.* **15**: 356–366.
16. Ulevitch, R. J., C. G. Cochrane, P. M. Henson, D. C. Morrison, and W. F. Doe. 1975. Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation. I. The role of complement. *J. Exp. Med.* **142**: 1570–1590.
17. Ulevitch, R. J., and C. G. Cochrane. 1978. Role of complement in lethal bacterial lipopolysaccharide-induced hypotensive and coagulative changes. *Infect. Immun.* **19**: 204–211.
18. Morrison, D. C., and R. J. Ulevitch. 1978. The effects of bacterial endotoxins on host mediation systems. *Am. J. Pathol.* **93**: 527–617.
19. Gwynne, J. T., D. Mahaffe, H. B. Brewer, Jr., and R. L. Ney. 1976. Adrenal cholesterol uptake from plasma lipoprotein: regulation by corticotropin. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 4329–4333.
20. Faust, J. R., J. L. Goldstein, and M. S. Brown. 1977. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured mouse adrenal cells. *J. Biol. Chem.* **252**: 4861–4871.
21. Kovanen, P. T., J. R. Faust, M. S. Brown, and J. C. Goldstein. 1979. Low density lipoprotein receptors in mouse adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured adrenocortical cells. *Endocrinology.* **104**: 599–609.