The Modification of Biophysical and Endotoxic Properties of Bacterial Lipopolysaccharides by Serum

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ABSTRACT Normal rabbit serum reduces the buoyant density of lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (d = 1.44 g/cm^3) and *Salmonella minnesota* R595 (d = 1.38 g/cm^3) to a value <1.2 g/cm^3. This density shift is associated with the inhibition of a number of endotoxic activities of the LPS; namely, the pyrogenic activity, the ability to produce an immediate neutropenia in rabbits, lethality in adrenalectomized mice, and anticomplementary activity. A qualitatively similar change in buoyant density was observed to occur after intravenous injection of the LPS into rabbits. Preliminary evidence suggests that the density shift does not occur as a result of the degradation of the glycolipid backbone of the LPS. These data suggest that the interactions of LPS with plasma (or serum) components leading to reduction in buoyant density may account for a major pathway of LPS detoxification.

INTRODUCTION

Direct interactions of bacterial lipopolysaccharide (LPS)[1] with humoral as well as cellular elements may lead to the production of injurious mediators responsible for the initiation of LPS-induced hemodynamic and coagulative changes (reviewed in 1). Therefore, the elucidation of the mechanisms of LPS-induced pathophysiologic changes may, in part, be accomplished by determining the specificity of LPS binding in the host. However, interactions of LPS with humoral, as well as cellular elements, may also lead to a progressive loss of biological activity of the LPS. This loss of activity has been termed detoxification (reviewed in 2). Thus, studies of LPS distribution in vivo designed to elucidate the pathogenic mechanism(s) of injury must also consider interactions of LPS that result in detoxification.

The mechanism of LPS detoxification that occurs in the vascular compartment is the subject of controversy and as yet, the plasma component(s) required have not been well characterized. The mechanisms proposed to account for LPS detoxification include either enzymatic degradation of LPS (2) or a "detergent-like" effect of plasma resulting in disaggregation of LPS with subsequent loss of biological activity (3). It has also been suggested that the complement system may participate in the detoxification of LPS in plasma (4, 5).

The studies described in this and subsequent reports examine the interaction of LPS from *Escherichia coli* 0111:B4 or *Salmonella minnesota* R595 with plasma and serum with radioiodinated preparations of 0111:B4 and R595 LPS as tracer molecules. We have recently described the preparation and characterization of ^{125}I-LPS with the following properties; (*a*) the radiolabel is covalently bound, (*b*) the specific activity is at least 2 μCi/μg, and (*c*) the biophysical and biologic properties of the radiolabeled LPS and unlabeled LPS are indistinguishable (6).

Experimental studies from this laboratory have shown that 0111:B4 and R595 LPS possess multiple endotoxic activities including the ability to initiate bone marrow-derived-cell mitogenesis, to stimulate murine peritoneal macrophages to become nonspecifically cytotoxic for tumor cells in vitro, to activate Hageman factor, to induce pyrogenic responses in rabbits, and to produce hypotensive shock and disseminated intravascular coagulation in rabbits and subhuman primates. Both of these LPS preparations contain a high proportion of the biologically active lipid A. However, they differ significantly in both the mechanism by which they activate complement and in their carbohydrate content. LPS 0111:B4, an activator of the alternative complement pathway, consists of lipid A, core, and o-antigen polysaccharide whereas LPS, R595, consists of lipid A and a trisaccharide of 2-keto-3-deoxyoctulosonate and activates the classical complement pathway (7).

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[1] *Abbreviations used in this paper*: KDO, 2-keto-deoxyoctulosonate; LPS, lipopolysaccharide; NRS, normal rabbit serum; SDS, sodium dodecyl sulfate.

In the studies described here we will examine the interactions of LPS with plasma (in vivo) and serum (in vitro). To do this we will characterize changes in the hydrodynamic properties of LPS induced by serum or plasma, isolate the modified LPS from LPS/serum mixtures, and study the biological activities of the modified LPS. In this and subsequent papers we will describe experiments that led to the formulation of the following minimal two-step scheme to explain the modifications of LPS by plasma (or serum) resulting in the detoxification of the LPS.

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

$$\text{Step II } (\text{LPS})_{\text{modified}} + \text{high density lipoprotein} \rightarrow (\text{LPS})_{d<1.2\text{g/cm}^3}. \quad (1)$$

LPS with $d < 1.2$ g/cm^3 is the detoxified form of the LPS.

METHODS

Rabbits

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

Reagents

Methyl-p-hydroxybenzimidate was purchased from Pierce Chemical Co., Rockford, Ill., and stored at 4°C with dessicant. Optical grade CsCl (Lot no. 70273) was obtained from Calbiochem, San Diego, Calif., reagent grade KBr (Lot no. 611864) was obtained from J. T. Baker Chemical Co., Philipsburg, N. J., pyrogen-free, sterile saline and distilled water were obtained from McGraw Laboratories, Irvine, Calif., and were used for preparing all solutions.

Lipopolysaccharide

The LPS preparations used for this study were prepared and kindly supplied by Dr. D. C. Morrison (Scripps Clinic and Research Foundation, La Jolla, Calif.). LPS was isolated from *E. coli* 0111:B4 by hot phenol extraction and molecular exclusion chromatography according to the method of Westphal and Jann (8) as modified by Morrison and Leive (9). The fraction of LPS with short antigenic side chains, termed 0111:B4, fraction II was used in these experiments. The concentration of LPS was determined by measurement of the dideoxyhexose sugar, colitose, in a colorimetric assay (10).

LPS internally labeled with [^{3}H]glucosamine and [^{14}C]galactose was extracted as above (9) from the J5 mutant of 0111:B4 *E. coli* grown in the presence of added [^{3}H]glucosamine and [^{14}C]galactose. The physical chemical properties of this [^{3}H]-, [^{14}C]LPS are indistinguishable from those of LPS prepared from *E. coli* 0111:B4 by the method of Morrison and Leive (9). We are especially grateful to Dr. Loretta Leive (National Institutes of Health, Bethesda, Md.) for the preparation of the *E. coli* J5 bacteria grown in the presence of the radioactive sugars.

LPS from *S. minnesota* R595 was extracted according to the method of Galanos et al. (11). This LPS consists of lipid A and a trisaccharide of 2-keto-3-deoxyoctulosonate (KDO). The presence of KDO was assayed for colorimetric assay by a modification (9) of the thiobarbituric acid assay of Cynkin and Ashwell (10). Solubilized preparations of R595 LPS were prepared by sonication of a 5-mg/ml LPS solution in the presence of 0.1% triethylamine followed by dialysis against sterile saline at 4°C.

Radioiodinated 0111:B4 or R595 LPS was prepared according to the method of Ulevitch (6). ^{125}I-LPS with a specific activity between 2 and 5 μCi/μg was employed for the experiments described in this paper. This material was stored at 4°C in the presence of 0.01% sodium azide. For some experiments LPS was radiolabeled with Na^{131}I (6).

Serum

Whole blood was collected from the medial ear artery of rabbits, allowed to clot in a glass tube for 60 min, 37°C and the serum removed after centrifugation at 10,000 *g* for 30 min 4°C. Serum from at least six rabbits was pooled, aliquoted, and stored at −70°C until needed. Frozen serum was thawed at 37°C and employed immediately for experiments described in this paper.

Analytical techniques to study LPS/serum interactions

Ultracentrifugation. Isopycnic density gradient ultracentrifugation was performed with CsCl solutions of an average *d* of 1.28 or 1.4 g/cm^3 as previously described (9). Samples were centrifuged to equilibrium in the Spinco model L-2 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) with the SW 50.1 rotor, 40,000 rpm for at least 48 h at 4°C, fractions collected (0.10 ml per fraction) and the densities calculated from the index of refraction with a refractometer.

Effect of LPS on core temperature and levels of circulating polymorphonuclear cells. Changes in core temperature induced by LPS injection were monitored with a model 44TH telethermometer from Yellow Springs Instrument Co., Yellow Springs, Ohio. Base-line temperatures were recorded for 1 h before LPS injection. Levels of peripheral blood polymorphonuclear cells were determined as previously described (12).

Lethality studies in mice. Adrenalectomized BALB/c AnCr mice (8–10 wk, 18–22 g) were used to determine the median lethal dose of either native LPS or serum altered LPS. Mice were anesthetized with ether, adrenalectomized, and allowed to recover for 24 h before use. Normal saline was substituted for drinking water for adrenalectomized mice. Sham operated and normal animals were also included in the study. Dilutions of the test samples were prepared in sterile saline and 0.2 ml samples injected into the tail vein. At least five mice were used for each concentration of LPS tested. Survivors were counted 24 h after injection and the data analyzed by the method of Reed and Meunch (13).

Complement activation. The measurement of serum hemolytic complement was performed as previously described (14). To compare the anticomplementary activity of native or serum-altered LPS we performed the following experiment. Pooled normal rabbit serum stored at −70°C was thawed at 37°C and equal volumes of the LPS sample and the serum were incubated for 30 min at 37°C. This solution was diluted (usually 1:15) and the total hemolytic complement remaining determined.

Sodium dodecyl sulfate (SDS) gel electrophoresis. 0.6 × 15 cm 12.5% polyacrylamide gels that contained 0.1% SDS and 8-M urea were prepared as described by Downer et al. (15) with two modifications. The final concentration of *N,N,N',N'*-tetramethylenediamine used was 1.7 μl/ml stock gel solution

and polymerization was initiated with 0.5 mg/ml gel solution of ammonium persulfate. Experimental samples (up to 100 μl) were added to 100 μl of 10% SDS, 8-M urea, and brought to 100° for 15 min before addition to the SDS polyacrylamide gel. After electrophoresis for 18 h at 1.5 mA/gel, gels were sliced and analyzed for radioactivity. Control experiments indicated that the ratio of SDS:LPS must be at least 16:1 (wt/wt) to achieve complete resolution of the "subunits" of the LPS by SDS/urea polyacrylamide gel electrophoresis.

In vivo studies of alterations of ^{125}I-LPS by plasma

Alterations of biophysical properties of LPS in vivo were examined as follows. A 12-cm, PE-50, polyethylene catheter was surgically introduced into the femoral artery of 2–2.5 kg rabbits and employed to collect arterial blood samples. A 0.5-ml sample of blood could be collected in less than 5 s through the catheter. Approximately 1 h after surgery a 1-ml solution of 100 μCi of ^{125}I-M-LPS and 2 mg of unlabeled 0111:B4 LPS was rapidly injected into the marginal ear vein. At different times after LPS injection, 0.5-ml blood samples were collected into 4.5 ml of saline, previously brought to 4°C, the diluted samples rapidly mixed by inversion, and the resultant solution maintained at 4°C until completion of the experiment. At the conclusion of the experiment the diluted blood samples were centrifuged at 3,500 rpm, 4°C for 60 min in an IEC International Refrigerated Centrifuge PR-6 (no. 243 rotor, International Equipment Co., Needham Heights, Mass.). The supernate was collected and solid CsCl added to achieve an average d of 1.28. These solutions were centrifuged at 40,000 rpm in an SW 50.1 rotor for 60 h, fractions collected, and the radioactivity present in each fraction determined.

RESULTS

It has been previously suggested that LPS may complex with serum lipoproteins and that this interaction produces a gradual loss of biological activity of the LPS (2, 16, 17). We reasoned that if indeed an LPS/lipoprotein interaction occurs a marked alteration in the buoyant density of the LPS might result. Initial experiments were therefore performed to determine the effect of normal rabbit serum (NRS) on the buoyant density of two different LPS preparations, namely the LPS isolated from either *E. coli* 0111:B4 or from *S. minnesota* R595. Equilibrium density ultracentrifugation in CsCl was employed to separate native and serum-altered LPS in the experiments described below.

Interaction of 0111:B4 LPS with serum

We first examined the effect of NRS on the buoyant density of 0111:B4 LPS. This LPS preparation is ≅50% lipid A by weight (9) and has a hydrated buoyant density of 1.44 g/cm^3. For this experiment, a 100 μl solution that contained a tracer amount of ^{125}I-LPS and 20 μg of unlabeled 0111:B4 LPS was added to 0.3 ml of pooled NRS and maintained at 37°C. At different times after the addition of LPS to serum an aliquot was removed, immediately diluted with 5 ml of cold CsCl

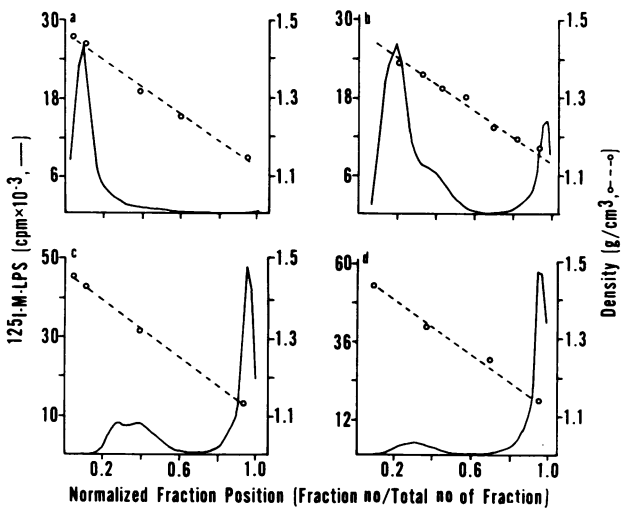

FIGURE 1 The effect of NRS on the buoyant density of *E. coli* 0111:B4 LPS. ^{125}I-LPS/serum mixtures were maintained at 37°C and then centrifuged in CsCl solutions for 60 h, SW 50.1 rotor at 40,000 rpm, 0.1 ml fractions were collected and analyzed for the distribution of ^{125}I-LPS as described in Methods. (*a*): ^{125}I-0111:B4 LPS before the addition to NRS. (*b*): ^{125}I-0111:B4 LPS 1 min after the addition to NRS. (*c*): ^{125}I-0111:B4 LPS 30 min after the addition to NRS. (*d*): ^{125}I-0111:B4 LPS 60 min after the addition to NRS. See text for experimental details.

(d = 1.28 g/cm^3), and maintained at 4°C.[2] After the final LPS/serum sample was diluted with CsCl, the resultant solutions were centrifuged at 40,000 rpm (SW 50.1 rotor) for 60 h. The presence of LPS in each gradient was determined by measurement of radioactivity and these data demonstrated that the buoyant density of ^{125}I-LPS undergoes a time-dependent shift from a d of 1.44 g/cm^3 to a value of less than 1.2 g/cm^3. Representative data from CsCl gradients for the ^{125}I-LPS before and at 1, 30, and 60 min after addition to NRS are shown in Fig. 1 (*a–d*, respectively). The appearance of intermediates with d between 1.35–1.41 g/cm^3 was consistently observed when 0111:B4 LPS/serum mixture was examined by CsCl ultracentrifugation. The concentration of these intermediates decreases with time as seen in Fig. 1*b–d*, suggesting that their formation may be important in the reaction sequence leading to the LPS with a $d < 1.2$ g/cm^3.

Increasing amounts of unlabeled LPS were added to a constant amount of ^{125}I-0111:B4 LPS to determine the capacity of NRS to effect the density shift. When 60 μg of 0111:B4 LPS is added to 1 ml of NRS (75% vol/vol) as described above ≅80% of the LPS appears at a $d < 1.2$ g/cm^3. Final 0111:B4 LPS concentrations in excess of 60 μg LPS/ml of NRS (75%) result in accumulation of LPS intermediates with $d > 1.2$ g/cm^3.

[2] The serum-induced density shift of 0111:B4 LPS does not occur at 4°C (Ulevitch, R. Unpublished data).

The ability of serum to modify the endotoxic activity of LPS has previously been shown to be markedly enhanced by the addition of chelators of divalent ions (18, 19). We therefore examined the effect of EDTA on the ability of NRS to alter the buoyant density of 0111:B4 LPS. Experiments were performed with 0111:B4 LPS and NRS that contained 10 mM EDTA (pH 7.5) exactly as described above. The kinetics of the NRS-induced alteration in the buoyant density of 0111:B4 were not enhanced or inhibited by prior addition of 10 mM EDTA to the NRS.

Interaction of R595 LPS with serum

The effect of NRS on the buoyant density of R595 LPS was next examined. This LPS consists of only lipid A and a trisaccharide of KDO and is therefore >90% lipid A. The hydrated buoyant density of R595 LPS is 1.38 g/cm^3 and as shown in Fig. 2*a* the ^{125}I-R595-LPS and unlabeled R595 LPS (detected by KDO assays) band in an identical position in a CsCl gradient. To compare the effect of NRS on R595 with that observed for 0111:B4 LPS we chose an amount of R595 LPS with a lipid A content equivalent to that found in 20 μg of 0111:B4 LPS. To do this a 100-μl solution that contained a tracer amount of ^{125}I-R595-LPS and 10 μg of unlabeled R595 LPS was added to 0.3 ml of NRS and maintained at 37°C. Samples were removed, diluted in cold CsCl, and centrifuged to equilibrium as described above. Data from CsCl gradients for the ^{125}I-R595-LPS before and at 1, 5, and 60 min after the addition of NRS are shown in Fig. 2. The reaction is essentially complete within 1 min after the addition of R595 LPS to serum. As shown in Fig. 2*a* the native R595 LPS has a hydrated buoyant density of 1.38 g/cm^3 and after the addition of serum a new peak appears at a $d < 1.2$ g/cm^3 (Fig. 2*b–d*). The predominance of the radioactivity remains at a $d = 1.38$ g/cm^3 and even after 60 min only 25% of the added radioactivity is found at $d < 1.2$ g/cm^3. No intermediates between $d = 1.38$ g/cm^3 and $d < 1.2$ g/cm^3 were observed. These data demonstrate that NRS can also produce a decrease in the buoyant density of R595 LPS, although the extent of this reaction is not as great as observed with 0111:B4 LPS.

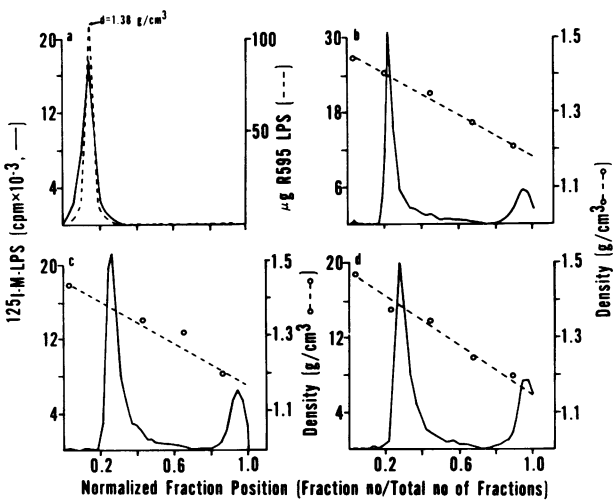

FIGURE 2 The effect of NRS on the buoyant density of *S. minnesota* R595 LPS. ^{125}I-LPS/serum mixtures were maintained at 37°C and centrifuged in CsCl as described in the legend for Fig. 1. (*a*): ^{125}I-R595 LPS before addition to NRS. The presence of added, unlabeled R595 LPS was determined by assays for KDO and these results are shown by the dashed line. (*b*): ^{125}I-R595 LPS 1 min after the addition to NRS. (*c*): ^{125}I-R595 LPS 5 min after the addition to NRS. (*d*): ^{125}I-R595 LPS 60 min after the addition to NRS. See text for experimental details.

We also examined the effect of prior addition of 10 mM EDTA on the interactions of R595 with serum. The experiment was performed exactly as described above except that the NRS contained 10 mM EDTA. The addition of EDTA was observed to markedly increase the amount of R595 LPS shifted to $d < 1.2$ g/cm^3. Within 1 min of incubation with NRS that contained 10 mM EDTA greater than 75% of the added LPS was found to band at a $d < 1.2$ g/cm^3. Representative data from CsCl gradients of R595 at 1 and 60 min after addition to EDTA-serum are shown in Fig. 3. Therefore in a marked contrast to experiments with 0111:B4 LPS, the prior addition of EDTA markedly enhances the NRS induced modifications of R595 LPS. In the presence of 10 mM EDTA, 1 ml of NRS (75% vol/vol), will convert at least 100 μg to R595 LPS to a buoyant density of <1.2 g/cm^3.

Because of the possible direct effect of EDTA on R595 LPS we incubated R595 LPS with 10 mM EDTA at 37°C. This pretreatment of R595 LPS with 10 mM EDTA followed by dialysis to remove EDTA was not sufficient to support the enhanced serum induced density shift observed when EDTA was added to NRS. Further the EDTA-treated R595 LPS banded in the same position as "native" R595 LPS in CsCl gradients.

Isotope dilution experiment

To further insure that the unlabeled and the radioiodinated LPS behave in an identical manner when added to NRS the following experiment was performed with 0111:B4 LPS. We first showed that the initial rate of the density shift was dependent upon the concentration of the added LPS up to a final concentration of 12.5 μg 0111:B4 LPS/ml of NRS (75%). No further increase in the initial rate of the density shift was observed when concentrations greater than 12.5 μg LPS/ml NRS were employed. We also demonstrated that the rate of the density shift was essentially linear during the first 15 min of the reaction.

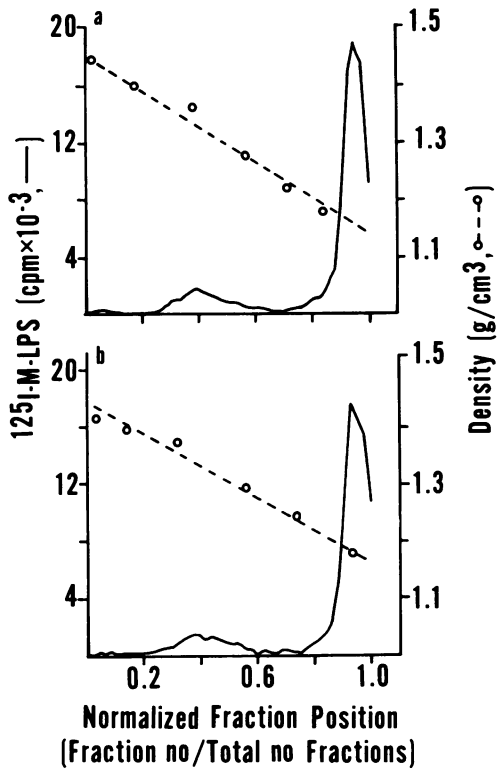

FIGURE 3 The effect of EDTA in the reduction in buoyant density of R595 LPS induced by NRS. ^{125}I-LPS/serum mixtures were maintained at 37°C and centrifuged in CsCl as described in the legend for Fig. 1. (*a*): ^{125}I-R595 LPS 1 min after the addition to NRS that contained 10 mM EDTA. (*b*): ^{125}I-R595 LPS 60 min after the addition to NRS that contained 10 mM EDTA. See text for experimental details.

Knowing these parameters, we next designed an isotope dilution experiment in which the amount of LPS shifted to a $d < 1.2$ g/cm^3 was determined for a series of solutions that contained 12.5 μg 0111:B4 LPS/ml NRS (75%) made up with varying amounts of ^{125}I-0111:B4 LPS and unlabeled 0111:B4 LPS. The ratio of ^{125}I-LPS:total LPS added varied over a 100-fold range for this experiment. The results of this experiment are shown in Table I. Over the range tested the amount of LPS shifted in 12 min at 37°C was independent of the ratio of ^{125}I-LPS:total LPS added. These data lend support to the proposal that the ^{125}I-LPS and the unlabeled LPS interact with serum in an identical manner. Other experimental evidence supporting this conclusion will be presented in a subsequent section in which the mechanism of the density shift is examined.

In vivo alteration of LPS

We next sought to determine if the density shift of LPS induced by serum also occurred in vivo. To do this we injected a mixture of radioiodinated and unlabeled

TABLE I
*A Comparison of the Behavior of ^{125}I-LPS and Unlabeled LPS in NRS**

^{125}I-LPS/^{125}I-LPS + unlabeled LPS‡	^{125}I-LPS at $d < 1.2$ g/cm^3
	% of total LPS in gradient
0.01	26
0.1	24
0.2	26
0.4	25
0.6	23
0.8	26
1.0	24

* Incubation of LPS + NRS, 37°C, 12 min and centrifuged in CsCl as described in Methods.
‡ The final concentration of LPS in the reaction mixture is 12.5 μg LPS/ml NRS (75%).

LPS into rabbits, removed blood samples at different times, and subsequently analyzed these samples by CsCl density ultracentrifugation as described in Methods.

Results obtained with 0111:B4 LPS are shown in Fig. 4 where the distribution of radioactivity in CsCl gradients for LPS before and at 0.25, 0.75, and 15 min after LPS injection is shown. These data indicate that at least within 0.25 min after injection of 0111:B4 LPS a reduction in the buoyant density of LPS occurs. Two major species of altered LPS are observed, namely one with a buoyant density of about 1.4 g/cm^3 and one with

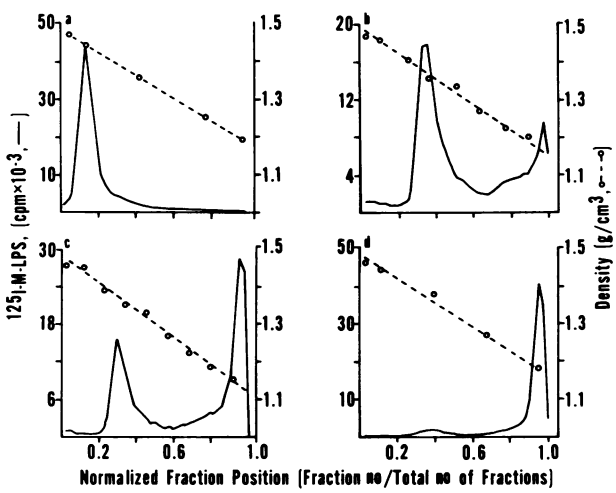

FIGURE 4 Changes in the buoyant density of 0111:B4 LPS produced in vivo after i.v. injection. Samples of blood were removed from rabbits at different times after LPS injection as described in Methods and centrifuged in CsCl as described in the legend for Fig. 1. (*a*): ^{125}I-0111:B4 LPS before the i.v. injection. (*b*): ^{125}I-0111:B4 LPS removed 0.25 min after the i.v. injection. (*c*): ^{125}I-0111:B4 LPS 0.75 min after the i.v. injection. (*d*): ^{125}I-0111:B4 LPS 15 min after the i.v. injection.

a $d < 1.2$ g/cm^3 as shown in Fig. 4*b*. The concentration of the former species disappears with time suggesting that this species of LPS may be an intermediate in the conversion to the low density form of 0111:B4 LPS. Calculations of the amount of LPS remaining in the circulation 30 min after injection indicated that at least 70% of that injected was present in the plasma.

Identical experiments performed with R595 LPS indicated that this LPS is also converted to a form with a $d < 1.2$ g/cm^3 in vivo (data not shown). However, in contrast to 0111:B4 LPS only 10% of the injected R595 LPS remained in the circulation 30 min after injection and this LPS was present as the species with $d < 1.2$ g/cm^3.

Isolation of altered LPS from LPS/serum mixtures

The previous experiments demonstrated that 0111:B4 as well as R595 LPS are altered by components of normal rabbit serum (in vitro) or by plasma (in vivo) so that the density of the LPS is reduced to <1.2 g/cm^3. To study the biological activity of the altered LPS we devised a method to isolate milligram quantities of the fraction of LPS with $d < 1.2$ g/cm^3 from LPS/serum mixtures. The method used to isolate 0111:B4 LPS with a $d < 1.2$ g/cm^3 from LPS/serum mixtures will be described below. Identical procedures were followed for R595 LPS except that the NRS contained 10 mM EDTA. To prepare the low-density form of 0111:B4 LPS we added 10 mg of 0111:B4 LPS and a tracer amount of ^{125}I-LPS to 200 ml of fresh NRS, maintained this solution of 37°C for 120 min, and added enough solid KBr to achieve a final average density of 1.28 g/cm^3. This solution was then centrifuged in a 60 Ti Rotor, 50,000 rpm for 36 h in a Beckman L-2 ultracentrifuge. The upper 20% of each tube was collected and dialyzed to remove free KBr. The dialyzed solutions were further purified by a second centrifugation in an SW 50.1 rotor (40,000 rpm, 48 h) after the addition of solid CsCl to achieve an average d of 1.28 g/cm^3. Fractions from each gradient were analyzed for the presence of LPS by measurement of radioactivity and the fractions containing LPS at a $d < 1.2$ g/cm^3 were pooled and dialyzed to remove free CsCl. The typical distribution of radioactivity in a CsCl gradient is shown in Fig. 5 and as shown at least 85% of the radioactivity present in the gradient is at $d < 1.2$ g/cm^3. No radioactivity was detected in the density region between 1.44–1.2 g/cm^3 suggesting the absence of unmodified LPS in these preparations.

The concentration of LPS was determined from measurements of radioactivity based upon the specific activity of the added LPS, e.g. ^{125}I-LPS, (counts per minute)/[LPS] × total or alternatively by measurement of colitose in the case of 0111:B4 LPS or KDO in the case of

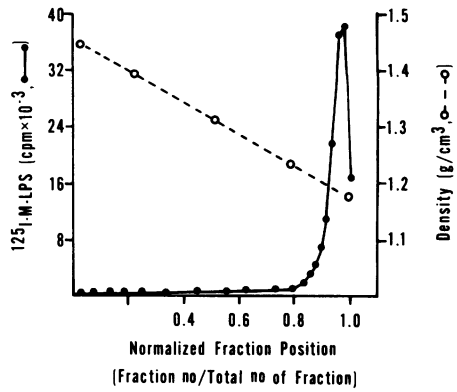

FIGURE 5 CsCl ultracentrifugation of the low-density form of 0111:B4 LPS ($d < 1.2$ g/cm^3) isolated from 0111:B4 LPS/serum mixtures by centrifugation in KBr and then in CsCl as described in the text.

R595 LPS. We also centrifuged NRS in an identical manner to obtain a fraction of serum proteins at a $d < 1.2$ g/cm^3 to be employed for control studies because the low density LPS peak also contains serum proteins with a buoyant density of <1.2 g/cm^3. Up to 10 mg of either LPS preparation was processed by this method and recoveries of LPS at a $d < 1.2$ g/cm^3 ranged between 40 and 60% after the second centrifugation.

Biological activity of LPS with $d < 1.2$ g/cm^3

Experiments to compare the endotoxic activity of native and serum-altered LPS were next performed, with 0111:B4 or R595 LPS with $d < 1.2$ g/cm^3 isolated from LPS/serum mixtures as described above. Ultracentrifugation of saline solutions of either 0111:B4 or R595 first in KBr, then in CsCl, as described above and finally recovering the native LPS after dialysis did not alter the biological properties of the LPS. Purified LPS, treated in this manner, was employed to measure the endotoxic activity of native LPS in the assays described below. We chose to use several assays which are sensitive indicators of the presence of native 0111:B4 or R595 LPS. These are (*a*) the pyrogenic response to LPS in rabbits, (*b*) the ability to produce an immediate neutropenia in rabbits, (*c*) toxicity in adrenalectomized mice, and (*d*) the ability to activate the classical pathway of complement. The results of these studies are described below.

Pyrogenic response and immediate neutropenia. We first determined that the intravenous injection of 50 ng of native 0111:B4 LPS routinely produced a biphasic rise of at least a 1–1.5°C in core temperature over a 5-h period. This amount of 0111:B4 LPS also produced an immediate neutropenia in the rabbits with at least 90% of the circulating neutrophils being removed from the circulation with 5 min of LPS injection. These data

were used for calculations of the extent of the loss of the activity in 0111:B4 LPS associated with the decrease in buoyant density.

Rabbits were injected with either 5 μg of native or low-density LPS and the temperature was measured during a 5-h period. The results of this experiment are shown in Fig. 6 and demonstrate that the low-density form of the 0111:B4 LPS was markedly inhibited in the ability to induce a pyrogenic response in rabbits. We also measured the levels of circulating neutrophils in rabbits injected with 5 μg of either native or the low-density form of 0111:B4 LPS. The immediate neutropenia induced by 0111:B4 LPS did not occur when the low-density form of LPS was injected. These data are shown in Fig. 7. Because as little as 50 ng of LPS (d = 1.44) produces a pyrogenic response as well as neutropenia, we can conclude that no more than 1% of both the pyrogenic and neutropenia-inducing activity of native 0111:B4 remains with the LPS at $d < 1.2$ g/cm³. The pyrogenic activity and the ability to induce a neutropenia in rabbits characteristic of native R595 LPS were also inhibited as a result of the reduction in buoyant density.

Toxicity in adrenalectomized mice. We next compared the toxicity of native and serum altered LPS ($d < 1.2$ g/cm³) in adrenalectomized BALBc/AnCr mice. BALBc/AnCr mice were adrenalectomized and 24 h after the surgery were injected with either native R595, native 0111:B4 LPS or the low-density form of these LPS preparations. The results of the lethality date are shown in Table II. These data indicate that the density shift results in a marked reduction in the lethality of R595 as well as 0111:B4 LPS. Controls for these experiments included adrenalectomized mice injected with saline employed as diluent and the serum

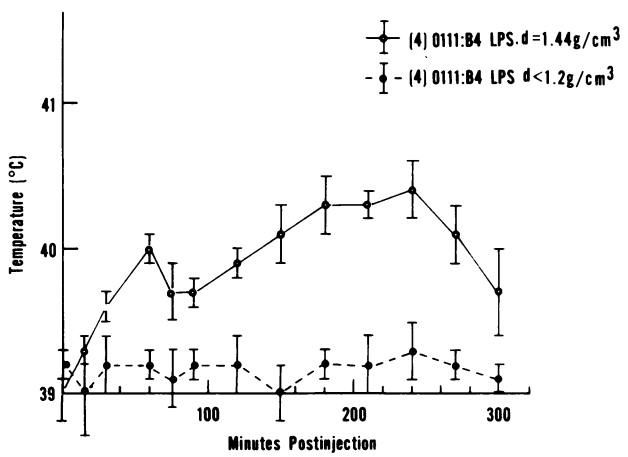

FIGURE 6 A comparison of the pyrogenic response of rabbits to 5 μg of native 0111:B4 LPS (d = 1.44 g/cm³) and 5 μg of serum-altered 0111:B4 LPS ($d < 1.2$ g/cm³). The data is expressed as the mean ± 1 SEM. See text for experimental details.

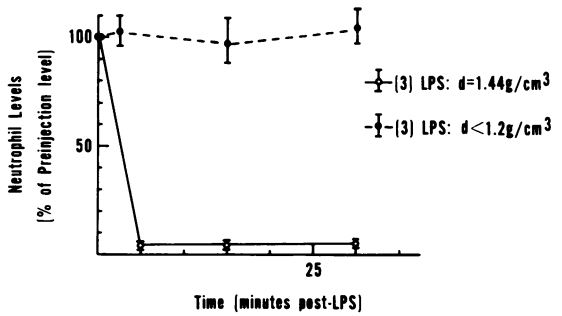

FIGURE 7 The effect of 5 μg of native 0111:B4 LPS (d = 1.44 g/cm³) and 5 μg of serum-altered 0111:B4 LPS ($d < 1.2$ g/cm³) on the levels of circulating neutrophils of rabbits. The data is expressed as the mean ± 1 SEM. See text for experimental details.

lipoprotein fraction with a $d < 1.2$ g/cm³ (−LPS) prepared from NRS by differential centrifugation in KBr and CsCl. No deaths were recorded in the control groups of animals.

Anticomplementary activity. R595 LPS has been shown to activate the classical pathway of complement (7). To further study the effect of the serum-induced density reduction on the endotoxic activity of R595 LPS we compared the anticomplementary activity of the low-density R595 LPS and native LPS. For this experiment we added different amounts of either native or serum-altered R595 LPS to normal rabbit serum, incubated these solutions at 37° and subsequently determined the level of hemolytic complement remaining as described in Methods. The amount of native R595 LPS required to reduce the hemolytic complement level 50% is 0.052 μg whereas at least 1.30 μg of the serum-altered R595 LPS is required to achieve a 50% reduction. The results of these experiments shown in Fig. 8 demonstrate that the anticomplementary activity of R595 LPS is reduced at least 47-fold as a result of the density shift. In control experiments we determined that the serum lipoproteins with $d < 1.2$

TABLE II
*Toxicity of 0111:B4 LPS and Serum-Altered 0111:B4 LPS in Adrenalectomized Mice**

LPS	Density	LD_{50}
	g/cm³	μg
0111:B4	1.44	0.1 (30)‡
R595	1.38	0.045 (19)
0111:B4	<1.2	2.6 (40)
R595	<1.2	0.28 (30)

* BALBc/AnCr mice were employed for this study and lethality was determined 24 h after the i.v. injection of LPS.
‡ The number of mice tested to determine the median lethal dose (LD_{50}).

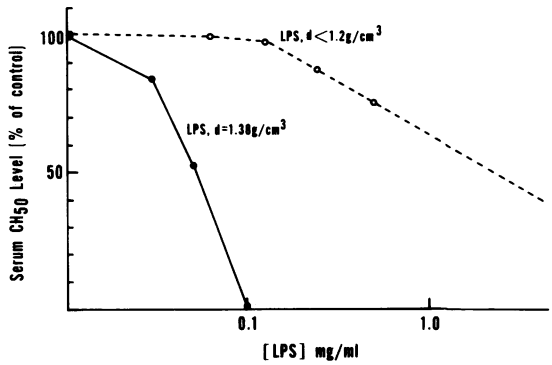

FIGURE 8 A comparison of the anticomplementary activity of native R595 LPS (d = 1.38 g/cm³) and serum-altered R595 LPS (d < 1.2 g/cm³). See text for experimental details.

g/cm³ (−LPS) were not anticomplementary but would markedly enhance the anticomplementary activity of a preparation of aggregated immunoglobulin (Ig)G. The mechanism of this effect is unknown and was not pursued in the present studies. However, this observation suggests that the residual anticomplementary activity of the serum-altered R595 LPS at high concentrations may be amplified by the effect of the lipoproteins present with the low-density LPS. Therefore the reduction in anticomplementary activity that results from the density shift may be greater than the 47-fold effect shown.

Mechanism of serum-induced density shift

We next sought to determine if the density shift resulted from an alteration in the structure of LPS produced by significant degradation of the glycolipid backgone. For example enzymatic hydrolysis of the ketosidic linkage between the KDO and the lipid A, resulting in the release of free lipid A, might account, in part, for the density shift observed. Two different experimental approaches were used in this preliminary examination of the mechanism of the serum-induced density shift.

In one approach we employed SDS/urea polyacrylamide gel electrophoresis to examine the size of the "subunits" of 0111:B4 LPS or R595 LPS before and after the addition of the LPS to NRS. The resolution of bacterial lipopolysaccharides into fractions of different electrophoretic mobility in the presence of SDS has previously been reported (20).

A preparation of ^{125}I-R595 or ^{125}I-0111:B4 LPS was added to NRS so that at least 80% of the added LPS would be banded at a d < 1.2 g/cm³ by CsCl density ultracentrifugation. The LPS/serum samples were centrifuged in CsCl and the low-density form of each LPS was pooled from individual gradients and dialyzed to remove free CsCl. A 100-μl sample of the low-density form of 0111:B4 or R595 LPS was added to 100 μl of 10% SDS, 8-M urea followed by the addition of an aliquot of the appropriate native ^{131}I-LPS. The samples were brought to 100°C for 15 min and electrophoresed and analyzed as described Methods. These results, shown in Fig. 9 for 0111:B4 LPS (top) and R595 LPS (bottom), demonstrate that the electrophoretic mobility of the native LPS, e.g. ^{131}I-LPS, and the serum-altered LPS, e.g., ^{125}I-LPS are identical. At least 85% of the added radioactivity were recovered in the individual gel slices for both ^{125}I- or ^{131}I-labeled R595 or 0111:B4 LPS. These data suggest that enzymatic degradation of the glycolipid backbone of LPS is not a likely mechanism to account for the density shift obtained for 0111:B4 as well as R595 LPS.

In a second approach we employed 0111:B4 LPS internally labeled with [^{3}H]glucosamine and [^{14}C]galactose. The ^{3}H-:^{14}C-ratio in this LPS reflects the relative proportion of lipid A because the [^{3}H]glucosamine is present in the lipid A, core, and 0-antigen region whereas the [^{14}C]galactose is present only in the core and 0-antigen portion of the LPS molecule. Thus if the density shift occurs as a result of degradation of the glycolipid backbone of LPS an alteration in the ^{3}H-:^{14}C-ratio would be detected. We added a 100 μl solution that contained 10 μg of internally labeled ^{3}H-, ^{14}C- 0111:B4 LPS to 0.3 ml of NRS, incubated this mixture at 37°C for 60 min and after dilution with cold CsCl centrifuged this to equilibrium as previously described. LPS incubated in saline was included as a control in this experiment. As shown in the upper panel

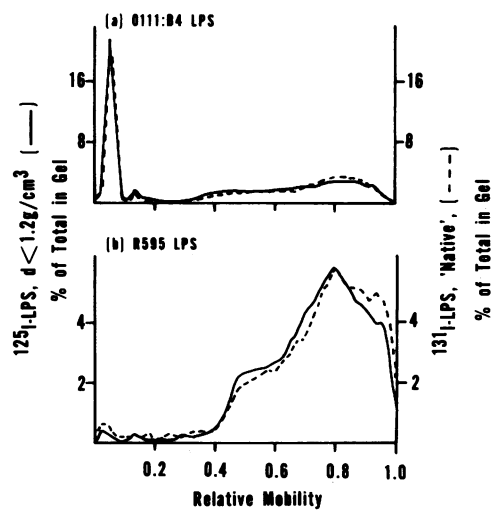

FIGURE 9 A comparison of the electrophoretic mobility of native and serum-altered LPS in SDS/urea polyacrylamide gel electrophoresis. (*a*): Native 0111:B4 LPS (d = 1.44 g/cm³) shown by the solid line (——) and serum-altered 0111:B4 LPS (d < 1.2 g/cm³) shown by the dashed line (---). (*b*): Native R595 LPS (d = 1.38 g/cm³) shown by the solid line (——) and serum-altered 0111:B4 LPS (d < 1.2 g/cm³) shown by the dashed line (---). See text for experimental details.

of Fig. 10, the ^{3}H-:^{14}C-ratio of the LPS (d = 1.44 g/cm^3) calculated for five individual gradient fractions is 1.34. After 60 min in NRS, >70% of the added [^{3}H], [^{14}C]LPS was found to band at $d < 1.2$ g/cm^3. As shown in the lower panel of Fig. 10 the ^{3}H-:^{14}C-ratio of the LPS peak was calculated to be 1.35. These data indicate that NRS only produces an 0.2% change in the ^{3}H-:^{14}C-ratio suggesting that no major degradation of the glycolipid backbone occurs.

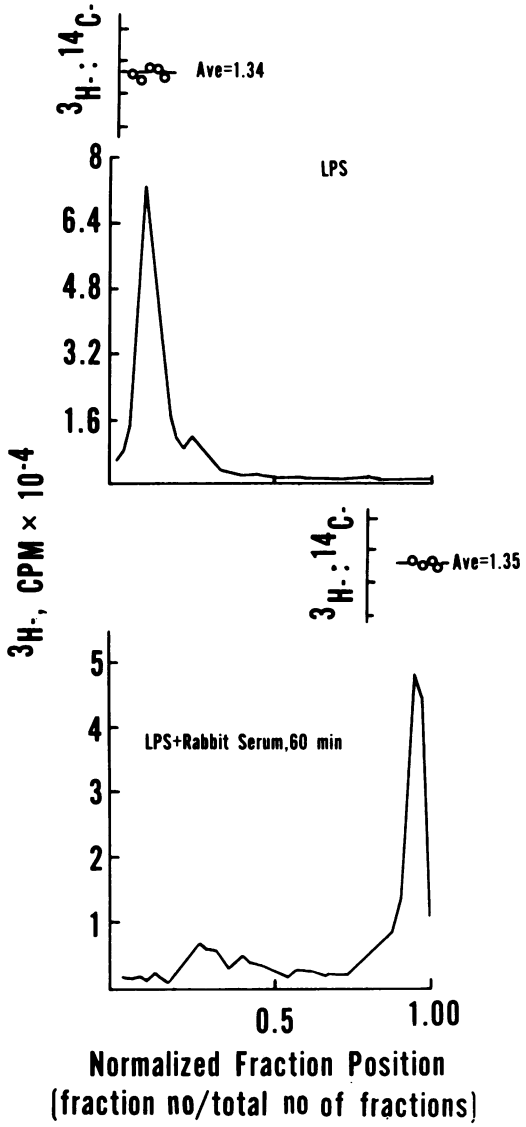

FIGURE 10 The effect of NRS on the buoyant density of 10 μg of [^{3}H], [^{14}C]0111:B4 LPS. Top: The distribution of radioactivity of [^{3}H], [^{14}C]0111:B4 LPS in a CsCl gradient after ultracentrifugation as described in Fig. 1. ^{3}H-Radioactivity is plotted in this figure with the ^{3}H-:^{14}C-ratio being 1.34 (Ave = average) across the LPS peak (d = 1.44 g/cm^3). Bottom: CsCl ultracentrifugation of 10 μg of [^{3}H], [^{14}C]0111:B4 LPS after 60 min incubation in NRS (75%). The ^{3}H-:^{14}C-ratio across the LPS peak with a $d < 1.2$ g/cm^3 is 1.35. Data shown for ^{3}H-radioactivity only.

Although not shown here we also determined that the rate of the density shift of 0111:B4 LPS produced by NRS at 37°C was identical when ^{125}I-0111:B4 LPS and [^{3}H], [^{14}C]0111:B4 LPS were compared. These data further support the conclusion that the ^{125}I-LPS and unlabeled LPS behave in an identical way when added to NRS.

DISCUSSION

These experiments demonstrate that normal rabbit serum can produce a marked reduction in the buoyant density of 0111:B4 as well as R595 LPS. This modification results in the loss of several endotoxic activities of native LPS, namely LPS-induced pyrogenicity, neutropenia induction, toxicity, and anticomplementary activity. We further showed that an identical decrease in the buoyant density of 0111:B4 as well as R595 LPS occurs after i.v. injection into rabbits. Therefore it appears that modification of the LPS by plasma (or serum) components resulting in a marked decrease in the buoyant density of the LPS may account for a major intravascular pathway of LPS detoxification.

LPS detoxification by serum has been studied by a number of investigators including Skarnes (2), Rudbach and Johnson (3), and Johnson and co-workers (4, 21). Previous studies have focused primarily on the characterization of the serum components involved, and to date, markedly differing data regarding the nature of the serum components responsible for LPS detoxification have been presented. For example Johnson et al. (21) have recently described the purification of an α-globulin capable of inactivating LPS. These authors indicate that this protein is neither a lipoprotein nor a serine esterase. In contrast, Skarnes has suggested that two α-globulins, both of which possessed nonspecific esterase activity and one of which was a lipoprotein are responsible for LPS detoxification (16, 17). Other studies (4, 5) have implicated C4, C5, and (or) C6 in LPS detoxification, none of which is a lipoprotein, an α-globulin, or is known to possess esterase activity. We have sought to clarify the problem by first characterizing the physical/chemical changes of the LPS induced by serum in vitro, and in plasma in vivo, and subsequently determining the effect of these changes on the biological activity of the modified LPS.

Modification of LPS by serum. Both 0111:B4 and R595 LPS are modified by serum components so that the buoyant density of the LPS is reduced to <1.2 g/cm^3. However, the characteristics of the LPS/serum interactions resulting in the density shift differ somewhat for these two LPS preparations. In vitro experiments with 0111:B4 LPS demonstrated that the serum-induced density shift requires 30–60 min to reach com-

pletion and that neither the rate nor extent of the reaction with serum is altered by the prior addition of 10 mM EDTA. In contrast, experiments with R595 LPS showed that the rate of the density shift is extremely rapid with greater than 90% of the reaction completed within 1 min after the addition of NRS. Furthermore the presence of 10 mM EDTA markedly enhances the extent of the density shift observed when R595 and serum are mixed. The EDTA effect may be explained by the studies of Galanos and Lüderitz (22) in which they described the marked effect of Ca^{++} and Mg^{++} on R595 LPS. Interaction of these divalent ions with R595, as well as other rough LPS preparations, results in the aggregation and precipitation of the LPS. In contrast these authors have also shown that smooth LPS preparations, e.g., 0111:B4 LPS, are relatively resistent to these interactions with divalent ions. The divalent cations present in NRS may aggregate and (or) precipitate the R595 LPS and consequently prevent the LPS/serum interactions necessary for the density shift. The addition of EDTA would prevent the interaction between divalent cations and R595 LPS and therefore increase the amount of LPS available for the density shift.

An explanation for the difference in the rate of the density shift for 0111:B4 and R595 LPS is not apparent. The data would be consistent with the proposal that the core and O-antigen polysaccharides of 0111:B4 LPS may stabilize this LPS and retard the rate of one or more of the serum-induced modifications required for the density shift. Experimental studies demonstrating a stabilizing effect of polysaccharide-polysaccharide interactions have been described (23). This possibility is currently under investigation.

In experiments not presented in this paper we also determined that normal human and normal mouse sera were qualitatively indistinguishable from NRS in the capacity to produce the density reduction of 0111:B4 as well as R595 LPS.[3] Because the modifications of these two different LPS preparations can occur in serum derived from at least three different sources we conclude that the density reduction may be a general mechanism for modifying the endotoxic activity of LPS in serum (or plasma).

Evidence was also presented here which demonstrated the occurrence of the time-dependent reduction in the buoyant density of 0111:B4 as well as R595 LPS in vivo. The in vivo reduction in the buoyant density of LPS appears to be at least qualitatively similar to that observed with serum, in vitro. This similarity suggests that the participation of fibrinogen or other coagulation components is not required for the reduction in buoyant density of the LPS.

Biological activity of serum-modified LPS. The low-density form of LPS was isolated from serum/LPS mixtures by equilibrium density ultracentrifugation. The fraction of modified LPS tested was shown to be devoid of any material with a $d > 1.2$ g/cm^3.

We demonstrated that the reduction in the buoyant density of 0111:B4 as well as R595 LPS results in the loss of several sensitive indicators of endotoxic activity. The ability of serum to abrogate the pyrogenic activity of LPS has been well documented and our findings are in good agreement with previously published reports (3, 24, 25). The abrogation by serum of the ability of LPS to induce a neutropenia has not been previously described.

In contrast to the complete inhibition of the pyrogenic response and neutropenia induction, the toxicity of the LPS was only partially diminished as a result of the density reduction. The median lethal dosage was increased 26-fold for 0111:B4 LPS and approximately sixfold for R595 LPS. Other studies that have examined the effect of serum on LPS-induced lethality have employed serum from species other than rabbits, used different LPS preparations, did not perform toxicity assays in the adrenalectomized mouse, and did not compute the median lethal dose of native and serum-treated LPS. Therefore, direct comparison of previous findings with our data is difficult. However, Johnson and Ward (4) reported a fourfold reduction in toxicity when *E. coli* 0111:B4 LPS (Difco Laboratories, Detroit, Mich.) was treated with NRS and subsequently injected i.p. into actinomycin D-treated mice. At least one other report has described a partial reduction, by serum, of the lethal effects of LPS while simultaneously demonstrating the complete reduction of the pyrogenic activity (25). Therefore our results obtained with serum-modified forms of LPS from *E. coli* 0111:B4 or *S. minnesota* R595 are in good agreement with those of previous studies of the serum-induced detoxification of bacterial lipopolysaccharides.

Mechanism of LPS detoxification. The observations regarding the reduction in buoyant density of LPS induced by serum or in the vascular compartment in vivo, are consistent with a mechanism requiring interaction of LPS with lipid components of serum or plasma. We have obtained experimental evidence to support the scheme shown in Eq. 1 and will present these data in detail in a subsequent publication. In brief, we have shown that the density shift does not occur in chemically delipidated serum, but that this activity can be fully reconstituted with the addition of physiologic concentrations of high-density lipoprotein. It is of interest to note that neither very low density nor low-density lipoprotein is effective in reconstituting the delipidated serum.[4]

[3] Ulevitch, R. Unpublished data.

[4] Ulevitch, R. J., and D. E. Weinstein. Manuscript in preparation.

Skarnes and his co-workers (2, 16, 17) have previously described a two-step mechanism for LPS detoxification which requires the participation of at least one lipoprotein. The mechanism proposed by Skarnes to account for the inactivation of LPS involves enzymatic degradation of the LPS. However direct experimental evidence to support this mechanism has yet to be presented. Recently Johnson et al. (21) have described the isolation of an LPS inactivator from human serum which appears to detoxify the LPS by disaggregation. These authors concluded that enzymatic degradation or modification of the LPS does not accompany the action of the LPS inactivator they have isolated. At present the relationship of the inactivator described by these investigators to that of the present study is uncertain.

In the present study, our results failed to support the hypothesis that enzymatic degradation of LPS accompanied serum- or plasma-induced detoxification of the LPS. Specifically the electrophoretic mobility in SDS of serum-altered R595 or 0111:B4 LPS ($d < 1.2$ g/cm^3) was unchanged when compared to the electrophoretic mobility in SDS of native LPS (Fig. 9). These peaks of radioactivity displayed on SDS polyacrylamide gels have been shown to correspond exactly to carbohydrate-containing species of 0111:B4 or R595 LPS after treatment of duplicate gels with periodic acid and subsequent staining with Schiff's reagent. The technique of electrophoresis of LPS in SDS has been shown to resolve LPS into subunits. Further, the electrophoretic mobility of the subunits has been shown to be a function of the amount of lipid A (20). The larger the ratio of lipid A:total LPS, (wt/wt), the farther the LPS will migrate into an SDS gel. Therefore if the density shift occurred as a result of the degradation of the glycolipid backbone an alteration in the electrophoretic mobility of the LPS subunits would be observed. Because we did not observe any alteration in electrophoretic mobility and because the recoveries of total radioactivity added to each gel were at least 85% of that added we conclude that no degradation occurred.

The size heterogeneity of LPS has been previously described for LPS from *E. coli* F492 and *Citrobacter* 393 (20). The chemical characteristics of the two peaks of radioactivity detected for 0111:B4 LPS are currently under investigation. The behavior of R595 LPS in the SDS/urea gel system is similar to that reported for LPS from a rough strain of *E. coli* F470 (20).

More compelling evidence for a mechanism of the density shift which does not require enzymatic degradation of LPS derives from the experiment performed with the [^{3}H], [^{14}C]0111:B4 LPS. If significant degradation of the core and(or) the O-antigen side chains of 0111:B4 LPS accounted for the density shift then a marked change in the ^{3}H-:^{14}C-ratio should have been observed when the native ($d = 1.44$ g/cm^3) and the serum-altered LPS ($d < 1.2$ g/cm^3) were compared. A more detailed analysis of the mechanism of the density shift is in progress. Preliminary evidence has been obtained with antiserum prepared against native 0111:B4 or R595 LPS which demonstrates that the native and the serum-altered LPS are immunologically identical. Because the major antigens are believed to be contained in the carbohydrate-containing portions of the molecule these data would further support the proposal that enzymatic degradation of the LPS does not account for the density reduction.

The species of 0111:B4 LPS with buoyant density between 1.35–1.41 g/cm^3 after exposure to plasma or serum (Figs. 1, 4) is of considerable interest and is under current investigation. In preliminary studies, conversion to the intermediate form was found to be essential for eventual transformation to the low-density form of LPS. Tests of the intermediate form of the LPS for pyrogenic activity in rabbits have shown them to be at least as effective as native 0111:B4 LPS ($d = 1.44$ g/cm^3). We believe these intermediates to represent a modified form of the LPS necessary to complete the interactions leading to the density shift, but not producing a loss of biological activity. The question of serum-induced modification of the LPS, before the density shift will be considered in a subsequent publication.[4]

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