

ISOLATION OF A LIPOPOLYSACCHARIDE-BINDING ACUTE PHASE REACTANT FROM RABBIT SERUM

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We have reported that human, murine, and lapine acute phase sera differ functionally from the respective normal sera in at least three ways (1, 2), insofar as interactions with bacterial LPS are concerned. First, the rate at which LPS binds to high density lipoprotein (HDL)¹ is much faster in normal sera than in acute phase sera. For example, in normal sera, the half time for binding of *Salmonella minnesota* Re595 LPS to HDL at 37°C is typically 2–3 min, whereas in acute phase sera, the same reaction can have a half time of up to 100 min. Second, when Re595 LPS is added to serum and spun to equilibrium in a CsCl density gradient, the LPS not bound to HDL is found at a density of 1.33 g/cm³ in normal sera, whereas in acute phase sera the LPS forms a complex with a density of 1.3 g/cm³. We refer to the form of LPS in acute phase serum as complex 1.3 (C.1.3). Finally, when sera containing LPS not yet bound to HDL are chilled rapidly to 4°C and dialysed against very low ionic strength buffer at 4°C an LPS-protein precipitate is formed from both normal and acute phase sera, but the LPS in the precipitate from normal serum redissolves readily in isotonic saline, while the LPS in the precipitate from acute phase serum does not. These phenomena have been seen after acute phase induction with either subcutaneous silver nitrate, intraperitoneal LPS, or casein (unpublished data). We have postulated the existence of a hitherto unrecognized acute phase reactant to explain these findings.

In this report, a purification of the acute phase reactant from acute phase rabbit serum (APRS) responsible for the phenomena noted above is described, as are some of the properties of the reactant. As shown by experiments reported herein, the acute phase reactant does bind directly to LPS; therefore, we refer to it as LPS-binding protein, or LBP.

Materials and Methods

Materials. Biosynthetically tritiated LPS ([³H]LPS) and unlabeled LPS were isolated from *Salmonella minnesota* Re595 as described previously (2, 3). Rabbit blood was collected

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¹ *Abbreviations used in this paper:* APRS, acute phase rabbit serum; ASD, sulfosuccinimidyl-2-(*p*-azido salicylamido)-1,3'-dithiopropionate; AU, absorbance unit; CRP, C-reactive protein; HDL, high density lipoprotein; LBP, LPS-binding protein; NRS, normal rabbit serum.

either by bleeding from the median ear artery or by heart puncture, allowed to clot at 37°C for 2–6 h and at 0°C overnight, centrifuged to remove clot fragments and cells, and the serum was stored frozen without preservative. APRS was collected 24 h after induction of an acute phase response by subcutaneous injection of 1 ml of 3% (wt/vol) silver nitrate in distilled water. Serum collected from noninduced rabbits was tested for normality before being used as normal rabbit serum (NRS). The initial test used was immunodiffusion vs. antiserum to rabbit C-reactive protein (CRP). Sera testing negative for CRP were further tested, by methods described below, to ensure a sufficiently rapid rate of binding of LPS to HDL. These precautions were instituted after observing that as many as one-half of newly acquired New Zealand White rabbits typically have detectable acute phase reactants in their sera.

Polyclonal rat antisera to LBP were raised in Lewis rats by intraperitoneal injection of each rat with 25 µg LBP in CFA, with 25 µg LBP in IFA at 3 wk, and with 10 µg LBP in buffer at 6 wk. Animals were bled by heart puncture under Innovar-Vet anesthesia and serum collected as described above. Immunoprecipitation experiments using these sera were performed by incubating rabbit serum together with varying volumes of antiserum for at least 3 h at 37°C or 4 h at 4°C. Precipitates were collected by centrifugation and washed twice with 50 mM phosphate buffer, 150 mM NaCl, and 0.1% Tween-20 (pH 7.4).

Unfractionated lipoproteins and delipoproteinated sera were prepared by ultracentrifugation. We added 13.23 g KBr to 35 ml serum, after which the serum was spun at 40,000 rpm in a 60 Ti (Beckman Instruments, Inc., Fullerton, CA) rotor for 36–60 h at 4°C. After fractionation, protein assay, and cholesterol assay (Cal Biochem-Behring, La Jolla, CA), the lipoproteins and serum proteins were separately pooled and dialysed extensively against 10 mM Hepes and 150 mM NaCl (pH 7.4). Finally, the delipoproteinated sera and the lipoproteins were brought to 75 and 25% of the original serum volume, respectively, by dilution or concentration, as required. Delipoproteinated sera and lipoproteins were recombined in a 3:1 ratio, respectively, to prepare lipoprotein-reconstituted sera.

LPS was coupled to sulfosuccinimidyl-2-(*p*-azido salicylamido)-1,3'-dithiopropionate (Pierce Chemical Co., Rockford, IL.) as described (4), and the resulting derivative (ASD-LPS) was radiolabeled with Na¹²⁵I using chloramine T (5) to yield ¹²⁵I-ASD-LPS. The product had a specific activity of 7.1×10^9 cpm/mg LPS, from which the incorporation of ¹²⁵I into LPS is calculated to be ~0.3 mol %. Unpublished data indicate that ¹²⁵I-ASD-LPS cosediments with LPS in CsCl gradients, ¹²⁵I-ASD-LPS is quantitatively taken up by HDL in NRS or APRS, and ASD-LPS has the same mitogenicity as LPS when tested with murine splenic B cells. Photolysis of ¹²⁵I-ASD-LPS was accomplished using a Rayonet photochemical reactor (Southern N.E. Ultraviolet Co., Middletown, CT) equipped with General Electric F8T5.BLB lamps with peak output at 370 nm. Reaction mixtures were exposed to this light source for 10 min at 4°C.

Methods. SDS-PAGE with staining by Coomassie Blue or periodic acid/Schiff reagent was performed by published procedures (6). Samples were reduced with 2-ME before SDS-PAGE, and gels contained 10% acrylamide. Concentrations of LPS and its derivatives were determined using the ketodeoxyoctanoate assay (7), with Re595 LPS as standard. Protein concentrations were determined by either the Folin (8) or BCA (Pierce Chemical Co.) reagents, using BSA as standard. All reactions of LPS or LPS derivatives with APRS or NRS were carried out at 10 µg/ml, unless otherwise noted.

Amino acid sequencing was carried out by the Protein Structure Core Laboratory of Scripps Research Foundation according to published procedures (9).

Kinetics of LPS Binding to HDL. The kinetics of LPS binding to HDL in serum were observed and quantitated by CsCl isopycnic density gradient ultracentrifugation. We added 0.4 ml of 0.4 M EDTA (pH 7.4) to 8 ml of rabbit serum, and warmed the mixture to 37°C in a water bath. At time 0, 0.4 ml of 200 µg/ml [³H]LPS in 0.02 M EDTA, pH 7.4, was added. At suitable times, 1.0-ml aliquots of the reaction mixture were removed and added to 3.8 ml of ice-cold 2.81 M CsCl, 0.15 M NaCl. These samples were then

spun to equilibrium for 16 h at 45,000 rpm in a TV-865 rotor (DuPont Co., Wilmington, DE) at 0–4°C. After centrifugation, the gradients were fractionated, the refractive index was measured if the density profile of the gradient was to be determined, and the [³H]-LPS in each fraction was determined by liquid scintillation counting. The efficiency of measuring [³H]LPS was independent of the amount of CsCl in each vial. After graphing the [³H]LPS profile for each gradient, the amount of radioactivity in the body of the gradient, i.e., not bound to the HDL that floats at the top of the gradient, was calculated as a percentage of the radioactivity recovered in the entire gradient. A logarithmic plot of this percentage as a function of the time of removal of the aliquot from the LPS serum reaction mixture yielded the $t_{1/2}$ for the binding of LPS to HDL.

Reconstitution Assay for LBP Activity. The basic method used during development of the purification procedure for LBP was a reconstitution assay in which fractions of acute phase serum were tested for their ability to reconstitute acute phase behavior in NRS. For a screening assay we mixed a sample of the material to be tested with 1.0 ml of NRS at 37°C for 30 min. [³H]LPS and EDTA were then added to 10 µg/ml and 20 mM, respectively, and the LPS-HDL binding reaction was allowed to proceed for 10 min at 37°C before addition of CsCl and centrifugation. The 10 min reaction time was chosen as a compromise between the times required for LPS to bind to HDL in NRS and APRS. Observation of a peak of [³H]LPS at a density of 1.30 g/cm³ signaled the presence of LBP activity. To more quantitatively assay LBP activity, we performed the reconstitution assay just described by assaying a series of different amounts of the sample, up to a maximum of 200 µl per ml NRS. After centrifugation and quantitation of the LPS in C.1.3, a plot of percent LPS as C.1.3 vs. log (sample volume) was made. One LBP unit is defined as that amount of LBP activity that causes 50% of the recovered LPS to be recovered as C.1.3 after 10 min. at 37°C. LBP activity in APRS was assayed similarly, except that the final volume of the NRS-APRS mixture was held constant and the final plot was then percent LPS as C.1.3 vs. log (percent APRS).

Purification of LBP. Two chromatographic procedures make up the purification procedure for LBP. Serum was first fractionated using Bio Rex-70 resin (Bio-Rad Laboratories, Richmond, CA). 50 ml of resin was equilibrated with 41 mM NaCl in 50 mM phosphate buffer, pH 7.3, containing 2 mM EDTA (phosphate/EDTA). 400 ml of APRS, containing 5 mM EDTA, was run over the column at ~65 ml/h. The column was then washed with column equilibration buffer overnight or until the absorbance (280 nm) of the eluate was <0.2 absorbance units (AU). Washing was continued with 220 mM NaCl in phosphate/EDTA, again until the absorbance was <0.2; this was followed by a linear gradient, formed from 60 ml each of 220 mM and 500 mM NaCl in phosphate/EDTA. Finally, the column was washed with 1 M NaCl in phosphate/EDTA. Pools of fractions to be tested for LBP activity were dialysed vs. 5 mM Hepes, pH 7.3, concentrated to 6 ml using PTGC (Millipore, Bedford, MA) or YM10 (Amicon Corp., Danvers, MA) membranes in an Amicon ultrafiltration cell, and any precipitate was removed by centrifugation. The second chromatographic step used HPLC (Perkin-Elmer Corp., Norwalk, CT), with a Mono-Q column (Pharmacia, Piscataway, NJ) as the adsorbent. Unless otherwise noted, the flow rate was 1 ml per minute. The column was equilibrated with 2 mM diethanolamine buffer, pH 8.3. Injection of the sample was followed immediately by a 15 ml gradient of 0–50 mM ammonium sulfate in 20 mM diethanolamine, pH 8.3. The gradient was then steepened, going in 15 ml from 50–333 mM ammonium sulfate in the same buffer. Finally the column was washed for 5 min at a flow rate of 2 ml per min with 333 mM ammonium sulfate, again in 20 mM diethanolamine, pH 8.3. Fractions were collected and, on the basis of the absorbance profile of the column eluate, pooled into three pools, dialysed against 5 mM Hepes buffer (pH 7.4), and concentrated to 2 ml.

Final separation of the last two components of the LBP-containing fractions from Mono-Q chromatography was accomplished by SDS-PAGE. After electrophoresis and light staining with Coomassie Blue, the protein bands were cut apart and recovered from the gel by electroelution (10).

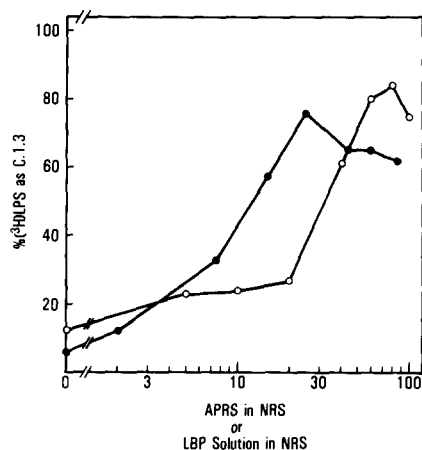


FIGURE 1. Examples of the NRS reconstitution assay for LBP activity. Percent of APRS in NRS (O) or reconstitution of NRS with LBP (\bullet). See text for details.

Results

NRS Reconstitution Assay. Two examples of the NRS reconstitution assay for LBP activity are shown in Fig. 1. In control experiments, no systematic dependence on the assay results was observed when smaller total assay volumes, i.e., 0.5 or 0.25 ml rather than the standard 1 ml, were used. Reproducibility of the assay was $\pm 20\%$.

Purification of LBP. Since LPS added to serum forms a complex with HDL, we determined whether an initial separation of the lipoproteins from APRS would be a useful first purification step. Mixing lipoproteins prepared from NRS or APRS with delipoproteinated NRS or APRS provided reconstituted sera with alternate sources of lipoproteins. As shown in Fig. 2, C.I.3 formed only when delipoproteinated APRS was used, and was independent of the source of the lipoproteins used to form the reconstituted serum. Therefore, we turned to whole serum as the starting material for LBP isolation.

Chromatography of APRS on Bio-Rex 70 proved to be a very effective initial step in purification of LBP. The absorbance profile of APRS or NRS passed over the column, as well as the fractions pooled for analysis, is shown in Fig. 3. When 400 ml APRS was passed over a 50-ml bed of Bio-Rex 70, the ability to form C.I.3 was largely removed. This can be seen in Fig. 4, where CsCl density gradient tests of the ability of the various pools to reconstitute NRS are shown, as are tests of the nonabsorbed proteins (flow-through). LBP activity was eluted only at salt concentrations above 300 mM NaCl, with the largest amount eluting in the 1 M NaCl wash, i.e., pool C, Fig. 4. Washing the column with 3M NaCl did not elute more LBP activity. Assay results for the three pools eluted from the Bio Rex-70 column are shown for a typical preparation of LBP (Table I). Overall, i.e., combining pools A, B, and C, some 32% of the LBP activity was recovered for an increase in specific activity of 927-fold by Bio-Rex 70 chromatography. When NRS rather than APRS was chromatographed on the same column, an almost identical protein elution profile was obtained, as shown in

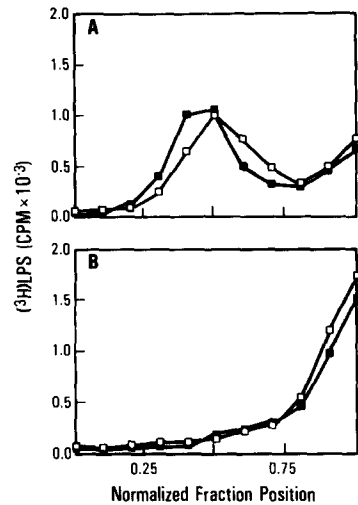


FIGURE 2. CsCl density gradient analyses for C.1.3 formation in delipoproteinated APRS (A) or delipoproteinated NRS (B) reconstituted with lipoproteins from NRS (■) or APRS (□).

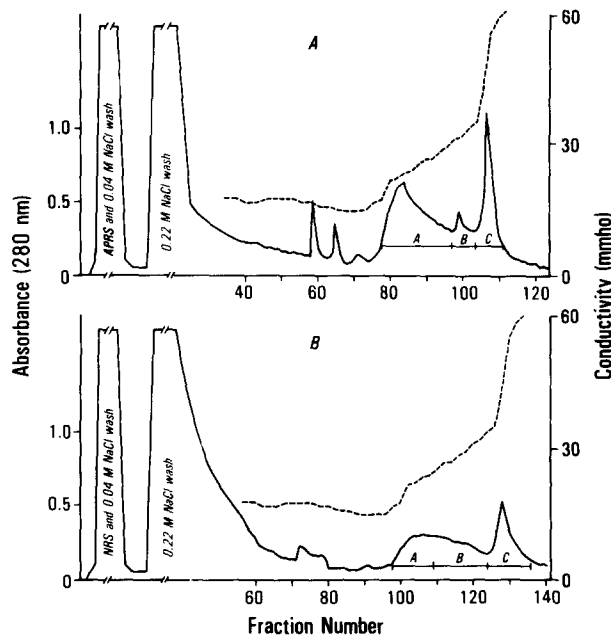


FIGURE 3. Ion exchange chromatography on Bio-Rex 70 resin of APRS (A) or NRS (B). Fractions pooled are denoted by the horizontal lines and letters within the graph. *Solid lines*, absorbance at 280 nm; *broken lines*, conductivity.

Fig. 3B, but none of the pooled fractions had any significant LBP activity (Fig. 4). SDS-PAGE gels of the "C" pools from APRS and NRS are shown in Fig. 5, lanes 3 and 6, respectively.

Further purification of LBP was accomplished with HPLC using a Mono-Q

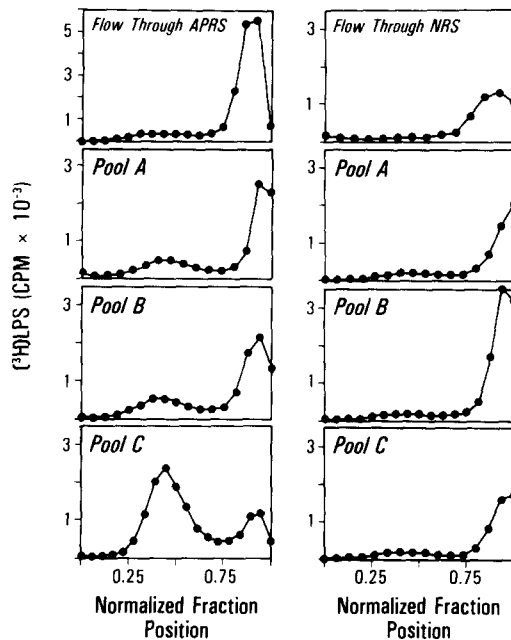


FIGURE 4. CsCl density gradient analyses of LBP activity in pools from Bio-Rex 70 chromatography of APRS (*left panels*) or NRS (*right panels*). Pools tested for activity are identified in Fig. 3. Not all gradients yielded exactly the same number of fractions. Thus, the abscissa normalized fraction position is used to standardize display of the gradients. A value of 0 represents the bottom of the gradient; 1 represents the top of the gradient.

column. When an aliquot of pool C from Bio-Rex 70 chromatography of NRS or APRS was run on the Mono-Q column and eluted with a gradient of ammonium sulphate, the absorbance profile of the eluate was as shown in Fig. 6. The profile obtained with Bio-Rex 70 pool C derived from APRS shows a peak eluting near ~ 20 min not seen in the profile obtained with pool C derived from NRS. Fractions containing the unique APRS-derived peak, as well as the analogous fractions from the NRS material, were assayed for LBP activity. As shown in Fig. 7, the unique protein peak from APRS does show LBP activity. All other fractions tested had no LBP activity. After pooling, dialysis, and concentration, 33% of the activity applied to the Mono-Q column was recovered, with a twofold increase in specific activity (see Table I). An SDS-PAGE gel of the LBP-containing peak is shown in Fig. 5, lane 8. From the mobilities of the two bands in lane 8, relative to the standards in lane 9, the apparent masses of the two proteins are 60.5 and 58 kD. Judging by staining intensity, the 60.5-kD band usually makes up 90% of the mixture, but has varied between 85 and 95%.

Final resolution of the two proteins present in the active pool from Mono-Q chromatography was accomplished by SDS-PAGE, then slicing the two bands apart after staining, and recovering the proteins by electroelution. The separation of the two bands is shown in Fig. 8. Amino acid sequence data, described below, suggest that both bands have very similar primary structures; this argues

TABLE I
Purification of LBP

Fraction	LBP			Sp Act	Total activity	Total protein
	Volume	Activity	Protein			
	<i>ml</i>	<i>U/ml</i>	<i>mg/ml</i>			
Starting material APRS	400	2.8	70	0.04	1,120	28,000
Bio-Rex-70 pools						
A	6	13.9	0.65	21.4	83	3.93
B	6	14.3	0.45	31.8	86	2.73
C	6	32.9	0.54	60.9	197	3.21
Combined results				37.1	366	9.87
Overall purification: 927-fold						
HPLC of Bio-Rex Pools						
A	2	4.6	0.12	38.3	9.2	0.24
B	2	15.1	0.20	75.5	30.2	0.40
C	2	40.6	0.44	92.3	81.2	0.88
Combined results				79.3	120.6	1.52
Purification of pool C through HPLC: 2,307-fold						
Overall purification: 1,982-fold						

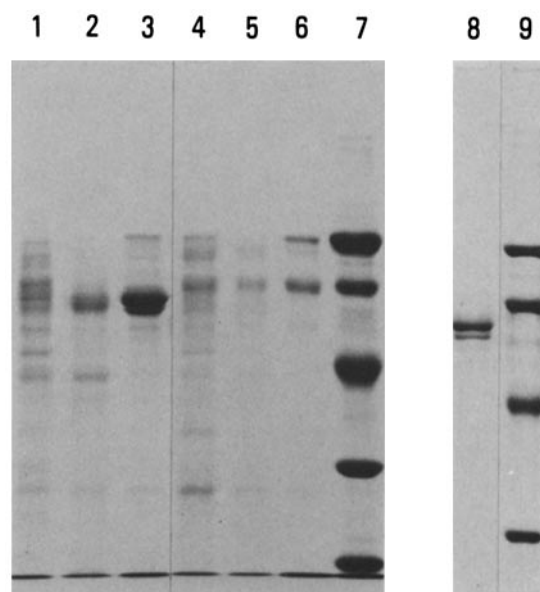


FIGURE 5. SDS-PAGE analyses of chromatography fractions. Lanes 1, 2, and 3 are pools A, B, and C, respectively, from Bio-Rex 70 chromatography of APRS. Lanes 4, 5, and 6 are pools A, B, and C, respectively, from Bio-Rex 70 chromatography of NRS. Lane 7, molecular mass markers: 94, 67, 43, 30, and 20.1 kD. Lane 8, 20-min fraction from Mono-Q chromatography of pool C, APRS. Lane 9, molecular mass markers: 94, 67, 43, and 30 kD.

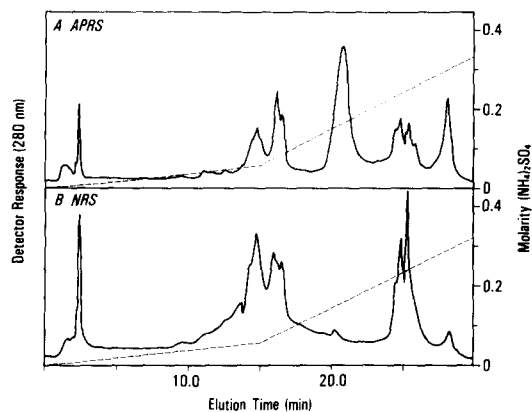


FIGURE 6. Ion exchange chromatography on Mono-Q resin of pool C from Bio-Rex 70 chromatography of APRS (A) or NRS (B). Solid lines, absorbance at 280 nm; broken lines, molarity of ammonium sulfate (1 ml fractions).

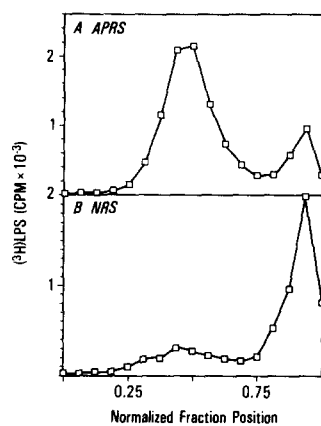


FIGURE 7. CsCl density gradient analyses of LBP activity in fractions eluting at 20 min from Mono-Q chromatography, as shown in Fig. 6. (A) Fractions collected from APRS; (B) fractions collected from NRS.

that both bands may be LBP. Both bands stain with periodic acid/Schiff reagent, thus they are both glycoproteins.

From the LBP-specific activities for APRS and the purest isolated LBP, the concentration of LBP in APRS is found to be 30–35 $\mu\text{g}/\text{ml}$.

Amino Acid Sequence Data. Partial amino acid sequence data were obtained for two preparations of LBP, the mixture of 60.5 and 58 kD proteins obtained from Mono-Q chromatography, and the 58 kD minor component purified by SDS-PAGE. Initially, material collected from Mono-Q chromatography was sequenced. Since this material consists of 85–90% of the 60.5-kD protein, these data reflect the sequence of the major component recovered from the column. The amino acid sequence of the first 39 amino acids from the *N*-terminus is shown in Fig. 9. With the exception of positions 1, 36, 38, and 39, all positions were determined in duplicate for two different preparations. Positions 1, 36, 38,

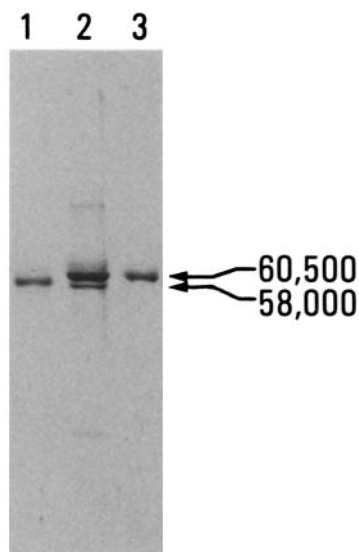


FIGURE 8. SDS-PAGE analysis of the 58,000 (lane 1) and 60,500 (lane 3) mol wt bands, separated electrophoretically from the mixture (lane 2) obtained after Mono-Q chromatography. See text for details.

1	10
Thr-Asn-Pro-Gly-Leu-Ile-Thr-Arg-Ile-Thr-	
11	20
Asp-Lys-Gly-Leu-Glu-Tyr-Ala-Ala-Arg-Glu-	
21	30
Gly-Leu-Leu-Ala-Leu-Gln-Arg-Lys-Leu-XXX-	
31	39
Gly-Val-Thr-Leu-Pro-Asp-Phe-Asp-Gly-	

FIGURE 9. Partial amino acid sequence for two preparations of LBP.

and 39 were identifiable in only one run of the sequenator. Position 30 did not yield an identifiable residue and may represent a site of glycosylation. Sequence data for the 58-kD minor protein was obtained for 36 residues. The sequence of the 58-kD protein agreed completely with that of the mixture of proteins, even to the indeterminate residue 30 (Fig. 9).

The 39 amino acid sequence was used to search for homologous sequences in the National Biomedical Research Foundation protein sequence database; we used the Wordsearch program from the University of Wisconsin Genetics Computer Group (Madison, WI). This procedure resulted in two matches: to sequences (Fig. 10) from human influenza virus b hemagglutinin precursor (11) and baker's yeast glyceraldehyde 3-phosphate dehydrogenase (12). We also used

5	LITRITDKGLEYYAAREGLLALQRKLGVTLP	35	LBP
459	LAVLLSNEGIIINSEDEHLLALERKLLKMLGP	489	INFLUENZA
8	RITDKGLEYYAAREGL.LALQRKLGXV	32	LBP
2	RIATNGFGRIGRLVLRLLALQRKDIIEV	27	BAKER'S YEAST

FIGURE 10. Amino acid sequence homologies found for LBP with human influenza virus b hemagglutinin precursor or baker's yeast glyceraldehyde-3-phosphate dehydrogenase.

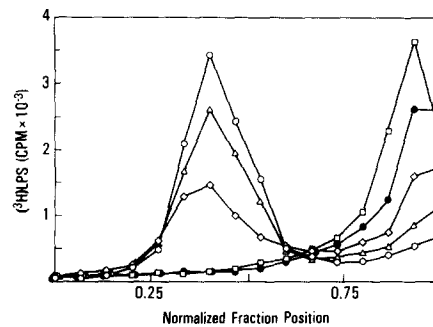


FIGURE 11. CsCl density gradient analysis for LBP activity of immunoprecipitate supernatants obtained from mixtures of APRS or NRS and rat anti-LBP antiserum. APRS, no antiserum (O); APRS, 1.7% (vol/vol) antiserum (Δ); APRS, 4.6% (vol/vol) antiserum (\diamond); APRS, 14% (vol/vol) antiserum (\square); NRS, 14% (vol/vol) antiserum (\bullet).

Wordsearch to look for homology between the sequences of LBP and rabbit CRP (13), human serum amyloid P (14), human serum amyloid a (15), syrian hamster female acute phase protein (16), human α -1-antichymotrypsin precursor (17), human α -1 acid glycoprotein (18), and the major acute phase α -1 glycoprotein of the rat (19). No significant homology was found with any of these acute phase reactants.

Depletion of APRS with Rat Polyclonal Anti-LBP. When examined by Ouchterlony gel diffusion, rat antisera were reactive with APRS- and LBP-containing fractions of APRS isolated by Bio Rex-70 and Mono-Q columns, but not with NRS or NRS fractions corresponding to the fractions isolated from APRS (data not shown). The antisera were then tested for their ability to immunoprecipitate LBP and simultaneously remove LBP activity from APRS. In these experiments, both NRS and APRS were mixed with immune and nonimmune rat sera. After precipitation the supernates were collected to determine their ability to form C.1.3 (Fig. 11), indicating the presence or absence of LBP activity, and the precipitates were saved for analysis by SDS-PAGE (Fig. 12). When the supernatants from the immunoprecipitates were tested for their ability to form C.1.3, the amount of C.1.3 seen was inversely proportional to the amount of immune serum added to the APRS (Fig. 11). Rat sera, immune or not, did not inhibit the binding of [3 H]LPS to HDL in NRS. When the constituent proteins of the immunoprecipitate were visualized by SDS-PAGE, only the mixtures of immune rat serum reacted with APRS yielded significant immunoprecipitates with bands

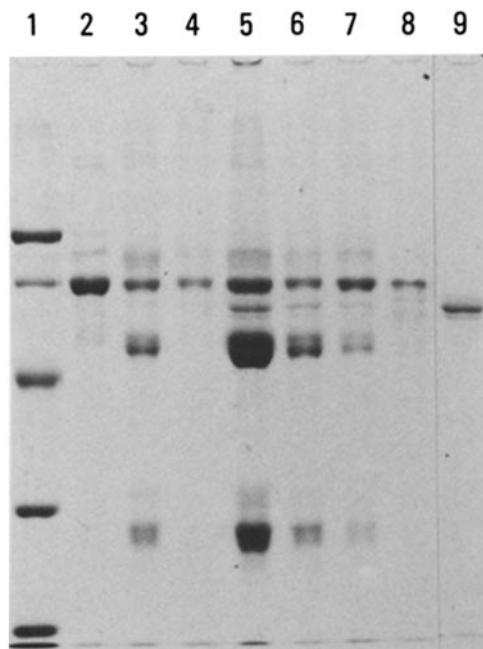


FIGURE 12. SDS-PAGE analyses of immunoprecipitates obtained from APRS or NRS and anti-LBP antisera. Lane 1, molecular mass (94, 62, 43, and 30 kD) markers; lane 2, NRS plus 14% (vol/vol) preimmune serum; lane 3, NRS plus 14% (vol/vol) antiserum; lane 4, APRS plus 14% (vol/vol) preimmune serum; lane 5, APRS plus 14% (vol/vol) antiserum; lane 6, APRS plus 4.6% (vol/vol) antiserum; lane 7, APRS plus 1.7% (vol/vol) antiserum; lane 8, APRS plus 0.6% (vol/vol) antiserum; lane 9, purified LBP.

corresponding to LBP (Fig. 12, lanes 5–7). The intensity of the LBP band in the immunoprecipitates correlated positively with the amount of immune serum added (Fig. 12, lanes 5–8), and it correlated negatively with the ability of the residual supernatant to form C.1.3 (see Figure 11). Bands other than LBP observed in the immunoprecipitates are attributable to rat albumin and rat heavy and light Ig chains. The combination of NRS with immune rat serum did yield very weak bands corresponding to rat Ig chains, presumably due to a minor contaminating specificity, (Figure 12, lane 3). Nonimmune rat serum reacted with either NRS or APRS yielded only bands corresponding to albumin (Figure 12, lanes 2 and 4). Thus immunoprecipitation of LBP from APRS decreased the ability of APRS to form C.1.3 in a dose-dependent manner.

Interaction of LPS with LBP. Two types of experiments were performed to determine whether LPS and LBP interact directly: immunoprecipitation of [^3H]LPS APRS in APRS as C.1.3 by anti-LBP sera, and delivery of ^{125}I to LBP by photolysis of ^{125}I -ASD-LPS in APRS as C.1.3.

In the immunoprecipitation experiments, [^3H]LPS and APRS (or NRS) were allowed to react at 37°C for 10 min and then were cooled to 0°C before rat anti-LBP was added. Unpublished data on the temperature dependence of LPS binding to HDL show that cooling to 4°C effectively stops the formation of LPS-HDL complexes. The [^3H]LPS content of a portion of the immunoprecipitate

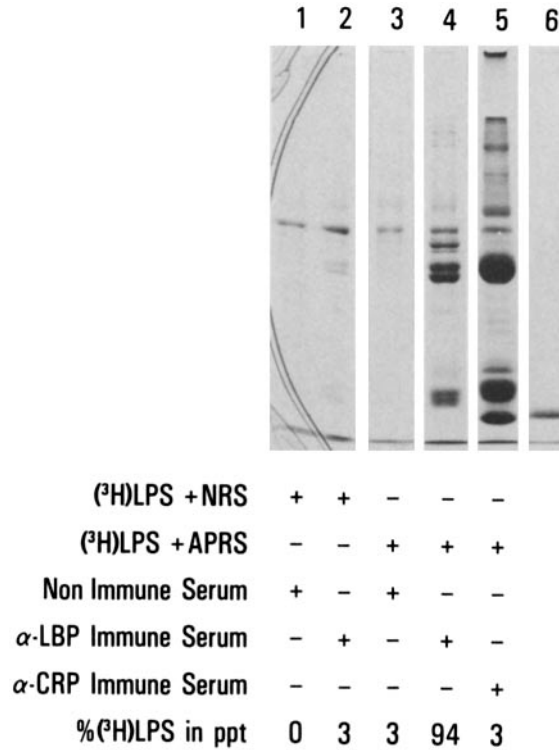


FIGURE 13. SDS-PAGE analyses of and quantitation of [^3H]LPS in immunoprecipitates obtained from mixtures of APRS or NRS and anti-LBP antisera. Reaction mixtures and [^3H]LPS precipitation shown below SDS-PAGE gel lanes. Lane 6, CRP.

was determined by liquid scintillation counting, while the remainder of the precipitate was taken for SDS-PAGE. Since LPS may bind nonspecifically to immune precipitates (20), immunoprecipitation of rabbit CRP from APRS by goat anti-CRP in the presence of ^3H -LPS was used as a control experiment. The SDS-PAGE analysis of the immunoprecipitates is shown in Fig. 13, together with the data for ^3H -LPS recovered with the immunoprecipitate. The results show a clear positive correlation between LBP precipitation and [^3H]LPS precipitation. However, in some control experiments as much as 25% of the ^3H -LPS was associated with the CRP-anti-CRP precipitate.

Further evidence for interaction of LPS with LBP was obtained through the use of ^{125}I -ASD-LPS. APRS (or NRS) was allowed to react with ^{125}I -ASD-LPS for 5 min at 37°C in the dark, chilled to stop further transfer to HDL, and photolysed. Anti-LBP antiserum was then added to collect LBP for SDS-PAGE and autoradiographic analysis. The results for this experiment and the control experiments are presented in Fig. 14. Lane 1 shows that ^{125}I -ASD-LPS photolysed in 20 mM EDTA and 150 mM NaCl (pH 7.4) does not yield a Coomassie Blue-stainable band (lane 1a), and the ^{125}I runs with the dye front (lane 1b). Lanes 2 and 3 show that ^{125}I -ASD-LPS mixed with immunopurified rabbit anti-LPS IgG (21) labels Ig heavy chains if photolysed after mixing (lane 3), but only very

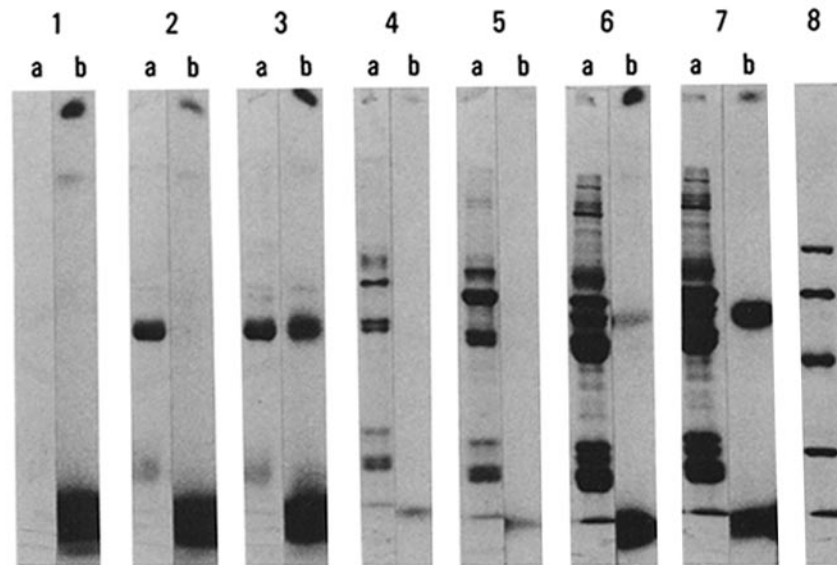


FIGURE 14. SDS-PAGE analysis of ^{125}I -ASD-LPS reaction mixtures. Lanes 1–3 are samples of the reaction mixtures applied directly to the gel. Lanes 4–7 are immunoprecipitates of the reaction mixtures precipitated with 14% (vol/vol) anti-LBP antiserum. Each pair of lanes, *a* and *b*, represents the Coomassie Blue–stained gel and the autoradiographic print, respectively. Lane 1, ^{125}I -ASD-LPS; lane 2, prephotolysed ^{125}I -ASD-LPS with immunopurified anti-LPS; lane 3, ^{125}I -ASD-LPS photolysed with immunopurified anti-LPS; lane 4, prephotolysed ^{125}I -ASD-LPS with NRS; lane 5, ^{125}I -ASD-LPS photolysed with NRS; lane 6, prephotolysed ^{125}I -ASD-LPS with APRS; lane 7, ^{125}I -ASD-LPS with APRS; lane 8, molecular mass markers: 94, 43, 30, and 20.1 kD.

weakly if also photolysed before mixing (lane 2). For lanes 1–3, aliquots of the reaction mixtures were applied directly to the gels. For lanes 4–7, the reaction mixtures were immunoprecipitated with anti-LBP before application to the gel. Lanes 4 and 5 shows that ^{125}I -ASD-LPS, whether photolysed only after mixture with NRS (lane 5) or also photolysed before mixture with NRS did not label any material immunoprecipitable with anti-LBP (i.e., lanes 4*b* and 5*b* are clear). Lanes 6 and 7 show that ^{125}I -ASD-LPS mixed with APRS labels LBP strongly if photolysed after mixing with APRS (lane 7), but labels LBP only weakly if also photolysed before mixing with APRS (lane 6).

Discussion

Purification of LBP. The two-step procedure for purification of LBP from APRS results in a 2,300-fold purification with a yield of 10% of the initial LBP activity. While the product is a mixture of two peptides, the peptides appear to be very closely related (see next section). It is likely that the two peptides we copurify as LBP are both present in APRS and are not generated during purification since immunoprecipitation of APRS also yields the two peptides. The initial chromatographic step in this procedure, chromatography on Bio Rex-70, provides a remarkable, nearly 1,000-fold purification of LBP in a single step. While the degree of purification of LBP is satisfactory, the yield of LBP activity

could be improved. Future development of an immunoassay for LBP will permit consideration of whether the low yield of activity is due to loss or inactivation of LBP, and should permit appropriate modifications to the purification procedure.

Our initial efforts to develop a purification of LBP provide some limits to possible alternative purification schemes. Previous publications (1) from this laboratory have described a method for the preparation of a euglobulin precipitate, which, when mixtures of LPS and APRS are used as the starting material, results in removal of the ability of the serum to form C.1.3. We assumed that LBP would be found in this precipitate and we showed reconstitution of NRS using the euglobulin precipitate. Ultimately, however, it was not possible to prepare a stable form of LBP from this source. Additionally, LBP activity is not recoverable from dextran or dextran/polyacrylamide gel filtration media.

Structural Properties of LBP. The two peptides that copurify as LBP have identical partial amino acid sequences, although these are clearly separable by SDS-PAGE. Whether the apparent size difference results from as yet unrevealed differences in amino acid sequence or from differential glycosylation, and whether the apparent size difference reflects any functional differences are all questions that are not now answerable. Given the structural similarity between the two peptides and their recovery in a somewhat variable ratio, it seems unlikely that LBP in serum is a heterodimer of the peptides we purify.

The available amino acid sequence data, when tested for homology against a wide variety of acute phase and normal proteins, do not reveal any clear correlations. The significance of some homology between portions of LBP and portions of human influenza virus b hemagglutinin precursor or baker's yeast glyceraldehyde-3-phosphate dehydrogenase is unclear, since there are no obvious functional similarities. Thus, although <5% of the sequence of LBP is available, LBP does not appear to be homologous to any known normal or acute phase protein.

In preliminary experiments, partial deglycosylation of LBP by peptide: *N*-glycosidase F (Genzyme, Boston, MA) has been seen. The pattern of multiple bands produced suggests a total of 3–4 carbohydrate chains of two different types.

Functional Properties of LBP. Experiments described earlier were designed to test the hypothesis that LBP and LPS interact directly as the means by which LBP exerts its effects. An alternate hypothesis was that in NRS, LPS uptake by HDL requires an as yet unknown component *X*. Binding of LBP to *X* could then block HDL uptake of LPS. The results clearly distinguish these two hypotheses. If LBP interacted with *X* rather than LPS, immunoprecipitation of LBP would coprecipitate *X* rather than LPS. Coprecipitation of LBP and LPS is the observed result. Likewise, photolysis of ¹²⁵I-ASD-LPS would not be expected to radiolabel LBP if LBP were interacting with *X* rather than LPS. Thus we conclude that LBP and LPS do directly interact.

Two sets of results argue that LBP is a component of C.1.3. In the first instance, NRS can be endowed with the ability to form C.1.3 by addition of purified LBP. In the second instance, APRS can be made incompetent to form C.1.3 by immunoprecipitation of LBP. Consequently, one may conclude that

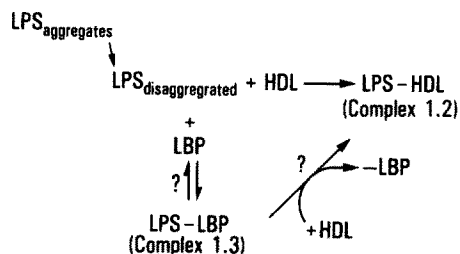


FIGURE 15. Schematic diagram of LPS interactions with HDL and LBP in serum.

C.1.3 includes LBP and LPS in direct contact. If there are other components of C.1.3 they are not sufficiently bound to coimmunoprecipitate and they are not acute phase reactants.

Fig. 15 summarizes our current understanding of the interactions of LPS with HDL and LBP in serum. An initial disaggregation step (22) allows binding of LPS to either LBP or HDL to form C.1.3 or C.1.2, respectively. However, since the final state of LPS in serum is C.1.2, C.1.3 must also dissociate LPS. Whether LPS transfers directly from LBP to HDL or goes via some other intermediate form is unknown.

We speculate that LBP probably plays some role in modifying the endotoxic effects of LPS. It is known that binding of LPS to HDL significantly alters the endotoxic activity of LPS in at least three ways. First, the time course of disappearance of a bolus dose of LPS from the circulation in vivo is biphasic (23); uncomplexed LPS and HDL-complexed LPS are removed from plasma with approximate $t_{1/2}$ of 2 min and 12 h, respectively. Second, the endotoxic activities of LPS and LPS-HDL complexes are different; the complexes are less pyrogenic and less able to activate complement, but can still induce lethal hypotensive shock and activate B cells and macrophages (24). The third consequence of LPS-HDL complexation is the cellular targeting of LPS and LPS-HDL complexes; stated simply, LPS itself is taken up by tissues containing macrophages (23), whereas LPS-HDL complexes are taken up by tissues metabolizing cholesterol, notably the adrenal gland (15). These observations underscore the point that the biochemistry and endotoxic activity of LPS are subject to modification by plasma proteins in important ways. Complexation of LPS to LBP provides a mechanism to modulate the endotoxic properties of LPS in ways not previously suspected.

With the benefit of hindsight, one might now say that several published studies (25–27) indicate a role for acute phase reactants, and possibly for LBP, in endotoxin tolerance or endotoxin inactivation. Since induction of tolerance involves administration of sublethal doses of endotoxin and endotoxin is an excellent acute phase inducer, induction of tolerance almost certainly also induces acute phase reactants. The study of Filkins (27) clearly shows an LPS detoxifying activity in serum after mild endotoxemia or trauma, either of which induce an acute phase response. It is intriguing to hypothesize that acute phase responses to injury include a constitutive response to the potential dangers of gram-negative

infection. The studies described here provide the basis for further analysis of this hypothesis.

Summary

This report describes the purification of an acute phase reactant from acute phase rabbit serum, which endows normal serum with the properties of acute phase serum, insofar as LPS is concerned. The acute phase reactant is referred to as LPS-binding protein, or LBP. LBP was purified ~2,000-fold by chromatography of acute phase serum on Bio-Rex 70 and Mono-Q resins. The resulting preparation consisted of two glycoproteins having molecular weights of 60,500 and 58,000; the two were obtained in a variable ratio, usually near 10:1, respectively. After separation by SDS-PAGE, the *N*-terminal 36 amino acid sequences of the two proteins were identical. From the *N*-terminal sequence, as well as other properties of LBP, LBP appears to be unrelated to any known acute phase reactants. The direct interaction of LPS and LBP was inferred from two types of evidence: first, immunoprecipitation of [³H]LPS from APRS by anti-LBP sera; and second, by the ¹²⁵I-labeling of LBP when APRS-containing ¹²⁵I-labeled 2-(*p*-azidosalicylamido)ethyl 1,3'-dithiopropionyl-LPS was photolysed.

The data presented here support the concept that the 60-kD glycoprotein we have termed LBP is a newly recognized acute phase reactant that may modulate the biochemical and biologic properties of LPS *in vivo*.

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