An Essential Role for Albumin in the Interaction of Endotoxin with Lipopolysaccharide Binding Protein and sCD14 and resultant cell activation

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Running title: Albumin facilitates endotoxin response
**SUMMARY**

Experiments utilizing endotoxin aggregates, lipooligosaccharides (LOS), isolated from metabolically labeled *Neisseria meningitidis* serotype group B, demonstrate that albumin is an essential component of lipopolysaccharide binding protein- (LBP) and sCD14-dependent 1) disaggregation of LOS and 2) LOS activation of human umbilical vein endothelial cells (HUVEC). Aggregates of LOS (LOS$_{agg}$) with an apparent $M_r \geq 2 \times 10^4$ kDa were isolated by gel sieving on Sephacryl HR S500 in buffered balanced salts’ solution $\pm$ albumin. Incubation of LOS$_{agg}$ with LBP and sCD14 promoted LOS$_{agg}$ disaggregation in an albumin-dependent fashion to complexes that contain LOS and sCD14, but no LBP, with an apparent $M_r \sim 60$ kDa (LOS:sCD14) as determined by Sephacryl S200 chromatography. Isolation by gel filtration of LOS$_{agg}$:protein aggregates formed by the interaction of LOS$_{agg}$ with either LBP or sCD14 alone revealed that the sequence of LOS-protein interactions as well as the step(s) at which albumin is necessary for the production of bioactive LOS:sCD14 were specific. Efficient generation of LOS:sCD14 required 1) interaction of LOS$_{agg}$ with LBP prior to interaction with CD14 and 2) the presence of albumin during the interaction of LBP with LOS$_{agg}$. Activation of HUVEC by LOS$_{agg}$, as measured by IL-8 production, required both LBP and sCD14 and was thirty times more potent in the presence of albumin. In contrast, LOS:sCD14 did not require additional LBP, sCD14, or albumin to activate HUVEC but depended on the presence of albumin for optimal solubility/stability once formed. The albumin effect is apparently specific, since neither ovalbumin nor gelatin substituted for albumin in facilitating LBP:sCD14-dependent disaggregation of LOS$_{agg}$ or activation of endothelial cells. These results indicate that albumin is an essential facilitator of LBP/sCD14-induced LOS disaggregation that is required for activation of endothelial cells by LOS$_{agg}$. 

**INTRODUCTION**

Host cell responses to Gram-negative bacteria are orchestrated by immediate recognition of and response to minute quantities of bacteria and/or bacterial products that mobilize defense mechanisms. Although normally protective, similar responses to higher concentrations of these products may provide the basis for much of the pathologic sequelae in certain invasive infections. For example, the pathogenicity and severity of infection provoked by *Neisseria meningitidis* (NMB) has been correlated with high circulating levels of meningococcal endotoxin, lipooligosaccharide (LOS) (1). LOS is a unique amphipathic glycolipid found in the outer leaflet of the outer membrane of NMB and related Gram-negative bacteria (2,3). LOS, like lipopolysaccharide (LPS), contains the bioactive lipid A moiety comprised of the disaccharide backbone of $\beta(1'\rightarrow6)$-linked D-glucoamine modified by O-phosphorylethanolamine and substituted with symmetrically arranged amide- and ester-linked 3-hydroxy substituted fatty acids (4-6).

Host cell response to LOS, like other endotoxins, is mediated through specific interactions with several host proteins including the lipopolysaccharide binding protein (LBP) and CD14 in either a membrane bound (mCD14) or soluble form (sCD14) to activate cells, such as macrophages, neutrophils, and endothelial cells, with the resultant release of a broad array of pro-inflammatory mediators (7-13). The cell protein(s) thought to be primarily responsible for triggering cell responses to endotoxin includes members of the Toll-like receptor protein family, most notably, Toll-like receptor 4 complexed to accessory proteins such as MD-2 (14-20). In addition to these protein interactions, it has been suggested by a number of earlier studies that serum albumin may be a factor in the host defense system by facilitating interactions of endotoxin with protein components responsible for cell activation and clearance (21-27).
Albumin facilitates endotoxin response

particular, interactions between the lipid A portion of endotoxins and albumin have been noted to be important in delivery of endotoxin to cell acceptors (21,24,26).

We have recently reported the generation of an acetate auxotroph of NMB that permits radiolabeling of LOS to high specific activity (28). The physical state of the highly radioactive LOS has been examined by gel filtration chromatography under conditions that produce material that can be used directly in bioassays at concentrations that are pathophysiologically relevant (28). These studies, as well as recent studies with lipopolysaccharide isolated from *E. coli* K12 (29), have shown that 1) activation of endothelial cells, as measured by production of IL-8, or 2) activation of blood leukocytes, as measured by lucigenin-enhanced chemiluminescence, can be correlated with changes in the physical and/or biochemical mode of presentation of LOS or LPS (28).

This gel filtration system has been utilized to isolate apparently homogeneous populations of LOS aggregates (LOS_{agg}) and protein:LOS aggregates/complexes (28,29). Subsequently, the bioactive form of LOS delivered to and responsible for response in mCD14-deficient endothelial cells consists of a complex of LOS with sCD14 that is significantly smaller in molecular size than is LOS_{agg}. Generation of this bioactive LOS containing complex is greatly facilitated by interaction with LBP and involves changes in physical state from an aggregate with an apparent molecular size greater than 2 x 10^4 kDa to an active complex with an apparent M_r \leq 100 kDa. Thus, cellular activation requires the transfer of the amphipathic glycolipid between complex hydrophobic environments: from endotoxin-rich aggregates or monolayers in the bacterial membrane to CD14 and subsequently to Toll-like receptor 4/MD-2 in a host cell membrane. When this transfer of endotoxin occurs across and within aqueous spaces, as it does with sCD14, a role for a lipid carrier might be anticipated. Previous studies have suggested that
Albumin facilitates endotoxin response

LBP can play a direct lipid transfer as well as LPS binding role (30-32). We now show that efficient LBP/sCD14-dependent disaggregation of LOS and activation of endothelial cells additionally require the presence of albumin. In summary, these findings indicate that albumin is essential for efficient fluid-phase interaction of endotoxin-binding proteins with LOS and subsequent generation of the bioactive species responsible for activation of cells deficient in mCD14. Such a role for albumin is consistent with its prominence in body fluids as a lipid carrier and its ability to bind endotoxin weakly.

EXPERIMENTAL PROCEDURES

Materials  Dr. Stephen Carroll, Xoma Corporation (Berkeley, CA) generously provided LBP, sCD14, and rabbit polyclonal anti-LBP antibodies. Sephacryl HR S500 was obtained from Amersham Biosciences Inc. (Piscataway, NJ). Human serum albumin (HSA) was endotoxin-free, 25% stock solution prepared by Baxter Healthcare Corp., Glendale, CA. Bovine serum albumin was purchased from Calbiochem (San Diego, CA). Electrophoresis grade gelatin, fatty acid-free human serum albumin, and ovalbumin were purchased from Sigma Corp. (St. Louis, MO). Polyclonal rabbit anti-CD14 antibodies (M305) directed against CD14 were purchased from Santa Cruz Biologicals, Santa Cruz, CA. Monoclonal murine antibodies to CD14 (MEM-18 and 18E12) were respectively purchased from Accurate Biochemicals and a gift from Johnson & Johnson Corp., New Brunswick, NJ. HUVEC, endothelial cell basal medium, fetal bovine serum, bovine brain extract, human endothelial growth factor, hydrocortisone and gentamicin were from Clonetics (San Francisco, CA). Bovine type 1 collagen was obtained from Collaborative Research Products. 96-well Optiplates were from Anthos Labtec Instruments (New Castle, DE). [1,2-14C] Acetic acid sodium salt (110 mCi/mmol) was purchased from Moraveck Biochemicals, Inc. (Brea, CA). 3H-LPS, purchased from List Biological Laboratories, Campbell,
Albumin facilitates endotoxin response

CA, was dissolved in endotoxin-free water to a final concentration of 100 µg/ml and sonicated twice on ice for 10 min. Aliquots were stored frozen at 70°C until needed.

Preparation of $^{14}$C-LOS from Neisseria meningitidis serogroup B

$^{14}$C-LOS from the mutant strain of encapsulated N. meningitidis serogroup B (NMB ACE-1) was metabolically labeled and isolated as previously described (28). Briefly, the acetate auxotroph strain NMB ACE-1 was generated by allelic exchange of the putative meningocococcal gene encoding the PDH E1 component ($pdhA$) with a plasmid containing a copy of $pdhA$ disrupted by insertion of a Km$^r$ cassette (28). The bacteria were cultured at 37°C in 5% CO$_2$/95% atmosphere on GC agar supplemented with 1X isovitalex. Radiolabeling was carried out in Morse's defined broth medium (33) supplemented with 1X isovitalex, 10 mM sodium bicarbonate and 1.5 mM sodium acetate. To obtain $^{14}$C-LOS of high specific radioactivity, 160 µCi/ml of [1,2-$^{14}$C]-acetate was added to the media during growth of meningococci to late log phase.

$^{14}$C-LOS was purified from radiolabeled bacteria by a modification of the hot phenol-water method (28). After ethanol precipitation, the $^{14}$C-LOS containing pellet was dried and resuspended in cold distilled water to an estimated concentration of 100 µg LOS/ml and then sonicated at room temperature for 15 min in a water bath sonicator. The composition of $^{14}$C-LOS was routinely analyzed for $^{14}$C-fatty acid content by thin layer chromatography and quantitation was performed by image analysis as described previously (28). The specific radioactivity of $^{14}$C-LOS was calculated from image and GC-MS analyses as described by Giardina et al (28).

Chromatography

Columns of Sephacryl HR S500 (1.5 cm x 18 cm) were pre-equilibrated in Hepes-buffered (10 mM, pH 7.4) Hanks’ balanced salts solution with divalent cations (HBSS$^+$) +/- 0.1% HSA. Aliquots of resuspended and sonicated $^{14}$C-LOS were diluted
Albumin facilitates endotoxin response

+- HSA and incubated at 37°C for 15 min before gel filtration chromatography. Chromatography on Sephacryl HR S500 of 14C-LOS, purified as described above, provides a population of 14C-LOS aggregates that elute in the void volume peak fractions, i.e., LOS\textsubscript{agg}, apparent M\textsubscript{r} \geq 2 \times 10^4 kDa. These fractions served as a source of 14C-LOS\textsubscript{agg} in subsequent experiments.

Chromatographed samples contained from 4 ng to 8 µg LOS\textsubscript{agg} in 0.2-0.5 ml of column buffer +/- 0.1% HSA. Fractions (1ml) were collected at a flow rate of 0.5 ml/min at room temperature. To prepare protein:LOS aggregates, samples were incubated with the indicated proteins for 15 min at 37°C before application to the column. Samples collected in the absence of albumin were used immediately to prevent loss of material due to nonspecific sticking. Aliquots of the collected [14C]-LOS fractions were analyzed by liquid scintillation spectroscopy using a Beckman LS liquid scintillation counter. Recoveries of the radiolabeled LOS\textsubscript{agg} +HSA ranged from 70-90%; however, recoveries of protein:LOS aggregates in the absence of albumin were lower (in the range of 50%-80%). To preclude contamination of purified LOS preparations, all solutions were pyrogen-free and sterile-filtered. The glass columns and connecting tubing were either autoclaved or washed extensively with 70% ethanol. After chromatography, selected fractions to be used in bioassays were pooled and passed through sterile syringe filters (0.22µm pore size) with greater than 90% recovery of labeled material in the sterile filtrate. Fractions were stored under sterile conditions at 4°C for more than 3 months with no detectable changes in chromatographic or functional properties.

The column was calibrated using 14C-oleate-labeled \textit{E.coli} PL-2 and 14C-acetate as markers of the void (10-11 ml) and inclusion volumes (25-26 ml), respectively, in column buffer with or without 0.1% albumin. Additional standards included blue dextran (est. M\textsubscript{r} 2000 kDa),
Albumin facilitates endotoxin response

thyroglobulin (650 and 1300 kDa, monomers and dimers, respectively), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa). Elution profiles of bacteria, blue dextran and acetate were unaffected by the presence of albumin in the column buffer.

Sephacryl S200 chromatography was performed on an AKTA FPLC system using a 1.6cm x 30cm column with a flow rate 0.5 ml/min in Hepes-buffered HBSS+; 1ml fractions were collected and evaluated for the presence of 14C-LOS by liquid scintillation spectroscopy. The column was equilibrated with BioRad gel filtration standards that include thyroglobulin (V₀), γ-globulin, ovalbumin, myoglobin, and vitamin B12 (Vᵢ).

Immunoblotting and Immunocapture  To demonstrate the presence of LBP or CD14 in isolated LOSₐgg:protein aggregates, samples of LOSₐgg + LBP or CD14 were incubated as described +/- HSA and the aggregates were isolated in the void volume of Sephacryl HR S500 columns equilibrated in Hepes-buffered HBSS⁺ (1.5cm x 6cm). Shorter columns were used to reduce losses of LOSₐgg during chromatography without albumin and allow efficient isolation of the large aggregates. Void volume fractions containing LOSₐgg were precipitated with trichloroacetic acid; the precipitated material was washed and then resuspended in SDS-PAGE sample buffer. Control samples containing only LBP or sCD14 with no LOS were also chromatographed and treated in a similar manner. In addition to these samples, control samples of LBP and sCD14 of varying concentrations were electrophoresed using an Amersham Pharmacia PhastGel System through either 12.5% or 10-15% acrylamide gels and transferred to nitrocellulose by semi-dry transfer using the same system. To block nonspecific background on the immunoblots, the nitrocellulose was washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 and then blocked with 3% BSA in the same buffer for 1 hr at 25°C. After washing, the blots were treated with the appropriate primary antibody (1:1000 rabbit anti-LBP or
Albumin facilitates endotoxin response

1:500 rabbit anti-CD14 serum) diluted in 1% BSA, PBS, 0.05% Tween-20 overnight at 25°C. After washing with PBS, 0.05% Tween-20, the blots were incubated with secondary antibody conjugated to horseradish peroxidase (donkey anti-rabbit IgG) for 1 hr at 25°C, washed with PBS, 0.05% Tween-20. Then the blots were developed using the Pierce SuperSignal substrate system.

To capture protein-containing aggregates/complexes of LOS, appropriate antibodies and controls (1 µg/well) were diluted in Hepes-buffered HBSS+, 1% HSA and applied to Nunc-Immuno Maxisorp plates in pH 9.6 bicarbonate buffer overnight at 25°C. The wells were rinsed with HBSS+, Hepes, 1% HSA three times and then incubated for 2 hr with each well filled with blocking buffer (Hepes, HBSS+, 1% HSA). The ligand to be captured by the antibody was then incubated overnight with shaking at 25°C. The supernatant was removed and an aliquot reserved for evaluation of the unbound LOS radioactivity. The wells were washed three times with HBSS+, Hepes, 1% HSA. Captured material was eluted with 2% SDS by incubation for 15 min at 37°C. The supernatant was removed and the recovery was evaluated by liquid scintillation spectroscopy.

Human cells HUVEC were routinely cultured on collagen-coated plates (Costar; Cambridge, MA) at 37°C, 5% CO₂ and 95% relative humidity in endothelial cell basal medium supplemented with 5% fetal bovine serum, 12 µg/ml bovine brain extract, 10 ng/ml human endothelial growth factor, 1 µg/ml hydrocortisone and 50 µg/ml gentamicin. Cells were subcultured and grown to confluence (~4-5 days). Cell monolayers were then washed twice with warm HBSS+ to remove traces of serum before adding experimental media. Experiments were done with cells between passages 2-6.
Albumin facilitates endotoxin response

**HUVEC activation assays** Cells in 48-well tissue culture plates were incubated for at least 6 hr at 37°C, 5% CO₂ and 95% humidity in Dulbecco’s Minimal Essential Medium and 0.1% albumin or, where indicated, without albumin or with 0.1% ovalbumin or gelatin with various concentrations of ¹⁴C-LOS ± LBP (0.2 µg/ml) and sCD14 (0.5 µg/ml). Activation of HUVEC was assessed by measuring accumulation of extracellular IL-8 by ELISA (34). Equivalent responses were observed with 0.1-0.5 µg/ml of LBP and 0.25-1.0 µg/ml sCD14.

**RESULTS**

**LOS<sub>agg</sub> isolated in the presence and absence of albumin.** We have previously described the purification of an apparently homogeneous population of LOS aggregates from NMB ACE1 with an apparent size ≥ 2 x 10⁴ kDa as determined by gel filtration (Sephacryl S500) in Hepes-buffered HBSS+ supplemented with 0.1% HSA (28). To examine the possible role of albumin in LBP/sCD14-dependent disaggregation of LOS<sub>agg</sub> and activation of HUVEC, we purified LOS<sub>agg</sub> in the absence of albumin. Neither the chromatographic profile of LOS nor the overall recovery of LOS was affected by the absence of albumin (Fig.1). The predominant population of LOS aggregates (LOS<sub>agg</sub>) formed in the absence or presence of albumin migrated with similar apparent M<sub>r</sub> (≥2 x 10⁴ kDa).

**Albumin is required for LBP/sCD14-dependent disaggregation of LOS<sub>agg</sub> and activation of HUVEC.** The isolation of LOS<sub>agg</sub> +/- albumin provided a source of LOS<sub>agg</sub> to examine the effect of albumin on the physical state of LOS-containing aggregates formed during the interaction of LOS<sub>agg</sub> with endotoxin-binding proteins and the role of albumin in cell activation by endotoxin. As we have previously shown (28), LOS<sub>agg</sub> formed and isolated in the presence of albumin (LOS<sub>agg</sub><sub>HSA</sub>) was quantitatively disaggregated when incubated with LBP, sCD14, and albumin
**Albumin facilitates endotoxin response**

(Fig. 2). In contrast, LOS<sub>agg</sub> formed and isolated in the absence of albumin (LOS<sub>agg</sub><sup>No HSA</sup>) and incubated with LBP and sCD14 without albumin was not significantly disaggregated (Fig. 2A).

The presence of albumin exerted similar influences on the activation of endothelial cells by LOS<sub>agg</sub> in the presence of LBP and sCD14. LOS<sub>agg</sub><sup>HSA</sup> isolated and incubated with LBP and sCD14 in the presence of albumin potently activated HUVEC whereas LOS<sub>agg</sub><sup>No HSA</sup> with LBP and sCD14 incubated in the absence of albumin did not (Fig. 2B). If albumin was added either *only* during the formation/isolation of LOS<sub>agg</sub> or introduced *only* during the incubation of cells with LBP and sCD14, substantial, but not optimal LOS-dependent cell activation was observed (data not shown). Different commercial sources of human albumin including fatty acid-free albumin, bovine serum albumin, and human serum albumin purified by gel filtration chromatography all had similar effects (data not shown).

The requirement for albumin is apparently specific: neither gelatin nor ovalbumin supported LOS-dependent cell activation (Fig. 3B) or LBP/sCD14-dependent disaggregation of LOS<sub>agg</sub> (Fig. 3A). Although the chromatographic profile of NMB ACE-1 LOS was similar in 0.1% ovalbumin (LOS<sub>agg</sub><sup>OVAL</sup>) and 0.1% HSA (compare Fig. 1 and 3A), the recovered LOS<sub>agg</sub><sup>OVAL</sup> was not significantly disaggregated during incubation with LBP and sCD14 in the presence of ovalbumin (Fig. 3A). Overall recoveries of <sup>14</sup>C-LOS either from the chromatographic step or from cell cultures were not appreciably different under the various experimental conditions. These findings strongly suggest that albumin is an essential co-factor for LBP/sCD14 dependent disaggregation of LOS<sub>agg</sub> and activation of endothelial cells.

Figure 4 illustrates that albumin is also needed for optimal LBP/sCD14-dependent activation of HUVEC by *E. coli* K12 (LCD25) lipopolysaccharide (LPS). Incubation of LPS<sub>agg</sub> + LBP, sCD14, and albumin produces disaggregation of LPS<sub>agg</sub> (29). However, recoveries of
Albumin facilitates endotoxin response

LPS\textsubscript{agg} and LPS\textsubscript{agg}:protein complexes during Sephacryl S500 chromatography in the absence of albumin were low (<30%) precluding a direct assessment of the role of albumin in LBP/sCD14-dependent disaggregation of LPS.

**LBP/sCD14-dependent disaggregation of LOS\textsubscript{agg} requires ordered interaction of LBP and sCD14 with LOS\textsubscript{agg}**. To better define where and when albumin is needed to facilitate LBP/sCD14-dependent disaggregation of LOS\textsubscript{agg}, we first examined whether the combined action of LBP and sCD14 in the presence of albumin on LOS\textsubscript{agg}\textsuperscript{HSA} required the simultaneous presence of these proteins or ordered sequential interactions with LOS\textsubscript{agg}\textsuperscript{HSA}. For this purpose, gel chromatography was employed to recover LOS\textsubscript{agg} exposed to either LBP or sCD14 alone. The aggregates formed with LOS\textsubscript{agg}\textsuperscript{HSA} were separated from any free protein that was not physically associated with LOS\textsubscript{agg}\textsuperscript{HSA}. In a manner similar to the recently described interactions of LPS\textsubscript{agg} with LBP (29), incubation of LOS\textsubscript{agg}\textsuperscript{HSA} with LBP alone had no direct disaggregating effects at the concentration utilized in these assays. However, LOS\textsubscript{agg} recovered after exposure to LBP (i.e., (LOS\textsubscript{agg}:LBP)\textsuperscript{HSA}; Fig.5A) was associated with LBP as determined by immunoblot (Fig.5 insert) and was disaggregated by subsequent treatment with sCD14 to an extent comparable to the disaggregation observed by simultaneous treatment of LOS\textsubscript{agg}\textsuperscript{HSA} with LBP, sCD14, and albumin (for comparison see Figs. 2A and 5A).

Treatment of LOS\textsubscript{agg}\textsuperscript{HSA} directly with sCD14 in albumin resulted in little or no disaggregation (Fig.5B) under the conditions and concentrations indicated. The addition of LBP (+albumin) to the recovered LOS\textsubscript{agg}\textsuperscript{HSA} treated with sCD14 (LOS\textsubscript{agg}:sCD14)\textsuperscript{HSA} also did not produce disaggregation of LOS\textsubscript{agg} (Fig.5B). The lack of disaggregation is consistent with the results of immunoblots of the sCD14-treated LOS\textsubscript{agg} that indicated no significant association of sCD14 with recovered LOS\textsubscript{agg}. Therefore, efficient LBP/sCD14-dependent disaggregation of
Albumin facilitates endotoxin response

LOS\textsubscript{agg} requires prior interaction of LOS\textsubscript{agg} with LBP in the presence of albumin before exposure to sCD14.

**Albumin is required during the interaction of LOS\textsubscript{agg} with LBP for subsequent sCD14-dependent disaggregation.** Because extraction of LOS from LOS\textsubscript{agg} by sCD14 occurred only after formation of LOS\textsubscript{agg}:LBP, we expected that this would be the step in which the presence of albumin would be most essential. However, as shown in Fig.5C, the absence of albumin during the exposure of LOS\textsubscript{agg} to LBP precluded subsequent sCD14 disaggregation of LOS\textsubscript{agg} even when albumin was introduced with sCD14. Immunoblots of LOS\textsubscript{agg} recovered after incubation with LBP +/- albumin indicated similar association of LBP with LOS\textsubscript{agg} in the presence or absence of albumin (compare blots in Fig. 5A inserts). Apparently the requirement for albumin during the interaction of LOS\textsubscript{agg} with LBP is not to permit LBP-LOS\textsubscript{agg} interactions, but rather to promote a presentation of LOS in LOS\textsubscript{agg}:LBP that facilitates sCD14-dependent disaggregation.

**Albumin is needed as a cofactor for LOS:sCD14 complex.** The known lipid-, including endotoxin-, binding properties of albumin introduced the possibility that albumin might be an essential constituent of the bioactive disaggregated complex. To test this possibility, we generated preparative amounts of the bioactive disaggregated complex (M\textsubscript{r} ~ 100 kDa) from the Sephacryl S500 chromatography for analysis on Sephacryl S200, a matrix with resolution more appropriate to the smaller size of the complex. The latter chromatography was carried out in Hepes-buffered HBSS+ without added albumin to permit simultaneous monitoring of the elution of \textsuperscript{14}C-LOS and the bulk albumin carried with the complex from the prior (S500) chromatography step. The \textsuperscript{14}C-LOS eluted as a very sharp and symmetric peak just after albumin (Fig.6A) suggesting that \textsuperscript{14}C-LOS was part of a complex with M\textsubscript{r} ~ 60 kDa and not physically associated with albumin. \textsuperscript{14}C-LOS in this complex was immunoprecipitated by a
Albumin facilitates endotoxin response

monoclonal antibody directed toward an epitope outside the endotoxin-binding site of CD14 (18E12; Fig. 6B). Neither an antibody whose epitope overlaps the endotoxin binding site of CD14 (MEM18; (35,36) nor an affinity-purified polyclonal antibody to LBP was able to capture the $^{14}$C-LOS containing complex. These findings suggest that the 60 kDa complex contains one molecule of LOS (4.8 kDa) and one molecule of sCD14 (ca. 50 kDa), i.e., LOS:sCD14 complex.

The peak $^{14}$C-LOS-containing fraction (LOS:sCD14) recovered from Sephacryl S200 chromatography potently activated HUVEC without the further addition of LBP and/or sCD14 in contrast to LOS$_{agg}$ (Fig.6C). Cell activation by this fraction was blocked by 18E12, but not by MEM18 or anti-LBP antibodies confirming that it is the LOS:sCD14 complex that is responsible for cell activation. Although albumin is not physically associated with the bioactive LOS complex, addition of albumin to this complex appears to be needed for solubility/stability of the complex and, hence, maximal cell activation (Fig.6C).

DISCUSSION

We have previously demonstrated that LOS isolated from an acetate auxotroph of Neisseria meningitidis can be resolved by gel sieving on Sephacryl S500 in the presence of 0.1% albumin to yield a population of LOS aggregates that can be disaggregated efficiently by interaction with LBP and sCD14 in a buffer system compatible with direct bioassay of isolated species at pathophysiologically relevant concentrations (28). The disaggregated LOS-containing complex is responsible for the maximal cell activation by LOS as measured by the production of IL-8 in HUVEC (28). In this study, we provide evidence that albumin is an essential component in the formation of the active LOS species. The chromatographic profile of LOS in the absence or presence of albumin is identical and generates a major species with $M_r > 2 \times 10^4$ kDa (Fig. 1). However, results presented here indicate that albumin is necessary for the disaggregation of
Albumin facilitates endotoxin response

LOS\textsubscript{agg} promoted by interaction with LBP and sCD14 (Fig. 2A) and for maximal cellular response to LOS in HUVEC as measured by IL-8 production (Fig. 2B).

Albumin has unique structural properties that include six hydrophobic sites responsible for its ability to bind fatty acids, hydrophobic drugs, bile acids, and steroid hormones (37-40). The ability of albumin to bind and transport long chain fatty acids accounts for the unexpected solubility of these compounds in plasma and permits their transport between different tissues and organs. Associations between endotoxin and albumin have been demonstrated in many studies and in diverse biological settings including whole serum (24,41-44). These interactions involve primarily the Lipid A region, most notably by increasing the solubility of isolated Lipid A (23,25,26,41,45). Reduced recoveries observed during chromatography with LPS in the absence of albumin would be consistent with these previous findings and support a role for albumin to facilitate the “solubility” of LPS in aqueous solutions.

An activating role of albumin on endotoxin activity in vitro and in vivo has been previously shown (23,43,46). Complexes of albumin with isolated lipid A or intact LPS are bioactive, especially when presented in disaggregated form (27,42,47-50). Our findings also show a striking parallel between albumin-dependent disaggregation of endotoxin and endotoxin-dependent cell activation (Figs. 2-4). However, we have shown by high resolution gel sieving chromatography on Sephacryl S200 and immunoassays that the bioactive complex is smaller than albumin (ca. 60 kDa) and likely contains one molecule each of sCD14 and LOS (“LOS:sCD14”) (Fig. 6). Whereas albumin is not associated with the bioactive complex in a high affinity interaction, nonetheless, albumin apparently is needed to maintain the solubility/stability of the bioactive species thereby facilitating its delivery to Toll-Like Receptor 4-dependent cell membrane acceptor systems\textsuperscript{1}. The formation of bioactive LPS-albumin
complexes in earlier studies was accomplished by using high concentrations of the reactants, long incubations times, EDTA and were generated in the absence of LBP and/or sCD14 (11,27,32,51-53). In contrast, under conditions more closely resembling physiological levels of endotoxin, LBP, sCD14 and albumin (0.1-1%), we observed virtually quantitative conversion of LOS$_{agg}$ to LOS:sCD14 within 15 min at $37^\circ C$. These findings strongly suggest that endotoxin:sCD14, not endotoxin-albumin, complexes are physiologically relevant. We suggest that albumin, instead, may function mainly to stabilize otherwise labile intermediates formed as endotoxin is extracted and transferred from a complex hydrophobic environment to specific protein acceptors across aqueous space.

More insight into the function of albumin has emerged by introducing albumin at various stages of LOS:protein interactions and isolating the intermediate species. From this experimental approach, we have obtained evidence that disaggregation of LOS$_{agg}$ required an ordered interaction with LBP, sCD14 and albumin. LBP, not sCD14, engaged LOS$_{agg}$ in a significant manner both in the presence or absence of albumin. But disaggregation to a bioactive form of LOS by interaction of sCD14 with the LBP:LOS$_{agg}$ occurred only when the association of LBP with LOS$_{agg}$ included exposure to albumin. Interactions of LOS$_{agg}$ with LBP must precede exposure to sCD14 since, even in the presence of albumin, sCD14 did not interact with LOS$_{agg}$ to form a stable intermediate that can be subsequently disaggregated. Therefore, LBP alone associated with aggregates of LOS in a way that permitted the transfer of individual endotoxin molecules to sCD14. This finding is consistent with the postulated catalytic role of LBP in that a single LBP: LOS$_{agg}$ provided a vehicle for generation of numerous LOS:sCD14 complexes. The fact that LBP efficiently catalyzed transfer of individual molecules of LOS to sCD14 only when LOS$_{agg}$:LBP was formed in the presence of albumin suggests an essential role
**Albumin facilitates endotoxin response**

for albumin in maintaining a physical presentation of \( \text{LOS}_{\text{agg}}:\text{LBP} \) needed for efficient extraction and transfer of individual molecules of LOS to sCD14. Given the highly amphipathic structure of LOS, it is very likely that lipophilic groups in Lipid A within \( \text{LOS}_{\text{agg}} \) are sequestered away from the external aqueous environment and are involved in intermolecular hydrophobic interactions that stabilize the large aggregates. Presumably LBP binding destabilizes endotoxin aggregates, at least in part, by inducing topological re-arrangements of endotoxin molecules that modify intermolecular interactions by exchanging attractive forces between individual LOS molecules to now include interactions between LOS molecules and LBP. The fact that albumin was needed at this stage to facilitate subsequent extraction and transfer of individual molecules of LOS to sCD14 suggests that the “active” configuration of \( \text{LOS}_{\text{agg}}:\text{LBP} \) is one in which lipophilic groups within Lipid A are more exposed (Fig. 7). In the absence of albumin, such a physical presentation would probably be unstable and lead to further re-arrangements that may then render \( \text{LOS}_{\text{agg}}:\text{LBP} \) refractory to disaggregation with sCD14 even when albumin is subsequently added (Fig. 5C; Fig. 7).

Therefore, while albumin may directly form bioactive complexes with LPS (Lipid A) under highly artificial conditions, we believe its more physiological role in endotoxin-dependent cell activation reflects its ability to associate, however transiently, with exposed Lipid A as endotoxin moves across aqueous spaces from a complex hydrophobic environment to specific protein acceptors. The requirement for albumin is apparently specific since neither ovalbumin nor gelatin was able to substitute for albumin in promoting disaggregation or cell activation. It is possible that this function could be fulfilled by other “lipid carriers”. However, it is also possible the weak endotoxin binding properties of albumin previously described (27,43,53) are ideally adapted and suited for this function. In either case, this is likely a natural function of albumin in
Albumin facilitates endotoxin response

body fluids given its abundance at these sites. Whether albumin is also needed to facilitate
transfer to acceptors that are themselves within complex hydrophobic environments (e.g.
mCD14, lipoproteins) remains to be determined. It is worth noting that, even in extensively
washed cells, albumin is still present suggesting a tight association of albumin with the cell
membrane and so implying the availability of albumin for participation in an exchange between
LBP and mCD14 if necessary (43,54).

Although all endotoxins have several highly conserved structural characteristics, there are
many structural variations among the broad array of Gram-negative bacterial endotoxin species
present in nature (5,2). These include differences in the number, structure, and site of linkage of
fatty acids within lipid A, the presence of other sub-stoichiometric substituents with lipid A and
inner carbohydrate core regions as well as the overall composition and length of the oligo- or
polysaccharide chain. Such structural variations may affect the solubility of endotoxin
aggregates and/or protein-endotoxin complexes in aqueous environments and, hence, the need
for albumin to maintain these aggregates in a dispersed form potentially reactive with
downstream host acceptors. A similar role for albumin in promoting LBP/sCD14-dependent
transformation of both E. coli K12 LPS and NMB LOS was observed (see figs. 2 and 4), but the
structural differences between these two endotoxin species may be too limited to adequately test
this hypothesis. It should be noted that the ability of albumin to facilitate transfer of
hydrophobic molecules in aqueous environments between proteins has been observed previously.
For example, albumin promotes a bi-directional transfer of cholesterol between cells and
extracellular proteins that contributes significantly to cholesterol efflux (55,56). This occurs
despite (because of ?) the low affinity of cholesterol for albumin. This effect is also albumin
specific and not replicated by either ovalbumin or gelatin. Therefore, it seems likely that the role
**Albumin facilitates endotoxin response**

described in this study will apply not only to NMB LOS and *E. coli* K12 LPS, but to a much broader array of endotoxin species.

In summary, the results presented in this paper indicate that albumin is an important cofactor in the formation and delivery of bioactive endotoxin-containing complexes to endotoxin-responsive cells. We propose that this role of albumin is conferred by its ability to transiently shield the hydrophobic portion of the Lipid A moiety from a hydrophilic environment and thereby permit more effective extraction and transfer of monomers of endotoxin to specific host targets. We have shown an essential role for albumin in the interaction of endotoxin with LBP and sCD14. Additional studies are needed to see if this role extends to other key acceptors in endotoxin-dependent cell activation (e.g. mCD14, Toll-like receptor 4, MD-2) or in the clearance and inactivation of endotoxin.
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**Albumin facilitates endotoxin response**


**Albumin facilitates endotoxin response**


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**FOOTNOTES**

The abbreviations used are: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HBSS+, Hanks’ balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cells; LBP, lipopolysaccharide binding protein; LOS, lipooligosaccharide; LOS<sub>agg</sub> lipooligosaccharide aggregates; NMB ACE-1, *Neisseria meningitidis* serotype B acetate auxotroph; PBS, phosphate buffered saline; sCD14, soluble CD14.

1K. Zaremba et al., unpublished observations.
**FIGURE LEGENDS**

**FIGURE 1.** Isolation of $^{14}$C-LOS aggregates by Sephacryl S500 HR chromatography +/- albumin. $^{14}$C-LOS was isolated from NMB ACE-1 by hot phenol-water extraction and ethanol precipitation as previously described (28). Aliquots of the purified $^{14}$C-LOS were incubated for 15 min at 37°C in Hepes-buffered HBSS$^+$ +/- 1% HSA before chromatography through Sephacryl S500 HR using the same buffer as described in Methods. The void volume peak fraction + albumin (LOS$_{agg}^{HSA}$) or -albumin (LOS$_{agg}^{No HSA}$) was utilized as a source of a relatively homogeneous population of LOS (LOS$_{agg}$). Data are expressed as percent of total $^{14}$C-LOS recovered. Total recovery of $^{14}$C-LOS was 70-90%.

**FIGURE 2.** LBP- and sCD14 dependent disaggregation of $^{14}$C-LOS and endothelial cell activation by $^{14}$C-LOS +/- albumin. (A) LOS$_{agg}^{HSA}$ or LOS$_{agg}^{No HSA}$ (100 ng LOS/ml, ca. 20 nM) was recovered after Sephacryl S500 chromatography (fraction 11, Fig. 1) and incubated for 15 min at 37°C with LBP (500 ng/ml, 10 nM) and sCD14 (5 µg/ml, 100 nM) in Hepes-buffered HBSS$^+$ +/- 1% HSA and then analyzed by gel filtration chromatography in HBSS$^+$ +/- 1% HSA as described in Methods. Results shown are representative of >3 experiments. Data are expressed as percent of total $^{14}$C-LOS recovered. Total recovery of $^{14}$C-LOS was 70-90% with albumin and 50-70% without albumin. (B) Cell activation of HUVEC by LOS$_{agg}^{HSA}$ or LOS$_{agg}^{No HSA}$ was measured as described in Methods. All samples contained LBP (100 ng/ml, 2 nM) and sCD14 (250 ng/ml, 5 nM). LOS aggregates were incubated for 20 hr with the proteins either in the presence (▲, ▼) or absence (▽, □) of albumin. The amount of LOS added was calculated from the experimentally determined specific activity of LOS. Data shown represent the means ± S.E. of the data from three or more experiments.
FIGURE 3. Determination of the specificity of albumin in endothelial cell activation and in LBP and sCD14-dependent disaggregation of LPS\textsubscript{agg}.  (A) \(^{14}\text{C}-\text{LOS}_{agg}\) were isolated by gel filtration chromatography (Sephacryl S500) as described in Methods in the presence of 0.1% ovalbumin and then a sample (\(\text{LOS}_{agg}^{\text{OVAL}}\), 100 ng/ml) was either re-chromatographed to check for homogeneity or incubated with 10nM LBP and sCD14 (5 µg/ml, ca. 100 nM) in Heps-buffered HBSS\(^+\), 0.1% ovalbumin for 15 min at 37°C before characterization by gel filtration chromatography in the same buffer. Results shown are representative of at least two experiments. Data are expressed as percent of total \(^{14}\text{C}-\text{LOS}\) recovered. Total recovery of \(^{14}\text{C}-\text{LOS}\) was >70%. (B) Cell activation of HUVEC by LOS\(_{agg}\) was measured as described in Methods. LOS\(_{agg}\) was isolated by Sephacryl S500 chromatography in Heps-buffered HBSS\(^+\). To samples containing LOS\(_{agg}\), LBP (100 ng/ml, 2 nM) and sCD14 (250 ng/ml, 5 nM) and 1% of the indicated proteins were added and incubated for 20 hr. The amount of LOS added was calculated from the experimentally determined specific activity of LOS. Data shown represent the means ± S.E. of the data from three or more experiments.

FIGURE 4. Specific potentiation of the LBP, sCD14-dependent activation of endothelial cells by LPS by serum albumin. Cell activation of HUVEC by LPS was determined by measurement of the accumulation of IL-8 as described in Methods. To samples containing LPS, LBP (100 ng/ml, 2 nM) and sCD14 (250 ng/ml, 5 nM) and 1% of the either human serum albumin or ovalbumin was added and incubated for 20 hr. The accumulation of IL-8 product represents the product generated from 0.3-1 ng/ml LPS. Control is IL-8 accumulation in the presence of albumin. Data shown represent the means ± S.E. of the data.
FIGURE 5. LBP/sCD14 dependent aggregation of $^{14}$C-LOS$_{agg}$ requires specific sequential exposure $^{14}$C-LOS$_{agg}$ to LBP, sCD14, and albumin. (A) Isolated LOS$_{agg}^{\text{HSA}}$ (Fig.1; 1 µg/ml, ca. 200 nM) was incubated with 100 nM LBP in Heps-buffered HBSS+, 1% HSA for 15 min at 37°C before characterization by gel filtration chromatography. LBP:LOS$_{agg}^{\text{HSA}}$ (100 ng LOS/ml, ca. 20 nM) was recovered after Sephacryl S500 chromatography (fraction 11) and then incubated for 15 min at 37°C + sCD14 (5 µg/ml; ca. 100 nM) in HBSS+, 1% HSA and then again analyzed by gel filtration chromatography. Results shown are representative of ≥ 3 experiments. Data are expressed as percent of total $^{14}$C-LOS recovered. Total recovery of $^{14}$C-LOS (from both columns) was >70%. Immunoblot analysis (insert) of isolated aggregates recovered after incubation of LOS$_{agg}^{\text{HSA}}$ + LBP indicate that the recovered void volume fraction contains LBP. Lane 1 represents 5 ng LBP; lane 2 represents LBP:LOS$_{agg}^{\text{HSA}}$; lane 3 is LBP:LOS$_{agg}^{\text{No HSA}}$ (see below C). No LBP was detected in the corresponding void volume fractions during chromatography of purified LBP without LOS$_{agg}$ nor was a signal detected in the void volume when LOS$_{agg}$ was chromatographed alone. (B) Purified LOS$_{agg}^{\text{HSA}}$ (100 ng/ml, ca. 20 nM) was incubated with sCD14 (5 µg/ml, ca. 100nM) in Heps-buffered HBSS+, 0.1% HSA and then analyzed by gel filtration chromatography. The peak fraction of “sCD14:LOS$_{agg}^{\text{HSA}}$” (100 ng LOS/ml) recovered after Sephacryl S500 chromatography (fraction 11) was then incubated for 15 min at 37°C with 10 nM LBP in Heps-buffered HBSS+, 1% HSA and then again analyzed by gel filtration chromatography. Results shown are representative of ≥ 3 experiments. Data are expressed as percent of total $^{14}$C-LOS recovered. Total recovery of $^{14}$C-LOS was >70%. Immunoblot analysis of the peak fraction demonstrated that less than 1% of the added sCD14 was associated with the LOS$_{agg}^{\text{HSA}}$ after column chromatography under these conditions (data
Albumin facilitates endotoxin response

not shown). (C) Isolated LOS\textsubscript{agg}\textsuperscript{No HSA} (Fig.1; 1 µg/ml, ca. 200 nM) was incubated with 100 nM LBP in Heps-buffered HBSS\textsuperscript{+} (no HSA) for 15 min at 37°C before characterization by gel filtration chromatography. (LBP:LOS\textsubscript{agg})\textsuperscript{No HSA} (100 ng LOS/ml, ca. 20 nM) was recovered after Sephacryl S500 chromatography (fraction 11) and then incubated for 15 min at 37°C + sCD14 (5 µg/ml, ca. 100 nM) in Heps-buffered HBSS\textsuperscript{+} with 1% HSA and then again analyzed by gel filtration chromatography. Results shown are representative of ≥ 3 experiments. Data are expressed as percent of total \textsuperscript{14}C-LOS recovered. Total recovery of \textsuperscript{14}C-LOS (from both columns) was >70%. Immunoblot analysis (insert above; lane 3) of isolated aggregates recovered after incubation of LOS\textsubscript{agg}\textsuperscript{No HSA} + LBP indicate that the recovered void volume fraction contains LBP.

**FIGURE 6.** Characterization of the bioactive LOS complex formed by LBP and sCD14-dependent disaggregation of LOS\textsubscript{agg}. (A) The product formed by disaggregation of LOS\textsubscript{agg} after treatment with LBP and sCD14 was isolated from Sephacryl S500 chromatography as described in Methods, concentrated using Amicon Centricon-10, and re-chromatographed on Sephacryl S200 (1.5 cm x 30 cm column) on Amersham Pharmacia AKTA FPLC in Heps buffered HBSS\textsuperscript{+}. The solid line represents \textsuperscript{14}C-LOS cpm recovered/fraction and the dashed line represents protein absorbance at 280 nm, i.e., elution of albumin remaining from the S500 chromatography column buffer. (B) The disaggregated LOS complex isolated from S500 was incubated in wells containing 1 µg of the absorbed antibodies as indicated (see Methods). The amount of \textsuperscript{14}C-LOS that was captured by interaction with the antibodies and remained associated after washing was determined as described in Methods and is expressed as % of total \textsuperscript{14}C-LOS added to the sample. Only mAb 18E12, an antibody that binds CD14 outside the endotoxin
Albumin facilitates endotoxin response

binding region and that does not capture aggregates of \( \text{LOS}_{\text{agg}}:\text{LBP} \), captures the disaggregated LOS complex. The data shown represent the mean ± S.E. of three experiments. (C) The effect of anti-CD14 antibodies and anti-LBP antibody on the ability of \( \text{LOS}:\text{sCD14} \) to activate endothelial cells was examined by pre-incubation of the \( \text{LOS}:\text{sCD14} \) complex with the indicated antibodies for 30 min at 37°C before addition to the cells. The effect of the absence of albumin added during the pre-incubation and activation of the cells was also examined. Results are expressed as % of control, i.e., production of IL8 formed by activation of cells with \( \text{LOS}:\text{sCD14} \). Data represent the mean ± S.E.M.

Figure 7. Model for the mode of interaction of albumin in LBP/sCD14-dependent disaggregation of \( \text{LOS}_{\text{agg}} \) and cell activation. Albumin is essential during the interaction of LBP with \( \text{LOS}_{\text{agg}} \) to produce a LBP:LOS\(_{\text{agg}}\) aggregate that presents LOS in such a way as to permit efficient transfer of LOS from the aggregate to a molecule of sCD14. The complex of \( \text{LOS}:\text{sCD14} \) formed, which is stabilized by the presence of albumin, is responsible for cell activation as monitored by the production of IL-8 by endothelial cells. The participation of albumin is as a transiently or loosely associated co-factor that permits efficient transfer/protection of the amphipathic LOS molecule between the endotoxin-binding proteins across an aqueous milieu.
Fig. 2
Fig. 3

A

% LOS recovered

Eluate (ml)

B

IL8, ng/well

[LOS_{agg}], ng/ml

- LOS, 0.1% ovalbumin
- Rechromatograph LOS_{agg}, 0.1% ovalbumin
- LOS_{agg}^{OVAL} + LBP + sCD14, 0.1% ovalbumin

- HSA
- Gelatin
- Ovalbumin
Fig. 4
Fig. 5
A

Sephacryl S200 of product from LOS$_{300} +$ LBP + sCD14

Absorbance

Eluate (ml)

B

Co-precipitation of $^{14}$C-LOS with anti-LBP and anti-CD14 antibodies

\[ \text{\% C. LOS co-ppt. (\%)} \]

Control IgG

anti-LBP

18 E12

MEM-18

C

% Control (IL8)

LOS

+HSA

HSA

IgG

anti-LBP

MEM18

18 E12

LOS:sCD14

Fig. 6
An essential role for albumin in the interaction of endotoxin with lipopolysaccharide binding protein and sCD14 and resultant cell activation
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