INFECTION AND IMMUNITY, Aug. 1993, p. 3209–3215
0019-9567/93/083209-07$02.00/0

Vol. 61, No. 8

Distribution of Bacterial Endotoxin in Human and Rabbit Blood and Effects of Stroma-Free Hemoglobin

ROBERT I. ROTH,[1,2]* FRANCINE C. LEVIN,[1] AND JACK LEVIN[1,2]

Department of Laboratory Medicine, University of California School of Medicine,[1] and the Veterans Administration Medical Center,[2] San Francisco, California 94121

Received 29 December 1992/Accepted 3 May 1993

Bacterial endotoxin (lipopolysaccharide [LPS]) is known to interact with numerous components of blood, including erythrocytes, mononuclear cells, platelets, neutrophils, lipoproteins, and plasma proteins. The relative affinities of LPS for these elements, and the distribution of LPS between them, are unknown. Cross-linked stroma-free hemoglobin (SFH), a potential substitute for erythrocyte transfusion, produces in vivo toxicity in animals consistent with significant LPS contamination. Therefore, we studied the distribution of LPS in human and rabbit blood and examined whether the presence of SFH altered LPS distribution. In either the presence or absence of SFH, LPS was associated predominantly with high-density lipoproteins and apoproteins. There was lesser binding to low- and very-low-density lipoproteins. Examination of the apoprotein pool by column chromatography and density centrifugation demonstrated that LPS in this fraction was predominantly protein bound. Binding of LPS to SFH resulted in dissociation of a portion of the LPS into low-molecular-weight complexes. Cell-bound LPS was only 2 to 16% of the total and was unaffected by SFH. The distribution among blood cells demonstrated predominant binding to platelets in human blood but predominant binding to erythrocytes in rabbit blood. Cellular distribution was not significantly altered by SFH.

Bacterial endotoxin (lipopolysaccharide [LPS]) is the cell wall component of gram-negative bacteria responsible for initiation of fever, cardiovascular shock, and disseminated intravascular coagulation during septicemia. Endotoxin can enter the peripheral circulation at sites of wounds or the portal circulation by absorption and/or translocation from the gastrointestinal tract (30). When endotoxin is administered parenterally to animals, much of the injected LPS is initially found in the cell-free fraction of plasma, with special affinity for high-density lipoproteins (HDL) (12). Prominent binding to HDL has been shown in vitro (28, 46). LPS association with protein also has clearly been demonstrated (41, 50). Nevertheless, it has been known for several decades that LPS, when introduced into the blood of experimental animals, is rapidly cleared from the circulation (19, 23, 25).

Interactions between LPS and circulating blood cells are of great interest since many of the deleterious effects of LPS during septicemia are the result of mediators released from inflammatory cells. LPS causes the release of a wide range of cell-derived substances, including cytokines (e.g., tumor necrosis factor [1, 7, 26, 43], interleukin-1 [36, 48, 49], and interleukin-6 [36, 48, 49]), eicosanoids (22), and procoagulants (e.g., tissue factor [34]). However, animal studies have resulted in substantially discordant descriptions of the distribution of LPS among the various types of circulating blood cells. Injected LPS has been reported to associate preferentially with buffy coat cells (3), platelets (19), platelets, monocytes, and polymorphonuclear leukocytes (4), and monocytes and polymorphonuclear leukocytes (23) or to be uniformly distributed between all cellular elements (24). LPS interaction with erythrocytes has been clearly demonstrated in vitro (38–40), although most in vivo animal studies of LPS distribution in blood have failed to demonstrate binding to erythrocytes. The quantitative distribution of LPS in human blood has not been reported.

There is experimental evidence that plasma proteins may influence the binding of LPS to other elements in blood, possibly by disaggregation of LPS (46) and/or by enhancement of the ability of LPS to bind to HDL (42). Alteration of LPS by protein binding could potentially affect the clearance of LPS and/or the ability of LPS to stimulate the release of effector molecules from circulating blood cells (37, 47). Cross-linked stroma-free hemoglobin (SFH) (defined in Addendum in Proof) is an oxygen-carrying protein being developed as an erythrocyte substitute for which endotoxin binding and a resultant synergistic toxicity are major concerns (51, 52). When used as a resuscitation fluid after trauma, 100 g or more of SFH would potentially be infused into a patient. Endotoxin is likely to be present in the circulation during resuscitation after trauma because of skin and gut wounds and/or ischemia of the gastrointestinal tract. In addition, the infused SFH may have been contaminated by LPS during its production (10). Therefore, the effect of SFH on the distribution of LPS in blood is an unknown but important variable during resuscitation therapy and of potentially great clinical relevance. The current study was undertaken to quantify the distribution of LPS in human blood and to determine if the distribution was altered by the presence of SFH.

MATERIALS AND METHODS

Reagents and labware. Percoll (adjusted to $d = 1.07$ or $d = 1.09$ g/ml) and Ficoll (adjusted to $d = 1.07$ g/ml) were purchased from Pharmacia LKB (Alameda, Calif.), and Mono-poly resolving medium was from Flow Laboratories (ICN Biomedicals, Inc., Costa Mesa, Calif.). Citrated blood tubes and Falcon centrifuge tubes (sterile, 15 ml) were obtained from Becton Dickinson (Mountain View, Calif.), and sterile phosphate-buffered saline (PBS) was from GIBCO Laboratories (Grand Island, N.Y.).

LPSs. ^{14}C-LPS [*Salmonella typhimurium* PR122(Rc),

* Corresponding author.

1 μCi/mg] was purchased from List Biologicals, Inc. (Campbell, Calif.). ^{125}I-LPS (1 μCi/μg) was prepared from *Escherichia coli* O55:B5 LPS B as described previously (44). Gamma counting was performed in an automatic gamma counter (LKB Instruments, Inc., Gaithersburg, Md.), and ^{14}C scintillation counting was performed, after samples were diluted 10-fold in fluor (formula A-989; NEN Research Products, Boston, Mass.), in an analytic liquid scintillation system (Tracor Analytic, Elk Grove Village, Ill.). ^{14}C was detected at 1.72×10^6 counts per minute (cpm) of ^{14}C per μCi by this instrument.

SFH. Human cross-linked SFH, prepared as described previously (53), was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco, Calif. Cross-linking between the alpha chains was produced by derivatization with bis-(3,5-dibromosalicyl)fumarate. The SFH (7-g/dl stock solution) used in these experiments was at a final concentration of 1.2 g/dl and contained less than 10 pg of endotoxin per ml (referenced to *E. coli* O55:B5 LPS B) as determined by the *Limulus* amebocyte lysate test (21).

Animals. Two- to three-kilogram New Zealand White female rabbits were purchased from Western Oregon Rabbit Co. (Philomath, Oreg.). C57BL mice (25 g) were purchased from Simonsen Laboratories, Inc. (Gilroy, Calif.).

Radiolabeled plasma protein preparation. Mice were injected intraperitoneally with 90 μCi of [^{35}S]methionine (NEN Research Products). Cell-free mouse plasma was obtained by centrifugation of anticoagulant-treated blood samples (3,000 × *g* for 20 min) obtained 16 h after injection. The resulting plasma contained 8.6×10^5 cpm/ml. By utilizing this protocol, radioactivity has been shown previously to be associated with plasma proteins (9).

Leukocyte and platelet counts. Leukocyte and platelet counts were determined with blood cell counters (Coulter Electronics, Inc., Hialeah, Fla.). Leukocyte differentials in human blood samples were obtained with an H1 particle counter (Technicon Instrument Corporation, Tarrytown, N.Y.). Leukocyte differentials in rabbit blood smears were determined by 500-cell manual counts of Wright-Giemsa-stained blood smears.

Fractionation of whole blood. The following procedure for the separation of the various types of blood cells was developed experimentally to provide concomitant (i) maximum recovery of platelets, mononuclear cells, polymorphonuclear cells, and erythrocytes, (ii) maximum purity of each cell preparation, and (iii) minimal cell damage. Unless otherwise stated, procedures for fractionation of human and rabbit blood were identical.

Blood samples were obtained after the subjects had fasted overnight. Five-milliliter samples of citrated blood were incubated with 0.6 ml of SFH or NaCl at room temperature for 10 min. Final SFH concentrations were 1.2 g/dl. These blood samples were then incubated with 25 μl of radiolabeled LPS (approximately 10^5 cpm) for an additional 15 min at room temperature and centrifuged in an Accuspin centrifuge (Beckman Instruments, Inc., Irvine, Calif.), with an AH-4 swinging-bucket rotor, at 600 × g for 3 min, to obtain platelet-rich plasma and a cell pellet. The plasma was then centrifuged at 1,300 × *g* for 20 min to obtain platelets, and the platelet pellet was washed three times with 5 ml of PBS at 600 × *g* for 3 min. The cell pellet from the initial whole-blood centrifugation was resuspended in PBS to 8 ml, layered over 3 ml of Ficoll (d = 1.070 g/ml), and centrifuged at 400 × g for 40 min. Mononuclear cells (monocytes and lymphocytes) were present in a band at the interface between the PBS (top) and the Ficoll (bottom) and were collected and washed once with 5 ml of PBS (250 × *g*) for 10 min. The pellet beneath the Ficoll (polymorphonuclear leukocytes and erythrocytes) was fractionated by either of the following procedures, with comparable results for recovery and purity. The Ficoll pellet was resuspended in an equal volume of platelet-free plasma and then layered on 3 ml of Mono-poly resolving medium (d = 1.140 g/ml) and centrifuged at 400 × *g* for 40 min. Polymorphonuclear leukocytes were present at the interface, and erythrocytes were present in the pellet at the bottom of the tube. The leukocyte band was washed with 5 ml of PBS (250 × *g* for 10 min) and then recentrifuged on 2 ml of Mono-poly resolving medium to further remove erythrocytes. Because of the subsequent unavailability of Flow Mono-poly resolving medium, a Percoll separation of the Ficoll pellet was also established. Human Ficoll pellet cells (see above) were layered on 3 ml of Percoll (d = 1.090 g/ml) and centrifuged at 400 × *g* for 15 min. Rabbit Ficoll pellet cells were layered on 3 ml of Percoll (d = 1.070 g/ml) and centrifuged at 400 × *g* for 15 min. Polymorphonuclear leukocytes, present at the plasma-Percoll interface, and erythrocytes in the pellet were washed once with 8 ml of PBS (250 × *g* for 10 min).

Erythrocyte-containing fractions exhibited quenching of both ^{125}I and ^{14}C cpm. Therefore, 0.1-ml aliquots of erythrocyte fractions were diluted 10-fold in water (final volume, 1 ml), and 1.0 ml of Solvable (NEN Research Products) was added. These mixtures were incubated at 60°C for 1 h, and then 0.3 ml of 25% H_2O_2 was added. After 30 min of additional incubation at room temperature, samples were pale yellow in color and could be analyzed for radioactivity. Recovery of a spiked radioisotope in preliminary experiments demonstrated >98% detection of the previously added radioactivity.

All cell preparations were cytocentrifuged (Shandon Southern Instrument Co., Sewickley, Pa.), and 200 to 500 cell differentials were performed to determine purity.

Separation of lipoproteins from cell-free plasma. Separation of platelets from human or rabbit plasma, as described above, yielded cell-free plasma which contained less than 0.1% of starting platelets and undetectable numbers of leukocytes or erythrocytes (determined by Coulter counter analysis and examination of Wright-Giemsa-stained smears). The cell-free plasma was subjected to sequential ultracentrifugation (35) at 4°C at plasma density (d = 1.006 g/ml), at a density of 1.063 g/ml (with KBr), and at a density of 1.21 g/ml (with KBr) for isolation of very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and HDL, respectively, and for isolation of apoproteins (i.e., plasma proteins remaining after sequential removal of all lipoproteins; d > 1.21 g/ml).

Fractionation of plasma apoproteins. Cell-free, lipoprotein-free proteins (d > 1.21 g/ml; see above) were dialyzed and concentrated in a concentration cell (Amicon Division, W. R. Grace & Co., Danvers, Mass.) with a 12-kDa-cutoff membrane for gel filtration chromatography on Sephadex G-150 (100 by 2.6 cm). Chromatography was performed in PBS at room temperature.

Fractionation of endotoxin complexes. Unbound LPS was separated from protein-LPS complexes (and free protein) by centrifugation through a cushion of 20% sucrose. A 1- to 3-ml sample was layered over 4 ml of 20% sucrose and centrifuged at 25,000 × *g* for 1 h at 20°C in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, Del.). Unbound LPS predominantly sedimented to the bottom of the

TABLE 1. Distribution of endotoxin in whole blood[a]

WB and endotoxin type	Endotoxin distribution						n^b
	% Total cpm		% cpm in cellular compartment				
	Plasma	Cells	PLTS	MNC	PMN	RBC	
Human WB							
^{125}I-*E. coli* O55:B5	98 ± 1	2 ± 1	77 ± 15	10 ± 10	3 ± 2	10 ± 10	9
^{14}C-*S. typhimurium*	96 ± 5	4 ± 5	55 ± 24	23 ± 5	14 ± 25	8 ± 6	5
^{14}C-*S. typhimurium* + SFH (1.2 g/dl)	96 ± 4	4 ± 4	37 ± 20	36 ± 14	15 ± 23	12 ± 10	9
Rabbit WB							
^{14}C-*S. typhimurium*	84 ± 16	16 ± 15	3 ± 3	15 ± 14	4 ± 3	78 ± 15	14
SFH (1.2 g/dl)	90 ± 10	10 ± 11	5 ± 5	23 ± 31	4 ± 4	68 ± 29	10
% Recovery of cells			49 ± 21	14 ± 10	62 ± 30	>99	

[a] Radiolabeled endotoxin was added to whole blood (WB), and the blood was then fractionated into cell-free plasma, platelets (PLTS), mononuclear cells (MNC), polymorphonuclear leukocytes (PMN), and erythrocytes (RBC). Endotoxin distributions are expressed as means ± SD. Recovery of added cpm was 98% (mean value).

[b] *n*, number of independent experiments.

tube under these conditions, whereas protein-LPS complexes remained above the sucrose layer.

RESULTS

Distribution of endotoxin in human blood. The in vitro distribution of endotoxin in human blood was studied, in the presence or absence of SFH (defined in Addendum in Proof), by using radiolabeled *E. coli* O55:B5 LPS B or *S. typhimurium* LPS. In the absence of SFH, almost all of either of the radiolabeled endotoxins (96 to 98%) was associated with cell-free plasma (Table 1). Because of the predominance of plasma-associated counts, it was necessary to develop a procedure for isolating blood cells free of plasma to accurately determine cell-bound LPS. The procedure for cell isolation described in Materials and Methods was tested with whole blood to which ^{35}S-labeled mouse plasma proteins (0.08 mCi; see Materials and Methods) had been added. Blood cells were isolated (see Materials and Methods) and ^{35}S cpm were measured to determine the percentage of plasma contamination in each cell pool. Platelets were shown to be associated with only 0.015% of the total plasma cpm initially added; mononuclear cells were associated with 0.008%; polymorphonuclear leukocytes were associated with 0.008%; and erythrocytes were associated with 0.001%. This level of plasma contamination was equivalent to only 1 to 3% of the cpm detected in the samples of isolated blood cells, thus ensuring that the endotoxin detected with cells was actually cell bound and did not represent contamination by plasma endotoxin. This extremely low level of plasma contamination of blood cell samples was accomplished by the extensive washing steps described in Materials and Methods; consequently, recoveries of cells were diminished because of the multiple wash steps. Recoveries of cells, from a total of 47 individual experiments (23 human and 24 rabbit), were as follows: platelets, 49% ± 21%; mononuclear cells, 14% ± 10%; polymorphonuclear leukocytes, 62% ± 30%; and erythrocytes, >99%.

In human blood, with both LPSs, 2 to 4% of the counts were cell associated (Table 1). In all experiments (n = 23), the majority of the cell-associated endotoxin cpm was found in the platelet pool. Distribution among the remaining cell types was quite variable between experiments. Blood from five normal human volunteers was utilized, and no reproducible differences in endotoxin distribution among the types of blood cells were detected between individuals. In addition, LPS distributions were the same in heparinized and citrate-treated blood samples from the same individual. Because of the predominance of platelet-associated cpm blood cell differentials were determined to ensure that cpm in the leukocyte and erythrocyte samples did not represent platelet contamination. Mononuclear cell preparations contained 76% ± 15% lymphocytes and monocytes, 1% ± 1% polymorphonuclear leukocytes, 17% ± 13% erythrocytes, and 6% ± 13% platelets (means ± standard deviations [SD], 15 experiments). Polymorphonuclear leukocyte preparations contained 65% ± 17% polymorphonuclear leukocytes, 34% ± 19% erythrocytes, 2% ± 7% platelets, and 0.1% ± 0.2% mononuclear cells (means ± SD, 15 experiments). The erythrocyte preparations contained greater than 99% erythrocytes, with less than 0.01% contamination with platelets. The low frequencies of platelets in the mononuclear leukocyte, polymorphonuclear leukocyte, and erythrocyte samples thus ensured that platelet-LPS contamination did not contribute to the presence of endotoxin in the preparations of the other types of circulating blood cells. Platelet-associated cpm contributed only 0.1% of the cpm in mononuclear cell preparations and 0.05% of the cpm in polymorphonuclear leukocyte preparations. Platelet preparations contained 99% ± 1% platelets (mean ± SD, 14 independent experiments).

The distribution of *S. typhimurium* endotoxin in human blood also was investigated in the presence of SFH. Almost all cpm (96%) were detected in cell-free plasma (Table 1), similar to the distribution observed in the absence of SFH. The distribution of endotoxin among the various types of blood cells (Table 1) demonstrated prominent binding to both platelets and mononuclear cells. In six of the nine experiments, most of the cell-associated cpm were again detected in the platelet pool. SFH appeared to produce an increase in the fraction of cell-bound LPS associated with mononuclear cells; however, because of the considerable variation of cellular distributions of endotoxin, differences between distributions in the presence and absence of SFH were not statistically significant.

Distribution of endotoxin in rabbit blood. The distribution of ^{14}C-labeled *S. typhimurium* endotoxin in rabbit blood, in the presence and absence of SFH, was determined in vitro. Almost all cpm were associated with the cell-free plasma (Table 1), similar to the distribution observed in human blood, although the total cell-associated endotoxin cpm were

TABLE 2. Endotoxin distribution in cell-free plasma[a]

Plasma and endotoxin type	Endotoxin distribution (% total cpm)				n^b
	VLDL	LDL	HDL	Apoproteins (lipoprotein-free pool)	
Human plasma					
S. typhimurium	2 ± 1	7 ± 1	68 ± 9	23 ± 7	4
S. typhimurium + SFH (1.2 g/dl)	2 ± 1	9 ± 7	67 ± 8	22 ± 3	4
Rabbit plasma					
S. typhimurium	3 ± 2	11 ± 11	50 ± 25	36 ± 3	4
S. typhimurium + SFH (1.2 g/dl)	2 ± 2	4 ± 4	53 ± 14	41 ± 14	4

[a] Radiolabeled endotoxin was added to whole blood in the presence or absence of SFH. Cell-free plasma was then prepared and fractionated by sequential ultracentrifugation steps into VLDL (d < 1.006 g/ml), LDL (d = 1.006 to 1.063 g/ml), HDL (d = 1.063 to 1.21 g/ml), and apoproteins (d > 1.21 g/ml; plasma proteins remaining after sequential removal of lipoproteins). Recovery of added cpm from the cell-free plasma was 85% (mean value). Endotoxin distributions are expressed as means ± SD.

[b] n, number of independent experiments.

in general greater in rabbit blood than in human blood. Sixteen percent of the total cpm was cell associated in the absence of hemoglobin (n = 14), and 10% was cell associated in the presence of hemoglobin (n = 10). However, this difference was not significant. The distribution among cells demonstrated that 78% of the endotoxin cpm in the absence of SFH (n = 14) and 68% of the endotoxin cpm in the presence of SFH (n = 10) were associated with the erythrocytes, a distinct difference from the predominance of platelet-associated endotoxin among the cells in human blood. The few erythrocytes that contaminated mononuclear cell preparations (17% of cells) and polymorphonuclear leukocyte preparations (34% of cells) contributed only 0.01 and 0.03% of the cpm in these cell preparations, respectively. Cell-associated endotoxin distributions in the presence and absence of SFH were not significantly different.

Distribution of endotoxin among the components of cell-free plasma. Cell-free plasma was fractionated by sequential ultracentrifugation steps, as described in Materials and Methods, into VLDL, LDL, HDL, and apoproteins (d > 1.21 g/ml). The distributions of ^{14}C-labeled *S. typhimurium* endotoxin among these components in both human and rabbit plasma samples and in the presence and absence of SFH are shown in Table 2. In both species, the relative magnitude of the distributions of endotoxin was HDL > apoproteins > LDL > VLDL.

Apoprotein fractions (d > 1.21 g/ml) from rabbit plasma samples containing ^{14}C-labeled *S. typhimurium* endotoxin were then fractionated by G-150 gel permeation chromatography. In the absence of SFH, all radioactivity eluted in the void volume (molecular mass, >200 kDa; Fig. 1). This pattern was detected in each of five independent samples chromatographed. Considerable A_{280} also was present in the void volume, and numerous protein bands were detected in this material by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (data not shown). In four apoprotein samples containing SFH, the majority of the cpm similarly was present in the void volume, although small amounts of radioactivity (5 to 24%) also were detected in the included volume (Fig. 2). SFH, as measured by A_{570}, was detected primarily in the included volume, although a small absorbance peak also was detected in the void volume. The retained peak of ^{14}C-endotoxin coeluted with the peak of SFH (Fig. 2). In contrast, ^{14}C-endotoxin alone eluted exclusively in the void volume (four experiments, data not shown). Thus, the endotoxin, which eluted in the included volume derived from samples containing SFH, was partially disaggregated.

Since the majority of cpm in the fraction of plasma with a density greater than 1.21 g/ml (i.e., which contained apoproteins) eluted in the void volume of Sephadex G-150, as did endotoxin alone, it was unclear whether endotoxin in this plasma fraction was protein bound. To distinguish unbound from bound endotoxin, sedimentation through sucrose was performed. Preliminary experiments demonstrated that unbound endotoxin sedimented through 20% sucrose (Table 3), under the conditions described in Materials and Methods, whereas plasma proteins or free SFH was predominantly less dense than the sucrose solution and, after centrifugation, remained in the aqueous layer above the sucrose cushion. Void volume fractions, from two samples to which SFH had not been added and two which contained SFH, were centrifuged over 20% sucrose. Endotoxin cpm in each of the four Sephadex G-150 void volume fractions remained predominantly in the layer above the sucrose, indicating that the endotoxin was comigrating with the proteins both in the presence and absence of SFH (Table 3). Endotoxin cpm from the included volume peak of a sample containing SFH similarly demonstrated that the endotoxin was primarily comigrating with protein in the layer above the sucrose (presumably SFH; compare Fig. 1 and 2).

To confirm the observation that endotoxin in the fraction of plasma with a density greater than 1.21 g/ml was protein bound, 11 additional apoprotein samples (5 without SFH and 6 with SFH) containing ^{14}C-LPS were generated. These fractions were then subjected to sedimentation through 20% sucrose without prior chromatography. Each of these samples demonstrated that the majority of endotoxin cpm was present in the aqueous phase above the sucrose cushion (Table 3). Most of the plasma proteins, as estimated by A_{280}, also were present in this top zone. These observations were consistent with a major decrease in density of endotoxin in the presence of plasma proteins, most likely due to a disaggregation of high-molecular-mass endotoxin macromolecules (typically greater than 1,000 kDa in the absence of protein) secondary to protein binding.

Since endotoxin was detected in the included volume of Sephadex G-150 chromatography in the presence of SFH (Fig. 2), it seemed likely that endotoxin had formed a specific complex with SFH. Therefore, we examined whether endo-

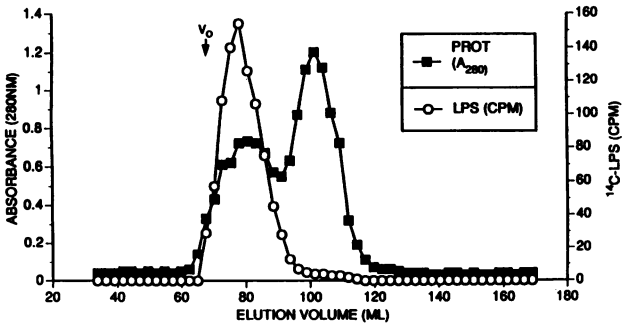

FIG. 1. Gel permeation chromatography of plasma apoproteins in absence of SFH. Plasma apoproteins (fraction with d of >1.21 g/ml from sequential density centrifugations of cell-free rabbit plasma) were chromatographed in the absence of SFH on Sephadex G-150. Proteins were monitored at A_{280} (closed squares), and endotoxin was monitored by determination of ^{14}C cpm (open circles). The void volume (V_0) is indicated.

toxin and SFH could form a stable complex in the absence of any other blood components. ^{14}C-labeled *S. typhimurium* LPS (6.8×10^4 cpm) was incubated with 0.5 ml of SFH (1.2 g/dl) at room temperature for 15 min. The mixture was then layered above 20% sucrose and centrifuged as described in Materials and Methods. Ninety-eight percent of the endotoxin cpm was detected in the SFH layer above the sucrose, 1.9% of the cpm was in the top three-fourths of the sucrose layer, and only 0.1% of the cpm was at the bottom of the sucrose layer. In contrast, a control tube of endotoxin in PBS demonstrated 97% of the endotoxin in the bottom fraction.

DISCUSSION

The relative affinities of LPS for specific cellular, lipoprotein, and apoprotein components of whole blood have not been described previously. Therefore, we performed in vitro studies of the distribution of endotoxin in samples of whole blood by using two purified, commonly studied enteric LPSs. In both human and rabbit blood, endotoxin associated primarily with the noncellular components of blood. Binding to HDL was greatest, followed by binding to apoproteins. Appreciable, although lesser, amounts of binding to LDL

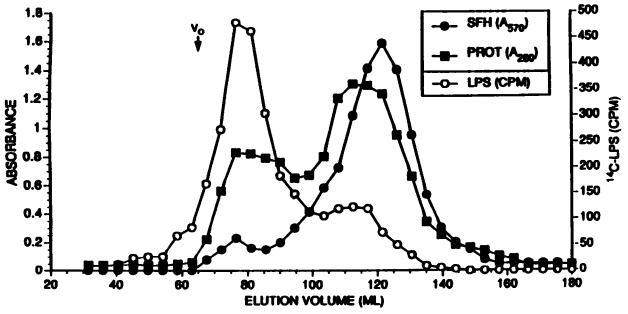

FIG. 2. Gel permeation chromatography of plasma apoproteins in the presence of SFH. Plasma apoproteins (fraction with d of >1.21 g/ml from sequential density centrifugations of cell-free rabbit plasma) were chromatographed in the presence of SFH (1.2 g/dl) on Sephadex G-150. Proteins were monitored at A_{280} (closed squares), SFH was monitored at A_{570} (closed circles), and endotoxin was monitored by determination of ^{14}C cpm (open circles). The void volume (V_0) is indicated.

TABLE 3. Sucrose sedimentation of apoproteins[a]

Fraction	Endotoxin distribution (% total cpm)		
	Top	Middle	Bottom
LPS alone (n = 4)	10 ± 1	4 ± 1	86 ± 13
G-150 V_0 fraction (apoproteins; n = 2)	77	12	11
G-150 V_0 fraction (apoproteins + SFH; n = 2)	82	2	6
G-150 included volume (apoproteins + SFH; n = 1)	87	8	5
Apoproteins (n = 5)	77 ± 10	17 ± 5	6 ± 2
Apoproteins + SFH (n = 6)	83 ± 6	13 ± 2	4 ± 2

[a] Apoproteins (d > 1.21 g/ml; plasma proteins remaining after sequential removal of lipoproteins) containing ^{14}C-LPS were obtained by centrifugation of cell-free rabbit plasma to remove blood cells and lipoproteins. These apoproteins, apoprotein fractions partially purified by Sephadex G-150 chromatography, and ^{14}C-LPS alone were centrifuged through 4 ml of 20% sucrose. After centrifugation at 25,000 × g for 1 h, cpm were determined in the layer above the sucrose cushion (top), the upper 3 ml of sucrose (middle), and the bottom 1 ml of sucrose (bottom). A_{280}s, as an estimation of protein concentration, also were measured in these sucrose layers and were 64 ± 7 (top), 12 ± 2 (middle), and 24 ± 5 (bottom). Recovery of cpm layered over sucrose in these studies was 83% (mean value). Means ± SD (when more than two independent experiments were performed) are shown.

and VLDL were observed. In human blood, cell-associated endotoxin was detected primarily with platelets. However, the platelet-bound cpm constituted only 1 to 2% of the total endotoxin cpm distributed throughout the blood. In rabbit blood, the cell-associated endotoxin was primarily bound to erythrocytes; this constituted 10 to 16% of the total endotoxin cpm distributed throughout the blood.

The demonstration of binding of LPS to platelets in humans, and to erythrocytes in rabbits, is potentially of relevance to the development of disseminated intravascular coagulation during endotoxemia. Several biological consequences of the interaction of LPS with mammalian platelets have been described previously, including the LPS-induced aggregation of human platelets (33), activation and secretion of platelet factor 3 (20), and secretion of 5-hydroxytryptamine (6). The latter two processes were described in rabbits (human platelets were not studied). Therefore, it is interesting that the endotoxins tested in our study had affinity for human platelets but not for rabbit platelets. Human platelets are less responsive to endotoxin than are rabbit platelets (29), but the pyrogenic response of humans to endotoxin exceeds that of rabbits (17). It is possible that these differences reflect species-specific cell membrane characteristics. The membranes of human platelets and megakaryocytes previously have been shown to differ biochemically from murine platelets and megakaryocytes by the presence of Fc receptors (and their absence on murine platelets and megakaryocytes) (32). In contrast, the murine cells demonstrate type 1 complement receptors, while the human cells do not (32). Whereas LPS binding to human platelets is likely to result in significant physiological effects on hemostasis, the significance of LPS binding to rabbit erythrocytes is less clear. However, a glycoprotein receptor on erythrocyte membranes that binds LPS has been described, and its potential role in the etiology of immune hemolysis during sepsis has been discussed (40).

Previous in vivo studies have demonstrated that much of administered LPS associates with HDL (12, 28, 46). The binding of high-molecular-weight LPS particles to HDL involves the dissociation of LPS (45), a process involved in

LPS detoxification, and has been shown to result in altered electrophoretic behavior of HDL (12). The role of an apoprotein factor(s) in LPS binding to HDL has been demonstrated (27, 42). LPS-binding protein appears to be one such apoprotein that is a mediator of LPS dissociation and binding to HDL (41). We also have shown prominent binding of LPS to HDL, as well as demonstrable, although lesser, binding to the other classes of lipoproteins. However, the lipoprotein binding of exogenously administered, isolated LPS may not totally mimic the distribution of shed LPS, associated with bacterial outer member structures, as demonstrated previously for the LPS of *S. typhimurium* (28) and *Neisseria meningitidis* (2). Shed LPS also may demonstrate variable lipoprotein binding depending on the presence or absence of bacterial membrane proteins (13). Although numerically less impressive, the binding to VLDL in our study also may be biologically significant. It has been shown previously that binding of LPS to triglyceride-rich lipoproteins (VLDL and chylomicrons) diminishes the potency of LPS in activation of the coagulation system in *Limulus* lysate (8). Furthermore, VLDL and chylomicrons protect mice from LPS-induced mortality (18). Since hypertriglyceridemia is one of the earliest abnormalities observed in blood during sepsis, this interaction between LPS and the triglyceride-rich lipoproteins may serve as a defense against endotoxemia.

The presence of SFH at a concentration of 1.2 g/dl did not appreciably alter the distribution of endotoxin among the various blood cell types, lipoproteins, and the apoprotein pool in either human or rabbit blood. However, since we demonstrated that endotoxin was able to bind to SFH, it is possible that the distribution of endotoxin among specific plasma proteins of the apoprotein pool was significantly altered by the presence of hemoglobin. Furthermore, in contrast to the binding of LPS to another apoprotein, aggregated immunoglobulin G, after which the density of the complex was identical to that of LPS alone (16), we have demonstrated that binding of LPS to SFH involves disaggregation of LPS (unpublished observations). Since the distribution of endotoxin among specific plasma proteins in the presence and absence of SFH is not known, further fractionation experiments will be required to determine if endotoxin binds to SFH preferentially. Several mammalian endotoxin-binding proteins in plasma have been documented previously, including a rabbit acute-phase LPS-binding protein (41), lysozyme (31), complement (5, 14), immunoglobulin (16), and albumin (15). We are now able to add SFH to this list of endotoxin-binding proteins.

The capacity of SFH to act as an endotoxin-binding protein may prove significant for the potential use of SFH as a blood substitute. Endotoxin contamination of SFH during its production and purification has been recognized (11; personal observations), and even low concentrations of endotoxin in preparations of SFH may prove clinically significant when liters of SFH solutions are infused into patients, especially those who are already hypotensive. It also is likely that endotoxemia will be present in many patients with trauma and shock who would be receiving this blood product. Gram-negative bacteremia associated with infection is another clinical situation in which endotoxin-hemoglobin binding may be a significant phenomenon. Endotoxin-hemoglobin complexes also may form as a result of the hemolysis that often occurs during the process of disseminated intravascular coagulation associated with gram-negative sepsis.

Importantly, our data indicate that the presence of SFH does not alter the association of endotoxin with mononuclear cells, an interaction which appears to play a critical pathophysiologic role in the production of the sepsis syndrome. However, further investigation will be required to determine if endotoxin-hemoglobin complexes differ from endotoxin alone in their ability to stimulate production or release of mononuclear cell factors involved in the response of the host to sepsis. Additional study also will be required to determine if endotoxin clearance from either the circulation or internal organs is altered by complex formation with hemoglobin.

ACKNOWLEDGMENTS

This work was supported, in part, by U.S. Army Medical Research and Development Command research contract log number 88270001/MIPR no. 90 MM0535. This work also was supported by research grant DK 43102 from the National Institute of Diabetes and Digestive and Kidney Diseases and by the Veterans Administration.

ADDENDUM IN PROOF

After the submission of this paper, it was determined that the term SFH (stroma-free hemoglobin) requires further definition in order to avoid confusion with the use of this term in the older literature. In this report, SFH refers to cell-free, ultrafiltered human hemoglobin free of detectable erythrocyte stroma and chemically cross-linked in order to improve intravascular persistence and prevent renal toxicity.

REFERENCES

1. **Beutler, B., and A. Cerami.** 1987. Cachectin: more than a tumor necrosis factor. N. Engl. J. Med. **316**:379–385.
2. **Brandtzaeg, P., K. Bryn, P. Kierulf, R. Ovstebo, E. Namork, B. Aase, and E. Jantzen.** 1992. Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. J. Clin. Invest. **89**:816–823.
3. **Braude, A. I., F. J. Carey, and M. Zalesky.** 1955. Studies with radioactive endotoxin. II. Correlation of physiologic effects with distribution of radioactivity in rabbits injected with lethal doses of E. coli endotoxin labelled with radioactive sodium chromate. J. Clin. Invest. **34**:858–866.
4. **Brunning, R. D., B. F. Woolfrey, and W. H. Schrader.** 1964. Studies with tritiated endotoxin. II. Endotoxin localization in the formed elements of the blood. Am. J. Pathol. **44**:401–409.
5. **Cooper, N. R., and D. C. Morrison.** 1978. Binding and activation of the first component of human complement by the lipid A region of lipopolysaccharides. J. Immunol. **120**:1862–1868.
6. **Des Prez, R. M., H. I. Horowitz, and E. W. Hook.** 1961. Effects of bacterial endotoxin on rabbit platelets. I. Platelet aggregation and release of platelet factors in vitro. J. Exp. Med. **114**:857–873.
7. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. **163**:1433–1450.
8. **Eichbaum, E. B., H. W. Harris, J. P. Kane, and J. H. Rapp.** 1991. Chylomicrons can inhibit endotoxin activity in vitro. J. Surg. Res. **51**:413–416.
9. **Evatt, B. L., and J. Levin.** 1969. Measurement of thrombopoiesis in rabbits using ^{75}selenomethionine. J. Clin. Invest. **48**: 1615–1626.
10. **Feola, M., J. Simoni, and P. C. Canizaro.** 1991. Quality control of hemoglobin solutions. I. The purity of hemoglobin before modification. Artif. Organs **15**:243–248.
11. **Feola, M., J. Simoni, P. C. Canizaro, R. Tran, and G. Raschbaum.** 1988. Toxicity of polymerized hemoglobin solutions. Surg. Gyn. Obstet. **166**:211–222.
12. **Freudenberg, M. A., T. C. Bog-Hansen, U. Back, and C. Galanos.** 1980. Interaction of lipopolysaccharides with plasma high-density lipoproteins in rats. Infect. Immun. **28**:373–380.
13. **Freudenberg, M. A., U. Meier-Dieter, T. Staehelin, and C.**

Galanos. 1991. Analysis of LPS released from *Salmonella abortus equi* in human serum. Microb. Pathog. **10:**93–104.
14. **Galanos, C., E. T. Rietschel, O. Luderitz, and O. Westphal.** 1971. Interaction of lipopolysaccharides and lipid A with complement. Eur. J. Biochem. **19:**143–152.
15. **Galanos, C., E. T. Rietschel, O. Luderitz, and O. Westphal.** 1972. Biological activities of lipid A complexed with bovine-serum albumin. Eur. J. Biochem. **31:**230–233.
16. **Ginsberg, M. H., and D. C. Morrison.** 1978. The selective binding of aggregated IgG to lipid-A-rich bacterial lipopolysaccharides. J. Immunol. **120:**317–319.
17. **Greisman, S. E., and R. B. Hornick.** 1969. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc. Soc. Exp. Biol. Med. **131:**1154–1158.
18. **Harris, H. W., C. Grunfeld, K. R. Feingold, and J. H. Rapp.** 1990. Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. J. Clin. Invest. **86:**696–702.
19. **Herring, W. B., J. C. Herion, R. I. Walker, and J. G. Palmer.** 1963. Distribution and clearance of circulating endotoxin. J. Clin. Invest. **42:**79–87.
20. **Horowitz, H. I., R. M. Des Prez, and E. W. Hook.** 1962. Effects of bacterial endotoxin on rabbit platelets. II. Enhancement of platelet factor 3 activity in vitro and in vivo. J. Exp. Med. **116:**619–633.
21. **Levin, J., and F. B. Bang.** 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. **19:**186–197.
22. **Lorenzet, R., J. Niemetz, A. J. Marcus, and M. J. Broekman.** 1986. Enhancement of mononuclear procoagulant activity by platelet 12-hydroxyeicosatetraenoic acid. J. Clin. Invest. **78:**418–423.
23. **Mathison, J. C., and R. J. Ulevitch.** 1979. The clearance, tissue distribution, and cellular localization of intravenously injected lipopolysaccharide in rabbits. J. Immunol. **123:**2133–2143.
24. **Maxie, M. G., and V. E. O. Valli.** 1974. Studies with radioactive endotoxin. III. Localization of ^{3}H-labeled endotoxin in the formed elements of the blood and detection of endotoxin in calf blood with the Limulus amebocyte lysate. Can. J. Comp. Med. **38:**383–390.
25. **Maxie, M. G., V. E. O. Valli, G. A. Robinson, R. B. Truscott, and B. J. McSherry.** 1974. Studies with radioactive endotoxin. I. Clearance of ^{51}Cr-labeled endotoxin from the blood of calves. Can. J. Comp. Med. **38:**347–366.
26. **Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, S. M. Wolff, and D. W. Wilmore.** 1988. Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. **318:**1481–1486.
27. **Munford, R. S., C. L. Hall, and J. M. Dietschy.** 1981. Binding of *Salmonella typhimurium* lipopolysaccharides to rat high-density lipoproteins. Infect. Immun. **34:**835–843.
28. **Munford, R. S., C. L. Hall, J. M. Lipton, and J. M. Dietschy.** 1982. Biological activity, lipoprotein-binding behavior, and in vivo disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. J. Clin. Invest. **70:**877–888.
29. **Nagayama, M., M. B. Zucker, and F. K. Beller.** 1971. Effects of a variety of endotoxins on human and rabbit platelet function. Thromb. Diath. Haemorrh. **26:**467–473.
30. **Nolan, J. P.** 1989. Intestinal endotoxins as mediators of hepatic injury—an idea whose time has come again. Hepatology **10:**887–891.
31. **Ohno, N., and D. C. Morrison.** 1989. Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity. J. Biol. Chem. **264:**4434–4441.
32. **Rabellino, E. M., R. L. Nachman, N. Williams, R. J. Winchester, and G. D. Ross.** 1979. Human megakaryocytes. I. Characterization of the membrane and cytoplasmic components of isolated marrow megakaryocytes. J. Exp. Med. **149:**1273–1287.
33. **Ream, V. J., D. Deykin, V. Gurewich, and S. Wessler.** 1965. The aggregation of human platelets by bacterial endotoxin. J. Lab. Clin. Med. **66:**245–252.
34. **Rickles, F. R., J. Levin, J. A. Hardin, and M. E. Conrad, Jr.** 1977. Tissue factor generation by human mononuclear cells: effects of endotoxin and dissociation of tissue factor generation from mitogenic response. J. Lab. Clin. Med. **89:**792–803.
35. **Roth, R. I., J. W. Gaubatz, A. M. Gotto, Jr., and J. R. Patsch.** 1983. Effect of cholesterol feeding on the distribution of plasma lipoproteins and on the metabolism of apolipoprotein E in the rabbit. J. Lipid Res. **24:**1–11.
36. **Schindler, R., J. Mancille, S. Endres, R. Ghorbani, S. C. Clark, and C. A. Dinarello.** 1990. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood **75:**40–47.
37. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. Science **249:**1429–1431.
38. **Springer, G. F., J. C. Adye, A. Bezkorovainy, and B. Jirgensons.** 1974. Properties and activity of the lipopolysaccharide-receptor from human erythrocytes. Biochemistry **13:**1379–1389.
39. **Springer, G. F., J. C. Adye, A. Bezkorovainy, and J. R. Murthy.** 1973. Functional aspects and nature of the lipopolysaccharide-receptor of human erythrocytes. J. Infect. Dis. **128:**S202–S212.
40. **Springer, G. F., S. V. Huprikar, and E. Neter.** 1970. Specific inhibition of endotoxin coating of red cells by a human erythrocyte membrane component. Infect. Immun. **1:**98–108.
41. **Tobias, P. S., K. Soldau, and R. J. Ulevitch.** 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. **164:**777–793.
42. **Tobias, P. S., and R. J. Ulevitch.** 1983. Control of lipopolysaccharide-high density lipoprotein binding by acute phase protein(s). J. Immunol. **131:**1913–1916.
43. **Tracey, K. J., H. Vlassara, and A. Cerami.** 1989. Cachectin/tumor necrosis factor. Lancet **i:**1122–1126.
44. **Ulevitch, R. J.** 1978. The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. Immunochemistry **15:**157–164.
45. **Ulevitch, R. J., and A. R. Johnston.** 1978. The modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. J. Clin. Invest. **62:**1313–1324.
46. **Ulevitch, R. J., A. R. Johnston, and D. B. Weinstein.** 1979. New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. J. Clin. Invest. **64:**1516–1524.
47. **Vosbeck, K., P. Tobias, H. Mueller, R. A. Allen, K.-E. Arfors, R. J. Ulevitch, and L. A. Sklar.** 1990. Priming of polymorphonuclear granulocytes by lipopolysaccharides and its complexes with lipopolysaccharide binding protein and high density lipoprotein. J. Leukocyte Biol. **47:**97–104.
48. **Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. J. Exp. Med. **169:**333–338.
49. **Waage, A., A. Halstensen, R. Shalaby, P. Brandtzaeg, P. Kierulf, and T. Espevik.** 1989. Local production of tumor necrosis factor α, interleukin 1, and interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. J. Exp. Med. **170:**1859–1867.
50. **Weiss, J., K. Muello, M. Victor, and P. Elsbach.** 1984. The role of lipopolysaccharides in the action of the bactericidal/permeability-increasing neutrophil protein on the bacterial envelope. J. Immunol. **132:**3109–3115.
51. **White, C. T., A. J. Murray, J. R. Greene, D. J. Smith, F. Medina, G. T. Makovec, E. J. Martin, and R. B. Bolin.** 1986. Toxicity of human hemoglobin solution infused into rabbits. J. Lab. Clin. Med. **108:**121–131.
52. **White, C. T., A. J. Murray, D. J. Smith, J. R. Greene, and R. B. Bolin.** 1986. Synergistic toxicity of endotoxin and hemoglobin. J. Lab. Clin. Med. **108:**132–137.
53. **Winslow, R. M., K. W. Chapman, and J. Everse.** 1991. Pilot scale production of pyrogen-free modified human hemoglobin for research. Biomat. Artif. Cells Immob. Biotechnol. **19:**503. (Abstract.)