

# Septin: A Factor in Plasma That Opsonizes Lipopolysaccharide-bearing Particles for Recognition by CD14 on Phagocytes

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## Summary

We have previously reported that lipopolysaccharide (LPS) binding protein (LBP) opsonizes endotoxin (LPS) for recognition by CD14 on phagocytes. Here we show that normal human plasma contains high titers of an activity that also binds LPS (Re, 595) and mediates recognition by CD14. Opsonization of LPS-coated particles with plasma enables the particles to be bound by phagocytes. Further, opsonization with plasma also enables subnanogram-per-milliliter concentrations of LPS to induce dramatic alterations in the function of leukocyte integrins on polymorphonuclear leukocytes and to induce secretion of tumor necrosis factor by monocytes, suggesting that opsonization by factors in plasma may be important in responses of cells to endotoxin. The opsonic activity in plasma appears distinct from LBP since it is not blocked by neutralizing antibodies against LBP. Surprisingly, the opsonic activity of plasma is not present in a single protein species, but at least two species must be combined to observe activity. Further, the opsonic activity of plasma for LPS is blocked by addition of protease inhibitors, suggesting that proteolytic activity or activities are required for opsonization. These properties are suggestive of the action of a protease cascade, but opsonic activity of plasma is not affected by blockade or depletion of either the complement or clotting cascades. We propose the name "septin" to describe this novel LPS-opsonizing activity in plasma.

Leukocytes recognize bacterial LPS (endotoxin) through several mechanisms (1). CD18 antigens (2) and the scavenger receptors (3) on leukocytes have been shown to bind particulate and dispersed LPS, respectively. This binding may result in uptake and degradation of LPS but is not associated with stimulation of the cells (3, 4). We have recently described a different means of recognizing LPS in which a soluble protein, rather than a membrane-bound molecule, performs the initial binding to LPS. LPS binding protein (LBP)<sup>1</sup> binds to both dispersions of LPS (5) and to LPS on the surface of particles (6). The resulting LPS/LBP complexes are recognized by the receptor CD14 on PMN (7), monocytes, and macrophages (MO) (8). "Opsonization" of bacteria with LBP enables phagocytes to bind and phagocytose the particles (6) in a CD14-dependent fashion (S. D. Wright, unpublished observations), thus resulting in clearance of LPS.

CD14 may also initiate the dramatic responses of leukocytes to picogram-per-milliliter concentrations of endotoxin.

PMN show the most rapid responses with priming for enhanced oxidative activity (9) and enhanced activity of leukocyte integrins (7) observed after 15–30 min of exposure to LPS. Both of these responses of PMN require LBP (7, 10) or serum (7, 11), and both can be blocked by anti-CD14 mAbs (7, and M. J. Pabst, personal communication). Monocytes and MO respond to LPS by synthesizing cytokines such as TNF, IL-1, and IL-6 within 1–2 h. TNF production by rabbit MO in response to sub nanogram-per-milliliter doses of LPS requires LBP (12), and blockade of CD14 on human monocytes prevents TNF production (8). These data suggest that CD14 is the principal mediator of responses to picogram-per-milliliter concentrations of LPS, and that LBP or other serum proteins are needed for recognition of LPS by CD14.

Here we have examined the proteins in serum or plasma that opsonize LPS-coated particles and mediate binding to CD14. We find that plasma from healthy human volunteers contains an enormous amount of this opsonic activity. The activity is not contained in a single protein fraction, but at least two species must be combined to duplicate the activity in plasma. Moreover, opsonic activity of plasma is completely inhibited by inhibitors of proteolysis. We suggest that LPS

<sup>1</sup> Abbreviations used in this paper: DFP, diisopropylphosphorofluoridate; LBP, LPS binding protein; MO, macrophage; NHP, normal human plasma.

is opsonized by the action of a novel protease cascade, and propose the name "septin" to define this activity.

## Materials and Methods

**Reagents.** Fresh frozen normal human plasma (NHP) from blood drawn into acid citrate dextrose and human fibronectin was supplied by the New York Blood Center. Human plasmas deficient in Hageman Factor, prekallikrein, factor IX, high molecular weight kininogen, and complement protein C5 were from Sigma Chemical Co. (St. Louis, MO); plasma deficient in protein C was from American Diagnostica (Greenwich, CT); and serum deficient in complement proteins C1, C2, and factor B were from Quidel (La Jolla, CA). Pefabloc SC (*p*-aminoethylbenzenesulfonyl fluoride) was from Centerchem Inc. (Stamford, CT); diisopropylphosphorofluoridate (DFP), PMSF, aprotinin, soybean trypsin inhibitor (SBTI), and benzamide were from Sigma Chemical Co.; and Chymostatin was from Calbiochem-Behring Corp. (La Jolla, CA). LPS (Re, 595) was from List Biologicals (Campbell, CA).

Goat anti-rabbit LBP and preimmune serum was a generous gift of Dr. R. Ulevitch (Scripps Institute, La Jolla, CA). IgG from this serum was purified by protein G chromatography. mAb 3C10 (13) against CD14 was also purified from ascites fluid by protein G chromatography.

Human LBP was purified from human ascitic fluid by a modification of the method of Tobias et al. (5). Ascitic fluid was first brought to 1 mM PMSF, 1 mM Pefabloc SC, 0.05% DFP, and 5 mM EDTA, and passed over BioRex 70 equilibrated in 50 mM phosphate, pH 7.3, 40 mM NaCl, and 2 mM EDTA. Proteins were eluted with a linear gradient of NaCl, and fractions were collected into the tubes containing Pefabloc SC and aprotinin. Fractions containing LBP were pooled and further purified by chromatography on a Mono Q column as described below. The LBP obtained by this procedure appeared as a single band on SDS gels of  $\sim 64,000 M_r$ , and NH<sub>2</sub>-terminal sequencing showed identity with the deduced sequence of human LBP (12).

**Cells.** Monocyte-derived MO were obtained by culturing purified human monocytes in Teflon beakers for 3–10 d as described (14). On the day of the experiment, MO were suspended in Dulbecco's PBS containing 0.5 mg/ml human serum albumin (Armour Pharmaceuticals, Kankakee, IL), 0.3 U/ml aprotinin, and 3 mM glucose (HAP buffer), and monolayers were obtained by incubating 2,500 MO in Terasaki wells for 45 min at 37°C. PMN were purified from fresh blood by the method of English and Anderson (15) and were suspended in HAP buffer. Monolayers were obtained by adding 10,000 PMN to Terasaki wells and incubating for 20 min at 37°C.

Production of TNF by monocytes was measured using freshly prepared PBMC. Preliminary studies showed that preparations of mononuclear cells containing platelets mounted a substantial response to LPS even in the absence of serum, suggesting that platelets may substitute for serum in this assay. We therefore used platelet-free preparations of fresh mononuclear cells obtained by a modification of the method of Pawlowski et al. (16). Blood was drawn into an equal volume of saline containing 5 mM EDTA and 20 U/ml heparin. After centrifugation on a Ficoll-Hypaque gradient, the overlying plasma and the mononuclear cell layers were separately recovered. After two low-speed washes, the mononuclear cells were suspended in the recovered autologous plasma and incubated for 10 min at 37°C. This step elutes bound platelets from the monocyte surface. Cells were then washed into RPMI 1640 containing 10  $\mu$ g/ml human  $\alpha_2$ -macroglobulin (Calbiochem-Behring Corp.), and 20,000 cells were added to each Terasaki well.

The human kidney cell line 293-S was stably transfected with

a full-length gene encoding CD14 (M. P and G. E. Mark, unpublished studies). These cells grow as a monolayer and are passaged by suspending in EDTA. The untransfected cells express no CD14 by FACS<sup>®</sup> analysis, but the transfectants express CD14 at levels comparable to those on monocytes. Monolayers were obtained by suspending the cells in HAP buffer and incubating 5,000 in fibronectin-coated Terasaki wells (17) for 60 min at 37°C.

Sheep erythrocytes (E) were coated with C3bi or LPS to yield EC3bi or ELPS by methods previously described (2, 6). The dose of LPS used to prepare the ELPS was 1  $\mu$ g/ $4 \times 10^7$  E. EC3bi were suspended in 2.5  $\mu$ M veronal buffer, pH 7.5, 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.15 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub> (DGVB<sup>2+</sup>), and ELPS were suspended in 5 mM veronal buffer, pH 7.5, 150 mM NaCl, 0.1% gelatin, 1 mM EDTA (EDTA-GVB<sup>2+</sup>).

**Opsonization of ELPS.** Opsonization of ELPS by plasma was measured by one of two procedures. In the first, monolayers of MO were washed into PBS containing 1 mM EDTA, then dilutions of plasma and  $5 \times 10^5$  ELPS were added. Wells were incubated for 15 min at 0°C to allow opsonization and settling of the E, then for 15 min at 21°C to allow binding to the MO. The cultures were next inverted for 20 min to allow gravity to gently remove nonadherent erythrocytes from the MO, and attachment of E was scored by phase contrast microscopy. EDTA was included throughout the assay to prevent clotting or deposition of complement, but similar results were obtained with serum diluted in buffers containing calcium and magnesium (Dulbecco's PBS).

In a second form of this assay, ELPS were opsonized by incubation with dilutions of plasma, washed, and then added to monolayers of MO. After 30 min at 21°C, cultures were inverted and washed as above. This "two-step" assay yielded titers 3–10-fold greater than the single-step assay above. For both forms of the assay, the data are presented as attachment index, the number of E bound per 100 MO. The opsonization detected by these assays is termed "septin activity," and the septin titer is the dilution of plasma needed for half-maximal rosetting. All experiments using these assays were repeated at least three times with NHP from different donors.

**Chromatography.** Bio-Rex 70 resin was equilibrated with 50 mM phosphate, pH 7.3, 40 mM NaCl, and 2 mM EDTA, then incubated batchwise for 18 h with a sixfold volume excess of neat human plasma containing 2 mM EDTA. The resin was then washed, poured into a column, and eluted with 50 mM phosphate, pH 7.3, 0.5 M NaCl. The eluate contained  $\sim 0.25\%$  of the protein in the starting material. Septin assays showed that opsonic activity was quantitatively adsorbed from the plasma by exposure to Bio-Rex, and that 50–70% of the activity was recovered in the eluate. The eluate from the Bio-Rex column was dialyzed against 20 mM Tris, pH 8.5, applied to a Mono Q column and eluted with a gradient of NaCl (see Fig. 4).

**Activation of CR3 on PMN.** The activation of the leukocyte integrin, CR3, was measured by determining the ability of PMN to bind EC3bi using one of two procedures. In the first, monolayers of PMN were washed into PBS containing 1 mM EDTA, and dilutions of LPS and plasma were added. EDTA was included in this step to insure that complement would not be activated by the LPS. After a 15-min incubation at 37°C, the monolayers of PMN were washed with PBS containing divalent cations,  $5 \times 10^5$  EC3bi were added, and cultures were incubated for 30 min at 37°C. Nonadherent EC3bi were removed by vigorous washing, and the attachment index was measured (7).

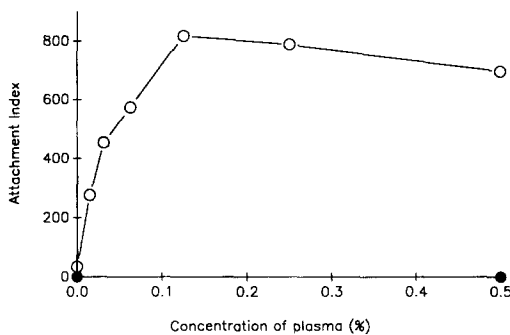
In a second form of this assay, PMN were activated by adherence to LPS-coated, opsonized culture wells. Terasaki wells were incubated for 2 h with 100 ng/ml LPS, washed thoroughly, then air dried. The surface were then opsonized by incubation with di-

lutions of plasma for the indicated times, and then washed in PBS. PMN were added to the wells and incubated for 15 min at 37°C. Activation of CR3 was measured by adding EC3bi and continuing the incubation for 20 min at 37°C.

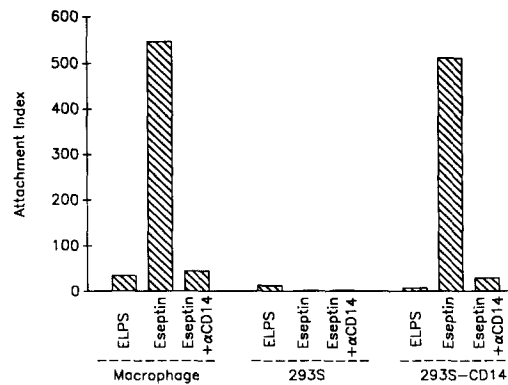
**TNF Secretion by Monocytes.** Production of TNF- $\alpha$  by freshly isolated monocytes in response to LPS was measured by one of two procedures. In the first, dilutions of LPS and NHS were added to mononuclear cells and incubated for 5 h at 37°C. TNF in the supernatant fluid was measured by ELISA in Terasaki plates as described (18). This assay employed an alkaline phosphatase-conjugated second antibody and was read on a fluorescence scanner (Cytofluor 2300; Millipore, Bedford, MA) using the fluorogenic substrate Attophos (JBL Scientific, San Luis Obispo, CA). In a second form of the assay, TNF production was induced upon interaction of monocytes with LPS-coated, opsonized culture wells as described above for PMN, with the exception that 20 ng/ml LPS was used for coating the substrates.

## Results

**Plasma Constituents Mediate Binding of LPS-coated Particles to MO.** Addition of human plasma to mixtures of ELPS and MO caused the ELPS to attach firmly to MO. All plasma samples tested (>30) caused comparable binding, with the attachment index rising from  $16.4 \pm 9.6$  for ELPS alone to  $496 \pm 192$  in the presence of 0.5% plasma ( $n = 10$ ). The binding of ELPS to MO observed in these studies is unlikely to be due to complement deposition on the ELPS or interactions of LPS with CD18 molecules, since the studies were performed in the presence of 1 mM EDTA (see Materials and Methods), conditions that block complement activity and the function of CD18 molecules (2, 14). The MO in these studies were taken from serum-containing cultures, indicating that pretreatment of the MO with serum does not enable recognition of ELPS. Rather, plasma mediates binding of LPS to MO by first interacting with the ELPS to form a stable complex. Incubation of ELPS with dilutions of plasma op-



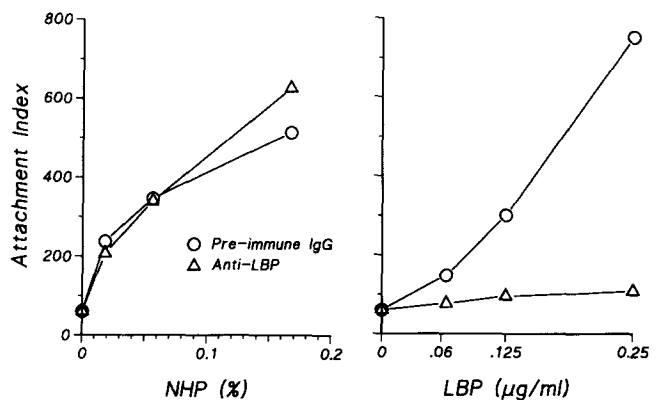
**Figure 1.** Plasma opsonizes ELPS for recognition by MO. Uncoated sheep E ( $0.5 \times 10^8$ /ml; ●) or LPS-coated E (ELPS; ○) were incubated with dilutions of NHP for 15 min at 21°C in PBS containing 1 mM EDTA. The E were washed twice and added to monolayers of human MO. After 30 min at 21°C, the monolayers were washed, and the attachment of ELPS to MO was measured by microscopy. Results are presented as attachment index, the number of erythrocytes bound per 100 MO, and are representative of three separate experiments.



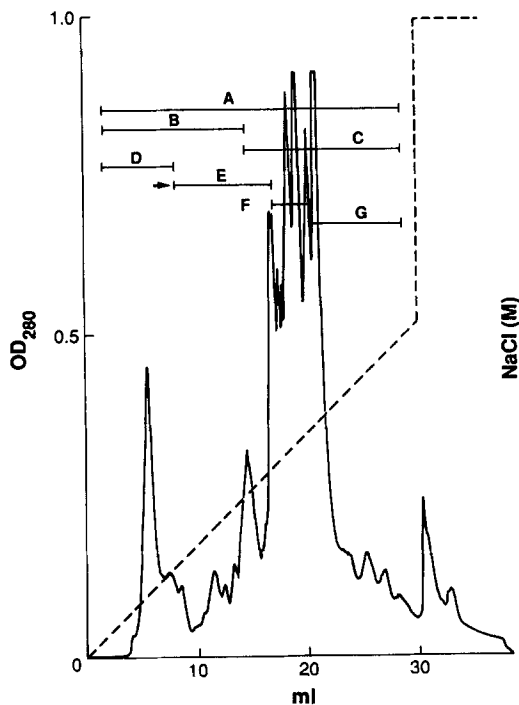
**Figure 2.** CD14 mediates binding of plasma-opsonized ELPS to MO. Monolayers of MO, 293-S cells, or 293-S cells stably transfected with CD14 were established in a 60-min incubation at 37°C. Where indicated, 10  $\mu$ g/ml of the anti-CD14 mAb 3C10 was added to the cells for 10 min at 5°C. Freshly washed ELPS or ELPS opsonized with NHP (0.5%, 15 min, 21°C) were then added to the monolayers, and attachment was scored after a 30-min incubation at 21°C. Additional studies showed that incubation of MO with antibodies against CD16, CD18, and HLA had no effect on binding of opsonized ELPS (not shown).

sonized the erythrocytes such that they bound strongly to MO in a subsequent incubation (Fig. 1). Plasma contains a great deal of opsonic activity for ELPS, with half-maximal binding observed in the two-step opsonic assay using plasma diluted  $\sim 4,000$ -fold (Fig. 1).

**ELPS Opsonized with Plasma Bind to CD14 on MO.** Binding of plasma-treated ELPS to MO is completely blocked by the addition of anti-CD14 mAb (Fig. 2), suggesting that CD14 is the receptor involved in recognition. The role of CD14 in binding opsonized ELPS was confirmed using transfection studies. Opsonized ELPS did not bind to untransfected 293-S cells, but bound avidly to 293-S cells stably transfected



**Figure 3.** Anti-LBP does not block the opsonization of ELPS by plasma. Anti-LBP IgG or preimmune IgG (50  $\mu$ g/ml final concentration) was added to dilutions of NHP or LBP, and these mixtures were added to monolayers of MO. ELPS were then added, and binding of ELPS to MO was measured as described in Materials and Methods. This study is representative of four separate experiments.



**Figure 4.** Septin activity is observed only in pools of fractionated protein. NHP was fractionated first on BioRex 70 resin and then applied to a Mono Q column and eluted with a gradient of NaCl. None of the individual 1-ml fractions had detectable septin activity. Aliquots of the fractions were pooled as shown and diluted to match the starting material. Essentially complete recovery of septin activity was observed in the pools labeled A and E, but activity in the other pools was undetectable. Septin titers (the dilution needed for half-maximal rosetting in the septin assay) were determined by graphic analysis of a threefold dilution series beginning at 1:30. Results were as follows: sample loaded on column, 250; fraction A, 230; fraction B, <30; fraction C, <30; fraction D, <30; fraction E, 250; fraction F, <30; fraction G, <30. Triplicate data points in the assay varied by <10%. Similar results were obtained in three separate experiments. Solid line, OD<sub>280</sub>; dashed line, NaCl concentration (M).

with the gene for CD14 (Fig. 2). Moreover, all binding to this transfectant could be blocked with anti-CD14 antibody. Thus, ELPS opsonized with plasma bind to CD14 on macrophages, as do ELPS opsonized with purified LBP (8).

*The Opsonic Activity of Plasma Appears Distinct from LBP.* LBP is an acute phase reactant reported to be present at low levels (~150 ng/ml) in the serum of healthy rabbits (19). To determine if LBP accounts for the large amount of opsonic activity in plasma of healthy volunteers, binding of ELPS was measured in the presence of anti-LBP antibody. While this antibody strongly blocked opsonization of ELPS by purified LBP, it had no effect on opsonization mediated by plasma (Fig. 3). This observation suggests that plasma contains an activity distinct from LBP that bridges LPS to CD14 on MO. We will refer to this opsonic activity as "septin."

*Septin Activity Requires More than One Protein.* Batch adsorption of plasma with Bio-Rex 70 resin (see Materials and Methods) quantitatively removed septin activity. The resin was poured into a column, washed, and eluted with a step gradient of NaCl. Septin activity was recovered in the eluate

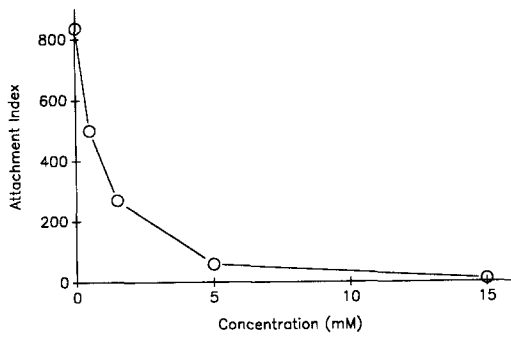
along with 0.25% of the applied protein. This fractionation step is similar to that described by Tobias et al. (5) for purification of rabbit LBP, and we have found that LBP in human ascites fluid is also enriched by this procedure. The partially purified septin was further fractionated on a Mono Q column (Fig. 4). To our surprise, none of the individual fractions eluted from this column contained significant septin activity. Pooling of all fractions, however, resulted in quantitative recovery of septin activity. Additional pooling studies indicated that the region of the gradient from 0.14 to 0.30 M NaCl (pool E) contained the septin activity, and this activity was not enhanced by addition of proteins pooled from other regions of the gradient. Exclusion of fractions at either margin of the active pool resulted in strongly decreased activity (not shown). These experiments indicate that septin activity is not produced by a single protein but that at least two distinct proteins must be simultaneously present to opsonize ELPS. LBP is unlikely to be among the proteins required for septin activity since LBP eluted in pool D (not shown).

*Opsonization with Septin Requires Proteolytic Activity.* Opsonization of ELPS was blocked by the addition of several well-characterized inhibitors of proteolysis to plasma (Table 1). Detailed studies on inhibition of septin activity were performed with Pefabloc SC, a water-soluble, covalent inhibitor of serine esterases. Inhibition of opsonization was virtually complete with 15 mM Pefabloc SC (Fig. 5), and complete inhibition of septin activity could be observed over a range of concentrations of plasma (Fig. 6). Importantly, neither Pefabloc SC (Fig. 6) nor any of the other inhibitors of pro-

**Table 1.** Protease Inhibitors Block Opsonization of ELPS by NHP

Inhibitor	IC <sub>50</sub> *
Pefabloc SC	2.5 ± 1.6 mM
DFP	0.65 mM
PMSF	0.21–0.40 mM
Chymostatin	0.05–0.08 mM
Aprotinin	43 ± 10 μg/ml
SBTI	33 ± 17 μg/ml
Benzamidine	>10 mM
LPS	15 ± 8 ng/ml

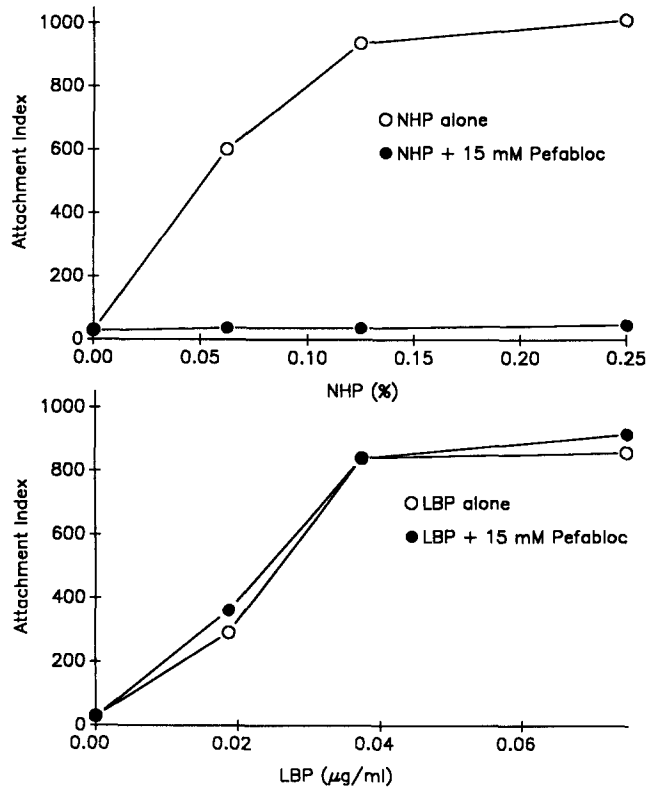
\* NHP was diluted to 0.1% in various concentrations of protease inhibitors and was then incubated for 15 min at 20°C with ELPS. The ELPS were washed, and their ability to adhere to macrophage monolayers was assessed. The concentration of inhibitor needed to block opsonization by a factor of two was determined by analysis of the resulting inhibition curves. Results are the range of two or the average ± SD of three experiments. None of the protease inhibitors blocked opsonization of ELPS by 0.03 μg/ml LBP in parallel experiments, suggesting that they did not contain high levels of LPS. Parallel studies indicated that LPS blocked opsonization of ELPS by LBP with an IC<sub>50</sub> of 16 ± 10 ng/ml.



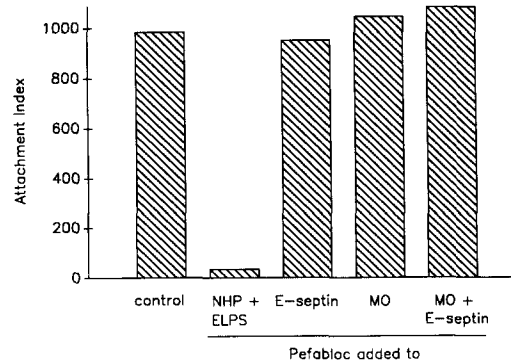
**Figure 5.** Pefabloc SC inhibits opsonization of ELPS by plasma. ELPS were incubated with 0.1% NHP for 10 min at 21°C in the presence of the indicated concentrations of Pefabloc SC. The treated erythrocytes were then washed thoroughly and incubated for 30 min with MO and attachment measured.

teolysis (Table 1) inhibited the opsonic activity of LBP. This finding indicates that the inhibition observed is not the result of endotoxin contamination of the inhibitors employed, and further indicates that the mechanism of action of sepsin is distinct from that of LBP.

The site of action of the protease inhibitors is in the plasma since pretreatment of MO or opsonized erythrocytes with



**Figure 6.** Pefabloc SC inhibits opsonization of ELPS by plasma but not by LBP. ELPS were incubated with the indicated concentrations of LBP or NHP in the presence or absence of 15 mM Pefabloc SC. After a wash, binding of cells to MO was measured.



**Figure 7.** Pefabloc SC blocks deposition of sepsin onto ELPS. ELPS incubated with 0.5% NHP showed strong binding to MO, and addition of 15 mM Pefabloc during this opsonization step blocked opsonization. Pefabloc had no effect on binding if added to MO, to ELPS pretreated with plasma (15 min, 15 mM, 21°C), or during the incubation of opsonized ELPS with MO.

Pefabloc SC caused no diminution of binding, but addition of inhibitor during opsonization with plasma strongly blocked the ability to bind MO (Fig. 7). Since opsonization was blocked by the classical serine esterase MO inhibitor DFP, and since opsonization occurs at neutral pH in the presence of EDTA, we suggest that a serine esterase is required for the action of sepsin, and we propose that the proteins that need to be combined to achieve sepsin activity represent protease and substrate.

Plasma contains an abundance of inhibitors that quickly inactivate proteolytic enzymes. Most proteases that act in this milieu are derived from inactive zymogens that are not sensitive to active-site protease inhibitors. To determine if sepsin enzymes in plasma are similarly insensitive, 10 mM Pefabloc SC was incubated with NHP for 15 min at 20°C, then removed by dialysis. A sepsin assay with 0.1% plasma treated in this way showed an attachment index of  $657 \pm 50$  vs.  $582 \pm 18$  for untreated, dialyzed plasma. Thus, sepsin enzymes in resting plasma are insensitive to the protease inhibitor, Pefabloc SC, and the active sites of the enzyme(s) necessary for sepsin activity are only made sensitive to Pefabloc SC upon interaction with LPS.

*Sepsin Activity Does Not Require the Action of the Complement or Clotting Cascades.* The complement cascade is known to opsonize particles through the action of several proteolytic enzymes, and a similar cascade of proteolytic events controls blood clotting. Several results, however, indicate that neither clotting nor complement cascades are involved in sepsin activity. Both the complement cascade and the clotting cascade are completely inhibited by removal of divalent cations, yet sepsin activity is measured in chelated buffers. Consumption of complement activity in serum using either classical pathway activators (IgG-coated particles) or alternative pathway activators (zymosan particles), or consumption of clotting factors by clotting of blood, had no effect on sepsin titers. Finally, normal sepsin titers were observed in sera deficient in several complement and clotting proteins (Table 2). These

**Table 2. Septin Is Not Related to Other Protease Cascades**

Septin activity is not depleted* by procedures that remove:	
Complement activity from serum	Clotting activity from plasma
EDTA (1 mM)	EDTA (1 mM)
Classical pathway depletion (EIgG) <sup>†</sup>	Clotting and conversion to serum
Alternative pathway depletion (zymosan) <sup>†</sup>	Depletion of factor XII (Hageman factor)
Depletion of C1, C2, C5, factor B	Depletion of factor IX
	Depletion of factor VII
	Depletion of prekallikrein
	Depletion of high molecular weight kininogen
	Depletion of protein C

\* All procedures caused less than a 50% loss in septin titre. Heating serum to 56°C for 60 min caused a 60% loss in septin titer.

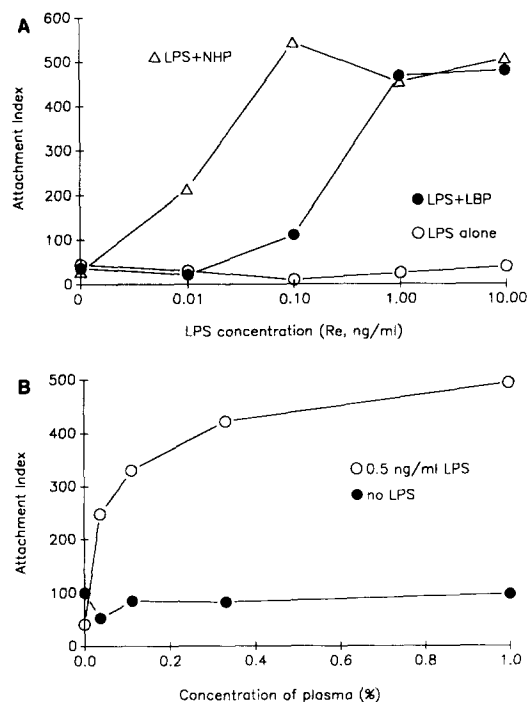
<sup>†</sup> Classical pathway and alternative pathway activity were depleted by incubating neat serum with  $2 \times 10^9$  EIgG or zymosan particles for 30 min at 37°C.

findings indicate that septin activity does not require an intact complement or clotting cascade. These findings do not, however, rule out the participation of individual enzymes from these cascades, such as the complement factors D and C1 in-

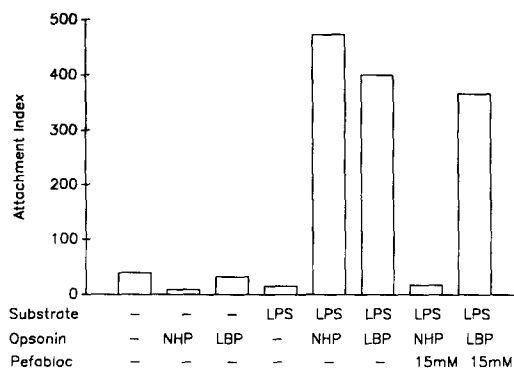
hibitor, or the clotting enzymes factors II, V, X, XI, plasmin, or plasminogen activator.

**Septin Mediates Stimulation of PMN by LPS.** We have previously shown that PMN respond to LPS/LBP complexes by upregulating the adhesive capacity of CR3 (7), the receptor for complement protein C3bi. To determine if septin is also capable of mediating stimulation of PMN by LPS, PMN were incubated with dilutions of LPS in the presence of LBP or NHP (Fig. 8 A). Concentrations of LPS alone as high as 10 ng/ml caused no activation of CR3. Addition of LBP enabled a half-maximal response to  $\sim 0.2$  ng/ml LPS, confirming our previous findings (7). NHP enabled a strong response to even lower concentrations of LPS, with  $\sim 0.01$  ng/ml being sufficient for a half-maximal response. The ability of NHP to enable this response to LPS was concentration dependent, with half-maximal activity observed at dilutions of  $\sim 1/3,000$  (Fig. 8 B). Activation of CR3 by LPS in the presence of NHP was completely blocked by anti-CD14 mAb 3C10 at 10  $\mu$ g/ml (data not shown). Since CR3 also binds constituents on the surface of endothelium (20, 21), the activation of this adhesion molecule may contribute to the neutropenia observed in animals exposed to endotoxin.

The above results indicate that NHP contains an activity that enables a CD14-dependent response of PMN to LPS, and the high titer of this activity in plasma suggests that it may result from septin rather than LBP. To determine if this activity has the characteristics of septin, we determined whether it could be stably deposited on LPS in a fashion that requires proteolytic activity. Tissue culture plastic surfaces were first coated with purified LPS and washed. Interaction of PMN with these coated surfaces caused no activation of CR3 (Fig. 9). Incubation of the LPS-coated surfaces with NHP or LBP, using conditions similar to those required for deposition of septin on the surface of ELPS, yielded a surface that caused strong activation of CR3 on adherent PMN. Generation of surfaces capable of activating PMN was abso-



**Figure 8.** Plasma enables PMN to respond to very low concentrations of LPS. A monolayer of PMN was established in a 20-min incubation at 37°C. (A) The cells were then incubated for 15 min at 37°C with NHP (0.25%) or LBP (0.5  $\mu$ g/ml), and dilutions of LPS in an EDTA-containing buffer. Cells were washed and the activity of CR3 was determined. (B) Monolayers of PMN were incubated in an EDTA-containing buffer for 15 min at 37°C in the presence or absence of 0.5 ng/ml LPS and dilutions of NHP, and CR3 activity determined as above.

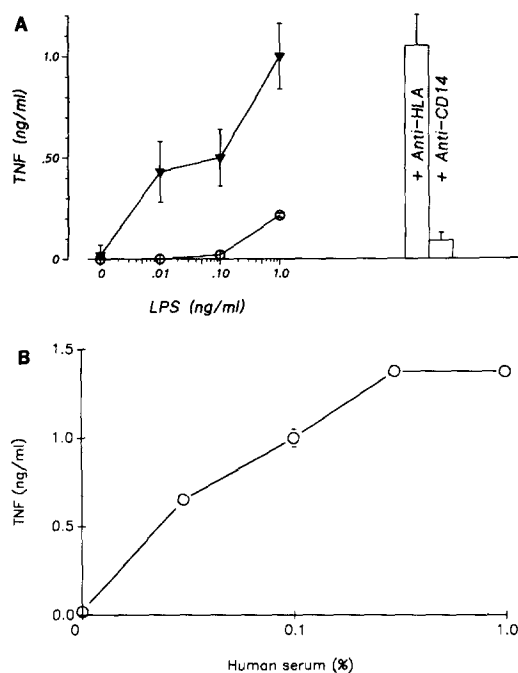


**Figure 9.** Plasma opsonizes LPS for recognition by PMN in a Pefabloc-sensitive fashion. Culture surfaces were coated with 100 ng/ml LPS as indicated. Surfaces were then washed and incubated for 10 min at 21°C with 0.2% NHP or 0.2  $\mu$ g/ml LBP diluted in the presence or absence of 15 mM Pefabloc SC. After a further wash, PMN were added, and the activation of CR3 was measured by the addition of EC3bi as described in Materials and Methods.

lutely dependent on the presence of both LPS and either plasma or LBP (Fig. 9), and the stimulation of PMN was completely blocked by mAbs against CD14 (not shown). These results suggest that stimulation of PMN by LPS requires deposition of serum proteins on LPS and subsequent interaction with CD14. Addition of Pefabloc SC during opsonization of the LPS surface completely inhibited the opsonization by NHP but had no effect on the action of LBP (Fig. 9). Thus, the factors in NHP that enable responses of PMN act by binding to LPS through a mechanism that requires proteolytic activity. We thus suggest that stimulation of PMN by LPS requires the factors in serum we have termed septin.

**Septin Mediates Stimulation of Monocytes by LPS.** Incubation of freshly isolated, platelet-free monocytes with LPS at a concentration up to 1 ng/ml LPS induced little TNF secretion, but addition of serum caused strong TNF synthesis in response to 0.01 ng/ml LPS (Fig. 10 A). Serum thus contributes a factor necessary for responses of monocytes to low levels of LPS.

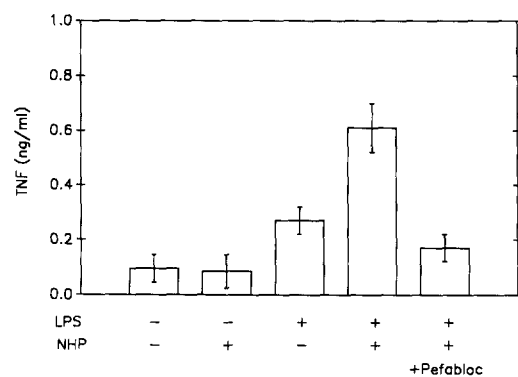
Several results suggest that septin is the factor in serum that enables responses of monocytes to concentrations of LPS <1 ng/ml. Induction of TNF by LPS was enabled by dilutions of serum >3,000-fold (Fig. 10 B), and this response was completely blocked by anti-CD14 mAb but not by an antibody against HLA (Fig. 10), confirming our previous finding with whole blood (8). Adherence of monocytes to a plastic surface coated with LPS induced modest TNF secretion, but opsonization of the surface with dilutions of plasma caused a much larger response (Fig. 11). Finally, the ability of plasma to opsonize LPS and enable secretion of TNF was blocked by the addition of Pefabloc SC during opsonization (Fig. 11). These results indicate that induction of TNF synthesis by MO requires prior interaction of plasma components with the LPS, and that interaction requires proteolytic activity.



**Figure 10.** Serum is necessary for induction of TNF by low levels of LPS. (A) Platelet-free mononuclear cells were incubated for 5 h with the indicated concentrations of LPS in the presence (▼) or absence (○) of 0.5% NHP, and TNF levels in the medium were measured. The histogram depicts responses to 0.1 ng/ml LPS, and 1% NHP in the presence of 20  $\mu$ g/ml W6/32 (anti-HLA) or 3C10 (anti-CD14). (B) Cells were incubated with dilutions of human serum and a fixed dose of LPS (0.1 ng/ml), and TNF levels in the medium were measured. Error bars represent SD calculated from triplicate data points, and in some cases are smaller than the symbol. These studies are representative of at least three separate experiments.

## Discussion

Here we define a novel biological activity present in serum and plasma. This activity, septin, opsonizes LPS-coated particles for recognition by CD14 on PMN, monocytes, and MO. Interaction of LPS/septin complexes with CD14 causes not



**Figure 11.** Plasma opsonizes LPS for recognition by monocytes in a Pefabloc-sensitive fashion. Culture surfaces were coated with 20 ng/ml LPS as indicated. Surfaces were then washed and incubated for 10 min at 21°C with 0.3% NHP diluted in the presence or absence of 15 mM Pefabloc SC. After a further wash, platelet-free mononuclear cells were added, and production of TNF was measured after a 5-h incubation. Error bars represent SD calculated from triplicate data points. This study is representative of more than six separate experiments.

only binding of the opsonized particle by leukocytes but also stimulation of the adhesive activity of PMN and production of TNF by monocytes. A similar requirement for serum to support responses of cells to LPS has been shown in several previous studies. Cohn and Morse (22) reported dramatic enhancement of microbicidal activity of PMN by LPS, but only if serum was also present. A requirement for serum was also observed for LPS-dependent priming of PMN for an oxidative burst (11), for LPS-dependent degranulation of PMN (23), for LPS-dependent production of cGMP in hepatocytes (24), and for LPS-dependent cytotoxicity of endothelial cells (25). Our data suggest that serum enables responses to LPS by supplying an opsonic activity that recognizes LPS and mediates interaction with CD14.

The mechanism by which septin opsonizes LPS involves participation of at least two protein species and requires proteolytic activity. These properties are suggestive of the action of a protease cascade. While it is clear that septin activity is not an attribute of either the clotting or the complement cascades, septin does resemble a protease cascade described in the hemolymph of arthropods. This cascade, which is initiated by endotoxin and results in the formation of a clot, is the basis of the "Limulus amoebocyte lysate" assay for endotoxin. The Limulus cascade is initiated by binding of LPS to the zymogen, factor C (26). Factor C then undergoes an autolytic cleavage that activates proteolytic activity against an additional zymogen, factor B. Two successive proteolytic steps eventuate in cleavage of a fibrinogen-like molecule that makes up the clot. It is reason-

able to speculate that septin may be evolutionarily related to the Limulus cascade.

The biological activities of septin appear similar to those of LBP in that both septin and LBP mediate binding of LPS-coated particles to MO (6), and both mediate stimulation of PMN (7, 10) and monocytes (8). They differ, however, in their distribution and mechanism of action. Septin activity requires at least two distinct protein species as well as proteolytic activity, while LBP is a single protein that binds LPS effectively in the presence of protease inhibitors. LBP is reported to be present in low levels in the serum of healthy rabbits and is only abundant after induction of the acute phase response. In contrast, septin activity is present at very high levels in plasma from healthy adults. Because of the preponderance of septin in normal plasma, we propose that septin represents the principal means for opsonization of LPS for recognition by CD14 in healthy hosts.

Septin appears necessary to enable responses to the low concentrations of LPS that occur in the blood during sepsis. With the exception of fulminant meningococcemia (27), LPS concentrations >0.1 ng/ml are practically never observed even in severely septic patients (28). While much higher concentrations of LPS (>10 ng/ml) can stimulate TNF production in monocytes and can stimulate mild activation of adhesivity in PMN, addition of septin is necessary for responses to concentrations of LPS <0.1 ng/ml. We thus believe that septin may represent an important, in vivo mechanism for responding to LPS.

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We thank Drs. Patricia A. Detmers, Leslie A. Leonard, and Thierry Calandra for critical reading of the manuscript.

This work was supported by U.S. Public Health Service grants AI-22003, AI-24775, and AI-30556. S. D. Wright is an Established Investigator of the American Heart Association.

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Received for publication 28 February 1992 and in revised form 5 May 1992.

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