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A Synthetic Lipopolysaccharide-Binding Peptide Based on Amino Acids 27–39 of Serum Amyloid P Component Inhibits Lipopolysaccharide-Induced Responses in Human Blood

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LPS-binding proteins in plasma play an important role in modifying LPS toxicity. Significant properties have already been attributed to the LPS-binding protein (LBP). It accelerates LPS toxicity as well as incorporation into high-density lipoproteins, leading to neutralization of LPS in serum. A search for other LPS-binding components in serum, using LPS-coated magnetic beads, revealed a new LPS-binding protein. N-terminal microsequencing identified this protein as serum amyloid P component (SAP). Purified SAP bound to smooth and rough types of LPS via the lipid A part. SAP inhibited the binding of FITC-labeled ReLPS (LPS from Salmonella minnesota strain R595) to human monocytes and the ReLPS-induced priming of the oxidative burst of human neutrophils only in the presence of low concentrations of LBP. In search for the LPS binding site of SAP, we found that pep27–39, a 13-mer peptide consisting of amino acids 27–39 of SAP, competitively inhibited the binding of LPS to SAP. In addition, pep27–39 significantly inhibited ReLPS-induced responses in phagocytes in the presence of serum, as well as in human whole blood. Carboxamidomethylated pep27–39 showed an even more pronounced reduction of the ReLPS-induced priming of phagocytes in human blood. Performing gel filtration of FITC-labeled ReLPS incubated with soluble CD14, we showed that SAP could not prevent binding of LPS to soluble CD14, in contrast to pep27–39. The ability of pep27–39 to antagonize specifically the effects of LPS in the complex environment of human blood suggests that pep27–39 may be a novel therapeutic agent in the treatment of Gram-negative sepsis. The Journal of Immunology, 1998, 161: 3607–3615.

Lipopolysaccharide is the major component of the outer membrane of Gram-negative bacteria. The specific interaction of LPS with soluble and cell surface localized components is a prerequisite for the orchestration of the immune response to a Gram-negative infection. Cell activation requires CD14, which is not only expressed on monocytes, macrophages, and neutrophils, but is also present in a soluble form in serum as soluble CD14 (sCD14). CD14 is the only known receptor for LPS that is able to transduce a signal, albeit probably indirectly (1, 2). CD14-negative cells, such as endothelial, epithelial, and smooth muscle cells, can be activated by LPS via the interaction of LPS with sCD14 (3). LPS-binding protein (LBP) is a lipid-transfer protein that promotes the movement of LPS from micelles to sCD14 (4). The interaction of LPS with sCD14 is markedly enhanced by LBP and initiates a cascade of cellular responses, which are necessary to fight Gram-negative infection, but under certain circumstances also lead to septic shock (5). LBP can also catalyze the movement of LPS to high-density lipoproteins (HDL), which neutralizes the capacity of LPS to stimulate cells (6, 7). There are more LPS-binding proteins known to play a role in LPS-mediated effects, several of which have been recognized among the antimicrobial arsenal secreted by neutrophils (8). Bactericidal/permeability-increasing protein (BPI), a cationic protein, not only exerts strong antimicrobial effects against Gram-negative bacteria, but is also potent in binding and detoxifying LPS (9, 10). Neutrophil granule components, such as cationic protein 18 (CAP18), lysozyme, lactoferrin, and azurocidin/CAP37, have also been described as LPS-binding proteins with a neutralizing capacity (8, 11, 12).

Serum amyloid P component (SAP) is a decameric serum glycoprotein composed of identical 25.5-kDa subunits noncovalently associated in two pentameric rings interacting face to face. It has been associated with all forms of amyloid deposits, for example with those in Alzheimer’s and Parkinson’s disease, Down’s syndrome, and Creutzfeldt-Jacob syndrome (13). It is described to protect amyloid deposits from proteolytic degradation in vivo (14). Recently, the participation of SAP in the pathogenesis of amyloidosis was demonstrated using mice with targeted deletion of the SAP gene (15). However, SAP has also been reported to inhibit Alzheimer β-peptide fibril formation in an in vitro model (16). Furthermore, it is present in the normal glomerular basement membrane covalently associated with collagen and is associated with elastic fibers in skin and blood vessels (17). SAP belongs to the family of pentraxins, lectin-like serum proteins, which have been stably conserved throughout vertebrate evolution. This protein has a 51% amino acid homology with C-reactive protein, the classical acute-phase protein found in humans. SAP is an acute-phase reactant in mice, while it is constitutively present in human serum at 40 µg/ml, with a maximum twofold increase during sepsis (14). SAP shows calcium-dependent binding to DNA (18), chromatin (19), and glycosaminoglycans such as heparin, heparan, and dermatan sulfate (20), and has been described to play a role in the complement cascade since it can bind to several complement components, such as C4b-binding protein, C1q, and C3bi (21, 22), and to immune complexes, probably via the F(ab′)2 fragment of IgG.
were obtained from Sigma (St. Louis, MO). Human rLBP was a generous gift from H. Lichtenstein (A mogła, Boulder, CO).

**Materials and Methods**

**Reagents**

LPS from *Salmonella minnesota* strain R595, *Escherichia coli* O111:B4, its Rc mutant J5, monophosphoryl lipid A, and LPS from *Salmonella typhimurium* and *S. typhimurium* TV119 (Ra), SL684 (Re), and SL1181 (Re) were obtained from Sigma (St. Louis, MO). Human rLBP was a generous gift from H. Lichtenstein (A mogła, Boulder, CO).

**Serum and plasma**

Blood was drawn from healthy human volunteers. Human serum was obtained after pooling the sera of three or more donors and stored until use at −70°C. Plasma was obtained from blood in heparinized or EDTA tubes. Fresh human serum was used for SAP isolation.

**Peptide synthesis**

A 13-mer peptide, pep27–39 (EKPLQNFQTLFCA), corresponding to amino acids 27–39 of SAP, and a scrambled peptide, pep27–39sc, were obtained from Genscript using standard Fmoc chemistry with in situ PyBop/1HMDA activation of the amino acids in a fivefold molar excess with respect to 2 μmol peptide PAL-PEG-PS resin (Perceptive Biosystems, Framingham, MA) was employed. Peptides were obtained as C-terminal amides after cleavage with 90 to 95% trifluoroacetic acid/scavenger mixtures. Peptides were dissolved in 50 mM HAc at a concentration of 5 mM and further diluted in 0.25 M Tris-HCl, pH 7.5, to a concentration of 0.6 mM. Before use in biologic assays, the peptides were further diluted in HBBS, containing 0.2% human serum albumin (HSA; Central Laboratory Blood transfusion, Amsterdam, The Netherlands). In some experiments, pep27–39 was carboxamidomethylated to prevent formation of dimers, as follows: Pep27–39 in 50 mM HAc was heated at 100°C for 2 min in 20 μl sample buffer (2% SDS, 2.5% DTT, 10% glycerol, 0.001% bromophenol blue, in 0.05 M Tris-HCl, pH 6.9) and detected by SDS-PAGE on 12.5%, 0.75 mm minigels. Gels were stained with Coomassie brilliant blue, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA), and sequenced on an Applied Biosystems protein sequencer model 470A. In other experiments, magnetic beads were coated with 1 mg/ml smooth type LPS from *S. typhimurium*. The beads (5 × 10⁹) were then incubated with 1 μg/ml LPS from *S. typhimurium*, LPS from *S. minnesota* R595, LPS from *E. coli* O111:B4, or from its Rc-mutant J5; washed three times with HBBS containing 0.05% Tween-20; and incubated with mouse anti-human SAP mAb clone 5 (Sigma) for 30 min at room temperature with a subsequent washing procedure. Detection of SAP binding to the beads was performed by incubating the beads for 30 min with FITC-labeled goat anti-mouse Ig (Becton Dickinson, Mountain View, CA) and analysis on a FACScan (Becton Dickinson).

**Isolation of SAP from serum**

Isolation of SAP from serum was performed as described by Skinner and Cohen (29) with modifications. Briefly, fresh human serum was centrifuged at 17,000 × g for 5 h at 4°C to remove the top lipid layer. The delipidated serum was then applied to a Sepharose 4B (Pharmacia, Upplands Väsby, Sweden) column, equilibrated with a calcium buffer (10 mM Tris·HCl, 0.01 M Tris·HCL, 2 mM CaCl₂, pH 7.8). SAP was eluted with an EDTA buffer (140 mM NaCl, 0.01 M Tris·HCL, 10 mM EDTA, pH 8) and applied to a gel-filtration column (Superdex 200; Pharmacia) equilibrated in the same EDTA buffer. Fractions containing SAP were concentrated in an Amicon filter system (10-kDa cutoff) and dialyzed against PBS or saline. Peptides were checked on SDSPAGE under reducing conditions and stained with Coomassie brilliant blue staining. The SAP concentration was determined by ELISA. Therefore, microtiter plates (96-well polyvinyll; Costar, Cambridge, MA) were coated overnight at 4°C with anti-human SAP mAb 5.4A (1 μg/ml; Monosan; Uden, The Netherlands) and blocked for 1 h at 37°C with PBS/0.05% Tween/4% BSA. Samples and a SAP standard (Calbiochem-Novabiochem, La Jolla, CA) were diluted in PBS/ Tween/1% BSA and incubated for 1 h at 37°C, followed by 1 h incubation with a second biotinylated anti-human SAP mAb 5.4A (1 μg/ml; Monosan; Sanbio). Peroxidase-labeled streptavidin (Southern Bio-technology, Birmingham, AL) was added, and after 1 h, the substrate composed of TMB (tetramethylbenzidine; Sigma) and H₂O₂ in 0.1 M acetate buffer was allowed to be converted for 10 min. To stop the enzymatic reaction, 2 N H₂SO₄ was added and the OD was determined using a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA) operating at 450 nm. In between incubations, the plate was washed five times with H₂O/0.05% Tween. LPS contamination of SAP preparations was about 10 to 20 ng/mg SAP, as determined by the Limulus amebocyte lysate assay (Chromogenix AB, Mölndal, Sweden).

**Cell isolation**

Human neutrophils and PBMC were isolated from heparinized blood drawn from healthy volunteers, as described by Troelstra et al. (30).

**Binding of fluorescein-labeled ReLPS to human monocytes**

FITC-labeled ReLPS (FITC-LPS) was prepared as described by Troelstra et al. (31), with a molar labeling efficiency of 1:1. For FITC-LPS binding studies, 2.5 mg/ml FITC-LPS was preincubated with increasing amounts of SAP (0–30 μg/ml) or pep27–39 (0–10 μM) for 0 to 30 min at 37°C in HBBS containing 0.2% HSA. Then LBP (10 ng/ml) and PBMC (6 × 10⁶ cells/ml) were added to the same buffer with the same washes, and the culture was incubated for 30 min at 37°C, and put on ice. Binding of FITC-LPS to monocytes was analyzed on a FACSscan, using forward and sideward scatter parameters to gate on monocytes. The results were expressed as the mean fluorescence of 10,000 cells. The percentage of inhibition of binding was calculated using the following formula: 1-(A-bgR/A-bgR+B-gbR) × 100%, where A is the mean fluorescence of cells incubated with FITC-LPS + LPS or pep27–39 and B is the mean fluorescence of cells incubated with FITC-LPS + LPS; bgR, the background fluorescence of cells incubated with FITC-LPS + SAP or pep27–39; and bgB, the background fluorescence of cells incubated with FITC-LPS alone.

**LPS-induced priming of human neutrophils**

This procedure has been described in detail elsewhere (30). Briefly, neutrophils (5 × 10⁷/ml) were added to a mixture of 1 ng/ml LPS alone or 1 ng/ml LPS with increasing amounts of SAP (0–30 μg/ml) or peptides (0–30 μM) in the presence of 1 to 100 ng/ml LBP in HBBS/1.8% HSA. In some experiments, increasing amounts of serum (0.1–3%) were used. In experiments using peptides, LPS and peptides were preincubated for 30 min before addition to the cells. Cells were incubated with the mixtures for 30 min at 37°C under constant agitation. Next, chemoluminescence response was measured in a luminometer (Autolumat LB 933, Berthold GmbH, Wildbad, Germany) after automated injection of FMLP (1 μM final concentration) and HBBS containing 180 μM luminal (Sigma). The chemoluminescence response was measured automatically over a period of
10 min. Data were analyzed with the AXIS software package (ExOxEmis, San Antonio, TX). Curves were obtained for all samples presenting the chemoluminescence response in cpm versus time. Absolute counts were obtained by calculating the area under the curve of the chemoluminescence response for 10 min. In experiments using human blood, 80 μl of human blood was incubated with 20 μl of LPS/peptide mixture for 30 min at 37°C. Then 900 μl PBS/0.5% glucose was added, and 100 μl of this mixture was used to measure the chemoluminescence response, as described. In some experiments, PMA (25 ng/ml) was used to activate the neutrophils or blood for a chemoluminescence response. In other experiments, TNF-α (1 nM) was used to prime neutrophils or blood for an enhanced FMLP response in the presence of peptides.

**J5-LPS ELISA**

J5-LPS was coated to 96-well flat-bottom plates (Greiner, Nürtingen, Germany) at a concentration of 1 μg/ml in PBS for 1 h at 37°C, with a subsequent overnight incubation at 4°C. The plate was washed five times with H₂O/0.05% Tween, and blocked for 1 h at 37°C with PBS/4% BSA/0.05% Tween. Then 0.3 μg/ml SAP was incubated with increasing concentrations of pep27−39 (0−30 μM) in HBSS/0.2% BSA/0.05% Tween for 1 h at 37°C. Subsequently, the binding of SAP was detected, as described for the SAP ELISA in Materials and Methods (isolation of SAP from serum), with the only exception of using HBSS/0.2% BSA/0.05% Tween as a dilution buffer for the second biotinylated anti-human SAP 5.4 antibody and the peroxidase-labeled streptavidin.

**FITC-LPS gel filtration**

To study the effect of SAP and pep27−39 on the LPS binding to recombinant sCD14 (rsCD14; kindly provided by Dr. Henri S. Lichenstein, Amsterdam), gel filtration of FITC-LPS in combination with on-line fluorescence detection was used. In principle, this system resembles the gel-shift assay described earlier by Hallman et al. (32) using 3H-labeled LPS. FITC-LPS alone forms self-quenching aggregates. This LPS aggregate will migrate as a molecule of about 500 kDa with a very low fluorescence signal. Addition of sCD14 will monomerize FITC-LPS, resulting in a rise in fluorescence of the rsCD14/FITC-LPS complex and a conformation of FITC-LPS with rsCD14. FITC-LPS (0.5 μg/ml) was incubated with 5 μg/ml rsCD14 and 100 ng/ml LBP with or without addition of 100 μg/ml SAP or 10 to 30 μM of pep27−39. Pep27−39 was preincubated with FITC-LPS for 30 min at 37°C before addition of rsCD14 and LBP. After 30-min incubation at 37°C, 100 μl of the mixture was loaded onto a Superdex TM 200 HR 10/30 column (Pharmacia) and run at a flow of 0.5 ml/min for 35 ml. The effect of SAP and pep27−39 on the binding of LPS to rsCD14 was also studied in the absence of LBP. In these experiments, the incubation time of rsCD14 and FITC-LPS was also 30 min. To determine the retention time of FITC-LPS, a sample of 50 μg/ml FITC-LPS alone was run. Fluorescence was recorded using a Perkin-Elmer (Norwalk, CT) LS30 lumino meter with excitation wavelength of 475 nm and emission set at 514 nm.

**Results**

**Detection of LPS-binding proteins in human serum and plasma**

Magnetic beads coated with LPS from *S. minnesota* strain R595 (ReLPS beads) were used to capture LPS-binding proteins from serum and plasma samples. Figure 1A shows the binding of three proteins, with $M_r$ values of approximately 70, 45, and 30 kDa, in the presence of 10% serum and heparin plasma, while hardly any proteins bound to the ReLPS beads in the presence of 10% EDTA plasma. Control nonReLPS-coated beads showed binding of the 70-kDa protein only (Fig. 1B). N-terminal amino acid sequencing of the blotted 45- and 70-kDa proteins showed 100% homology with the α- and β-chain of complement component C3bi, respectively. The 30-kDa protein yielded a sequence of 17 amino acids that was 100% homologous with that of the N-terminal sequence of human SAP. To check whether binding of these proteins to ReLPS was specific, we incubated ReLPS beads in 10% serum in the presence of increasing amounts of free ReLPS. Figure 2 shows that in the presence of 10 or 100 μg/ml free ReLPS, binding of the 30-kDa protein to the beads was inhibited, suggesting competition between free LPS and LPS coated on the beads for binding to SAP. Additional experiments showed that purified SAP, isolated from human serum, also bound to ReLPS beads. This binding could be inhibited by the addition of free ReLPS (data not shown).

These data suggest that SAP has a specific binding capacity for LPS. As shown in Figure 3, experiments performed with beads coated with smooth type LPS from *S. typhimurium* demonstrated that purified SAP can also bind to a smooth type of LPS. Competition experiments with other rough and smooth types of LPS from *E. coli* and other *Salmonella* strains showed that SAP not only exhibits a specific binding to ReLPS from *S. minnesota*, but it also specifically binds to all other tested forms of LPS, including monophosphoryl lipid A.

**SAP inhibits binding of FITC-LPS to human monocytes**

The finding that SAP specifically binds to LPS prompted us to investigate the effect of SAP on the LPS-induced effects on phagocytes. For this purpose, we studied the effect of purified SAP on LPS binding to monocytes. FITC-LPS was preincubated with various amounts of SAP, whereas the binding of FITC-LPS to monocytes was studied by flow cytometry. As shown in Figure 4,
preincubation of FITC-LPS with SAP dose dependently inhibited the binding of FITC-LPS to monocytes up to 90%. This effect was already evident at a concentration of 1 μg/ml SAP. To study kinetics of SAP binding to LPS, FITC-LPS was preincubated for 0 to 30 min with 10 μg/ml SAP, and the competence of FITC-LPS to bind to monocytes was analyzed. Even without preincubation, SAP inhibited binding of FITC-LPS to monocytes to the same extent as was achieved after 30 min of preincubation (data not shown). To exclude the possibility that SAP prevents LPS binding to the monocytes, via binding to the monocytes itself, a control experiment was performed. Therefore, PBMC were preincubated with SAP for 30 min, and washed three times to remove unbound SAP. Subsequent incubation of the PBMC with FITC-LPS, and analysis on the FACS, showed that preincubation of PBMC with SAP did not inhibit binding of FITC-LPS to monocytes.

**SAP inhibits LPS-induced priming of human neutrophils**

To evaluate the effects of SAP on LPS toxicity in a functional assay, we investigated whether SAP could inhibit LPS-induced priming of neutrophils. Neutrophils were primed with 1 ng/ml LPS for an enhanced FMLP response in the presence of 1 ng/ml LBP for 30 min. Addition of SAP revealed a dose-dependent inhibition on LPS-induced priming of neutrophils with a 70 to 80% inhibition at 30 μg/ml SAP (Fig. 5). However, the inhibitory effect of SAP on LPS-induced priming of neutrophils was profoundly reduced when higher concentrations of LBP were used (Fig. 6). In the presence of serum concentrations over 0.1%, the inhibitory effect of SAP on LPS-induced priming was strongly reduced as well (data not shown).

**SAP peptide, pep27–39, inhibits binding of SAP to LPS**

In literature, a peptide, comprising the amino acids 27–39 of the SAP sequence, was described to interfere with the binding of SAP

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**FIGURE 3.** Flow-cytometric analysis of SAP binding to various LPS types and monophosphoryl lipid A. Magnetic beads were coated with smooth type LPS from *S. typhimurium*. Binding of 1 μg/ml SAP to the beads was tested in the presence of increasing amounts (1–100 μg/ml) of monophosphoryl lipid A, smooth and rough types of LPS from *S. typhimurium*, LPS from *S. minnesota* R595, and LPS from *E. coli* O111:B4 and its Rc mutant JS. SAP binding was determined by incubating the beads with an anti-human SAP mAb and then FITC-labeled goat anti-mouse Ig. Beads were analyzed on a FACScan. Binding of SAP to the beads without the addition of one of the aforementioned free types of LPS represents 100% SAP binding. Data represent the mean of two separate experiments.

**FIGURE 4.** Flow-cytometric analysis demonstrating inhibition of FITC-LPS binding to monocytes by SAP. Human monocytes were incubated with FITC-LPS (2.5 ng/ml) in the presence or absence of increasing amounts of SAP for 30 min. FITC-LPS binding was assessed by FACScan analysis. FITC-LPS binding to monocytes is shown in the presence of 10 ng/ml LBP without SAP (A), and with 1 (B), 3 (C), 10 (D), and 30 (E) μg/ml SAP. The background binding of FITC-LPS in the absence of LBP is shown in all figures (thin lines). The data are representative of five separate experiments. Percentage of inhibition of FITC-LPS binding to monocytes is shown in the presence of increasing amounts of SAP ± SEM (n = 5) (F).
to some of its ligands. Therefore, we tested whether this peptide (pep27–39) could interfere with the binding of SAP to LPS as well. A microtiterplate was coated with J5-LPS and incubated with SAP in the presence of increasing amounts of pep27–39. Figure 7 shows that the binding of SAP to J5-LPS was completely inhibited by 30 μM of pep27–39. A control peptide, comprising the same amino acids in a scrambled order (pep27–39scr), did not interfere with the binding of FITC-LPS to monocytes (Fig. 8). A control experiment testing the possibility that binding of pep27–39 to the monocytes would influence subsequent binding of FITC-LPS, as we performed earlier for SAP, showed that prebound pep27–39 did not influence the binding of FITC-LPS to the monocytes. Testing the effect of pep27–39 on the LPS-induced priming of neutrophils in the presence of increasing concentrations of LBP, it was found that 3 μM of pep27–39 profoundly reduced the LPS-induced priming of neutrophils, even at high concentrations of LBP (Fig. 9). Also in the presence of serum, 30 μM of pep27–39 was able to almost completely inhibit the LPS-induced priming of neutrophils (1% serum; percentage of inhibition, 90.2 ± 11.7;

Pep27–39 neutralizes LPS in human serum and blood

Pep27–39 was anticipated to inhibit binding of FITC-LPS to monocytes. In a flow-cytometric assay, as little as 0.1 μM of pep27–39 inhibited the binding of FITC-LPS to monocytes by 50%, while a complete inhibition was reached at a concentration of 3 μM of pep27–39. The scrambled peptide, pep27–39scr, did not interfere with the binding of FITC-LPS to monocytes (Fig. 8). A control experiment testing the possibility that binding of pep27–39 to the monocytes would influence subsequent binding of FITC-LPS, as we performed earlier for SAP, showed that prebound pep27–39 did not influence the binding of FITC-LPS to the monocytes. Testing the effect of pep27–39 on the LPS-induced priming of neutrophils in the presence of increasing concentrations of LBP, it was found that 3 μM of pep27–39 profoundly reduced the LPS-induced priming of neutrophils, even at high concentrations of LBP (Fig. 9). Also in the presence of serum, 30 μM of pep27–39 was able to almost completely inhibit the LPS-induced priming of neutrophils (1% serum; percentage of inhibition, 90.2 ± 11.7;

FIGURE 5. Chemoluminescence assay illustrating inhibition of LPS-induced priming of the oxidative burst in human neutrophils by SAP. Human neutrophils were incubated with 1 ng/ml ReLPS in the presence or absence of increasing amounts of SAP for 30 min, after which the FMLP-induced chemoluminescence was measured. Effects of SAP on LPS priming of neutrophils are shown with LPS together with increasing amounts of SAP (0–30 μg/ml), all in the presence of 1 ng/ml LBP. Background luminescence was measured with 1 ng/ml LPS without addition of LBP. The data are representative of four separate experiments (A). Percentage of inhibition of the 10-min integral (AUC) of the LPS-induced priming of neutrophils is shown in the presence of increasing amounts of SAP ± SEM (n = 4) (B).
As pep27–39 contains a Cys residue, it will spontaneously form dimers. To investigate the effect of dimerization of pep27–39 on its LPS-inhibitory effects, the free sulfhydryl group of the Cys residue was blocked by carboxamidomethylation. In human whole blood, 30 μM of pep27–39 decreased the LPS-induced priming of neutrophils to about 50% compared with the chemoluminescence response in the presence of 1 ng/ml LPS alone. Carboxamidomethylated pep27–39 was even more efficient in inhibiting the LPS-induced priming of human blood (Fig. 10). The peptide with the scrambled sequence, pep27–39scr, showed no effect. In addition, carboxamidomethylated pep27–39 was more potent than pep27–39 in the inhibition of FITC-LPS binding to monocytes (IC50 of 0.03 μM compared with 0.1 μM). Control experiments using TNF-α as the primer for the FMLP-induced oxidative burst of human blood did not show any inhibitory effects of pep27–39 or carboxamidomethylated pep27–39. The activation of human blood by PMA was not affected by either of the peptides (data not shown). This indicates that (carboxamidomethylated) pep27–39 specifically antagonizes the LPS-induced priming of neutrophils and that this inhibitory effect was not caused by nonspecific cytotoxicity.

SAP-derived peptide, pep27–39, but not SAP, inhibits binding of LPS to rsCD14

To study the effect of SAP and pep27–39 on the LPS binding to rsCD14, we used a FITC-LPS gel-filtration technique in which we determined the capacity of rsCD14 to bind FITC-LPS, by monitoring the change in retention time of FITC-LPS on a gel-filtration column, in the presence of SAP or pep27–39. In Figure 11, we show that FITC-LPS runs at about 7 ml, just after the void volume of this column, representing 500 kDa (Fig. 11A). A 30-min preincubation of FITC-LPS with rsCD14 and LBP resulted in a shift of fluorescence from this quenched fluorescence signal at 7 to 14 ml (60 kDa), the place at which rsCD14 elutes from the column (Fig. 11B). Addition of SAP did not decrease the 60-kDa signal (data not shown). Even without LBP, SAP could not inhibit binding of FITC-LPS to rsCD14 (Fig. 11F). Lower concentrations of rsCD14 did not result in a retention time shift, so that the effect of SAP could not be tested at these concentrations of rsCD14. However, preincubation of FITC-LPS with 30 μM of pep27–39 very potently inhibited the binding of FITC-LPS to rsCD14, as is shown by the disappearance of the 60-kDa peak (Fig. 11D). The concentrations of FITC-LPS in Figure 11, B to G, are 10-fold lower then in Figure 11A. Therefore, the 500-kDa peak, representing the quenched form of FITC-LPS aggregates, is not visible in the lower panels. Figure 11, E and G, show that pep27–39 also prevents binding of LPS to rsCD14 in the absence of LBP.

Discussion

In literature, SAP is described to bind many ligands, although no clear biologic function has been ascribed to it yet (19, 20, 22,
In our study, using magnetic beads coated with ReLPS, we identified SAP as a new LPS-binding protein present in human plasma. Competition experiments showed that SAP specifically binds to rough as well as smooth types of LPS; however, the affinity of SAP for smooth types of LPS seems less as compared with rough types of LPS (Fig. 4). This can be partly explained by differences in m.w. of the LPS, but the affinity of SAP to smooth types of LPS could also be lower because of sterical hindrance caused by the oligosaccharide chain of smooth LPS. Monophosphoryl lipid A was a very potent inhibitor for the binding of SAP to LPS, indicating that SAP binds to the lipid A part of LPS.

We showed that SAP profoundly inhibited LPS responses in human granulocytes in the presence of low concentrations of LBP. This interference of LPS binding to CD14 by SAP was not the result of direct SAP binding to CD14, thereby inhibiting LPS/CD14 interactions, as preincubation of monocytes with SAP did not affect subsequent binding of LPS to the cells (data not shown). SAP was not able to neutralize LPS in serum or human blood. However, we demonstrated that SAP binds to LPS in the presence of serum, suggesting a role for SAP binding to LPS in vivo. Not much is known about the fate of LPS in vivo. To date, LPS has been described to bind either (s)CD14 or HDL, after entering the circulation, via interaction with LBP (4, 6). In the circulation, LPS-binding proteins as BPI and CAP18 cannot play a role in the immediate binding of LPS since they are constituents of neutrophil granules and are supposed to play a role in LPS neutralization only at specific sites of inflammation (8, 10, 35). Because the binding of LPS to HDL is a slow process (6, 36) and we have shown that binding of SAP to LPS occurs rapidly, we propose that, on entering the circulation, LPS is immediately captured by SAP. Although SAP is not able to neutralize LPS in vivo directly, it could serve as a carrier protein to transport LPS to the liver for rapid detoxification, and thus indirectly contribute to LPS clearance. Experiments in SAP-knockout mice are needed to further investigate the exact role of SAP binding to LPS in endotoxemia.

It has been shown that SAP can interact with phagocytes (37, 38). It can prime neutrophils (37) and enhance macrophage listericidal activity (39, 40). Furthermore, it has been described that substrate-bound SAP can activate C3b and C3bi receptors of monocytes (38). Since SAP can bind to phagocytes, bacteria (41, 42), and complement components (21–24), it might serve a role as an opsonin, potentiating phagocytosis of C3- or SAP-coated pathogens. The direct interaction with LPS on bacteria clearly fits in this model.

In search for the LPS-binding region of SAP, we found a SAP peptide, pep27–39, which could compete for the binding of SAP to LPS. This 13-mer synthetic SAP peptide, comprising the amino acids 27–39 of SAP, was described to interfere with the interaction of SAP with heparin and C4b-binding protein (21, 33, 43). In addition, a 12-mer synthetic peptide that corresponds to amino acids 27–38 was reported to support cell attachment (44). We showed that pep27–39 was able to inhibit LPS responses in human phagocytes even in the presence of human blood. Carboxaminomethylation of pep27–39, which prevents formation of dimers via blockage of the free sulfydryl groups of Cys residues, resulted in a peptide that was about 4 times more active. In other studies concerning cell attachment, it was shown...
that 83% of the initial activity of the SAP peptide, pep27–38, was confined to a hexapeptide, pep33–38 (44). When heparin binding was studied, pep33–38 was even found to have 10-fold higher activity than pep27–38 (43). However, in the present study, investigating the interactions between SAP and LPS, the hexapeptide, pep33–38, was about 40-fold less active than pep27–39 in binding to LPS (data not shown).

The LPS-binding motifs of several LPS-binding proteins have already been described. LPS-binding motifs for Limulus anti-LPS factor (LALF), BPI, and LBP show high sequence similarity with an alternating series of positively charged and hydrophobic residues with a proposed ability to produce an amphipathic loop that binds to the lipid A part of LPS (45, 46). In addition, CAP18 shows an LPS-binding region that contains a high number of basic and hydrophobic residues (47). We show a new LPS-binding motif within SAP, which does not contain the usual stretches of positively charged residues that are found in other LPS-binding proteins (Fig. 12).

Much to our surprise, a peptide of only 13 amino acids mimicked the effect of the whole protein in binding to and neutralization of LPS. Even more surprising, this peptide inhibited LPS-induced responses in human blood, whereas SAP did not. We propose that at least one reason for this phenomenon is a competition between SAP and LBP for binding to LPS, as we showed that increasing LBP concentrations abolished the inhibiting effects of SAP. Pep27–39, in contrary, still was able to inhibit LPS responses in the presence of high concentrations of LBP. This could be due to the fact that the pep27–39 sequence within the SAP molecule appears not to be readily available, as it is partly situated on a β-strand under a short α-helix (14). Once liberated from the rest of the protein, pep27–39, better than SAP, competes with LBP for binding to LPS. Another explanation might be the fact that SAP is a rather large protein. Therefore, pep27–39, only 13 amino acids in size, will be more capable than SAP in binding all LPS molecules in an LPS aggregate, and thereby more efficiently shield them from the action of LBP.

Until now, we described the effects of SAP on LPS binding to membrane-bound CD14 (mCD14). Using the ability of FITC-LPS to increase its fluorescence signal and shift its retention time via FITC-LPS binding to rsCD14, we showed that SAP was not able to prevent FITC-LPS binding to rsCD14, even in the absence of LBP, while it could inhibit binding of LPS to mCD14. sCD14, just like LBP, acts as a lipid transfer molecule. In contrast to mCD14, sCD14 does not need LBP to bind LPS, although LBP accelerates binding of LPS to sCD14 (32). We could also demonstrate this accelerated LPS binding to sCD14 by LBP in our gel-filtration assay, as the fluorescence peak in the presence of LBP was about fivefold higher as compared with the fluorescence peak when no LBP was added. As SAP was not able to interfere in the binding of LPS to rsCD14, we hypothesize that rsCD14 competes with SAP for binding to LPS, just as LBP does. The finding that pep27–39 is still capable of interfering with the binding of LPS to rsCD14 strengthens this hypothesis. We can thus conclude that SAP competes with both LBP and sCD14 for binding to LPS, but that SAP-derived peptide pep27–39 is capable of preventing LPS binding to mCD14 as well as sCD14.

We discovered SAP as a novel LPS-binding protein in human plasma. As SAP did not neutralize LPS responses in human blood, its role in the pathophysiology of Gram-negative infections has yet to be elucidated. However, a 13-mer peptide, pep27–39, derived from SAP was found to bind to LPS. Its carboxamidomethylated form was even more potent in binding to LPS. The ability of pep27–39 to antagonize specifically the effects of LPS in the complex environment of human blood suggests that pep27–39 may be a novel therapeutic agent in the defense against Gram-negative sepsis.

We are currently investigating the capacity of other SAP-derived peptides to bind and neutralize LPS.

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