Structural Basis for Interactions between Lung Surfactant Protein C and Bacterial Lipopolysaccharide*

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In the respiratory tract, recognition of bacterial endotoxin (lipopolysaccharide, LPS) is a critical step of the innate host defense system directed against invading pathogens. Secretions of the airways contain proteins that have direct antimicrobial activity (lysozyme, lactoferrin, defensins, and cathelicidins) as well as complement factors and surfactant proteins that contribute to host defense. The hydrophobic surfactant protein C (SP-C) recognizes LPS (Augusto, L., Le Blay, K., Auger, G., Blanot, D., and Chaby, R. (2001) Am. J. Physiol. 281, L766–L785). In the present study, using synthetic analogs of SP-C, we demonstrate that the palmitoyl residues of SP-C are not required for the interaction with LPS and that both the hydrophilic and hydrophobic regions of SP-C are required for specific binding of a radiola- beled rough-type LPS. In addition, using LPS submitted to different chemical treatments as well as synthetic analogs of the lipid A moiety of LPS, we established that the terminal phosphate group at the reducing end of the lipid A disaccharide in configuration is of crucial importance for recognition by SP-C. The N-linked fatty acyl chain on the reducing glucosamine of lipid A also takes part in the interaction. Dipalmitoyl phosphatidylcholine is not specifically required for the LPS-binding activity of SP-C, although a lipid environment significa ntly increases the binding. These results provide a basis for experiments on the role of SP-C in presentation of LPS to alveolar cells and for the design of drugs for the management of endotoxin-induced lung injury.

Lungs are constantly exposed to a great number of microorganisms and represent a common route of entry of pathogens into the body. Infection of the lung is one of the most frequent causes of morbidity and death. Many pathogens also release constituents or produce factors that further damage the host defense system, thus facilitating spread of the organisms. The lipopolysaccharide (LPS), also termed endotoxin, of the outer membrane of Gram-negative bacteria can initiate a cascade of biochemical and cellular events leading to multiple organ dysfunction and death (1). The lung is often involved in such organ failure, which can lead to acute respiratory distress syndrome with a high (over 50%) mortality rate (2).

To prevent these deleterious effects, the host has developed an innate, non-adaptive defense system, which includes factors that detect bacterial products and prepare their recognition by cellular receptors, and a family of pattern-recognition receptors that interact with common bacterial motifs. Some of the recognition molecules are present on the surface of different cell types (CD14, TLR2, and TLR4), whereas others are soluble proteins (LBP, conglutinin, CL-43) (3). In the lungs, the alveolar fluid contains regulatory molecules that are capable of sensing the environment and amplifying or dampening inflammatory responses. Lung surfactant, which covers the epithelial lining of the alveoli, contains several components that are active participants in the innate defense system. Two hydrophilic components, SP-A and SP-D, are lung collectins that can modulate the function of phagocytic cells (4). SP-A and SP-D can interact with a wide range of microorganisms and their components (endotoxin, lipoteichoic acid, and mannans), and a role in pattern recognition has been proposed for these collectins (5). Particularly, it has been shown that both SP-A and SP-D can bind LPS (6, 7). SP-A- and SP-D-deficient mice have grossly normal lung function and longevity (8–10), but SP-A null mice are more susceptible to lung infections than wild type mice (11).

The hydrophobic surfactant protein SP-B is mainly involved in the surface tension-lowering activity of pulmonary surfactant. Inherited deficiency of SP-B in human infants (12) and knockout of SP-B in mice (13) inevitably result in severe respiratory distress syndrome and death. The second hydrophobic surfactant protein, SP-C, is produced exclusively by type II alveolar epithelial cells, whereas SP-A, SP-B, and SP-D can also be produced by non-ciliated bronchiolar epithelial (Clara) cells (14), SP-A is expressed in rat intestine (15), SP-D is found on other extra-pulmonary mucosal surfaces (16), and SP-A and SP-D are expressed in the Eustachian tube (17).

Characterization of the structure of SP-C has revealed many uncommon features (18), but the function of this peptide in vivo remains unclear. SP-C null mice show no signs of respiratory dysfunction, but the resistance to lung infections was not tested in the initial report (19). SP-C may play an important role in the defense capacities of pulmonary surfactant, because its presence in aerosolized surfactant improves pulmonary function in endotoxin-induced lung injury (20). In this connection, we demonstrated recently (21) that lipopolysaccharides of different bacterial origins interact with lipid vesicles containing SP-C and that this binding is saturable, temperature-dependent, and related to the concentrations of SP-C and LPS. In this study, our goals were to characterize which regions of LPS and...
SP-C are involved in this interaction and to determine the role of a lipid environment in the binding of the two molecules.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Synthetic dipalmitoyl-L-α-phosphatidylcholine, egg yolk L-α-phosphatidylcholine (type XV-E), cholesterol, tripalmitin, bovine serum albumin, porcine trypsin, 8-anilino-1-naphthalenesulfonic acid (ANS), and LPS from *Salmonella minnesota* (rough mutant Re595). LPS from *S. minnesota* Re595 was oxidized (150 min, 20 °C) with sodium periodate (3 × 10⁻² M). After destruction of the oxidant with 1 M ethyleneglycol, aldehyde groups were reduced (18 h at 4 °C) with an ice-cold solution of NaBH₄ (0.46 GBq, 481 GBq/mmol) in 200 μl of ice-cold borate buffer (0.05 M acetic acid, pH 9.5). Excess sodium borohydride was destroyed with 5 μl of acetic acid. After two washings (centrifugation at 100,000 g for 10 min) in 400 μl of an ice-cold-water-ethanol mixture (1:1, v/v), the radiolabeled [3H]LPS (9 × 10⁶ cpm/μg, 2 × 10⁷ cpm/pmol) was stored at −20 °C until use. Nitric oxide production induced by 2 and 5 ng of this radiolabeled material in mouse macrophages was not significantly different from that induced by the same concentrations of unlabeled LPS, indicating that the bioactivity of the LPS was not modified by the radiolabeling procedure.

**Purification of SP-C—**Mouse and rabbit SP-C were prepared as described previously (21). Briefly, crude surfactant was isolated from the bronchoalveolar lavage of 5-10-week-old Swiss mice, or adult rabbits, on a NaCl/NaBr density gradient, as described by Katyal et al. (23). To determine if this observation can be extended to SP-C from other origins, rabbit SP-C was isolated from Merck (Darmstadt, Germany). The liquid scintillation reagent Aqualyte was from Amersham Biosciences (Buckinghamshire, England). All HPLC solvents (LiChrosolv grade) were from Merck (Darmstadt, Germany). The liquid scintillation reagent Aquasol was from Baker (Deventer, The Netherlands).

**Preparation of Tritium-labeled LPS—**The labeling was done by a modification of the procedure of Watson and Riblet (22): A sample (2 mg) of LPS from *S. minnesota* Re595 was oxidized (150 min, 20 °C) with sodium periodate (3 × 10⁻² M). After destruction of the oxidant with 1 M ethyleneglycol, aldehyde groups were reduced (18 h at 4 °C) with an ice-cold solution of NaBH₄ (0.46 GBq, 481 GBq/mmol) from Amersham Biosciences (Buckinghamshire, England). The liquid scintillation reagent Aquasol was from Baker (Deventer, The Netherlands).

**Critical Micelle Concentration of Synthetic Glycolipids—**The ability of 8-anilino-1-naphthalenesulfonic acid (ANS) to become highly fluorescent in the presence of a methyl palmitate standard (from Sigma Chemical Co.).

**Fluorometric Estimation of Amino Acids—**Samples (200 pmol) of natural SP-C and synthetic peptides, mixed with synthetic PC and recovered at the top (1 ml) of the density gradient used in the LPS binding assay, were centrifuged for 90 min at 100,000 g to remove salt. Salts were lyophilized and hydrolyzed (105 °C, 72 h) in 400 μl of 6 M HCl. After evaporation under vacuum, the dried material was dissolved in 100 μl of a 1 M borate buffer (pH 10.6). Amino acids in the hydrolysates were estimated by reaction with a fluorogenic reagent (32). Fractions (10 μl) of these samples were added to borate buffer (40 μl) consisting of 5 M Triethylamine and 75 mM NaCl (pH 7.4) and removal of phospholipids by extraction with a mixture of diisopropyl ether/1-butanol (3:2, v/v), the hydrophobic surfactant components were isolated at the interface. SP-C was isolated from this mixture by reverse-phase HPLC.

Porcine SP-C was prepared as described previously (24, 25). Briefly, minced porcine lungs were washed with saline, and the mixture was filtered through 50 μm of cells and debris. The surfactant was pelleted by centrifugation. The pellet was extracted with chloroform/methanol (2:1, v/v), and the lipid extract obtained was then purified by chromatography over Lipidx 5000 in ethylene chloride/methanol (1:4, v/v), Sphadex LH-60 in chloroform/methanol/0.1 M HCl (19:18:2, v/v), and finally by reverse-phase HPLC over a C₁₈ column, using aqueous ethanolic or methanolic mobile phase and a linear gradient of 2-propanol for elution.

**Reverse-phase HPLC—**Samples of the hydrophobic material extracted from mouse or rabbit surfactant (five mice or 0.2 rabbit equivalents) were dissolved in 100 μl of solvent A (0.2% trifluoroacetic acid and 75% methanol in water) and applied on a C₁₈ column from Waters (μBondapak C₁₈, 10 μm, 300 × 3.9 mm). Analysis was carried out at a flow rate of 0.7 ml/min, first with solvent A for 10 min and then with a linear gradient (2.5% per min for 30 min) of solvent B (0.1% trifluoroacetic acid in 2-propanol) in solvent A. Absorbance of the effluent was monitored at 225 nm.

**Synthetic Lipid A Analogs and Peptides—**The syntheses of the monooctasaccharide-derivated lipid A analogs, performed by D. Charon and M. Mondhare, have been described previously (26). Synthetic analogs of human and mouse SP-C were prepared as described earlier (27–31). Briefly, peptides were synthesized by t-Boc chemistry, cleaved from the synthesis resin with hydrogen fluoride, and purified with the reverse-phase HPLC system described by Gustafsson et al. (25). Palmitoylation of the serine hydroxyl groups of SP-C(Leu) was performed by addition of palmitoyl chloride to the peptide dissolved in neat trifluoroacetic acid, quenching with aqueous NH₄OH, removal of excess acid by chromatography over Lipidx 5000 in ethylene chloride/methanol (1:4, v/v), and final peptide purification by reverse-phase HPLC (30, 31).

**LPS Binding Assays—**Binding to SP-C inserted into lipid vesicles was performed as described previously (21): Glass tubes containing phosphatidylcholine (PC) (0.3 mg) alone or mixed with SP-C or peptid analogs were evaporated to dryness. After sonication with a solution of BSA (60 μl, 1 mg/ml in 0.15 M NaCl), [3H]LPS (3.6 × 10⁶ cpm, 290 μl in 0.15 M NaCl) was added and the mixture was incubated (2 h, 20 °C) under gentle rotation. The mixture was adjusted to 1.185 g/ml by addition of 350 μl of a solution of 1.1% NaCl and 46% NaBr. A discontinuous gradient was prepared by sequential addition of 23% NaCl (0.7 ml), the sample mixture (0.7 ml, 20% NaCl (0.7 ml), and 8% NaCl (0.2 ml). After centrifugation (65,000 × g, 90 min), the radioactivity of collected fractions was determined by liquid scintillation. Vesicles with bound radiolabeled LPS were recovered at the top (1 ml) of the density gradient, whereas unbound [3H]LPS was found at the bottom. The LPS binding index (LBI) of a compound is defined as the radioactivity recovered at the top (1 ml) of the density gradient, expressed as a percentage of the total radioactivity recovered. A second LPS binding assay based on the binding of [3H]LPS to SP-C-coated plates was also designed: Solvent-resistant (polypropylene) microplates were coated with a mixture of trypsin and cholesterol (100 μg/well each). On the dried film, a solution (100 μl) of SP-C(LKS) (200 pmol) in a chloroform/methanol (1:1) mixture was added. After evaporation, the film was incubated at room temperature with [3H]LPS (360,000 cpm), in the presence or absence of unlabeled LPS, in a binding medium (100 μl) containing bovine serum albumin (50 μg) in saline. After 3 h of incubation, the plates were washed three times with 100 μl of saline, and the remaining bound radioactivity was measured.

**GLC Assay of Palmitate**—The amount of palmitate released from SP-C by methyl palmitate treatment was determined by gas-liquid chromatography. After incubation (150 min, 37 °C) of 80 pmol of murine SP-C (one mouse equivalent) with 0.6 μl triethylamine and 300 μl of a chloroform/methanol mixture (1:1, v/v), the pH was adjusted to 2 with HCl. The mixture was dried, and free fatty acids were methylated with diazomethane and analyzed by gas-liquid chromatography on an HP-5 capillary column (30 m × 0.32 mm). A two-step temperature gradient starting at 130 °C (3 °C/min for 10 min, followed by 4 °C/min for 20 min) was used. The amount of methyl palmitate was determined by comparison of the integrated area of the corresponding peak with that of a methyl palmitate standard (from Sigma Chemical Co.).

**RESULTS**

**SP-C from Different Species Binds LPS—**We reported previously that mouse SP-C can interact with bacterial lipopolysaccharides (21). To determine if this observation can be extended to SP-C from other origins, rabbit SP-C was isolated from surfactant recovered from bronchoalveolar lavage of rabbit lung and purified by HPLC (compound eluted between 26 and 28 min), as described for the preparation of mouse SP-C. Porcine SP-C was prepared from minced porcine lungs by chromatography in organic solvents (24). Fractions of these compounds giving HPLC peaks of similar intensities (167 pmol according
to quantitative amino acid analysis of mouse SP-C) were incorporated into PC/BSA vesicles, and the binding of \(^{3}H\)LPS to the vesicles was measured as described previously (21). We found that the LPS binding indexes of 167 pmol of mouse, rabbit, and porcine SP-C were of 80.4 ± 5.1%, 79.5 ± 6.0%, and 66.1 ± 9.1%, respectively. These results show that LPS can interact with SP-C produced by different animal species.

**Influence of Chemical Treatments of \(^{3}H\)LPS on the Binding to Mouse SP-C**—Tritium-labeled LPS was submitted to chemical treatments generally used for selective removal of particular residues such as mild alkaline, hydrazine, and aqueous hydrogen fluoride. Karibian et al. (34) established that fatty acyl chains linked at the C3 and C3' positions of lipid A are more labile than esters at other positions and can be selectively released by a mild alkaline treatment (0.25 M NaOH at 37 °C for 10–25 min depending on the LPS used). Treatment with hydrazine (37 °C, 30 min) is often used to remove selectively all ester-linked fatty acids (35, 36), and treatment with aqueous HF (4 °C, 48 h) is classically used to completely dephosphorylate LPS preparations (36, 37). To examine the influence of fatty acid and phosphate residues of LPS on the interaction between LPS and SP-C, \(^{3}H\)LPS was submitted to the three treatments mentioned above, and the binding of the resulting LPS derivatives to PC/BSA vesicles containing SP-C was determined. The results (Table I) show that LPS was still fully recognized by SP-C after removal of the fatty acyl chains at C3 and C3'. The interaction was reduced, but persisted at rather high levels, after complete removal of ester linked fatty acids. In contrast, LPS dephosphorylated by HF treatment was no more recognized by SP-C.

**The Palmitoyl Residues of Mouse SP-C Are Not Required for Binding LPS**—One specific feature of SP-C is that it contains two palmitoyl chains covalently attached via S-ester bonds to cysteine residues of the polypeptide chain. S-ester bonds are easily cleaved by mild alkaline treatment (38). To determine whether the SP-C palmitoyl residues play a role in the interaction with LPS, HPLC-purified mouse SP-C (2 mouse equivalents) was incubated for 150 min at 37 °C with 300 μl of 0.6 M triethylamine in a chloroform-methanol (1:1, v/v) mixture (lower panel) or deacylated by incubation (150 min, 37 °C) with 300 μl of 0.6 M triethylamine in a chloroform-methanol (1:1, v/v) mixture (upper panel), were analyzed by HPLC as described under “Experimental Procedures.”

![Fig. 1. Analysis of deacylated mouse SP-C. Samples of HPLC-purified mouse SP-C (2 mouse equivalents), either untreated (upper panel) or deacylated by incubation (150 min, 37 °C) with 300 μl of 0.6 M triethylamine in a chloroform-methanol (1:1, v/v) mixture (lower panel), were analyzed by HPLC as described under “Experimental Procedures.”](image)

**TABLE I Interacting Structures of LPS and SP-C**

| Vesicles of PC/BSA | Treatment of \(^{3}H\)LPS | LPS binding index |
|-------------------|-------------------------|------------------|
| Without SP-C      | None                    | 9.6 ± 1.2        |
| With 60 pmol of SP-C | None                | 82.6 ± 5.5       |
| 0.25 M NaOH, 37 °C, 25 min | 71.3 ± 5.8         |
| 95% Hydrazine, 37 °C, 30 min | 51.6 ± 4.0         |
| 48% HF, 4 °C, 48 h   | 11.2 ± 0.1            |

...continued
the amino acid content of the recovered fractions, determined and recovered at the top of the density gradient, as assessed by C(LKS) and SP-C/BR, also bind LPS. It should be noted that groups of SP-C do not play a role in the LPS-binding capacity of the results mentioned in the preceding paragraph, the palmitoyl residues, the last 13 amino acids of the polypeptide, play a critical role in LPS binding. In contrast, vesicles containing even lower amounts (200 pmol) of peptides SP-C(Leu) and SP-C(LKS), which contain the hydrophobic region, bind LPS as efficiently as those with natural porcine SP-C. Because SP-C-(1–21) was the only synthetic peptide that did not bind LPS when used at the same concentration as that the four others, we analyzed vesicles in which higher amounts of this peptide were incorporated.

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The results in Fig. 6 show that, in addition to the homologous LPS, only two of the seven glycolipids, PPDm2B and M13, induced a high level of inhibition (over 60%) of the binding of \(^{3}H\)LPS to SP-C(LKS). Compound M6, which is structurally similar to the non-reducing moiety of lipid A (with a glucosamine phosphorylated on C4), is a poor inhibitor. Ester-linked fatty acids do not seem to play a determinant role, because the inhibition potency of M6 is not significantly higher than that of M19g (with only two O-acyl chains) and P4Dm2 (without O-acyl chains). Compound PPDm2B, which mimics both the reducing (glucosamine 1-phosphate) and the non-reducing (glucosamine 4-phosphate) moieties of lipid A, is the most efficient inhibitor. It appears, however, that the phosphate group on C1 plays a determinant role, because compound P4Dm2, which bears an O-methyl group instead of the C1 O-phosphate residue, is inactive. This is confirmed by the observation that the disaccharide-based glycolipid D2, which is not phosphorylated on C1, is also a poor inhibitor. Therefore, it clearly appears from these data that the terminal glucosamine 1-phosphate group of the LPS molecule is a major binding site of SP-C(LKS).

Another important feature is the \(\alpha/\beta\) anomery of the glucosamine residue: at 230 nmol/ml, compound M13, the \(\alpha\) anomer of N-acylated glucosamine 1-phosphate, exhibits high inhibition potency, whereas compound M14, its \(\beta\) anomer, is completely unable to interact with SP-C(LKS) (Fig. 6A). To confirm the importance of the anomery of glucosamine, the inhibition potencies of M13 and M14 were re-examined at different concentrations. We found (Fig. 6B) that at 115 and 230 nmol/ml, M13 inhibited the binding of \(^{3}H\)LPS (37 and 60% inhibition, respectively), whereas M14 did not (0 and 2% inhibition, respectively). Three other phosphorylated sugars were also examined: \(\alpha\)-D-glucose-1-phosphate, \(\beta\)-D-glucose 1-phosphate, and \(\alpha\)-D-N-acetylglucosamine 1-phosphate. We found (Fig. 6B) that the \(\alpha\) and \(\beta\) anomers of glucose 1-phosphate are both inactive. On the other hand, the \(\alpha\) anomer of N-acetylglucosamine 1-phosphate exhibits a significant inhibitory activity, although lower than that of compound M13. Two conclusions can be drawn from this observation. The first is that a \(\beta\)-phosphate residue and a vicinal R-CO-NH group are both required for the interaction with SP-C, and the second is that a long N-acyl chain (as that of the 14-carbon fatty acid of compound...
M13 is more efficient than a short one (as in the 2-carbon acetyl group of \( \alpha\)-N-acetylglucosamine 1-phosphate).

Because LPS and synthetic partial structures derived thereof contain a hydrophilic and a hydrophobic region, it was important to determine if the amphipathic properties of these molecules play a role in the interaction with SP-C. One of the best parameters describing this property is the critical micelle concentration (CMC), defined as the concentration over which the molecules arrange as micelles. CMC, which is directly related to the surface tension of the solution and, thus, to the surfactant properties of the compounds, can be measured by a fluorometric method based on the property of ANS embedded in the different glycolipids during the incubation of \(^{3}H\)LPS with void vesicles of PC does not significantly modify the nonspecific binding of LPS itself, the three structural analogs of lipid A were detected at the bottom of the gradient and did not form floating vesicles (data not shown). Furthermore, we found that the presence of the different glycolipids during the incubation of \(^{3}H\)LPS with vesicles of PC does not significantly modify the nonspecific binding of the radiolabeled ligand (data not shown). These results assess that the inhibition of the binding of \(^{3}H\)LPS to SP-C(LKS) induced by the competitors, are the mean ± S.D. of duplicates.

**FIG. 6. Influence of synthetic glycolipids and carbohydrate derivatives on the binding of \(^{3}H\)LPS to SP-C(LKS).** Vesicles of PC/BSA (300 μg/ml) containing 84 pmol of SP-C(LKS) were preincubated (2 h, 20 °C) with a fixed concentration (230 nmol/ml) \( \alpha\)-acetylglucosamine 1-phosphate \((\alpha\)-acetylglucosamine 1-phosphate \( \beta\)-1-phosphate derivative) and M6 (4-phosphate derivative) were poor inhibitors. Therefore, to determine if a phosphorylated lipid is required, or if LPS binding can occur in the presence of a non-phosphorylated lipid, or even in the absence of any lipid in the environment. To address this question we designed a new LPS binding test on solvent-resistant (polypropylene) microplates. In the first experiment the wells, uncoated or coated with a tripalmitin-cholesterol mixture (100 μg each), were

**Because the most efficient inhibitors, PPDm2B and M13, were active at concentrations higher than their CMCs and, thus, as micelle suspensions, it was important to determine the position of these micelles after the density gradient used to remove unbound \(^{3}H\)LPS. Suspensions of PPDm2B, M13, and M14 (4 mM, 350 μl) were centrifuged in a NaCl/NaBr gradient containing 200 μl ANS, and vesicles were localized by the fluorescence of collected fractions. We found that similar to LPS itself, the three structural analogs of lipid A were detected at the bottom of the gradient and did not form floating vesicles (data not shown). Furthermore, we found that the presence of the different glycolipids during the incubation of \(^{3}H\)LPS with void vesicles of PC does not significantly modify the nonspecific binding of the radiolabeled ligand (data not shown). These results assess that the inhibition of the binding of \(^{3}H\)LPS to floating SP-C(LKS)-loaded vesicles, induced by the presence of glycolipids, is really due to their competitive interaction with SP-C(LKS).

**Interaction between Synthetic Analogs of Lipid A and Mouse SP-C.—To assess that the results obtained with the synthetic peptide SP-C(LKS) apply to SP-C of natural origin, we performed similar inhibition experiments with vesicles bearing HPLC-purified mouse SP-C. We restricted the study to one synthetic analog of the lipid A region that is phosphorylated on C4 (compound M6) and one that is phosphorylated on C1 (compound M13). The \( \beta\) anomer of M13 (compound M14) was also used for comparison. The results (Table III) were very similar to those obtained with SP-C(LKS); natural SP-C interacts mainly with N-acetylated glucosamine bearing an \( \alpha\)-1-phosphate group. Compounds M14 (\( \beta\)-1-phosphate derivative) and M6 (4-phosphate derivative) were poor inhibitors.

**Role of a Phospholipid Environment in the Interaction between LPS and SP-C—**In all the experiments described above, we measured the interaction of LPS with vesicles containing SP-C and phosphatidylcholine (PC). Because the phosphate group of LPS appears to play an important role in the binding, we wished to examine the role of PC (which also contains a phosphate group) and to determine if a phosphorylated lipid is required, or if LPS binding can occur in the presence of a non-phosphorylated lipid, or even in the absence of any lipid in the environment. To address this question we designed a new LPS binding test on solvent-resistant (polypropylene) microplates. In the first experiment the wells, uncoated or coated with a tripalmitin-cholesterol mixture (100 μg each), were
TABLE III

Influence of unlabeled glycolipids on the binding of [3H]LPS to mouse SP-C

Vesicles of PC/BSA (300 μg/60 μg) containing 84 pmol of HPLC-purified mouse SP-C were preincubated (2 h, 20 °C) with or without different unlabeled glycolipids (230 nmol/ml, 350 μl). The vesicles were then reincubated (2 h, 20 °C) after addition of 21 μg/ml [3H]LPS (6.7 × 10^5 cpm). The radiolabeled LPS bound to the vesicles was recovered at the top (1 ml) of an NaCl/NaBr gradient. The LBI value obtained with radiolabeled LPS bound to empty vesicles of PC/BSA was 9.6 ± 1.2%. Results are the mean ± S.D. of duplicates.

| Unlabeled compound | LPS binding index |
|--------------------|------------------|
| None               | 82.6 ± 5.5       |
| M6                 | 59.9 ± 0.4       |
| M13                | 31.3 ± 1.4       |
| M14                | 67.0 ± 0.7       |

A fourth experiment, carried out on a film of neutral lipids in the presence of increasing amounts of unlabeled LPS (2 μg/ml) inhibited efficiently the binding of [3H]LPS (210,000 cpm) alone (A) and in the presence or absence of unlabeled LPS (100 μg) (B). Bound radioactivity remaining on the plates after three washings with 100 μl of saline was determined. Results are the mean ± S.D. of triplicates.

FIG. 8. Role of neutral lipids on the binding of LPS to SP-C(LKS)-coated plastic. Polypropylene wells were coated with SP-C(LKS) (various amounts in panel A; 200 pmol in panel B) in the presence or absence of a tripalmitin-cholesterol mixture. The dried film was incubated for 3 h at room temperature with [3H]LPS (360,000 cpm) alone (A) and in the presence or absence of unlabeled LPS (100 μg) (B). Bound radioactivity remaining on the plates after three washings with 100 μl of saline was determined. Results are the mean ± S.D. of triplicates.

coated again with SP-C(LKS) (200 pmol). The dried film was incubated for 3 h at room temperature with [3H]LPS, in the presence or absence of an excess of unlabeled LPS (100 μg). After three washings, the remaining bound radioactivity was measured. The results in Fig. 8A show that, with or without a film of neutral lipids, [3H]LPS binds to the SP-C(LKS)-coated wells and that the binding of LPS is dose-dependently related to the amount of SP-C(LKS). The saturation values of the two curves were similar, suggesting that similar amounts of SP-C(LKS) are bound to the wells in the presence or absence of neutral lipids. This was confirmed by amino acid estimation of bound SP-C(LKS); after addition of 200 pmol of the peptide into the wells, we found that 200 ± 37 pmol and 194 ± 10 pmol were bound in the presence or absence of neutral lipids, respectively. In both cases, a specific binding (inhibited by 100 μg of unlabeled LPS) was observed (Fig. 8B). It is noteworthy that inhibition was higher in the presence of the neutral lipid mixture, although the amounts of coated SP-C(LKS) and of [3H]LPS were similar. This means that the specific binding of LPS to SP-C(LKS) is markedly enhanced in a lipid environment. Using this technique, we also examined the LPS binding to SP-C(1–21). In line with the data in Fig. 3, we found again that LPS binding can be observed only with high amounts (>400 pmol) of SP-C(1–21) (data not shown), confirming the important role of the C-terminal region of SP-C in the interaction with LPS.

In a third set of experiments performed in the environment of neutral lipids, it was found that the binding of LPS to different amounts of SP-C(LKS) is not affected by the presence of PC, a phospholipid that is abundant in natural surfactant (Fig. 9).

A fourth experiment, carried out on a film of neutral lipids in the presence of increasing amounts of unlabeled homologous LPS (Fig. 10), indicated that even low concentrations of unlabeled LPS (2 μg/ml) inhibited efficiently the binding of [3H]LPS to SP-C(LKS). Overall, these experiments suggest that LPS can bind directly and specifically to SP-C(LKS), that this specific binding is increased in a lipid environment, and that PC is neither required nor unfavorable for binding.

DISCUSSION

In a previous study (21) we set up a procedure for analysis of the binding of LPS to hydrophobic peptides associated with lipid vesicles, and we demonstrated that the hydrophobic component SP-C of mouse pulmonary surfactant interacts specifically with LPS. It was then important to identify the structural elements of SP-C and LPS that are involved in the interaction between these molecules. In the first part of this study, we show that the binding of LPS is not restricted to murine SP-C but occurs also with porcine and rabbit SP-C and with synthetic peptide analogs of porcine and human SP-C. This suggests that the phenomenon is of general physiological relevance. Using the synthetic peptides, we have also shown that the capacity to bind LPS is independent of the two palmitoyl residues of SP-C and requires both the hydrophilic region and the C-terminal hydrophobic region of the peptide. Native SP-C, the leucine-substituted full-length analogs thereof, and SP-C/BR all contain a C-terminal helix with a size that is suited to traverse a phospholipid bilayer (27, 28, 40, 41), whereas SP-C(1–21) is too short to span a phospholipid bilayer. A possible explanation for the observed LPS-binding capacities of the different SP-C peptides is that the N-terminal region, which contains polar and basic residues (Fig. 2), is involved in peptide-LPS interactions whereas the C-terminal non-polar region is important for correctly localizing the peptide in a phospholipid bilayer.

In the second part of this study we focused on the structure of LPS to identify which structural elements of this ligand are involved in the interaction with SP-C. Using chemically modified lipopolysaccharides and synthetic lipid A analogs, we established that the phosphate residue at position 1 of the reducing glucosamine unit, when present in the α configuration, is required for an interaction with SP-C. We have also shown that...
the N-linked fatty acid chain present on the same glucosamine unit takes a small part in LPS/SP-C interactions. On the other hand, as shown with synthetic lipid A analogs, the amphipathic character of the LPS molecule seems irrelevant. These results have some analogies with those reported for other proteins that bind LPS. Particularly, the prominent role of the charged phosphates of LPS for its interaction with various proteins has been recognized. This is particularly the case with polymyxin B, lysozyme, the limulus anti-LPS factor, and with the two structurally related mammalian proteins that share high affinity binding to LPS: bactericidal/permeability-increasing protein and lipopolysaccharide-binding protein. For example, polymyxin B and lysozyme bind electrostatically to the phosphate groups of lipid A, as shown by analysis of phosphate vibration (42). Concerning LPS-binding protein and bactericidal/permeability-increasing protein, the interactions of these proteins with LPS seems to be a complex multisystem process involving, on one hand, the lipid-binding pockets of these molecules (which can accommodate the apolar moiety of LPS but with little specificity) and, on the other hand, positively charged groups (between residues 90 and 103) (43) leading to electrostatic interactions with phosphorylated sugar groups of LPS (44).

Concerning SP-C, positive charges in the hydrophilic region of the peptide are probably involved. However, the interaction with the 1-phosphate group of LPS is certainly not exclusively electrostatic, because the spatial position of the phosphate is of crucial importance (absence of interaction with β-glucosamine 1-phosphate or glucosamine 6-phosphate derivatives). The importance of the anomeric configuration of glucosamine may rely on the distance between the phosphorus and nitrogen atoms. In the α anomer, their spatial proximity allows an interaction that maintains a short distance between these two atoms. This is impossible in the β anomer where the distance between phosphorus and nitrogen is much larger (Fig. 11). Therefore, SP-C may recognize a molecular substructure consisting of a close spatial arrangement of nitrogen and phosphorus, similar to that of phosphoramidates.

The 1-phosphate group of the reducing glucosamine unit, which is required for the binding to SP-C, is also involved in many of the pathophysiological effects of LPS. It has been suggested by Kirikae et al. (45) that the fatty acid chains of lipid A are critical for induction of cellular responses such as production of cytokines, whereas the phosphorylated disaccharide headgroup is essential for binding to cell receptors. Furthermore, in vivo studies have shown that dephosphorylation of LPS by endogenous alkaline phosphatase contributes to a better survival of rats in a septicemia model (46) and that the absence of the terminal phosphate group from the reducing end of the lipid A disaccharide abolished its ability to cause pulmonary disorders in guinea pigs after inhalation (47). Therefore, the interaction of SP-C with this particular phosphate group may block or modify some of the physiological responses to LPS in the lungs.

In the third part of this study, we addressed the question of a possible role of dipalmitoylphosphatidylcholine (DPPC), a major component of lung surfactant, in the interaction between SP-C and LPS. Because our LPS-binding test was performed on vesicles containing DPPC and because the phosphate group of DPPC can interact with SP-C, it was conceivable that such associations may influence the vesicle association of the peptide and hence the binding to LPS. By a technique based on the adsorption of SP-C analogs on plastic wells, we found phospholipids are not required, and a direct binding of LPS to SP-C can occur in the absence of any additional molecule. However, a better binding was observed in a lipid environment, obtained by addition of neutral lipids. Because LPS and SP-C are both amphipathic molecules, the role of a lipid environment could be to concentrate and provide an optimal presentation of the two molecules.

The physiological importance of the interaction between...
SP-C and LPS in the alveolar space remains to be established. One possibility is that the role of SP-C is to neutralize inhaled LPS. Another hypothesis is that SP-C could act as a shuttle and allow the binding of LPS to appropriate receptors on alveolar cells. Although no interaction between SP-C and alveolar macrophages has been described so far (48), a receptor involved in recycling of SP-C is present on type-2 epithelial pneumocytes (49). These different hypotheses deserve close scrutiny in further studies.

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