Binding of surfactant protein A to the lipid A moiety of bacterial lipopolysaccharides

J. Freek VAN IWAARDEN,*†‡, Jacqueliën C. PIKAAR,*†, Janet STORM,* Ellen BROUWER,† Jan VERHOEF,† Ronald S. OOSTING,*† Lambert M. G. VAN GOLDE* and Jos A. G. VAN STRIJP†

*Laboratory of Veterinary Biochemistry, P.O. Box 80176, 3508 TD Utrecht, The Netherlands and †Eijkman–Winkler Laboratory of Medical Microbiology, Utrecht University, Utrecht, The Netherlands

Surfactant protein A (SP-A) enhances the phagocytosis of opsonized and non-opsonized bacteria by alveolar macrophages, but it is not known with which component of the bacterial surface it associates. We investigated the interaction of SP-A with lipopolysaccharides (LPS), which are important biologically active constituents of the outer membranes of Gram-negative bacteria. Flow cytometry was used to study the binding of fluorescein isothiocyanate-labelled SP-A either to LPS of various chain lengths coupled to magnetic beads or to Gram-negative bacteria. The binding of SP-A to LPS-coated beads was saturable, both time- and concentration-dependent, and required both Ca^{2+} and Na^+ . SP-A bound to the lipid A moiety of LPS and to LPS from either the Re-mutant of *Salmonella minnesota* or the

J5-mutant of *Escherichia coli*. In contrast, it did not bind to O111 LPS of *E. coli*, suggesting that SP-A binds only to rough LPS. The binding of SP-A to LPS was not affected by mannan and heparin or by deglycosylation of the SP-A, indicating that the carbohydrate-binding domain and the carbohydrate moiety of SP-A are not involved in its interaction with LPS. We also observed saturable and concentration-dependent binding of SP-A to the live J5 mutant of whole *E. coli*, but not to its O111 mutant. In addition, Re LPS aggregated in the presence of SP-A, Ca^{2+} and Na^+ . We conclude that SP-A associates with LPS via the lipid A moiety of rough LPS and may be involved in the anti-bacterial defences of the lung.

INTRODUCTION

Pulmonary surfactant protein A (SP-A), one of the most abundant proteins of pulmonary surfactant, is synthesized and secreted by bronchiolar Clara cells and alveolar type-II cells [1–4]. Under denaturing and reducing conditions the relative molecular mass of SP-A is 28000–35000, depending on the degree of glycosylation [3]. A short N-terminal region of 7–10 amino acids is followed by 72 amino acids in a collagen-like sequence [5]. The remaining C-terminus, which consists of 149 amino acids, has sequence similarity with a number of Ca^{2+} -dependent lectins such as surfactant protein D (SP-D) and the plasma proteins mannose-binding protein and conglutinin [1,6,7]. The native form of SP-A is assembled into a complex of approx. 18 similar subunits [8–10] resembling the structure of C1q of the complement system [8]. The function of SP-A in the alveolar space is not known with certainty, but studies *in vitro* have implicated SP-A to be involved in (i) the formation of tubular myelin in the presence of phospholipids, Ca^{2+} and surfactant protein B [11]; (ii) the adsorption of surfactant lipids to an air–liquid interface [12,13]; and (iii) the recycling of surfactant by type-II cells [14,15]. Due to its lectin property of binding to mannose residues [16] and its structural similarities with C1q, conglutinin and mannose-binding proteins, which play a role in the defence system in plasma [17–21], SP-A may have a similar function in the alveoli. Indeed SP-A has been reported (i) to stimulate alveolar macrophages to produce oxygen radicals [22]; (ii) to enhance phagocytosis of serum-opsonized erythrocytes [23], opsonized [22] and non-opsonized bacteria [24], and viruses [25]; and (iii) to stimulate chemotaxis [26]. However, little information is available about the structures on the outside of pathogens that SP-A can recognize. The aim of the present study

was to investigate the interaction of SP-A with lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria. Therefore, binding studies were performed with fluorescein isothiocyanate (FITC)-labelled SP-A, various LPS mutants coupled to magnetic beads, and Gram-negative bacteria using flow cytometry. In addition, aggregation of LPS in the presence of SP-A was monitored by spectrophotometry.

MATERIAL AND METHODS

Chemicals

Hepes acid was from BDH Chemicals (Poole, Dorset, U.K.); FITC (isomer I), yeast mannan, n-octyl β -D-glucopyranoside and heparin were from Sigma (St. Louis, MO, U.S.A.).

Pulmonary SP-A

SP-A was isolated from bronchoalveolar lavage fluid of patients with alveolar proteinosis as previously described [16]. SP-A was dissolved in 5 mM Hepes buffer, pH 7.4, (1 mg/ml) and stored in small aliquots at -70°C . The labelling of SP-A with FITC was performed as described elsewhere [27] and did not affect the properties of SP-A such as mannose binding [16], self-aggregation [28], lipid aggregation [28], and stimulation of alveolar macrophages measured as lucigenin-dependent chemiluminescence [22].

FITC-labelled SP-A was deglycosylated using N-glycosidase F (Boehringer 913782) as described previously [28], except for one minor modification, 0.2% Triton X-100 was replaced by 6 mM n-octyl β -D-glucopyranoside. The detergent was removed by dialysis of the reaction mixture against distilled water for 48 h. No glycosylated SP-A could be detected after incubation using SDS/PAGE under reducing conditions. The amount of FITC

Abbreviations used: SP-A, surfactant protein A; SP-D, surfactant protein D; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; HBBS, Hanks balanced salt solution supplemented with BSA and 0.1% azide.

† To whom correspondence should be addressed.

associated with SP-A after deglycosylation, as determined spectrophotometrically at 492 nm, was not altered.

Bacteria

Escherichia coli O111:B4 (O111) and its Rc mutant (J5) were grown overnight in Mueller–Hinton broth at 37 °C. Subsequently, the bacteria were washed three times with 140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4 , 10 mM Hepes, 2 mM MgSO_4 , 6 mM glucose, 2 mM CaCl_2 and 0.1% gelatin (w/v), pH 7.4, (buffer A) by centrifugation (1600 g for 10 min at room temperature). The bacteria were suspended in buffer A to a final concentration of 5×10^8 cells/ml as determined by spectrophotometry.

LPS

LPS was isolated and analysed as described before [29] from the following strains: *Salmonella minnesota* strain R595 (Re-LPS), *E. coli* O111:B4 (O111-LPS) and its Rc mutant (J5-LPS). Lipid A used in this study was derived from *S. minnesota* R595 and was purchased from Sigma (St. Louis, MO, U.S.A.).

Coating of magnetic beads with LPS

The coating of magnetic beads with LPS was performed as described previously [29]. Briefly, magnetic beads (4×10^7 tosyl-activated Dynabeads; M-450, Dynal A. S., Oslo, Norway) were incubated for 48 h at room temperature with 0.1 M sodium carbonate buffer, pH 9.5, with or without 1 mg/ml LPS dispersed by sonication. Thereafter, the beads were collected with a magnet and washed four times with Hanks balanced salt solution supplemented with BSA and 0.1% (w/v) azide (HBBS). The presence of LPS bound to the beads could be demonstrated using specific antisera such as rabbit anti-(O111-LPS) and mouse anti-(lipid A) monoclonal antibody 8-2 [30] for smooth and rough LPS respectively.

Fluorimetric binding assay

Beads (1×10^7 /ml of HBBS), coated with or without LPS (control beads), were washed three times with a buffer consisting of 5 mM Hepes/50 mM NaCl, pH 7.4, supplemented with 0.1% (w/v) BSA (buffer B). Subsequently, the beads were incubated with a buffer consisting of 5 mM Hepes/50 mM NaCl/1% BSA, pH 7.4, to prevent aspecific binding of proteins, for 1 h at 37 °C on a rotator. After washing with buffer B, the beads were suspended in buffer B supplemented with 5 mM CaCl_2 (incubation buffer; 1×10^6 beads/ml), unless otherwise stated. A sample of bead suspension (0.01 ml) was incubated with FITC–SP-A and incubation buffer (final reaction volume: 0.3 ml) at 37 °C. After 1 h the incubation was terminated by washing the beads three times with 0.4 ml of buffer B. The beads were suspended in 0.25 ml of buffer B. The bead-associated fluorescence, a measure for the amount of SP-A bound to the beads, was determined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.) as described elsewhere [29].

To study the binding of FITC–SP-A to whole bacteria, 0.1 ml of bacteria (5×10^8 /ml), either *E. coli* O111 or J5, was incubated with the indicated concentrations of FITC–SP-A (adjusted to a total volume of 0.2 ml using buffer A) at 37 °C for 30 min. Subsequently, the bacteria were washed three times with buffer A at 4 °C, suspended in 0.15 ml of buffer A, and then fixed by addition of 0.15 ml paraformaldehyde (2%, w/v). The bacteria-

associated fluorescence was determined as described elsewhere [31].

Aggregation of LPS by SP-A

Re-LPS (25 µg/ml) was suspended in 5 mM Tris/150 mM NaCl buffer, pH 7.4, by sonication. To this suspension SP-A (10 µg/ml) was added. Subsequently, the reaction mixture was transferred to reference and sample cuvettes (final volume 1 ml). The aggregation was initiated by the addition of CaCl_2 (final concentration 0.5 mM) to the sample cuvette and monitored at 37 °C and 400 nm using an Aminco DW-2a spectrophotometer.

RESULTS

Binding of SP-A to Re-LPS beads

Approximately three to four times as much FITC–SP-A was bound to beads coated with Re-LPS as to control beads (Figure 1a), indicating that SP-A binds specifically to Re-LPS. The binding was maximal after 30 min of incubation (Figure 1b) and at an SP-A concentration of 25 µg/ml (Figure 1a). At an SP-A

Figure 1 Concentration- and time-dependent binding of FITC–SP-A to Re-LPS-coated beads and control beads

(a) Control beads (▲) or Re-LPS-coated beads (●) were incubated with the indicated concentrations of FITC–SP-A for 1 h at 37 °C. Binding of FITC–SP-A to the beads is expressed as the amount of fluorescence associated with the beads (mean fluorescence). Observations were expressed as mean $\pm$ S.D. of four experiments. (b) Re-LPS-coated beads were incubated with 20 µg/ml FITC–SP-A for the indicated time intervals at 37 °C. Binding is expressed as the percentage of binding of FITC–SP-A to control beads. A representative experiment of three experiments is shown.

Figure 2 Influence of cations on the binding of FITC-SP-A to Re-LPS-coated beads

Re-LPS-coated beads were incubated with 20 $\mu\text{g/ml}$ FITC-SP-A at 37 °C for 1 h in the presence of either 0 mM NaCl (■), 50 mM NaCl (▲), or 150 mM NaCl (●) and the indicated CaCl_2 concentrations. Binding is expressed as the total amount of bead-associated fluorescence. A representative experiment of three experiments is shown.

Figure 3 Influence of polysaccharides on the binding of FITC-SP-A to Re-LPS-coated beads

(a) Re-LPS-coated beads were incubated with 20 $\mu\text{g/ml}$ FITC-SP-A for 1 h at 37 °C in the presence of various concentrations of heparin (◆) or mannan (▲). Binding is expressed as the percentage of FITC-SP-A binding to control beads. Observations were expressed as mean $\pm$ S.D. of four experiments. (b) Re-LPS-coated beads were incubated with either FITC-SP-A (●) or deglycosylated FITC-SP-A (■) for 1 h at 37 °C. Binding is expressed as the percentage of FITC-SP-A or deglycosylated FITC-SP-A bound to control beads. A representative experiment of three experiments is shown.

Table 1 Binding of FITC-labelled SP-A to various species of LPS coupled to beads

Beads were coated with lipid A or with one of the following LPS species: O111-LPS, J5-LPS, or Re-LPS. Control beads and coated beads were then incubated with 20 $\mu\text{g/ml}$ FITC-labelled SP-A for 1 h at 37 °C as described in the Materials and methods section. Binding is expressed as percentage of the amount of FITC-labelled SP-A bound to control beads. Values are given as means $\pm$ S.D. of five experiments.

	Binding of FITC-labelled SP-A (% of control)
Lipid A	320 $\pm$ 64
Re-LPS	380 $\pm$ 68
J5-LPS	290 $\pm$ 52
O111-LPS	120 $\pm$ 40

concentration of 50 $\mu\text{g/ml}$ less SP-A associated with the LPS-coated beads than at 25 $\mu\text{g/ml}$. This may be explained by the occurrence of self-aggregation of SP-A [28] at 50 $\mu\text{g/ml}$, which was observed by analysis of the sample using flow cytometry (results not shown). At lower SP-A concentrations hardly any SP-A aggregates could be detected.

Influence of cations on the binding of FITC-SP-A to Re-LPS-coated beads

In the absence of Na^+ and Ca^{2+} very little specific binding of FITC-SP-A to Re-LPS was observed (Figure 2). Both cations are required for binding, since in the absence of Na^+ little binding of FITC-SP-A was detected upon raising the Ca^{2+} concentration and, vice versa, when no Ca^{2+} was present increasing the Na^+ concentration did not give rise to binding of SP-A to Re-LPS. However, at the highest Na^+ concentration tested, less Ca^{2+} was required for maximal binding of SP-A than at lower Na^+ concentrations. In addition, more SP-A was found associated with LPS under these conditions.

Effect of polysaccharides on the binding of FITC-SP-A to Re-LPS coated beads

To study whether the carbohydrate-binding moiety of SP-A or the carbohydrate moieties of SP-A are involved in the binding of FITC-SP-A to Re-LPS the following experiments were designed. Binding of FITC-SP-A was performed in the presence of mannan (a mannose-containing polysaccharide that has been shown to bind to the carbohydrate-binding domain of SP-A) or heparin (a negatively charged polysaccharide that blocks the binding of SP-A to herpes simplex virus) [27]. Neither mannan nor heparin influenced the binding of FITC-SP-A to Re-LPS beads (Figure 3a). Also removal of the whole carbohydrate moiety of FITC-SP-A by enzymic digestion using N-glycosidase F hardly affected the binding of SP-A to Re-LPS-coated beads (Figure 3b).

Characterization of the SP-A-binding domain of LPS

To locate the SP-A-binding domain of LPS, binding studies were performed using LPS with various chain lengths, ranging from lipid A, the smallest size, via Re-LPS and J5-LPS, which consist of lipid A moiety and variable parts of the core region (J5-LPS > Re-LPS), to O111 which has complete core and O-antigen domains. As shown in Table 1, FITC-SP-A binds to lipid A, Re-LPS and J5-LPS but not to O111-LPS.

Figure 4 Aggregation of Re-LPS induced by SP-A

Re-LPS (25 $\mu\text{g/ml}$) (■) or SP-A (10 $\mu\text{g/ml}$) (▲) or Re-LPS (25 $\mu\text{g/ml}$) plus SP-A (10 $\mu\text{g/ml}$) (●) was transferred to reference and sample cuvettes (final volume 1 ml) and kept at 37 °C. Subsequently, CaCl_2 (0.5 mM final concentration; indicated with an arrow) was added to the sample cuvette. The change in turbidity, a measure for the aggregation, was expressed in arbitrary units. A representative experiment of four experiments is shown.

Figure 5 Binding of FITC-SP-A to J5 and O111 *E. coli*

Bacteria (5×10^7), either strain J5 (●) or O111 (■), were incubated with the indicated concentrations of FITC-SP-A for 30 min at 37 °C. Binding is expressed as the total amount of bacteria-associated fluorescence. Observations were expressed as mean $\pm$ S.D. of three experiments.

Aggregation of Re-LPS induced by SP-A

The interaction of SP-A with free LPS was also studied using aggregation studies (Figure 4). No aggregation of Re-LPS was observed in the absence of SP-A (■; Figure 4) and only a little aggregation of SP-A was observed without LPS, which is probably due to self-aggregation of SP-A induced by Ca^{2+} [28]. However, when SP-A and Re-LPS were added together and Ca^{2+} ions were introduced into the reaction mixture, aggregation was initiated, reaching a maximum after approx. 4 min (●; Figure 4).

Binding of FITC-SP-A to live Gram-negative bacteria

To assess whether SP-A can recognize LPS of live bacteria binding studies were performed with FITC-SP-A and either the J5 or O111 strains of *E. coli*. FITC-SP-A bound with high affinity to the J5 mutant of *E. coli* (●; Figure 5), reaching a

plateau at approx. 20 $\mu\text{g/ml}$, whereas no binding of SP-A to O111 strain of *E. coli* was observed (■; Figure 5).

DISCUSSION

This study demonstrates that SP-A binds to rough LPS in the presence of Ca^{2+} and Na^+ (Figures 1 and 2). The SP-A concentration required for optimal binding to Re-LPS (approx. 25 $\mu\text{g/ml}$) is similar to the SP-A concentration measured in bronchoalveolar lavage of humans [32]. Therefore, it is likely that enough SP-A will be present *in situ* to bind LPS.

Binding studies in the presence of mannan or heparin (Figure 3a) or with deglycosylated SP-A (Figure 3b) indicated that neither the carbohydrate-binding region nor the carbohydrate moiety of SP-A are involved in its binding to LPS. The nature of the domain of SP-A that is involved in the interaction with LPS is not known, but the lipid-binding region of SP-A, which binds to phospholipids [33] and glycolipids [34,35], could be a candidate. LPS consists of lipid A, a relatively conserved core oligosaccharide and a terminal polysaccharide of variable length and composition that comprises the O-specific antigen domain [36]. SP-A binds to the lipid A part of LPS, to rough LPS derived from the Re mutant (which consists of lipid A and two 3-deoxy-D-manno-octulosonic acid residues) and to the Rc mutant containing a larger part of the core oligosaccharide than the Re mutant. Interestingly, no binding is observed to smooth O111-LPS coupled to beads (Table 1) or to live O111 bacteria (Figure 5). This LPS has complete core oligosaccharide and O-antigen regions, and its terminal oligosaccharide moiety may mask lipid A thereby preventing its interaction with SP-A. Such a masking effect has been observed for various antibodies directed against lipid A.

The lipid A parts of the bacteria, *E. coli* and *S. minnesota*, investigated in this study are similar to those of important Gram-negative lung pathogens such as *Pseudomonas aeruginosa* and *Haemophilus influenzae*. The major differences between these lung pathogens and the bacteria used in the present study reside in the O-antigen and core regions. Therefore, we speculate that SP-A also binds to the lipid A regions of *P. aeruginosa* and *H. influenzae*, particularly as clinical isolates of these bacteria contain rough LPS.

Until now only two of the mammalian C-type lectins were shown to interact with rough LPS, i.e. the plasma protein mannose-binding protein [37] and the alveolar protein SP-D [38]. Both proteins appear to associate with rough LPS via their carbohydrate-binding domain. SP-D may aggregate Gram-negative bacteria in the alveolar space via recognition of the core region of rough LPS [38]. As suggested in the present study, SP-A may bind and aggregate LPS via the lipid A moiety. However, it can not be excluded that SP-A may also interact with the O-antigen of those Gram-negative bacteria that are highly enriched in mannose residues, as was shown for mannose-binding protein [39]. The amount of SP-A in the rat lung is approx. 100 μg and that of SP-D 10 μg [40]. However, roughly five to ten times less SP-D is needed to bind and aggregate Gram-negative bacteria [38] and to stimulate alveolar macrophages [22,41]. The localization of SP-A and SP-D in the alveoli also differs. SP-A is predominantly associated with pulmonary surfactant whereas SP-D is largely present in the aqueous phase [42]. Whether SP-A and SP-D are both needed, act in concert, or under some conditions even inhibit each other in their putative role in the alveolar defence, is not known at present, but is an intriguing topic for future research.

The interaction of SP-A with Gram-negative bacteria (or LPS) may have several implications *in vivo*, such as for instance: (i)

opsonization of Gram-negative bacteria for phagocytosis by alveolar macrophages, as was demonstrated earlier for herpes simplex virus [25]; (ii) aggregation of these bacteria, thereby preventing adherence to the alveolar epithelium; and (iii) facilitating mucociliary clearance of the bacteria, as was suggested for SP-D [38]. In analogy with LPS-binding protein [43], binding or complex formation of SP-A with 'free' LPS, released from dividing or dying Gram-negative bacteria, could also affect the LPS-induced synthesis and secretion of cytokines like tumour necrosis factor by alveolar macrophages.

In conclusion, our results demonstrating binding of SP-A to the lipid moiety of rough LPS underscore earlier suggestions [22,23,32] for a role of this protein in the antibacterial defences of the lung.

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