Surfactant Protein D Binds to Mycobacterium tuberculosis Bacilli and Lipoarabinomannan via Carbohydrate-Lectin Interactions Resulting in Reduced Phagocytosis of the Bacteria by Macrophages

J. Scott Ferguson, Dennis R. Voelker, Francis X. McCormack and Larry S. Schlesinger

http://www.jimmunol.org/content/163/1/312

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References This article cites 63 articles, 42 of which you can access for free at:
http://www.jimmunol.org/content/163/1/312.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Surfactant Protein D Binds to Mycobacterium tuberculosis Bacilli and Lipoarabinomannan via Carbohydrate-Lectin Interactions Resulting in Reduced Phagocytosis of the Bacteria by Macrophages

J. Scott Ferguson,* Dennis R. Voelker,‡ Francis X. McCormack,§ and Larry S. Schlesinger*†

Surfactant protein-D (SP-D) is a collectin produced in the distal lung airspaces that is believed to play an important role in innate pulmonary immunity. Naive immunologic responses to Mycobacterium tuberculosis (M.tb) are especially important in the lung, since entry of this inhaled pathogen into the alveolar macrophage is a pivotal event in disease pathogenesis. Here we investigated SP-D binding to M.tb and the effect of this binding on the adherence of M.tb to human macrophages. These studies demonstrate specific binding of SP-D to M.tb that is saturable, calcium dependent, and carbohydrate inhibitable. In addition to purified SP-D, SP-D in bronchoalveolar lavage fluids from healthy donors and patients with alveolar proteinosis also binds to M.tb. Incubation of M.tb with SP-D results in agglutination of the bacteria. In contrast to its binding to M.tb, SP-D binds minimally to the avirulent Mycobacterium smegmatis. SP-D binds predominantly to lipoarabinomannan from the virulent Erdman strain of M.tb, but not to the lipoarabinomannan from M. smegmatis. The binding of SP-D to Erdman lipoarabinomannan is mediated by the terminal mannosyl oligosaccharides of this lipoglycan. Incubation of M.tb with subagglutinating concentrations of SP-D leads to reduced adherence of the bacteria to macrophages (62.7% of control adherence ± 3.3% SEM, n = 8), whereas incubation of bacteria with surfactant protein A leads to significantly increased adherence to monocyte-derived macrophages. These data provide evidence for specific binding of SP-D to M. tuberculosis and indicate that SP-D and surfactant protein A serve different roles in the innate host response to this pathogen in the lung. The Journal of Immunology, 1999, 163: 312–321.

Mycobacterium tuberculosis (M.tb) infects nearly one third of the world’s population and is the leading cause of mortality due to an infectious disease (1). Factors that affect the pathogenesis of tuberculosis are complex and not fully defined. M.tb on droplet nuclei is inhaled into the alveoli of the lung where macrophages and surfactant are located. M.tb enters and can survive in the macrophage, whereas many other pathogens ingested by these phagocytes are killed. It is likely that M.tb encounters pulmonary surfactant components before entry into the macrophage and that these surfactant components influence the M.tb—macrophage interaction.

Pulmonary surfactant is a heterogeneous multimolecular complex, composed of phospholipids and associated proteins, that lines the alveolar space. The collectin proteins of surfactant, surfactant protein-A (SP-A) and surfactant protein-D (SP-D), are produced and secreted by alveolar macrophage and that these surfactant components influence the M.tb—macrophage interaction.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication September 25, 1998. Accepted for publication April 8, 1999.

Copyright © 1999 by The American Association of Immunologists

0022-1767/99/S02.00

Abbreviations used in this paper: M.tb, Mycobacterium tuberculosis; SP-A, surfactant protein A; SP-D, surfactant protein D; CRD, carbohydrate recognition domain; LAM, lipoarabinomannan; AraLAM, LAM from Mycobacterium smegmatis; CHO, Chinese hamster ovary; ABTS substrate, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid); GaRigG-HRP, HRP-conjugated goat anti-rabbit IgG; BAL, bronchoalveolar lavage; CBAL, concentrated BAL; WLL, whole lung lavage; HSA, human serum albumin; MDM, monocyte-derived macrophages; Amax, absorbance at 405 nm; PPD, purified protein derivative; RPMI, RPMI 1640 medium with L-glutamine; AM, alveolar macrophage.

1 Abbreviations used in this paper: M.tb, Mycobacterium tuberculosis; SP-A, surfactant protein A; SP-D, surfactant protein D; CRD, carbohydrate recognition domain; LAM, lipoarabinomannan; AraLAM, LAM from Mycobacterium smegmatis; CHO, Chinese hamster ovary; ABTS substrate, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid); GaRigG-HRP, HRP-conjugated goat anti-rabbit IgG; BAL, bronchoalveolar lavage; CBAL, concentrated BAL; WLL, whole lung lavage; HSA, human serum albumin; MDM, monocyte-derived macrophages; Amax, absorbance at 405 nm; PPD, purified protein derivative; RPMI, RPMI 1640 medium with L-glutamine; AM, alveolar macrophage.

2 Address correspondence and reprint requests to Dr. Larry S. Schlesinger, SW 54 GH, Division of Infectious Diseases, Department of Internal Medicine, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242. E-mail address: larry-schlesinger@mail.int-med.uiowa.edu

3 Abbreviations used in this paper: M.tb, Mycobacterium tuberculosis; SP-A, surfactant protein A; SP-D, surfactant protein D; CRD, carbohydrate recognition domain; LAM, lipoarabinomannan; AraLAM, LAM from Mycobacterium smegmatis; CHO, Chinese hamster ovary; ABTS substrate, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid); GaRigG-HRP, HRP-conjugated goat anti-rabbit IgG; BAL, bronchoalveolar lavage; CBAL, concentrated BAL; WLL, whole lung lavage; HSA, human serum albumin; MDM, monocyte-derived macrophages; Amax, absorbance at 405 nm; PPD, purified protein derivative; RPMI, RPMI 1640 medium with L-glutamine; AM, alveolar macrophage.

4 Received for publication September 25, 1998. Accepted for publication April 8, 1999.

Copyright © 1999 by The American Association of Immunologists

0022-1767/99/S02.00
SP-A enhances the interaction between M. tb and macrophages (14, 23, 24). Previous work from our laboratory has demonstrated that SP-A, through a direct interaction with the macrophage, enhances macrophage phagocytosis of virulent M. tb potentially by up-regulating mannose receptor activity (14).

Mature monomeric SP-D is a 43-kDa polypeptide that oligomerizes to form four trimers that are covalently associated at their N termini. The resultant ~516-kDa dodecameric protein has a cruciform shape with the CRDs arranged peripherally at the end of long stalks (9). SP-D binds to carbohydrates such as maltose, glucose, and mannose but binds poorly to amino sugars (25). The role of SP-D in the microbe-host cell interaction is a growing area of interest. SP-D has been shown to bind to certain bacteria, influenza virus, Cryptococcus neoformans, Pneumocystis carinii, and Aspergillus fumigatus through lectin interactions (26–31). These interactions can result in aggregation of the microorganism (26, 27, 29, 31, 32) and altered interactions with host cells (26, 30, 31, 33).

Although SP-A and SP-D are members of the same protein family, they differ significantly in size and tertiary structure, and their recognition of microorganisms is not identical. Additionally, SP-A is ~10-fold more abundant and is more tightly associated with surfactant phospholipids than SP-D (34). Together, these differences suggest that SP-A and SP-D may have different roles in the innate immune response of the host, potentially at different sites in the lung.

The aim of the present study was to investigate the interaction between M. tb and SP-D and to determine the impact of this interaction on the adherence of M. tb to human macrophages. Our studies demonstrate that SP-D binds to M. tb, but not the avirulent Mycobacterium smegmatis, and that the M. tb cell wall lipoglycan lipoarabinomannan (LAM) serves as a major binding molecule for SP-D. The SP-D–M. tb interaction reduces the adherence of bacteria to human macrophages. Thus, these studies provide evidence for different roles of SP-A and SP-D in the innate immune response to M. tb in the lung.

Materials and Methods
Reagents
RPMI 1640 medium with l-glutamine (RPMI) was purchased from Life Technologies (Grand Island, NY). Middlebrook 7H9 broth was purchased from BBL, Microbiology Systems (Becton Dickinson, Cockeysville, MD). 7H11 agar was purchased with Bacto Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI), oleic acid albumin dextrose catalase enrichment medium (Difco), 0.1% casine enzymatic hydrolyse (Sigma, St. Louis, MO), and glycerol (Difco), as described (35), and dispensed into 100 × 15-mm bacteriologic petri dishes. Auramine-iodine stain (Difco) was purchased. Formaldehyde solution 37% w/w and potassium permanganate were purchased from Sigma. 2,2′-Azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate and AHRP-conjugated goat anti-rabbit IgG (GeRigG-HRP) were purchased from Bio-Rad (Hercules, CA).

Growth and preparation of bacteria
Lyophilized M. tb Erdman strain (ATCC 35801) was obtained from American Type Culture Collection (ATCC, Manassas, VA), reconstituted, and used as described (36). Briefly, for each experiment, aliquots of frozen stock in 7H9 broth were thawed. The bacteria were then cultured for 9–11 days on 7H11 agar, scraped from the culture plate into microfuge tubes containing buffer or medium and two 4-mm glass beads, pulse vortexed briefly (1–2 s), and allowed to settle over 30 min. The upper bacterial wash was removed. The suspension was then removed to a second tube. After a second settling for 10 min, an aliquot from the second tube was transferred to a third tube and allowed to settle for an additional 10 min. The top portion of this aliquot was used in the experiments. The concentration of bacteria (1–2 × 10^8 bacteria/ml) and the degree of clumping (≤10%) were determined by counting in a Petroff-Hausser chamber.

Recombinant rat SP-D, expressed in Chinese hamster ovary (CHO) cells, and recombinant rat SP-D deficient in hydroxylation of proline residues expressed in SP-D (SP-D hyp ) were prepared and characterized as described previously (37, 38). The experiments were performed using the CHO cell-expressed SP-D except where otherwise stated. SP-A was prepared from whole lung lavage fluid from a patient with alveolar proteinosis as described previously (39). The endotoxin concentration of the collectin preparations was determined by the Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD) and found to be 0.82 pg/ml for CHO cell-expressed SP-D, 40.8 pg/ng for SP-D hyp and 1.5 pg/ng for SP-A. To produce anti-SP-D antiserum, a New Zealand White rabbit was immunized and boosted with 50 μg purified SP-D hyp in IFA over a 3-wk interval. After 1 mo, serum was harvested. The anti-SP-D antiserum did not react with M. tb or with SP-A by ELISA (not shown) and reacted only with SP-D in alveolar lavage fluids by Western blotting (Fig. 1). Lipoarabinomannan (LAM), from the virulent Erdman strain of M. tb, and rabbit anti-LAM Ab were prepared and characterized as described (40). M. smegmatis-type LAM (AraLAM) was provided by Dr. Patrick Brennan and colleagues (Colorado State University, National Institute of Allergy and Infectious Diseases, National Institutes of Health contract N01-AI-25147). 2,2′-Azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate and AHRP-conjugated goat anti-rabbit IgG (GeRigG-HRP) were purchased from Bio-Rad (Hercules, CA).

Preparation of human bronchoalveolar lavage fluid
Normal human volunteers who were not cigarette smokers and were purified protein derivative (PPD) negative underwent bronchoalveolar lavage (BAL) with ~200 ml of saline using a previously described procedure (41) that has been approved by the University of Iowa Institutional Review Board for human subjects. After removing cellular material and passing through a 0.22-μm filter, the BAL was concentrated (CBAL) 10-fold using a Centricon-10 concentrator (W.R. Grace, Danvers, MA). BAL, CBAL, and the low m.w. filtrate of the CBAL were stored in 1-ml aliquots at −70°C until the day of use. Whole lung lavage (WLL) fluid (the first 3
liters of an ~21-liter total lavage) was obtained from a patient with alveolar proteinosis, who was undergoing therapeutic lavage, and stored at ~70°C.  

Isolation and preparation of human macrophages

Monolayers of monocyte-derived macrophages (MDM) were prepared from healthy PPD-negative human volunteers as described (42). Briefly, mononuclear cells were isolated from heparanized blood on Ficoll gradients and then plated onto Chromerge-cleaned glass coverslips or plastic coverslips for 2 h at 37°C (1×10⁶ MDM/coverglass). The wells were washed with RPMI and then used in the experiments. For experiments with human alveolar macrophages (AM), healthy PPD-negative donors underwent BAL as described above. The AM were collected by centrifugation and washed in RPMI (14). Cell viability (~93%) was assessed using Trypan blue exclusion, and the percentage of macrophages present (~90%) was determined using a differential cell stain (Leukostat, Fisher Scientific, Pittsburgh, PA). The cells were then resuspended in RPMI with 10% autologous serum and plated onto Chromerge-cleaned glass coverslips at 1×10⁶ AM/coverglass. After 2 h at 37°C in 5% CO₂ and air, the monolayer cells were fixed with 10% formaldehyde and then used in the experiments. Monolayer integrity and density were monitored by inverted phase-contrast microscopy throughout the experiments.

Radio labeled SP-D binding experiments

SP-D expressed in CHO cells was metabolically labeled with Tran³⁵S-label (ICN) as described (38). M.tb was incubated in 50 mM tris-HCI, 150 mM NaCl (pH 7.5) (TBS) and 1 mg/ml human serum albumin (HSA) in a pre-blocked microtiter plate (5% HSA in TBS overnight at 4°C) with various concentrations of [³⁵S]SP-D in the presence and absence of Ca²⁺, excess unlabeled protein, EDTA, or competing carbohydrates for 30 min at 37°C. At the end of the incubation period, the pellets were washed three times with ice-cold TBS containing 2 mM CaCl₂ (TBS-C) and transferred to scintillation fluid for liquid scintillation counting in a beta counter (Beckman, Irvine, CA). The sp. act. of [³⁵S]SP-D ranged from 50 to 1500 cpn/μg. The mean ± SD of the cpm bound to triplicate pellets was calculated, and the amount in nanograms bound per 5×10⁵ bacteria was determined by dividing the cpm bound per pellet by the sp. act. of the protein. Counts obtained in the absence of M.tb or the presence of 10 mM EDTA were subtracted out in each experiment (~30 cpn).

Bacterial ELISA

In experiments designed to determine the saturable binding of SP-D to M.tb, M.tb (at 5×10⁵ bacteria in 10 μl TBS) were dried in the wells of a microtiter plate (DYNATECH, Chantilly, VA) (36), followed by exposure to ultraviolet light for 2 h to kill viable bacteria. After blocking with TBS-C containing 3% BSA and 0.2% Triton X-100 overnight at 4°C, the wells were then incubated with increasing concentrations of SP-D in blocking buffer in the presence or absence of 100 mM malonate for 60 min at room temperature. After washing away unbound SP-D, the wells were incubated with a 1:20,000 dilution of anti-SP-D antiserum in blocking buffer for 2 h at room temperature, washed, and then incubated with 1:3000 dilution of goat anti-rabbit IgG-HRP. Color development with HRP substrate was determined in a microplate spectrophotometer, and the mean ± SD of the absorbance for triplicate wells of each concentration was calculated.

M.tb adherence assay

M.tb was incubated in RPMI containing 20 mM HEPES, and 1 mg/ml HSA (pH 7.2) (RHI) in the presence or absence of SP-D or SP-A for 4 h at 37°C. Following the incubation period, the M.tb/collectin mixture was added to MDM or AM monolayers on glass coverslips and incubated for 2 h at 37°C in 5% CO₂ and air. After washing away nonadherent bacteria, the coverslips were fixed in 10% formaldehyde, and bacteria were stained with auramine-rhodamine. The macrophages were counterstained using 5% polysporin (Boehringer Mannheim, Mannheim, Germany). The LAMs were adhered to a microtiter plate as above and then incubated with sodium metaperiodate and then DIG-biotin labeled anti-S. typhimurium LPS (2 or 3 μg/ml) and purified Erdman strain LAM or ArabLAM (2 or 3 μg/ml) were added and denatured by boiling in 20% SDS and 10% 2-ME and resolved in 10% SDS-PAGE (43) or 10% polyacrylamide, 0.96% bisacrylamide SDS-PAGE (40). The gels were stained using the periodate silver method (44) or transferred to nitrocellulose. After blocking nonspecific sites with 1% BSA and 0.05% Tween 20 in TBS-C, the nitrocellulose membranes were incubated with 5×10⁵ cpm/ml [³⁵S]SP-D in blocking buffer in the presence and absence of 10 nM EDTA overnight at 4°C, washed extensively, sprayed with Enhance (DuPont, Boston, MA), and exposed to x-ray film (Eastman Kodak, Rochester, NY). Additional nitrocellulose membranes were Western blotted with anti-Erdman LAM primary Ab and GoRiG-HRP secondary Ab (40). The Western blots were developed using 4-chloro-1-napthol (Bio-Rad) and hydrogen peroxide (Mallinckrodt, Paris, KY).

Exomannosidase treatment of LAM

Erdman LAM and ArabLAM (1 μg/ml in carbonate-bicarbonate buffer, pH 9.6) were adhered to wells of a microtiter plate (DYNATECH) overnight at 37°C. After washing, plate-bound LAM was incubated with 0.1 M sodium acetate buffer (pH 5.5), or with buffer containing 0.18 μM exomannosidase for 34 h at 37°C to remove the terminal mannosyl oligosaccharides, as described previously (45). After blocking with TBS-C containing 0.05% Tween 20, the wells were incubated with 4 μg/ml SP-D⁹⁹⁰ in blocking buffer for 1 h at room temperature in the presence and absence of EDTA, malonate, or N-acetylglucosaminase. After washing, the wells were sequentially incubated with anti-SP-D Ab or anti-LAM Ab for 2 h at room temperature, followed by GoRiG-HRP. Color development with HRP substrate was stopped by 1% oxalic acid. The absorbance in individual wells was determined in a microtiter photometer, and the mean ± SD of triplicate wells was determined. Equivalent coating of wells with the LAMs was determined using the Dig-Glycan Detection Kit (Boehringer Mannheim, Mannheim, Germany). The LAMs were adhered to a microtiter plate as above and then incubated with sodium metaperiodate and then DIG-succinyl-e-amidoacparic acid hydrazide. After blocking, the wells were then incubated with alkaline phosphatase-conjugated anti-DIG Ab and developed with ABTS substrate.
Electron microscopy

MDM monolayers on duplicate plastic coverslips were incubated with \( M.t.b \) in the presence or absence of SP-D as described above. After 2 h at 37°C, the monolayers were washed with RPMI, then fixed in 2.5% glutaraldehyde, stained, and sectioned as described (42). The number of attached vs internalized bacteria were determined by counting greater than 20 consecutive MDM cross-sections in each monolayer under transmission electron microscopy.

Statistics

For each experiment, a ratio of experimental results to control (expressed as fold increase or percentage decrease) was obtained, and the mean ratio was then tested for a significant difference from 1 using \( t \) statistics. Statistical significance was defined as a \( p < 0.05 \).

Results

SP-D binds specifically to \( M.t.b \) in a saturable, calcium-dependent, and carbohydrate-inhibitable manner

To determine the specific interaction between SP-D and \( M.t.b \), and to characterize this interaction, SP-D–\( M.t.b \)-binding studies were conducted in the presence and absence of various inhibitors. SP-D bound to \( M.t.b \) in a specific and saturable manner. Ten-fold excess unlabeled SP-D inhibited 2 \( \mu \)g/ml \( [35 \text{S}] \)SP-D binding by 63% ± 8% (mean ± SEM, \( n = 3 \)). \( [35 \text{S}] \)SP-D binding to \( M.t.b \) in suspension was concentration dependent and appeared to be saturable at low concentrations of SP-D; however, at higher concentrations, bacterial aggregation (see below) limited the analysis. Thus, saturable binding was determined by ELISA with \( M.t.b \) adsorbed to the plastic wells to avoid the confounding variable of bacterial agglutination. The binding of SP-D to \( M.t.b \) was found to be saturable in a range between 2–4 \( \mu \)g/ml (Fig. 2). A double reciprocal plot of these data gives an approximate dissociation constant of 7.15 \( \times \) 10\(^{-10} \) M, assuming that the molecular mass of SP-D is 516,000 daltons and that the reaction represents a simple bimolecular interaction. These data provide strong evidence that SP-D binds to \( M.t.b \) in a specific manner and that there is a finite number of binding sites available on the bacteria for interactions with SP-D.

The carbohydrate-rich outer surface of \( M.t.b \) suggested that SP-D may bind to \( M.t.b \) via its CRD. To examine this possibility, we determined whether SP-D binding was calcium dependent and carbohydrate inhibitable. \( [35 \text{S}] \)SP-D binding to \( M.t.b \) was negligible (<1 ng) in the absence of calcium ions or the presence of EDTA (data not shown). These results suggested that SP-D binding to \( M.t.b \) is dependent on divalent cations. To examine whether the cation requirements for the binding of SP-D to \( M.t.b \) were specific for calcium ions, we determined whether other divalent cations could substitute for calcium (Fig. 3). By ELISA, SP-D binding was undetectable (<0.05 A\(_{405 \text{ nm}}\)) in the presence of 0.2 mM EDTA and 5 mM MgCl\(_2\), MnCl\(_2\), or SrCl\(_2\). However, SP-D binding in the presence of 5 mM CaCl\(_2\) and 0.2 mM EDTA was similar to SP-D binding in the presence of 5 mM CaCl\(_2\) and the absence of EDTA. These data provide evidence that the binding of SP-D to \( M.t.b \) is cation dependent and exhibits a specific requirement for calcium.

To assess the carbohydrate specificity of SP-D binding to \( M.t.b \), assays were performed in the presence of increasing concentrations of selected carbohydrates with a fixed concentration of \( [35 \text{S}] \)SP-D (Fig. 4). Maltose, glucose, and mannose were strong inhibitors of SP-D binding. The concentrations of these carbohydrates required to reduce the binding of SP-D to \( M.t.b \) by 50% were derived from nonlinear regression curves of the data shown in Fig. 3 and were found to be 0.97 mM for maltose, 1.21 mM for glucose,
and 4.61 mM for mannose. N-acetylgalactosamine did not inhibit SP-D binding at the concentrations tested. These data are consistent with the published carbohydrate specificity of the CRD of SP-D (25). These data provide evidence that the binding occurs between carbohydrate moieties on the surface of *M. tb* and the CRD of SP-D.

SP-D in BAL fluid binds to *M. tb*

Since SP-D is located in alveolar surfactant and *M. tb* gains entry to the human host via the airspace, we examined the ability of human SP-D in surfactant-containing pulmonary lavage fluid to bind to *M. tb*. The binding of SP-D contained in BAL fluid, CBAL fluid, and WLL fluid from a patient with pulmonary alveolar proteinosis was assessed by ELISA (Table I). Significant binding of SP-D was detected from BAL, CBAL, and WLL compared with buffer control. There was no binding detected using a low m.w. filtrate of CBAL. These results support the concept that SP-D contained in alveolar lining fluids can bind to *M. tb*.

SP-D agglutinates *M. tb*

SP-D has been found to agglutinate certain strains of *E. coli* bacteria, influenza virus, *Cryptococcus neoformans*, and *A. fumigatus*, and this is believed to be an important host defense property of the collectin (27, 29, 31, 33). We examined for SP-D-mediated agglutination of *M. tb* using a fluorescence microscopy assay (Fig. 5). Aggregation of some *M. tb* bacilli was seen under control conditions, consistent with the tendency of this microorganism to clump. However, at concentrations of SP-D $\geq$ 5 $\mu$g/ml, larger and more numerous aggregates were readily apparent in all fields visualized. EDTA and maltose but not N-acetylgalactosamine inhibited the agglutinating effect of SP-D. These results indicate that calcium-dependent lectin interactions of SP-D with *M. tb* lead to agglutination of the bacteria.

Table I. SP-D in BAL fluids binds to *M. tb*

<table>
<thead>
<tr>
<th>Lavage Fluid</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.245 ± 0.080</td>
<td>0.131 ± 0.005</td>
</tr>
<tr>
<td>BAL</td>
<td>0.103 ± 0.009</td>
<td>0.132 ± 0.019</td>
</tr>
<tr>
<td>CBAL</td>
<td>0.513 ± 0.016</td>
<td>0.628 ± 0.031</td>
</tr>
<tr>
<td>CBAL filtrate</td>
<td>0.011 ± 0.016</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>WLL</td>
<td>0.624 ± 0.050</td>
<td>0.510 ± 0.081</td>
</tr>
</tbody>
</table>

$M. tb$ (2.5 $\times$ 10$^7$) was incubated with 1 $\mu$g/ml SP-D$^{35}$S (control) or 170 $\mu$l of BAL, 10-fold CBAL, the low m.w. filtrate of the CBAL, or WLL from a patient with pulmonary alveolar proteinosis in the presence of TRS, 1 mg/ml HSA, and 2 mM calcium for 60 min at 37°C (final reaction volume 200 $\mu$l). The pellets were then washed three times in ice-cold buffer, and 100-$\mu$l aliquots were allowed to evaporate to dryness in the wells of a 96-well microtiter plate (8 $\times$ 10$^6$ bacteria/well). After blocking, SP-D binding was detected by ELISA as in Fig. 2. Shown is the mean A$_{405 \text{nm}}$ ± SD of triplicate groups in two experiments.
M. tb LAM serves as a major binding molecule for SP-D

We performed [35S]SP-D lectin-blotting studies to identify specific molecules on M. tb that serve as binding molecules for the protein (Fig. 6A, panel a, lane 1). The [35S]SP-D was incubated with reduced, SDS solubilized M. tb preparations separated by SDS-PAGE and immobilized on nitrocellulose. SP-D binding was predominantly to a region of the blot that corresponds with the known electrophoretic mobility and appearance of LAM (46), a major cell wall-associated lipoglycan of M. tb. There was also a small degree of SP-D binding to a lower molecular size M. tb component consistent with the location of lipomannan, another mannosylated lipoglycan (47). Based on these results, we next examined whether SP-D binds to purified M. tb LAM. SP-D bound to immobilized purified Erdman LAM in the presence of calcium (Fig. 6A, panel a, lane 2; and Fig. 6B, panel b, lane 1), but not in the presence of 10 mM EDTA (Fig. 6B, panel c).

Compared with M. tb LAM, [35S]SP-D bound minimally to immobilized, purified AraLAM, the LAM type from the avirulent M. smegmatis (Fig. 6B, panel b, lane 2). These data indicate that M. tb LAM is a major SP-D-binding molecule on the surface of M. tb and suggest that SP-D binds specifically to LAM exposed on pathogenic but not avirulent mycobacteria.

SP-D binds to the terminal mannosyl units of M. tb LAM

M. tb LAM and AraLAM differ with respect to the terminal α-(1→2)-linked mannosyl oligosaccharides (“mannose caps”) that are present on M. tb LAM (46, 48). Based on the known affinity of SP-D for mannose and the observation that SP-D binds to M. tb LAM but not AraLAM, we hypothesized that SP-D binds to the mannose caps of M. tb LAM. To test this hypothesis, we used an ELISA to examine the binding of SP-D to immobilized, intact LAM in the absence and presence of various carbohydrates, and to M. tb LAM devoid of the mannosyl caps (Fig. 7). SP-D bound to immobilized Erdman LAM, consistent with the lectin-blotting studies. Maltose markedly inhibited SP-D binding to LAM, whereas the inhibition observed with N-acetylglucosamine was much less, consistent with the known carbohydrate-binding specificity of the CRD. The maltose-inhibitable SP-D binding was abolished by treatment of M. tb LAM with exomannosidase, an enzyme that removes α-(1→2)-linked oligomannosyl units. Binding of SP-D to AraLAM was minimal compared with Erdman LAM, and the small amount of maltose-inhibitable binding observed was not influenced by treatment of AraLAM with exomannosidase. These data provide evidence that SP-D binds to the terminal mannosyl oligosaccharides of M. tb LAM. The adsorption of Erdman LAM and AraLAM to the plates was comparable, based on the detection of carbohydrate by the Digoxigenin-Glycan Assay. The absorbance at 405 nm was 0.212 ± 0.038 for Erdman LAM and 0.217 ± 0.003 for AraLAM (mean ± SD, triplicate wells, n = 2). Additional control experiments using a specific polyclonal Ab to LAM demonstrated that exomannosidase treatment did not reduce the amount of LAM bound to the microtiter plates (data not shown).

SP-D binds minimally to the avirulent M. smegmatis

All mycobacteria have carbohydrates associated with their outer surface. However, it is likely that these complex carbohydrates differ with respect to the amount expressed on the surface and the
specific residues included in the overall structure of the complex carbohydrate. To determine whether SP-D binds to \textit{M. smegmatis}, an avirulent species of mycobacteria and the source for AraLAM, we used a bacterial ELISA. By comparison with binding to \textit{M. tb}, SP-D bound minimally to \textit{M. smegmatis} (Table II). In like fashion, by lectin blotting using 2 \textsuperscript{[35}S\textsuperscript{]} SP-D was observed (data not shown). These results suggest that the binding of SP-D to \textit{M. tb} is dependent upon specific carbohydrate structures exposed on the surface of \textit{M. tb} that are not present on the surface of \textit{M. smegmatis}. Inasmuch as these results resemble those obtained with \textit{M. tb} LAM and AraLAM, they further support the concept that \textit{M. tb} LAM serves as a major binding molecule for SP-D in the context of whole bacteria.

**SP-D reduces the adherence of \textit{M. tb} to human macrophages**

SP-D has been reported to be an opsonin for \textit{A. fumigatus} (31) and influenza virus (33). We have previously demonstrated that SP-A increases the phagocytosis of Erdman \textit{M. tb} by human macrophages (14). Since we determined that SP-D binds to \textit{M. tb} LAM, we hypothesized that this binding would interfere with the recognition of LAM by the macrophage mannose receptor and thereby reduce \textit{M. tb} adherence to macrophages. Alternatively, SP-D binding to \textit{M. tb} could provide an alternative route of entry for the bacterium by serving as an opsonin that is recognized by a collectin receptor on the macrophage (49). To examine these possibilities, we performed fluorescence microscopy experiments to assess the adherence of \textit{M. tb} to PMN in the presence and absence of SP-D (Fig. 8 and Table III). SP-D at a subagglutinating concentration (0.5 \(\mu\)g/ml) did not demonstrate opsonic activity. In fact, it reduced the number of bacteria that adhered to macrophages (Fig. 8). In experiments in which an SP-D/\textit{M. tb} mixture was added to PMN, SP-D caused a 37.3\% \(+ 3.3\%\) (mean \(+ \text{SEM}\), \(n = 8\), \(p < 0.001\)) reduction in the adherence of bacteria compared with control. This effect was retained if the SP-D/PMN mixture was washed before incubation with PMN (37.7\% \(+ 1.8\%\) reduced adherence, mean \(+ \text{SEM}\), \(n = 4\)), but lost if the PMN were incubated with SP-D and washed before adding bacteria (Table III). These results indicate that the reduced adherence is dependent on the SP-D/PMN interaction and not due to an SP-D-only interaction of PMN. In contrast, SP-A increased the adherence of \textit{M. tb} to macrophages (Fig. 8). Washing away excess collectin from the monolayers before adding bacteria did not attenuate the increased \textit{M. tb} adherence (Table III). These results provide evidence that an interaction between SP-A and the macrophage plays an important role in the increased adherence of \textit{M. tb}, consistent with our previous observations (14). To control for LPS contamination in our collectin preparations, LPS was included in some experiments at the concentration found in the collectin preparations. There was no effect by LPS alone on the adherence of \textit{M. tb} to macrophages (Fig. 8).

**Table II. SP-D binds minimally to \textit{M. smegmatis} compared to \textit{M. tb}**

<table>
<thead>
<tr>
<th>Condition</th>
<th>\textit{M. smegmatis}</th>
<th>\textit{M. tb}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-D/Ca\textsuperscript{2+}</td>
<td>0.160 (+ 0.09)</td>
<td>0.993 (+ 0.020)</td>
</tr>
<tr>
<td>SP-D/EDTA</td>
<td>0.085 (+ 0.09)</td>
<td>0.121 (+ 0.033)</td>
</tr>
<tr>
<td>SP-D/Ca\textsuperscript{2+}/maltoose</td>
<td>0.106 (+ 0.006)</td>
<td>0.177 (+ 0.021)</td>
</tr>
<tr>
<td>SP-D/Ca\textsuperscript{2+}/GalNAc</td>
<td>0.132 (+ 0.019)</td>
<td>0.659 (+ 0.009)</td>
</tr>
</tbody>
</table>

\(*\) Bacteria \((2.5 \times 10^7)\) were incubated with 4 \(\mu\)g/ml SP-D in the presence of 2 mM calcium, or 10 mM EDTA, or the presence of 2 mM calcium and 100 mM maltoose or N-acetylglactosamine (GalNAc) for 60 min at 37°C. The pellets were then washed three times in ice-cold buffer, and 100-\(\mu\)l aliquots were allowed to evaporate to dryness in the wells of a 96-well microtiter plate \((8 \times 10^6\) bacteria/well\). After blocking, SP-D binding was detected by ELISA as in Fig. 2. Shown is the mean absorbance \(+ SD\) of triplicate wells in an experiment representative of two.

**FIGURE 8.** SP-D reduces the adherence of \textit{M. tb} to macrophages, whereas SP-A enhances bacterial adherence. \textit{M. tb} \((1.5 \times 10^7)\) in RHH was incubated in the presence or absence of 0.5 \(\mu\)g/ml SP-D, 10 \(\mu\)g/ml SP-A, or 10 ng/ml LPS for 1 h at 4°C on a rotating platform. The mixture was allowed to return to room temperature and then was added to MDM on triplicate glass coverslips for 2 h at 37°C in 5% CO\textsubscript{2} and air. Nonadherent bacteria were then washed away, and the coverslips were fixed in 10% formaldehyde. The bacteria were stained with auramine-rhodamine and visualized using phase-contrast and fluorescent microscopy. The number of bacteria per MDM was enumerated by counting \(\pm 100\) consecutive MDM per coverslip. The percentage of control adherence was calculated by dividing the mean number of bacteria/MDM in the presence of collectin or LPS by the control value (mean number of bacteria/MDM in the absence of collectin and LPS) \times 100. The control level of bacteria/MDM was 2.07 \(+ 0.8\) (mean \(+ \text{SEM}\), \(n = 8\)). Shown is the mean \(+ \text{SEM}\) of triplicate determinations for four to eight independent experiments. **, \(p < 0.05\) vs control.

Since it is possible that the results obtained using MDM may differ from those seen with alveolar macrophages (50), we performed experiments to determine whether SP-D also reduces the adherence of \textit{M. tb} to human AM. SP-D (0.5 \(\mu\)g/ml) was incubated with \textit{M. tb} and then added to AM monolayers as described for MDM. SP-D reduced the adherence of \textit{M. tb} to AM by 53.1\% \(+ 2.0\%\) (mean \(+ \text{SEM}\), \(n = 2\), \(p < 0.05\)). The number of bacteria per AM in the control condition was 2.2 \(+ 0.5\) (mean \(+ \text{SEM}\), \(n = 2\)).

**Table III. The inhibition of \textit{M. tb} adherence to macrophages by SP-D results from the SP-D-MDM interaction, whereas the enhancement of \textit{M. tb} adherence to macrophages results from the SP-A-macrophage interaction**

<table>
<thead>
<tr>
<th>Collectin</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-D</td>
<td>53.3 (+ 1.3)*</td>
<td>94.5 (+ 4.5)</td>
</tr>
<tr>
<td>SP-A</td>
<td>152.3 (+ 5.4)*</td>
<td>170.2 (+ 13.1)*</td>
</tr>
</tbody>
</table>

* Monolayers of MDM were incubated with the indicated collectins (SP-D: 0.5 \(\mu\)g/ml; SP-A: 10 \(\mu\)g/ml) for 1 h at 37°C in 5% CO\textsubscript{2} and air. \((A)\) \textit{M. tb} \((1 \times 10^9)\) was then added to the MDM, or \((B)\) the MDM were washed prior to adding bacteria. After 2 h, the nonadherent bacteria were washed away, and the coverslips were then assessed for bacterial adherence by fluorescence microscopy. The number of bacteria per MDM for the control condition was 1.82 \(+ 0.31\) (mean \(+ \text{SEM}\), \(n = 3\)). Shown is the percentage of control bacterial adherence (number of bacteria per MDM in test group/number of bacteria per MDM in control group) \times 100) performed in the absence of collectin (mean \(+ \text{SEM}\) of triplicate determinations from three independent experiments. **, \(p < 0.05\) versus control.
To determine whether the reduced bacterial adherence due to SP-D correlated with decreased phagocytosis, we performed a transmission electron microscopy experiment to distinguish attached from internalized bacteria. No attached bacteria were seen in the consecutive MDM sections examined in all groups. SP-D reduced the number of internalized bacteria per MDM cross-section (control: 1.64 and 2.24 bacteria/section on two coverslips; SP-D: 0.20 and 0.78 bacteria/section and two coverslips), and the percentage of MDM cross-sections on two coverslips with internalized bacteria (control: 34.0 and 28.6%; SP-D: 8.7 and 6.6%). These results combined with the fluorescence microscopy data demonstrate that SP-D reduces the phagocytosis of M.tb by MDM.

**Discussion**

The recent increase in tuberculosis cases in the United States and worldwide has triggered research efforts aimed at better defining the interactions between the causative bacterium M.tb and the host immune system. Tuberculosis is a respiratory infection and, as such, the critical early interaction between the inhaled organism and the host is with components of the alveolar compartment. The outcome of this interaction will determine infection vs resolution. Within this context, M.tb will encounter surfactant when inhaled on droplet nuclei into the alveolus. It is likely that the interaction between M.tb and surfactant components alters the ultimate fate of the bacterium. However, our understanding of the molecular details and the importance of this initial interaction is limited.

The surface of M.tb is composed primarily of carbohydrates and lipid (51). Since SP-D has been described to bind to other pathogens (27, 29–31, 52) and is felt to play an important role in the innate immune response in the lung, we investigated the potential for SP-D to bind to M.tb and the influence of this interaction on the adherence of M.tb to human macrophages. In this report, we show that the binding of SP-D to M.tb is specific, that SP-D in human BAL fluids binds to M.tb, that SP-D binds to LAM on the surface of Erdman M.tb via the mannose caps on Erdman LAM, and that the SP-D–M.tb interaction reduces the adherence of M.tb to human macrophages. Furthermore, we show that SP-D binds minimally to the avirulent mycobacterial species, M. smegmatis.

Our data provide evidence that the binding of SP-D to M.tb is specific. Saturable binding was demonstrated in a range of concentrations of SP-D that are likely to be below the actual concentration of SP-D in the normal airspace, estimated to be 50–90 μg/ml (53, 54). Saturable binding indicates that there is a finite number of binding sites available for SP-D on the surface of M.tb. We were unable to perform Scatchard analysis using radiolabeled protein and M.tb in a suspension system since higher concentrations of SP-D caused aggregation of SP-D and agglutination of M.tb. However, the estimated dissociation constant derived from our solid-phase assay (7.15 × 10⁻¹⁰ M) indicates that SP-D binds with high avidity to the surface of M.tb. This type of analysis is limited by the fact that SP-D is an oligomeric protein with the capability for multivalent binding.

We found that the binding of SP-D to M.tb is calcium dependent and carbohydrate inhabitable. These data indicate that SP-D binds to the surface of the bacteria via its CRD. However, it is possible that mycobacterial lectins may participate in some of the observed binding, presumably by binding to the N-linked sugar of SP-D (55–57). Although we cannot completely exclude that a mycobacterial lectin binds to SP-D, calcium-dependent mycobacterial lectins have not been described. Furthermore, the carbohydrate inhibition profile that we observed is consistent with the published carbohydrate specificity of SP-D (25). Thus, it is likely that the majority of the binding of SP-D to M.tb observed in our studies is via the CRD of the protein.

Our lectin-blotting studies indicate that LAM from the virulent Erdman strain of M.tb is a major binding molecule for SP-D. LAM is a complex lipoglycan from the cell wall of many mycobacteria and is believed to be an important virulence determinant for M.tb (58–63). LAM serves as a ligand for the mannose receptor on macrophages and mediates the phagocytosis of virulent M.tb strains (40, 42, 45). The fatty acid tail of LAM serves as an anchor, leaving the carbohydrate portion of the molecule exposed on the surface (51, 64, 65). This carbohydrate portion is composed of an α-(1→6)-linked mannose core with short branching α-(1→2)-linked mannose side chains, followed by a large branching arabino- furan segment. For the described M.tb LAMs, these arabinofuran branches are 40% to 70% “capped” with approximately one to three α-(1→2)-linked mannosyl units. The presence of these mannose caps on M.tb LAM contrasts sharply with AraLAM, which lacks mannose capping and is ~10% capped with inositol phosphate (48). This structural difference is believed to be responsible for the observation that M.tb LAM stimulates much less TNF-α production by macrophages than AraLAM (66). The mannose cap structure on M.tb LAM also mediates the binding of LAM to the mannose receptor (45).

Three lines of evidence indicate that SP-D binds to the mannose caps of M.tb LAM. First, removal of the caps with exomannosidase abolished maltose-inhibitable binding of SP-D to LAM. Exomannosidase cleaves mannose units that are joined in the α-(1→2) position, the structure of the mannose caps. Second, when compared with Erdman LAM, SP-D bound only minimally to AraLAM, which does not contain mannose caps. The small degree of maltose-inhibitable binding to AraLAM (Figs. 6B and 7) may be the result of SP-D binding to the inositol phosphate caps since SP-D can bind to inositol and phosphatidylinositol (38). Third, exomannosidase did not affect the binding of SP-D to AraLAM. This result suggests that the mannose core region and its branching mannose side chains are not involved in the interaction between SP-D and LAM.

Other carbohydrates present on the surface of M.tb may also participate in the binding of SP-D. We observed a small degree of binding to a region of the M.tb lectin blot consistent with lipo- mannan, another lipoglycan of M.tb. Free complex carbohydrates such as mannan, arabinomannan, and glucan, which have been described on the surface of M.tb (67), may potentially interact with SP-D. Our methods are less likely to detect these binding interactions, however, since some of these carbohydrates may not be resolved by SDS-PAGE.

We were unable to detect significant binding of SP-D to M. smegmatis, the source of AraLAM. There are at least three possibilities to account for this observation. First, it is possible that SP-D may bind only to the mannose-capped LAM of M.tb, which is lacking on the surface of M. smegmatis. Second, the avirulent M. smegmatis may have less surface-exposed carbohydrate for SP-D binding compared with M.tb (68). Along this line, we have observed much smaller amounts of LAM from solubilized M. smegmatis compared with Erdman M.tb by Western blotting (our unpublished observations). Third, there may be subtle differences in the spatial presentation of the exposed carbohydrates between the two species that result in reduced binding to M. smegmatis. The marked difference between SP-D binding to pathogenic and non-pathogenic mycobacteria invites speculation that the human host has developed a mechanism to identify virulent bacteria that possess unique carbohydrate surface structures. These possibilities warrant further study.
Agglutination of microorganisms is thought to be an important innate host defense mechanism. Agglutinated bacilli may be cleared more readily from the respiratory tract by the mucociliary system or may have altered interactions with host cells (13, 27). We were unable to assess macroscopic agglutination using spectrophotometry because of the self-aggregating property of M.tb, which did not allow us to observe additional macroscopic agglutinating effects of the collectin above control (our unpublished observations). Our data demonstrate, however, that SP-D causes macroscopic agglutination of M.tb. The effect of macroscopic agglutination on the host response to pathogens has not been determined. For M.tb, it is possible that microscopic agglutination may lead to altered phagolysosomal fusion (69) or entry of the bacterium into the host cell.

The determinants of entry of M.tb into macrophages is an active area of investigation. These bacteria survive in the intracellular environment of the macrophage whereas many other pathogens are killed. Within this context, mechanisms that enhance entry of M.tb into macrophages may be detrimental to the host, whereas mechanisms that reduce entry may be protective, at least for the initial exposure of the host to the bacterium. Molecules that regulate innate immunologic responses play an important role in this interaction. We have previously reported that SP-A enhances the phagocytosis of M.tb by human MDM and alveolar macrophages via up-regulation of macrophage mannose receptor activity (14). Others have also reported a potential opsonin effect of SP-A for M.tb (22–24).

The studies on SP-A in this report are consistent with our prior report (14). In contrast, subagglutinating concentrations of SP-D reduce macrophage adherence and phagocytosis of M.tb. Thus, the functions of SP-A and SP-D appear to differ. One prior study demonstrated that SP-A mediated phagocytosis of Gram-negative bacteria by alveolar macrophages, but SP-D did not (13). SP-D has been shown to bind to AM (70, 71). A putative receptor on human macrophages has been described recently (49), raising the possibility that there is a pathway for SP-D-mediated uptake of microorganisms. However, microorganisms differ with respect to their predominant mechanism of entry into host cells. Altering these mechanisms by the addition of a collectin, Ab, or complement is likely to change these pathways in unique ways. M.tb is phagocytosed via complement receptors and the mannose receptor found on macrophages (72). On the basis of the results of our studies, we speculate that SP-D inhibits the LAM–mannose receptor interaction. This effect of SP-D may override any potential enhancing effect of a SP-D–macrophase interaction for M.tb uptake. Further studies are needed to examine this possibility.

In summary, we found that SP-D binds to M.tb in a specific manner. This binding is likely mediated by the terminal mannosyl oligosaccharides of LAM and the CRD of the protein. The presence of SP-D reduces the adherence of M.tb to human macrophages, whereas SP-A leads to enhanced adherence. These studies indicate that SP-D and SP-A influence the M.tb–mononuclear phagocyte interaction in the lung and are consistent with the hypothesis that these two collectins serve different roles in innate immunologic responses to respiratory pathogens. Ongoing studies are addressing the net effect of combinations of surfactant proteins in the presence and absence of lipid.

Acknowledgments

We thank Thomas Kaufman and Jennifer Ufnar for their expert technical assistance, and Dr. Stephen McGowan for assistance with the BAL.
protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus. Biochem. J. 323:395.


