Surfactant Protein D Binds to *Mycobacterium tuberculosis* Bacilli and Lipoarabinomannan via Carbohydrate-Lectin Interactions Resulting in Reduced Phagocytosis of the Bacteria by Macrophages[1]

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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 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)*[3] 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 type II epithelial cells and nonciliated bronchiolar (Clara) cells (2, 3). These proteins are calcium-dependent carbohydrate-binding proteins that contain an N-terminus with cysteines that participate in covalent oligomerization, a collagen-like region that imparts pronounced linear character to the molecules, and a highly conserved carbohydrate recognition domain (CRD) that is necessary for carbohydrate binding. SP-A and SP-D are highly homologous to the collectins mannose binding protein and bovine conglutinin, respectively (4–6). Both SP-D and SP-A have been implicated in innate immunity of the host (5, 7–9). The observation that mice homozygous for null alleles of SP-A are more susceptible to infection from group B streptococci supports this concept (10).

SP-A is an oligomeric glycoprotein composed of 28- to 35-kDa polypeptide subunits that form a complex of six trimers through covalent and noncovalent interactions (8). SP-A enhances phagocytosis through opsonization of microorganisms and/or up-regulation of phagocyte receptor function (11–16) and may also induce reactive oxygen intermediate formation and chemotaxis by macrophages (17, 18). Receptors for SP-A on macrophages have been described (19–21), one of which has been implicated in the effect of SP-A on the increased uptake of *Mycobacterium bovis*-bacillus Calmette Guérin into rat bone marrow-derived macrophages (22).

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 prepared with Bacto Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI), oleic acid albumin dextrose catalase enrichment medium (Difco), 0.1% casein enzymatic hydrolysate (Sigma, St. Louis, MO), and glycerol (Difco), as described (35), and dispensed into 100 × 15-mm bacteriologic petri dishes. Auramine-rhodamine stain (Difco) was purchased. Formaldehyde solution 37% w/w and potassium permanganate were purchased from EM Science (Gibbstown, NJ). Tran^{35}S-label was purchased from ICN (Costa Mesa, CA). Ficoll-sodium diatrizoate (Ficoll) was purchased from Pharmacia Fine Chemical (Piscataway, NJ). Chromerge was purchased from Fisher Scientific (Pittsburgh, PA). α-D-mannosidase (exomannosidase) was purchased from V-labs (Covington, LA). Maltose, mannose, glucose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine were purchased from EY Laboratories (San Mateo, CA). LPS from *Escherichia coli* 055:B5 strain was purchased from Sigma, suspended in endotoxin-free deionized water at 1 mg/ml, and stored frozen at −20°C.

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 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
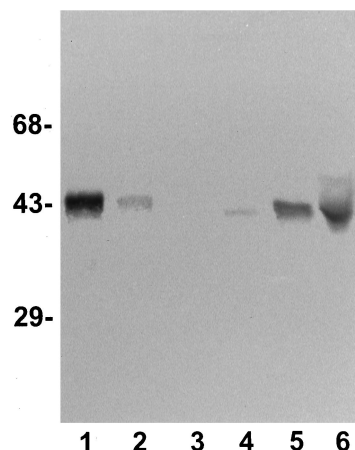

FIGURE 1. SP-D antiserum is specific for SP-D. Purified recombinant SP-D, APP SP-A, or lavage fluids were resolved by 10% SDS-PAGE, transferred to nitrocellulose, blocked, then incubated with a 1:20,000 dilution of anti-SP-D antiserum followed by GαRIgG-HRP and developed using 4-chloronapthol and H$_2$O$_2$. M.w. markers are shown on the *left*. *Lane 1*, 50 ng SP-D; *lane 2*, 12.5 ng SP-D; *lane 3*, 200 ng SP-A; *lane 4*, 50 μl normal BAL fluid; *lane 5*, 50 μl concentrated BAL fluid; *lane 6*, 50 μl alveolar proteinosis lavage fluid.

bacteria/ml) and the degree of clumping (≤10%) were determined by counting in a Petroff-Hausser chamber. *M. smegmatis* strain MC^{2}6 was provided by Dr. Steven Clegg (Department of Microbiology, University of Iowa). *M. smegmatis* was prepared as above except that the bacteria were grown for a period of 3–4 days and then harvested. After counting in a Petroff-Hausser chamber, the *M. smegmatis* suspensions were diluted to a concentration of 1–1.5 × 10^8 bacteria/ml and stored frozen at −70°C until the day of the experiment.

Collectin proteins, lipoglycans, and Abs

Recombinant rat SP-D, expressed in Chinese hamster ovary (CHO) cells, and recombinant rat SP-D deficient in hydroxylation of proline residues expressed in *Sf*9 cells (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/ng 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, but not with purified SP-A or SP-A 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 NO1-AI-25147). 2,2′-Azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS substrate) and AHRP-conjugated goat anti-rabbit IgG (GαRIgG-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 Centriprep-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 heparinized blood on Ficoll gradients and then were cultured in Teflon wells (Savillex, Minnetonka, MN) for 5 days in the presence of 20% autologous serum. On the day of each experiment, the cells were removed from the Teflon wells and washed, then resuspended in RPMI containing 10% autologous serum. The MDM were then plated onto Chromerge-cleaned glass coverslips or plastic coverslips in 24-well tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) for 2 h at 37°C (1×10^5 MDM/coverslip). 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 ($\geq$93%) was assessed using Trypan blue exclusion, and the percentage of macrophages present ($\geq$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 1×10^5 AM/coverslip. After 2 h at 37°C in 5% CO_2 and air, the monolayers were washed with RPMI and then used in the experiments. Monolayer integrity and density were monitored by inverted phase-contrast microscopy throughout the experiments.

Radiolabeled SP-D-binding experiments

SP-D expressed in CHO cells was metabolically labeled with Tran^{35}S-label (ICN) as described (38). *M.tb* was incubated in 50 mM tris-HCl, 150 mM NaCl (pH 7.5) (TBS) and 1 mg/ml human serum albumin (HSA) in preblocked microfuge tubes (5% HSA in TBS overnight at 4°C) with various concentrations of [^{35}S]SP-D in the presence and absence of Ca^{2+}, 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 $CaCl_2$ (TBS-C) and transferred to scintillation fluid for liquid scintillation counting in a beta counter (Beckman, Irvine, CA). The sp. act. of [^{35}S]SP-D ranged from 50 to 1500 cpm/ng. The mean $\pm$ SD of the cpm bound to triplicate pellets was calculated, and the amount in nanograms bound per 5×10^5 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 ($\leq$30 cpm).

Bacterial ELISA

In experiments designed to determine the saturable binding of SP-D to *M.tb*, bacterial suspensions (5×10^5 bacteria in 100 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 maltose 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 a 1:3000 dilution of GαRIgG-HRP for 2 h at room temperature. After washing, ABTS substrate (Bio-Rad) was added. The substrate development was stopped after 20 min by 1% oxalic acid, and the absorbance of individual wells was determined at 405 nm in a microplate spectrophotometer (Benchmark, Bio-Rad). The mean $\pm$ SD of triplicate wells for each concentration of SP-D was calculated. The background ($\leq$0.15 absorbance units) for each concentration of SP-D was defined as the absorbance of wells incubated with SP-D in the absence of *M.tb* and was subtracted out in each case. The absorbance of wells containing *M.tb* that were incubated with primary and secondary Abs but not SP-D was below background levels ($\leq$0.05 absorbance units). In experiments designed to examine the divalent cation requirements for SP-D binding to *M.tb*, the wells were incubated with a fixed concentration of SP-D in the presence or absence of 0.2 mM EDTA, and in the presence or absence of 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, or $SrCl_2$. SP-D binding was then detected by ELISA as above.

In ELISA experiments to further characterize the binding of SP-D to mycobacteria, *M.tb* or *M. smegmatis* (2.5×10^7) was incubated in TBS-C containing 1 mg/ml HSA with SP-D, lavage fluids, or buffer control in the presence or absence of EDTA, maltose, and *N*-acetylgalactosamine for 1 h at 37°C. The reaction volume was 200 μl. The bacteria were extensively washed in ice-cold TBS-C, resuspended in 300 μl buffer, pipetted into

triplicate wells (100 μl/well) of a 96-well microtiter plate (Becton Dickinson), and allowed to dry overnight. The plates were then treated with UV light for 2 h. The wells were blocked in TBS-C containing 5% BSA at room temperature for 2 h and then sequentially incubated with primary and secondary Abs in TBS-C containing 0.5% BSA and processed as described above. The mean $\pm$ SD of the absorbance for triplicate wells of each condition was calculated. The absorbance of buffer control wells (devoid of SP-D or BAL) was subtracted out in each case. At concentrations of SP-D $\leq$ 5 μg/ml, the results from the two types of ELISAs were similar.

Bacterial agglutination

M.tb was incubated with increasing concentrations of SP-D or the control protein HSA in TBS-C in the presence or absence of EDTA, maltose, and *N*-acetylgalactosamine for 1 h at room temperature. Aliquots of the reaction mixture were dried on glass coverslips, fixed in 10% formaldehyde, and stained with auramine-rhodamine. Agglutination was assessed by examining $\geq$30 consecutive fields per coverslip under fluorescence microscopy at 1250$\times$ magnification.

Lectin blotting

M.tb (2×10^7) and purified Erdman strain LAM or AraLAM (2 or 3 μg each) were reduced and denatured by boiling in 2% SDS and 10% 2-ME and resolved by 10% SDS-PAGE (43) or 15% 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 5×10^5 cpm/ml [^{35}S]SP-D in blocking buffer in the presence and absence of 10 mM 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 an anti-Erdman LAM primary Ab and GαRIgG-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 AraLAM (1 μg/well 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 U/ml 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-D^{hyp} in blocking buffer for 1 h at room temperature in the presence and absence of EDTA, maltose, or *N*-acetylglucosamine. After washing, the wells were sequentially incubated with anti-SP-D Ab or anti-LAM Ab for 2 h at room temperature, followed by GαRIgG-HRP. Color development with HRP substrate was stopped by 1% oxalic acid. The absorbance of individual wells was determined in a microplate spectrophotometer, and the mean $\pm$ 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-ϵ-amidocaproic acid hydrazide. After blocking, the wells were then incubated with alkaline phosphatase-conjugated anti-DIG Ab and developed with ABTS substrate.

M.tb *adherence assay*

M.tb was incubated in RPMI containing 20 mM HEPES, and 1 mg/ml HSA (pH 7.2) (RHH) in the presence or absence of SP-D or SP-A for 1 h at 4°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% CO_2 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% potassium permanganate. The mean number ($\pm$SD) of cell-associated bacteria per macrophage on three (three or nine in the case of AM) coverslips per condition was determined by counting $\geq$100 consecutive macrophages per coverslip using phase-contrast and fluorescence microscopy at 1250$\times$ magnification (42).

Additional adherence assays were conducted as above except that either the bacteria were washed before adding the mixture to coverslips, or the MDM monolayer was incubated with collectin, then washed before adding bacteria. Additionally, LPS was used in some experiments to determine the effect of the contaminating LPS of the collectins.
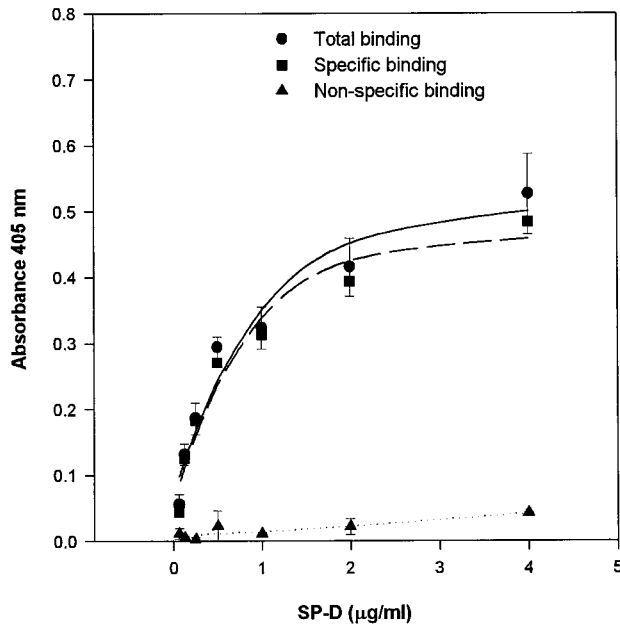
FIGURE 2. SP-D demonstrates saturable binding to *M.tb*. *M.tb* (5 × 10⁵) was dried in the wells of a microtiter plate and incubated with 0.0625 μg/ml to 4.0 μg/ml SP-D. Bound SP-D was detected by ELISA using rabbit anti-SP-D antiserum as primary Ab and GαRIgG-HRP as the secondary Ab. "Nonspecific binding" was defined as the absorbance at 405 nm ($A_{405\ nm}$) of wells that contained 100 mM maltose and was subtracted from the total $A_{405\ nm}$ to calculate the "specific binding." The solid, dashed, and dotted lines represent the mathematical curve fit of the total, specific, and nonspecific binding, respectively, and are derived from the experimental data points shown. Shown is the mean ± SEM of triplicate determinations from three independent experiments.

Electron microscopy

MDM monolayers on duplicate plastic coverslips were incubated with *M.tb* 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% gluteraldehyde, stained, and sectioned as described (42). The number of attached vs internalized bacteria per MDM cross-section and the percentage of MDM cross-sections that contained 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.tb *in a saturable, calcium-dependent, and carbohydrate-inhibitable manner*

To determine the specific interaction between SP-D and *M.tb*, and to characterize this interaction, SP-D−*M.tb*-binding studies were conducted in the presence and absence of various inhibitors. SP-D bound to *M.tb* in a specific and saturable manner. Ten-fold excess unlabeled SP-D inhibited 2 μg/ml [³⁵S]SP-D binding by 63% ± 8% (mean ± SEM, n = 3). [³⁵S]SP-D binding to *M.tb* 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.tb* adsorbed to the plastic wells to avoid the confounding variable of bacterial agglutination. The binding of SP-D to *M.tb* was found to be sat-
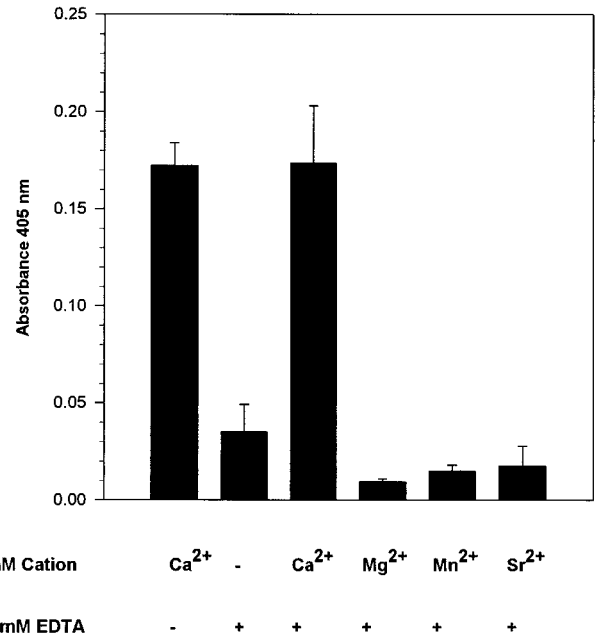

FIGURE 3. SP-D binding to *M.tb* is calcium dependent. *M.tb* (5 × 10⁵) was dried in the wells of a microtiter plate. After blocking, the wells were incubated with 1 μg/ml SP-D in blocking buffer in the presence or absence of 5 mM CaCl₂, MgCl₂, MnCl₂, or SrCl₂ and the presence or absence of 0.2 mM EDTA as indicated for 60 min at room temperature. Bound SP-D was detected by ELISA using rabbit anti-SP-D antiserum as the primary Ab and GαRIgG-HRP as the secondary Ab. Shown is the mean ± SEM of triplicate determinations from three independent experiments.

urable in a range between 2–4 μg/ml (Fig. 2). A double reciprocal plot of these data gives an approximate dissociation constant of 7.15×10^{-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.tb* 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.tb* suggested that SP-D may bind to *M.tb* via its CRD. To examine this possibility, we determined whether SP-D binding was calcium dependent and carbohydrate inhibitable. [³⁵S]SP-D binding to *M.tb* 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.tb* is dependent on divalent cations. To examine whether the cation requirements for the binding of SP-D to *M.tb* 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 $A_{405\ nm}$) in the presence of 0.2 mM EDTA and 5 mM MgCl₂, MnCl₂, or SrCl₂. However, SP-D binding in the presence of 5 mM CaCl₂ and 0.2 mM EDTA was similar to SP-D binding in the presence of 5 mM CaCl₂ and the absence of EDTA. These data provide evidence that the binding of SP-D to *M.tb* is cation dependent and exhibits a specific requirement for calcium.

To assess the carbohydrate specificity of SP-D binding to *M.tb*, assays were performed in the presence of increasing concentrations of selected carbohydrates with a fixed concentration of [³⁵S]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.tb* 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,
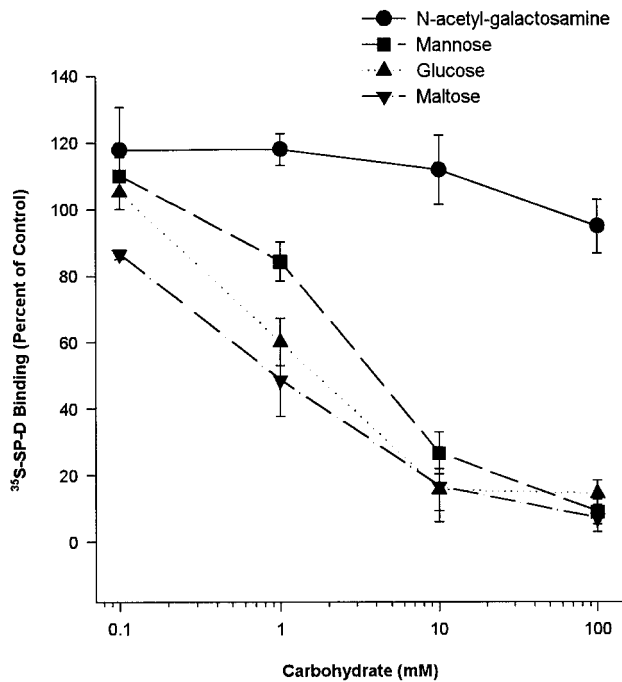
FIGURE 4. SP-D binding to *M.tb* is carbohydrate inhibitable. *M.tb* (5×10^5) was incubated with 4 μg/ml [^{35}S]SP-D in the absence or presence of increasing concentrations of *N*-acetylgalactosamine, mannose, glucose, or maltose for 30 min at 37°C. After centrifugation, the washed pellets were transferred to scintillation fluid for liquid scintillation counting. The results are expressed as the mean percentage of control [^{35}S]SP-D binding $\pm$ SEM of triplicate determinations from three independent experiments.

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.

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

Lavage Fluid	Expt. 1	Expt. 2
Control	0.245 ± 0.080	0.131 ± 0.005
BAL	0.103 ± 0.009	0.132 ± 0.019
CBAL	0.513 ± 0.016	0.628 ± 0.031
CBAL filtrate	0.011 ± 0.016	0.006 ± 0.002
WLL	0.624 ± 0.050	0.510 ± 0.081

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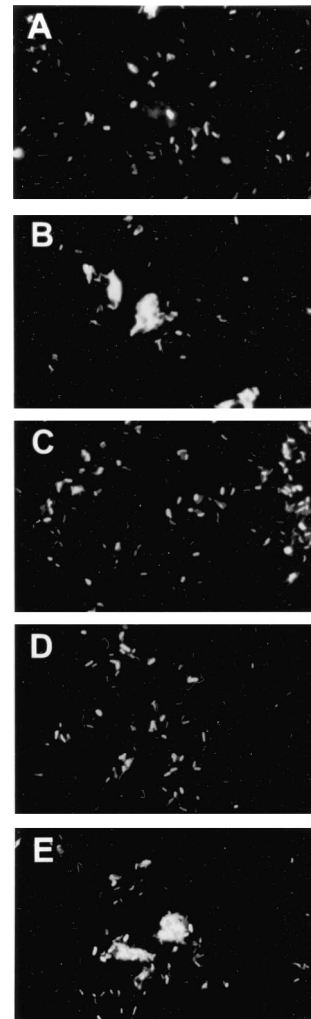

FIGURE 5. SP-D agglutinates *M.tb*. *M.tb* (1×10^8/ml) in TBS-C containing 1 mg/ml HSA was incubated in the absence (*A*) or presence (*B*) of 10 μg/ml SP-D, and the presence of 10 μg/ml SP-D and 100 mM maltose (*C*), 10 mM EDTA (*D*), or 100 mM *N*-acetylgalactosamine (*E*). Aliquots were dried on glass coverslips, fixed with 10% formaldehyde, and stained with auramine-rhodamine; $\geq$30 consecutive fields were visualized by fluorescence microscopy. Shown are representative fields (final magnification, $\times$1100) of one experiment (*n* = 3).

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 μg/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.
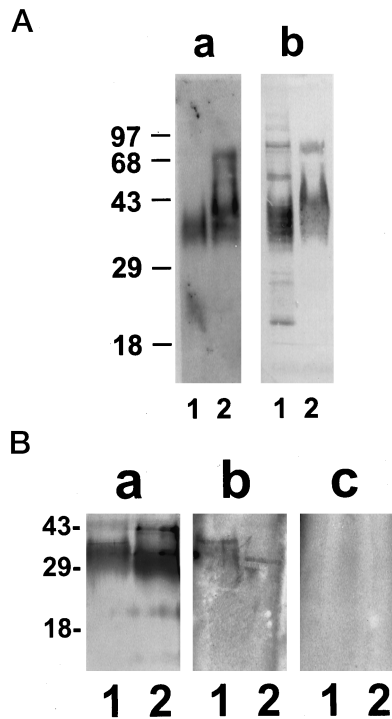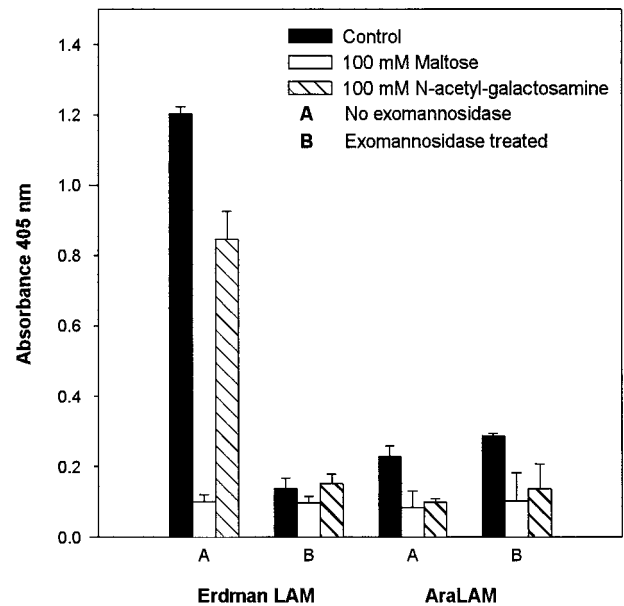
FIGURE 7. SP-D binds to the terminal mannosyl oligosaccharides of *M.tb* LAM. Erdman LAM and AraLAM were adhered to the wells of a microtiter plate and incubated in sodium acetate buffer (pH 5.5) in the absence or presence of 0.18 U/ml exomannosidase as indicated to cleave α-(1→2)-linked mannosyl oligosaccharides. After washing, the wells were incubated with 4 μg/ml SP-D[hyp] in the absence (control), or presence of 10 mM maltose, or 10 mM *N*-acetylglucosamine, and SP-D binding was determined by ELISA using anti-SP-D antiserum. Shown is the mean ± SD of triplicate wells in a representative experiment of two.

FIGURE 6. SP-D binds to *M.tb* LAM. *A*, 2×10^7 *M.tb* (*lane 1*) or 3 μg Erdman LAM (*lane 2*) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were then subjected to [^{35}S]SP-D lectin-blotting (*panel a*) or anti-LAM Western-blotting (*panel b*). The m.w. are indicated on the *left*. Erdman LAM appears as a wide band at ~35 kDa. A small amount of SP-D binding is also visualized at ~20–25 kDa, consistent with the known apparent m.w. of lipomannan. *B*, Two micrograms of Erdman LAM (*lane 1*) or AraLAM (*lane 2*) were resolved by 15% SDS-PAGE and periodate acid silver stained (*panel a*), or transferred to nitrocellulose and subjected to lectin blotting in the presence of 2 mM CaCl$_2$ (*panel b*), or 10 mM EDTA (*panel c*). Shown is one experiment representative of three.

M.tb *LAM serves as a major binding molecule for SP-D*

We performed [^{35}S]SP-D lectin-blotting studies to identify specific molecules on *M.tb* that serve as binding molecules for the protein (Fig. 6*A*, *panel a*, *lane 1*). The [^{35}S]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. 6*A*, *panel a*, *lane 2*; and Fig. 6*B*, *panel b*, *lane 1*), but not in the presence of 10 mM EDTA (Fig. 6*B*, *panel c*).

Compared with *M.tb* LAM, [^{35}S]SP-D bound minimally to immobilized, purified AraLAM, the LAM type from the avirulent *M. smegmatis* (Fig. 6*B*, *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 .212 ± .038 for Erdman LAM and .217 ± .003 for AraLAM (mean ± SEM, 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

Table II. *SP-D binds minimally to* M. smegmatis *compared to* M.tb[a]

Condition	*M. smegmatis*	*M.tb*
SP-D/Ca^{2+}	0.160 ± .009	0.993 ± .020
SP-D/EDTA	0.085 ± .009	0.121 ± .033
SP-D/Ca^{2+}/maltose	0.106 ± .006	0.177 ± .021
SP-D/Ca^{2+}/GalNAc	0.132 ± .019	0.659 ± .009

[a] Bacteria (2.5×10^7) were incubated with 4 μg/ml SP-D in the presence of 2 mM calcium, or 10 mM EDTA, or the presence of 2 mM calcium and 100 mM maltose or *N*-acetylgalactosamine (GalNAc) for 60 min at 37°C. The pellets were then washed three times in ice-cold buffer, and 100-μl aliquots were allowed to evaporate to dryness in the wells of a 96-well microtiter plate (8×10^6 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.

specific residues included in the overall structure of the complex carbohydrate. To determine whether SP-D binds to *M. smegmatis*, an avirulent species of mycobacteria and the source for AraLAM, we used a bacterial ELISA. By comparison with binding to *M.tb*, SP-D bound minimally to *M. smegmatis* (Table II). In like fashion, by lectin blotting using 2×10^7 *M. smegmatis*, no binding of [^{35}S]SP-D was observed (data not shown). These results suggest that the binding of SP-D to *M.tb* is dependent upon specific carbohydrate structures exposed on the surface of *M.tb* that are not present on the surface of *M. smegmatis*. Inasmuch as these results resemble those obtained with *M.tb* LAM and AraLAM, they further support the concept that *M.tb* LAM serves as a major binding molecule for SP-D in the context of whole bacteria.

SP-D reduces the adherence of M.tb *to human macrophages*

SP-D has been reported to be an opsonin for *A. fumigatus* (31) and influenza virus (33). We have previously demonstrated that SP-A increases the phagocytosis of Erdman *M.tb* by human macrophages (14). Since we determined that SP-D binds to *M.tb* LAM, we hypothesized that this binding would interfere with the recognition of LAM by the macrophage mannose receptor and thereby reduce *M.tb* adherence to macrophages. Alternatively, SP-D binding to *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 *M.tb* to MDM in the presence and absence of SP-D (Fig. 8 and Table III). SP-D at a subagglutinating concentration (0.5 μg/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/*M.tb* mixture was added to MDM, SP-D caused a 37.3% ± 3.3% (mean ± SEM, $n = 8$, $p < 0.001$) reduction in the adherence of bacteria compared with control. This effect was retained if the SP-D/*M.tb* mixture was washed before incubation with MDM (37.7% ± 1.8% reduced adherence, mean ± SEM, $n = 4$), but lost if the MDM 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−*M.tb* interaction (SP-D binding to *M.tb*), and not due to an SP-D−MDM interaction. In contrast, SP-A increased the adherence of *M.tb* with macrophages (Fig. 8). Washing away excess collectin from the monolayers before adding bacteria did not attenuate the increased *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 *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 *M.tb* to macrophages (Fig. 8).
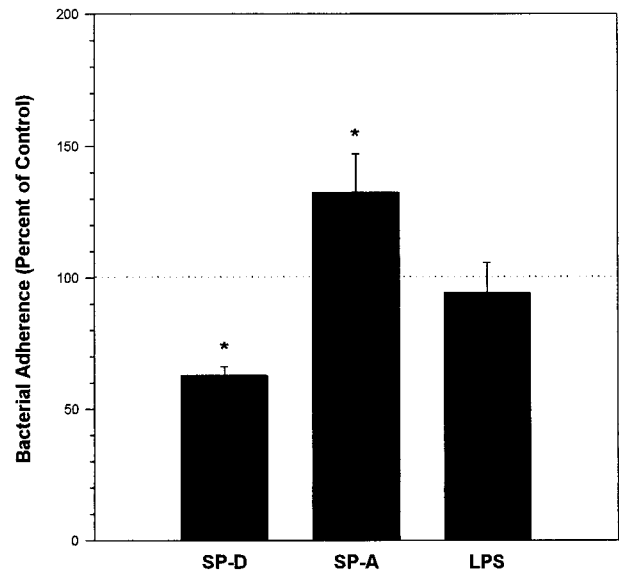

FIGURE 8. SP-D reduces the adherence of *M.tb* to macrophages, whereas SP-A enhances bacterial adherence. *M.tb* (1.5×10^7) in RHH was incubated in the presence or absence of 0.5 μg/ml SP-D, 10 μg/ml SP-A, or 10 ng/ml LPS for 1 h at 4°C on a nutating platform. The mixture was allowed to return to room temperature and then was added to MDM on triplicate glass coverslips for 2 h at 37°C in 5% CO$_2$ 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 ≥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) × 100. The control level of bacteria/MDM was 2.07 ± 0.8 (mean ± SEM, $n = 8$). Shown is the mean ± 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 *M.tb* to human AM. SP-D (0.5 μg/ml) was incubated with *M.tb* and then added to AM monolayers as described for MDM. SP-D reduced the adherence of *M.tb* to AM by 53.1% ± 2.0% (mean ± SEM, $n = 2$, $p < 0.05$). The number of bacteria per AM in the control condition was 2.2 ± 0.5 (mean ± SEM, $n = 2$).

Table III. *The inhibition of* M.tb *adherence to macrophages by SP-D results from the SP-D-*M.tb *interaction, whereas the enhancement of* M.tb *adherence to macrophages results from the SP-A-macrophage interaction*[a]

Collectin	A (%)	B (%)
SP-D	53.3 ± 1.3*	94.5 ± 4.5
SP-A	152.3 ± 5.4*	170.2 ± 13.1*

[a] Monolayers of MDM were incubated with the indicated collectins (SP-D: 0.5 μg/ml; SP-A: 10 μg/ml) for 1 h at 37°C in 5% CO$_2$ and air. (A) *M.tb* (1×10^6) 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 ± 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] × 100) performed in the absence of collectin (mean ± 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 inhibitable. 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 mannan core with short branching α-(1→2)-linked mannose side chains, followed by a large branching arabinofuran 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. 6*B* 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 mannan 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 lipomannan, 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 nonpathogenic 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 microscopic agglutination of *M.tb*. The effect of microscopic 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−macrophage 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.

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