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Pulmonary Surfactant Protein A Mediates Enhanced Phagocytosis of *Mycobacterium tuberculosis* by a Direct Interaction with Human Macrophages[1]

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During initial infection with *Mycobacterium tuberculosis*, bacteria that reach the distal airspaces of the lung are phagocytosed by alveolar macrophages in the presence of pulmonary surfactant. Here we have examined the role of surfactant-associated protein A (SP-A) in phagocytosis of the virulent Erdman strain of *M. tuberculosis* by human monocyte-derived macrophages (MDMs) and human alveolar macrophages (HAMs). Macrophage monolayers incubated with soluble SP-A from alveolar proteinosis patients (APP SP-A[4]) and recombinant rat SP-A (SP-A^{hyp}) demonstrated enhanced adherence of *M. tuberculosis*, 82 ± 17% and 49 ± 18%, respectively. Removal of SP-A from monolayers by washing before adding bacteria did not diminish the enhanced adherence. Fluorescence microscopy demonstrated that washed monolayers contained intracellular rather than surface-bound SP-A. These studies indicated a direct interaction between SP-A and the macrophage in mediating enhanced adherence of *M. tuberculosis*. Consistent with this interpretation, macrophage monolayers formed on human or rat SP-A (substrate SP-A) demonstrated enhanced adherence of *M. tuberculosis* to their apical surface (APP SP-A and native rat SP-A increased *M. tuberculosis* adherence by 102 ± 16% and 102 ± 25%, respectively). Electron microscopy demonstrated increased numbers of phagocytosed bacteria in APP SP-A-treated MDM cross-sections. SP-A proteins devoid of carbohydrate failed to enhance *M. tuberculosis* adherence to macrophages. In contrast, heat-denatured APP SP-A enhanced adherence of bacteria equivalent to that of intact glycoprotein. Thus, the carbohydrate moieties of SP-A appear to be critical in the SP-A-macrophage interaction. Finally, mannan and anti-mannose receptor Ab completely inhibited the enhanced phagocytosis of *M. tuberculosis* observed with APP SP-A, providing evidence for up-regulation of macrophage mannose receptor activity. These studies implicate SP-A as an important modulator of alveolar macrophage function that results in an enhanced potential for *M. tuberculosis* to gain access to its intracellular niche. ***The Journal of Immunology*, 1995, 155: 5343–5351.**

During infection, the respiratory pathogen *Mycobacterium tuberculosis* is inhaled into the alveoli of the lung, where it is phagocytosed by and multiplies within mononuclear phagocytes. Thus, the interaction between *M. tuberculosis* and the alveolar lining, particularly alveolar macrophages and surfactant, represents the initial contact of this bacterium with the host immune system. The factors involved in this unique interaction are likely to be important in disease pathogenesis but are poorly understood. Lung surfactant functions primarily to reduce surface tension forces of the alveolar lining and prevents atelectasis (1). It is composed primarily of phospholipids but also contains 5 to 10% protein. Surfactant protein A (SP-A)[4] is a major nonserum protein constituent of surfactant, produced by alveolar type II cells, that is involved in surfactant lipid homeostasis (1). It is a 28- to 36-kDa glycoprotein containing one or two *N*-linked oligosaccharide attachment sites, a collagen-like domain, and a carbohydrate recognition domain (CRD). The oligosaccharides of SP-A have been implicated as important in some of the functions of the protein (2–4). SP-A assembles into an oligomer of up to 18 subunits (5, 6), resulting in a high valency of binding sites for diverse sets of molecules, including surface receptors on macrophages and alveolar type II cells and surface components of microorganisms (6).

Recent studies have shown that SP-A can augment phagocytosis by up-regulation of phagocyte receptor function (7). Since SP-A is an important constituent of the lung airspace, and we have previously determined that phagocytosis of *M. tuberculosis* is a receptor-mediated process (8, 9), we explored the role of SP-A and its carbohydrate moieties in regulating phagocytosis of the virulent Erdman strain of *M. tuberculosis* by human monocyte-derived macrophages (MDMs) and human alveolar macrophages (HAMs). The purpose of this study was to examine 1) the influence of SP-A

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[4] Abbreviations used in this paper: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; APP, alveolar proteinosis protein; DIG, digoxigenin; PBS, Dulbecco's PBS with Ca^{2+} and Mg^{2+} ions; HAM, human alveolar macrophage; HSA, human serum albumin; MDM, monocyte-derived macrophage; PD, PBS without Ca^{2+} and Mg^{2+}; x-phosphate/NBT, 6-bromo-4-chloro-3 indolyl-phosphate/nitroblue tetrazolium chloride.

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proteins in solution on *M. tuberculosis* phagocytosis; 2) the influence of substrate-bound SP-A proteins in *M. tuberculosis* phagocytosis; and 3) the role of the carbohydrate moieties of SP-A in mediating its effects. Our results demonstrate that SP-A interacts with macrophages to enhance phagocytosis of *M. tuberculosis* and that the oligosaccharides of SP-A play a critical role in this interaction.

Materials and Methods

Buffers, reagents, and media

Dulbecco's PBS with Ca^{2+} and Mg^{2+} ions (PBS) (Life Technologies, Grand Island, NY), PBS without Ca^{2+}, and Mg^{2+} (PD) (Cancer Center, University of Iowa, Iowa City, IA) and RPMI 1640 medium with L-glutamine (RPMI) (Life Technologies) were purchased. RPMI medium was used alone or with 20 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO), pH 7.2, and 1 mg/ml human serum albumin (HSA) (Calbiochem Corp., La Jolla, CA). 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 Chemical Co.), and glycerol (Difco), as described (10), and dispensed into 100 × 15-mm bacteriologic petri dishes. No antibiotics were added to media in any of the experiments. Formaldehyde solution, 37% w/w (Fisher Scientific Company, Fairlawn, NJ); auramine-rhodamine stain (Difco); and potassium permanganate (EM Science, Gibbstown, NJ) were purchased. Radioisotopes were purchased from ICN ([^{35}S]methionine; Costa Mesa, CA) and Amersham (^{125}I-labeled Bolton-Hunter reagent; Arlington Heights, IL).

SP-A proteins

The SP-A proteins used in this study were developed and purified as described (11–13). In brief, bronchoalveolar lavage was utilized to obtain SP-A from patients with alveolar proteinosis (APP SP-A) and from healthy volunteers (SP-A) (12). Rat SP-A was purified from silica-treated Sprague-Dawley rat lungs (12). Recombinant SP-A (which is deficient in hydroxyproline content and hence designated SP-A^{hyp}) was produced from SF-9 insect cells following infection with a recombinant baculovirus containing a 1.6-kb cDNA for rat SP-A (13). Nonglycosylated recombinant SP-A protein was synthesized from infected SF-9 cells cultured in the presence of tunicamycin (SP-A^{hyp,TM}) (13). Recombinant SP-A proteins devoid of oligosaccharides at one or both of the consensus sequences for glycosylation were generated by amino acid substitutions at the Asn^{1} site (SP-A^{hyp,thr1}), the Asn^{187} site (SP-A^{hyp,ser187}) or both sites (SP-A^{hyp,thr1,ser187}). Carbohydrate-deficient SP-A proteins retain structural and biologic functions including oligomerization, aggregation of phospholipid liposomes, binding to immobilized carbohydrate, inhibition of lipid secretion from type II cells, and competition for receptor occupancy on type II cells (13).

Macrophages

Blood was obtained from four healthy adult volunteers who were purified protein derivative skin test negative for tuberculosis, had no clinical history of tuberculosis, and no known exposure to tuberculosis cases. PBMC were isolated from heparinized blood on Ficoll-sodium diatrizoate (Pharmacia Fine Chemical, Piscataway, NJ) gradients and were then cultured in Teflon wells (Savillex Corp., Minnetonka, MN) for 5 days in the presence of 20% autologous serum (1.5–2.0 × 10^6 mononuclear cells/ml) (9). On the day of each experiment, PBMC were removed from Teflon wells, washed extensively, and the MDM fraction was purified by adherence to Chromerge (Fisher Scientific Co.)-cleaned glass coverslips (2 h) in 24-well, flat-bottom plates (Linbro, Flow Laboratories, McLean, VA) in the presence of medium containing RPMI-HEPES-HSA; 1.5 × 10^6 mononuclear cells were added to each well.

Human alveolar macrophages were obtained from healthy volunteers who were not cigarette smokers and had negative skin tests for tuberculosis. Bronchoalveolar lavage was performed using a previously described procedure that has been approved by the Institutional Review Board for human subjects (14). Alveolar macrophages were isolated from the bronchoalveolar lavage fluid as described (15), except that all solutions used for washing and resuspending the cells did not contain antibiotics. The alveolar macrophages were used in experiments on the day of isolation. 5.0 × 10^5 macrophages were added to each well.

Bacteria

Lyophilized *M. tuberculosis* Erdman strain (ATCC 35801) was obtained from American Type Culture Collection (ATCC, Rockville, MD), reconstituted, and used as described (8). Briefly, for each experiment, aliquots of frozen stock in 7H9 broth were thawed, cultured for 9 days on 7H11 agar, scraped from agar plates, suspended in RPMI-20 mM HEPES, vortexed briefly (5–6 pulses) with two glass beads, and allowed to settle over 30 min. The upper bacterial suspension (devoid of clumps) was removed to a second tube. The concentration of the bacterial suspension (approximately 3 × 10^8/ml) was further adjusted for use in experiments.

Modification of SP-A proteins

Using a modification of published protocols (4), the *N*-linked oligosaccharides of APP SP-A were enzymatically removed by treatment with *N*-glycosidase F (1 U enzyme/25 μg APP SP-A) (Boehringer Mannheim Corp., Indianapolis, IN). The reaction was conducted in deglycosylation buffer at 37°C for 16 h. The deglycosylation buffer contained 20 mM sodium phosphate, pH 7.4, 6 mM *n*-octyl-β-D-glucopyranoside (Sigma Chemical Co.), and 10 mM EDTA (Mallinkrodt, Paris, KY). The reaction mixture was then dialyzed extensively against 10 mM phosphate buffer, pH 7.4, over 24 h (dialysis tubing m.w. cutoff of 12,000–14,000; Spectrum Medical Industries, Inc., Los Angeles, CA) and the retentate was concentrated using Centriprep-10 (Amicon, Danvers, MA). Sham-treated APP SP-A, prepared exactly as above except for the omission of *N*-glycosidase F, was used as the control protein in *M. tuberculosis* adherence assays that compared the activity of intact APP SP-A to deglycosylated APP SP-A. Final protein concentrations were determined with the bicinchoninic protein assay kit (BCA) (Pierce) using BSA as a standard.

Recombinant SP-A was metabolically labeled with ^{35}S (13) (sp. act., 35 cpm/ng). APP SP-A, deglycosylated APP SP-A, and rat SP-A were labeled with ^{125}I, using the Bolton-Hunter technique (13, 16) (sp. act., 350 cpm/ng, 160 cpm/ng, and 447 cpm/ng, respectively). All radiolabeled proteins were determined to be >95% TCA precipitable before use and were free of radiolytic damage as determined by SDS-PAGE and autoradiography. In experiments with heat-denatured SP-A, SP-A was heated at 100°C for 10 min (17, 18), cooled, and used immediately in experiments.

Analysis of SP-A proteins

SP-A proteins were analyzed by SDS-PAGE and by immunoblots. For gel analysis, SP-A proteins were separated by standard 12.5% SDS-PAGE (19) under reducing conditions and stained by silver stain (20).

For immunoblot experiments, the digoxigenin (DIG) glycan detection kit (Boehringer Mannheim Corp.) was used to detect the presence or absence of carbohydrate moieties on APP SP-A and deglycosylated APP SP-A. Initially, the proteins were incubated with sodium metaperiodate and then DIG-succinyl-E-amidocaproic acid hydrazide was covalently linked to the oxidized carbohydrate moieties. After separation by SDS-PAGE, as above, the proteins were transferred to nitrocellulose. The membrane was exposed to alkaline phosphatase-conjugated anti-DIG Ab and developed with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride (x-phosphate/NBT).

M. tuberculosis *adherence assay with SP-A in solution*

MDM or HAM monolayers on glass coverslips in tissue culture wells were incubated with various concentrations of SP-A proteins in the presence of RPMI, 20 mM HEPES, and HSA for 60 min. *M. tuberculosis* was then added to the monolayers for 120 min. In certain experiments, the monolayers were washed extensively with RPMI to remove SP-A before adding bacteria. Subsequently, the monolayers were washed and fixed in formalin, and total cell-associated bacteria were stained with auramine-rhodamine (9). The mean number (±SD) of adherent bacteria per macrophage on duplicate or triplicate coverslips was determined by counting ≥100 consecutive macrophages per coverslip using phase contrast and fluorescence microscopy.

Fluorescent staining of SP-A in macrophages

MDM monolayers on glass coverslips in tissue culture wells were incubated with APP SP-A in solution or HSA (10 μg/ml) for 60 min, and the monolayers were washed extensively to remove SP-A exactly as described above. The monolayers were then fixed in 3.3% formalin for 10 min or 100% methanol for 3 min. Methanol disrupts the cell membrane to allow Ab staining of total cell-associated SP-A, whereas with formalin fixation, Abs stain surface SP-A only (21). Nonspecific macrophage binding sites were then blocked with 2% BSA in PD for 120 min, the monolayers were washed, and polyclonal rabbit anti-human SP-A (1:2000) in 0.2% BSA in PD was added for 20 min. After further washing, FITC-conjugated goat anti-rabbit Ab (1:100; Cappel, Westchester, PA) was added for 20 min. Subsequently, the coverslips were washed and examined using phase and fluorescent microscopy.

M. tuberculosis *adherence assay with SP-A on a substrate*

SP-A or HSA (control) was covalently linked to glass coverslips that had been coated with poly-L-lysine (Sigma Chemical Co.) and 2.5% glutaraldehyde (EM Science) in tissue culture wells using a technique previously described for covalent linkage of proteins (22). Twelve micrograms of SP-A or HSA was added to the coverslips for 30 min before washing. Unreacted aldehyde groups were blocked with 0.2 M glycine, pH 7.4. Macrophages were plated on these substrates for 120 min. After washing, macrophages in monolayer culture were then incubated with *M. tuberculosis* for 120 min and adherent bacteria enumerated exactly as in the assay with SP-A in solution.

To determine the amount of SP-A bound to the substrate, radiolabeled SP-A proteins were covalently linked to glass coverslips as described above. For the determination of bound ^{35}S recombinant SP-A, 12, 6, or 3 μg of protein of known specific activity was added to the substrate and washed six times with PD, and the coverslips were counted in a beta counter (Beckman L5330I; Beckman, Fullerton, CA). For the determination of bound ^{125}I SP-A proteins (APP SP-A, deglycosylated APP SP-A, and rat SP-A), 1 μg of each radiolabeled protein was used with 11, 6, or 2 μg of unlabeled protein on the substrate. Coverslips were counted in a Beckman gamma counter (5500B). Six washes were required to reduce the number of counts in the fluid wash to <10% of the counts on the coverslip.

To determine the influence of soluble mannan or anti-mannose receptor Ab on *M. tuberculosis* adherence, MDMs were plated on a substrate of HSA or SP-A as above, washed and preincubated with mannan (4 mg/ml) for 60 min, heat-inactivated polyclonal anti-mannose receptor Ab (1:100 final dilution, kindly provided by Virginia Shepherd, Vanderbilt University, Nashville, TN) for 20 min, or heat-inactivated normal rabbit serum (1:100, control). *M. tuberculosis* was then added to monolayers and adherent bacteria enumerated as above. As additional controls in these experiments, certain MDM monolayers were incubated with unopsonized zymosan (3.5×10^6/well, positive control) (23), as described (24), or sheep erythrocytes coated with IgG (E-IgG) (5.0×10^6/well, negative control), as described (25).

In adherence assays with SP-A in solution or on a substrate, the viability of macrophages in monolayer culture in the presence of SP-A proteins was >95%, as assessed by trypan blue exclusion. The density of macrophage monolayers in the presence of SP-A proteins (assessed by nuclear staining (26)) was equivalent to the density observed in the presence of HSA (data not shown).

Electron microscopy assay to assess ingestion of M. tuberculosis *by macrophages in the presence of SP-A*

MDMs were adhered to a substrate of APP SP-A or HSA (control) on plastic coverslips (Wako Chemical Co., Dallas, TX). The monolayers were then incubated with *M. tuberculosis* exactly as described above before being prepared for electron microscopy (EM). Macrophages on plastic coverslips were fixed with 2.5% glutaraldehyde (EM grade, TAAB Laboratories Equipment LTD, Berkshire, England), in 0.1 M cacodylate buffer, pH 7.2, for 2 h, and then washed with the same buffer three times. Following incubation with 1% osmium tetroxide (Stevens Metallurgical Corp., New York, NY) and 1.5 potassium ferrocyanide (Fisher Scientific Company) in 0.1 M cacodylate buffer, cells were washed three times with the same buffer, dehydrated through ethanol series up to 100%, and infiltrated with the mixture of Eponate 12 (Ted Pella Inc., Redding, CA) and 100% ethanol (1:1). After infiltration with 100% Eponate 12 for 8 to 12 h, cells were embedded by placing a Beem capsule filled with fresh Eponate 12 on top of the coverslips with cell side up and polymerized in a 60 to 65°C oven.

After the resin was cured, coverslips were peeled off from Beem capsules. En face sections were cut at 90 μm and stained with 5% uranyl acetate for 12 min and lead citrate for 8 min. Sections were examined and photographed on the Hitachi H-7000 transmission electron microscope (Hitachi Instruments, Inc., San Jose, CA). The number of bacteria associated with >25 consecutive cross-sections of different MDMs in each treatment group was enumerated.

Statistics

A two-tailed Student's *t*-test was used for analyzing differences between test groups and control groups.

Results

SP-A in solution enhances adherence of M. tuberculosis *to MDMs*

To determine the influence of SP-A on adherence of *M. tuberculosis* to macrophages, we incubated MDMs in monolayer culture with SP-A in solution before adding bacteria. In initial studies, we used APP SP-A because of the abundance of purified material available from these patients (27). APP SP-A in solution enhanced adherence of *M. tuberculosis* to macrophages in a dose-dependent fashion (Fig. 1). At the maximal level of enhancement (≥5 μg/ml), APP SP-A significantly enhanced *M. tuberculosis* adherence by 82 ± 17% compared with HSA control (mean ± SE, $n = 7$, $p < 0.01$).

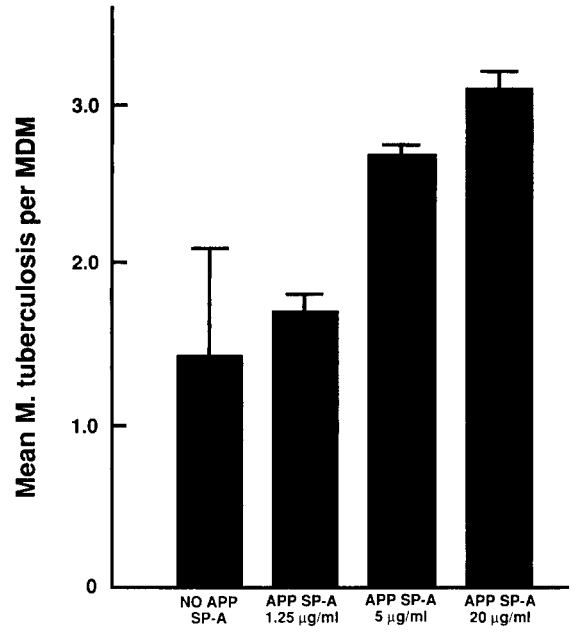

FIGURE 1. Human APP SP-A in solution enhances *M. tuberculosis* adherence to MDMs in a dose-dependent fashion. MDMs in monolayer culture were incubated with Erdman *M. tuberculosis* (1×10^6) in the presence of the indicated concentrations of APP SP-A and serum-free medium (RPMI, HEPES, HSA) or medium alone. SP-A was added 60 min before adding bacteria. Adherence was evaluated by fluorescence microscopy and the mean number of bacteria per MDM was calculated. Data are the mean ± SD of triplicate coverslips in a representative experiment.

We next determined the influence of several other SP-A proteins in the adherence of *M. tuberculosis* to MDMs. We studied purified recombinant SP-A ($SP\text{-}A^{hyp}$), SP-A from healthy donors, and rat SP-A. When macrophage monolayers were incubated with each of these glycoproteins in solution before adding bacteria, $SP\text{-}A^{hyp}$ significantly enhanced *M. tuberculosis* adherence by 49 ± 18% (mean ± SE, $n = 7$, $p < 0.02$), whereas the results obtained with the other proteins were variable, yielding a mean level of adherence that was not significantly different from HSA control (4 ± 9%, $n = 4$, enhanced adherence for SP-A from healthy donors; and 7 ± 14%, $n = 4$, enhanced adherence for rat SP-A).

We next determined whether the enhanced level of adherence of *M. tuberculosis* seen with APP SP-A in solution required the presence of the protein throughout the adherence assay. To accomplish this, we extensively washed macrophage monolayers to remove APP SP-A before the addition of bacteria and then compared the resulting level of bacterial adherence with that seen under conditions in which APP SP-A was present throughout the assay. Removal of APP SP-A before adding bacteria did not diminish the enhanced level of bacterial adherence. When APP SP-A was removed, the level of bacterial adherence was increased 97 ± 8% (mean ± SEM, $n = 2$) compared with HSA control. Similarly, when APP SP-A was not removed in separate wells of the same

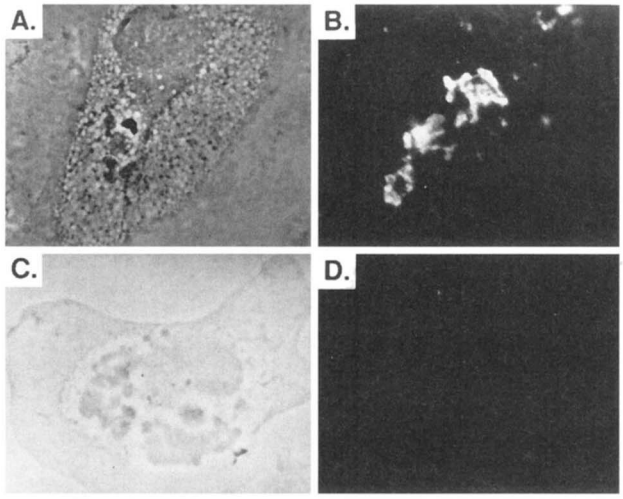

FIGURE 2. APP SPA is localized inside macrophages. MDMs in monolayer culture were incubated with APP SP-A in solution (10 μg/ml) for 60 min. The monolayers were washed extensively to remove non-cell-associated SP-A (*A–D*), and the cells were fixed with either 100% methanol (*A* and *B*) or 3.3% formalin (*C* and *D*). After washing, the monolayers were incubated with BSA, washed again, stained with polyclonal rabbit anti-human SP-A Ab and then, after further washing, with FITC-conjugated goat anti-rabbit Ab. The macrophages were examined for SP-A by phase (*A* and *C*) and fluorescence microscopy (*B* and *D*). The scant peripheral fluorescence after formalin fixation (*D*) is not well visualized.

experiment, the level of bacterial adherence was increased 76 ± 7% (mean ± SEM, $n = 2$).

Although this experiment suggested that a specific interaction between APP SP-A and macrophages was important in mediating SP-A's effect, it did not rule out the possibility that SP-A remained on the macrophage cell surface after washing and bound *M. tuberculosis* directly. To address this issue, we stained macrophage monolayers for SP-A, after methanol or formalin fixation, to determine the cellular location of SP-A after extensively washing the monolayer. Methanol-fixed cells demonstrated abundant fluorescent staining in the cell interior, whereas cells fixed in formalin displayed minimal peripheral staining of extremely low intensity (Fig. 2). Control experiments in which the monolayer was not washed confirmed that SP-A exposed to formalin retained its antigenicity, and monolayers incubated with HSA rather than SP-A demonstrated no fluorescent staining (data not shown). These results indicated that SP-A in solution had been internalized after the 1 h preincubation period and that washing macrophage monolayers resulted in nearly complete removal of surface-bound SP-A. This experiment provided further evidence for a direct interaction between SP-A and macrophages in mediating SP-A's effect in our assay and prompted us to perform substrate-bound SP-A studies.

MDMs plated on a substrate of SP-A demonstrate enhanced adherence of M. tuberculosis

To further ascertain whether a direct interaction between SP-A and macrophages leads to the enhanced adherence of *M. tuberculosis*, we plated MDMs on a substrate of SP-A (basal surface of the cell) and then added *M. tuberculosis* to assess adherence to the apical surface of the cell. Using radiolabeled APP SP-A, rat SP-A, and SP-A^{hyp}, we determined that the substrate maximally contained 217 ± 32 ng of covalently-bound SP-A per coverslip (mean ± SEM, $n = 5$) without significant differences between proteins. Substrate-bound human or rat SP-A significantly enhanced *M. tuberculosis* adherence to macrophages when compared with HSA control (Table I). APP SP-A enhanced *M. tuberculosis* adherence to a greater degree (102 ± 16%, mean ± SEM, $n = 10$, $p < 0.001$) than did SP-A from healthy donors (32 ± 10%, mean ± SEM, $n = 6$, $p < 0.02$). Rat SP-A and SP-A^{hyp} enhanced *M. tuberculosis* adherence by 102 ± 25% ($n = 4$, $p < 0.05$) and 50 ± 7% ($n = 4$, $p < 0.02$), respectively. Thus, similar to the condition in which APP SP-A or recombinant SP-A was added to macrophage monolayers in solution, substrate-bound APP SP-A, and SP-A^{hyp} demonstrated significant enhancement of *M. tuberculosis* adherence. However, in contrast to the soluble SP-A studies, substrate-bound SP-A from healthy donors and rat SP-A also led to significant enhancement of *M. tuberculosis* adherence. These studies provided further evidence that the enhancement of *M. tuberculosis* adherence seen with SP-A is mediated by a direct interaction between SP-A and macrophages. They also indicated that surface ligation by SP-A is sufficient for enhancing MDM activity.

Table I. *MDMs plated on a substrate of SP-A with intact carbohydrate moieties but not a nonglycosylated form of protein (SP-A^{hyp,TM}) demonstrate enhanced adherence of* M. tuberculosis [a]

Substrate	*M. tuberculosis* per MDM (Mean ± SD)	% Increase in *M. tuberculosis* Adherence
HSA	1.82 ± 0.75	
APP SP-A	4.57 ± 0.61	151
Boiled APP SP-A	4.58 ± 0.03	152
RAT SP-A	3.49 ± 0.42	92
SP-A^{hyp}	3.63 ± 0.03	99
SP-A^{hyp,TM}	1.94 ± 0.00	7

[a] MDMs were plated on glass coverslips coated with the respective human or rat SP-A proteins. MDMs in monolayer culture were washed, incubated with Erdman *M. tuberculosis*, and bacterial adherence was measured as in Figure 1. Data are the mean ± SD of triplicate coverslips from a representative experiment (of up to 10 independent experiments).

Because the assay used to assess bacterial adherence could not distinguish between attachment and ingestion of bacteria, we used electron microscopy to determine whether the enhanced adherence of *M. tuberculosis* seen with SP-A reflected enhanced attachment or ingestion (phagocytosis). Electron microscopy revealed that >95% of bacteria associated with macrophage cross-sections in the adherence assay were intracellular (rather than surface attached), both in the control and SP-A treatment groups. After exposure to APP SP-A, the mean number of intracellular bacteria/macrophage cross-section was enhanced 91 ± 17% (mean ± SEM, $n = 3$, $p < 0.05$) compared with control cross-sections (Fig. 3). Thus, exposure to SP-A enhances the number of phagocytosed *M. tuberculosis* by macrophages.

Deglycosylation of SP-A abolishes the ability of this protein to mediate enhanced phagocytosis of M. tuberculosis *by MDMs*

We next explored the domains of SP-A that mediate the enhanced adherence of *M. tuberculosis* to macrophages. Previous studies have indicated that the interaction between SP-A and macrophages is in part mannose dependent; however, the role of the carbohydrate moieties of SP-A in this interaction was not directly addressed in these studies (28, 29). To study the potential influence of the carbohydrate moieties of SP-A in mediating the ability of this glycoprotein to enhance *M. tuberculosis* adherence, we initially used two variant forms of SP-A: deglycosylated APP SP-A and SP-A$^{hyp.TM}$. APP SP-A was deglycosylated using *N*-glycosidase F and recombinant SP-A$^{hyp.TM}$ was synthesized without *N*-linked carbohydrates by tunicamycin treatment of the host insect

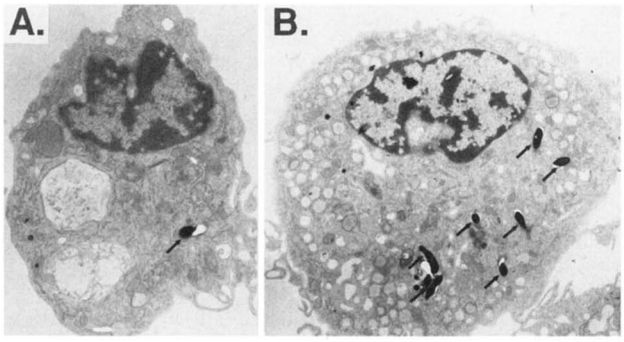

FIGURE 3. MDMs plated on a substrate of SP-A demonstrate enhanced ingestion of *M. tuberculosis*. MDMs were plated on plastic coverslips coated with HSA or APP SP-A. After 120 min, MDM monolayers were washed, incubated with *M. tuberculosis* for 120 min, washed, fixed, and prepared for electron microscopy. *A*, Representative cross-section of a HSA-treated MDM. The cross-section contains one bacterium (arrow) that is intracellular. *B*, Representative cross-section of a SP-A-treated MDM. Several intracellular bacteria (arrows) are seen. *A*, ×10,000. *B*, ×8,000.

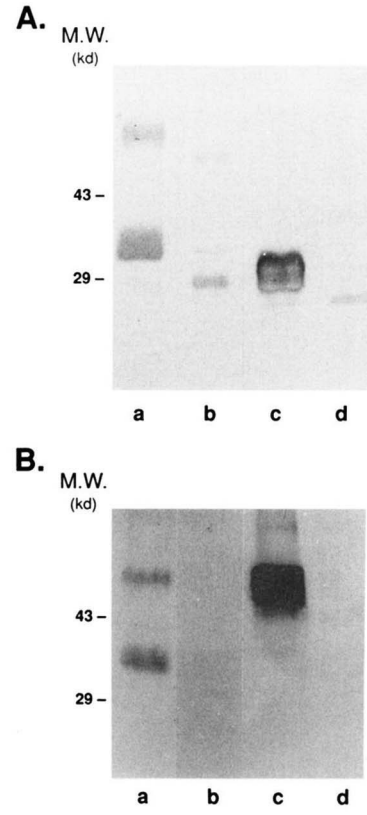

FIGURE 4. *A*, Electrophoretic analysis of SP-A proteins. Proteins (2 μg) were separated by 12.5% SDS-PAGE under reducing conditions and stained by silver stain. APP SP-A (*a*), deglycosylated APP SP-A (*b*), SP-A^{hyp} (*c*) and SP-A^{hyp,TM} (*d*) are compared. *B*, Immunoblot demonstrating complete removal of *N*-linked glycosylation units of APP SP-A. Intact and deglycosylated APP SP-A were labeled with DIG-succinyl-E amidocaproic acid hydrazide. Electrophoretic separation of proteins was performed as in *A*, and proteins were transferred to nitrocellulose. The membrane was exposed to anti-DIG Ab conjugated with alkaline phosphatase and developed with x-phosphate/NBT. APP SP-A (*a*), deglycosylated APP SP-A (*b*), fetuin (*c*, positive control), and creatinase (*d*, negative control) are compared.

cells. The absence of carbohydrates on these proteins was confirmed by the predicted mobility shift on SDS-PAGE (Fig. 4*A*). Additionally, immunoblot analysis of APP SP-A, in which the carbohydrate moieties were covalently labeled with DIG, was performed to verify the absence of carbohydrates on deglycosylated APP SP-A (Fig. 4*B*). The absence of carbohydrates on the variant recombinant SP-A proteins used in these studies has been demonstrated previously (13). In contrast to sham-treated APP SP-A and SP-A^{hyp}, deglycosylated APP SP-A and SP-A^{hyp,TM} failed to enhance *M. tuberculosis* adherence to macrophages when used as proteins in solution ($-8 \pm 20\%$, $n = 2$ and $-10 \pm 25\%$, $n = 3$, respectively, when compared with HSA) or when placed on a substrate ($-1 \pm 8\%$, $n = 5$, and $-4 \pm 14\%$, $n = 4$, respectively, when compared with HSA) (Table I). In substrate experiments, to determine whether deglycosylation of APP SP-A influenced the ability of this protein to bind to the substrate, we compared directly the substrate binding of radiolabeled intact and deglycosylated APP SP-A (equal moles of these proteins were added to the substrate). Deglycosylated APP SP-A bound to the substrate to a greater extent than the intact glycoprotein (275 ± 82 ng for deglycosylated APP SP-A; 153 ± 62 ng for intact APP SP-A; mean ± SEM, n = 3). Thus, although nearly twice as much deglycosylated APP SP-A bound to the substrate as APP SP-A, there was no augmentation of adherence of *M. tuberculosis* to macrophages by the deglycosylated protein. These results provided evidence that attached carbohydrate is important in mediating SP-A's effect in our assay.

To assess further the importance of the carbohydrate moieties of SP-A in mediating enhanced adherence of *M. tuberculosis*, we compared adherence of *M. tuberculosis* to macrophages plated on a substrate of SP-A^{hyp} to that of macrophages plated on a substrate of mutant SP-A proteins in which glycosylation was prevented at one or both oligosaccharide attachment sites. Substitutions in the consensus sequences for glycosylation were produced by site-directed mutagenesis, to prevent attachment of carbohydrate to asparagine residues (asn^{1}, asn^{187}). Macrophages plated on SP-A^{hyp} demonstrated significantly enhanced adherence of *M. tuberculosis* (75 ± 12%, mean ± SEM, n = 4), comparable to the results shown earlier. In contrast, macrophages plated on the nonglycosylated SP-A^{hyp,thr1,ser187} did not demonstrate enhanced adherence of bacteria compared with controls (7 ± 11%, mean ± SEM, n = 4). Thus, two different variant recombinant SP-A proteins devoid of carbohydrate (SP-A^{hyp,TM} and SP-A ^{hyp,thr1,ser187}) failed to enhance *M. tuberculosis* adherence to macrophages. Macrophages plated on substrates of mutant SP-A proteins devoid of *N*-linked carbohydrate at either the Asn^{1} site (SP-A^{hyp,thr1}) or the Asn^{187} site (SP-A^{hyp,ser187}) gave variable results (24 ± 15% enhancement of adherence with SP-A^{hyp,thr1} and 0 ± 24% enhancement of adherence with SP-A^{hyp,ser187}). These results suggest that glycosylation at both consensus sequences of SP-A is necessary to optimally enhance the adherence of *M. tuberculosis* to macrophages.

In other experiments, we compared adherence of *M. tuberculosis* to macrophages plated on a substrate of intact APP SP-A to macrophages plated on a substrate of APP SP-A that had been heat-denatured under conditions that abolish activities of the CRD and disrupt the structure of the collagen region, while maintaining intact carbohydrate moieties (17, 18). Under these conditions, heat-denatured SP-A mediated enhanced adherence of *M. tuberculosis* to macrophages to the same extent as did intact APP SP-A (104 ± 28% enhancement of bacterial adherence with intact APP SP-A and 114 ± 23% enhancement of bacterial adherence with boiled APP SP-A; mean ± SEM, n = 3) (Table I). Taken together, these studies demonstrate a critical role for the carbohydrate moieties of SP-A in mediating its interaction with macrophages that leads to enhanced phagocytosis of *M. tuberculosis*.

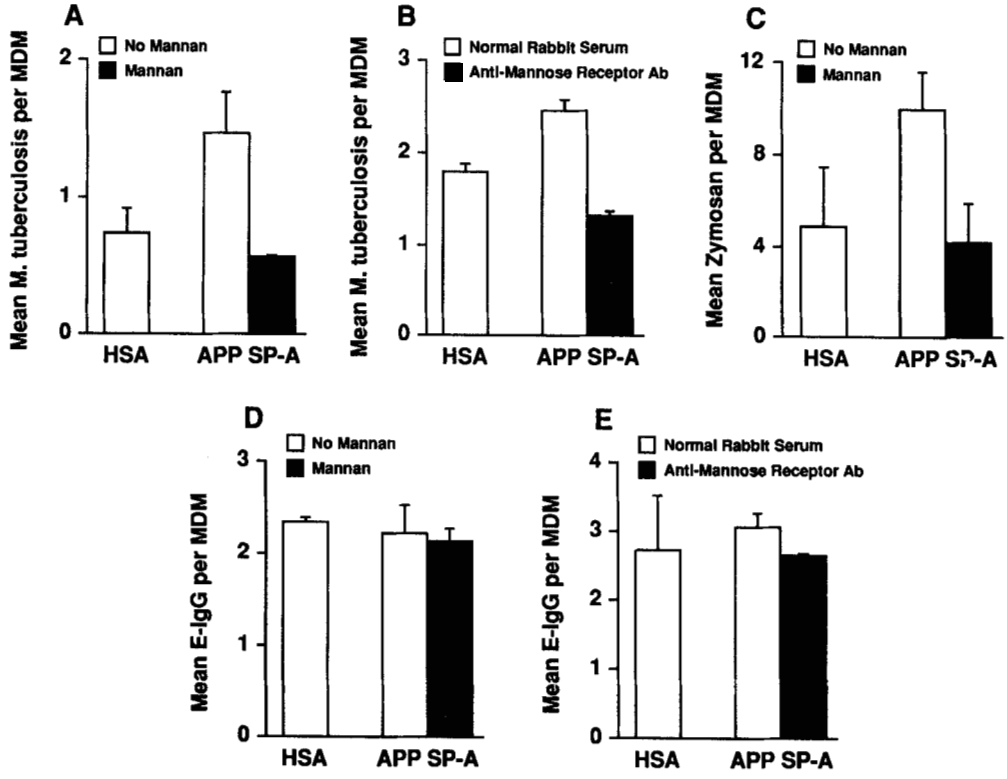

FIGURE 5. Enhanced phagocytosis of *M. tuberculosis* by MDMs plated on a substrate of APP SP-A is inhibited by soluble mannan and anti-mannose receptor Ab. MDMs were plated on glass coverslips coated with APP SP-A or HSA (control) for 120 min and washed. Certain wells containing MDMs plated on APP SP-A were then incubated with soluble mannan or anti-mannose receptor Ab before incubation with Erdman *M. tuberculosis* (*A* and *B*), unopsonized zymosan (*C*), or E-IgG (*D* and *E*). Bacterial adherence was measured as in Table I, and adherence of zymosan and E-IgG was measured by light microscopy. Data (mean ± SD) are representative of at least two independent experiments for each condition.

Soluble mannan and anti-mannose receptor Ab abolish the enhanced phagocytosis of M. tuberculosis *by MDMs plated on a substrate of APP SP-A*

Our previous studies have demonstrated a role for the macrophage mannose receptor in the phagocytosis of virulent *M. tuberculosis* strains, including the Erdman strain (9). To explore one potential mechanism for the enhanced phagocytosis of *M. tuberculosis* by macrophages exposed to SP-A, we preincubated MDMs that were plated on a substrate of APP SP-A with soluble mannan or anti-mannose receptor Ab before adding *M. tuberculosis* (Fig. 5). Preincubation of MDMs with either blocking reagent completely inhibited the enhancement seen with APP SP-A in three independent experiments. Similar results were seen with the positive control particle, unopsonized zymosan (Fig. 5; mean level of enhancement was 83 ± 23%, mean ± SEM, $n = 2$). In contrast, the adherence of E-IgG to Fc receptors was not influenced by APP SP-A in the presence or absence of these blocking reagents in our assay (Fig. 5; mean level of enhancement was 3 ± 4%, mean ± SEM, $n = 5$). These results provided evidence for up-regulation of macrophage mannose receptor activity as a major mechanism for the enhanced phagocytosis of *M. tuberculosis* by MDMs exposed to APP SP-A.

SP-A in solution or on a substrate enhances adherence of M. tuberculosis *to HAMs*

To determine whether SP-A also mediates enhanced adherence of *M. tuberculosis* to HAMs, we first incubated macrophage monolayers with APP SP-A in solution before adding bacteria. Similar to the results obtained with MDMs, APP SP-A in solution enhanced adherence of *M. tuberculosis* to HAMs in a dose-dependent fashion with a maximal effect at ≥5 μg/ml (data not shown). At 5 to 10 μg/ml, APP-SP-A enhanced *M. tuberculosis* adherence by 72 ± 10% (mean ± SEM, $n = 2$). We next examined whether APP SP-A on a substrate enhanced adherence of *M. tuberculosis* to HAMs. Substrate-bound APP SP-A enhanced *M. tuberculosis* adherence by 54 ± 18% (mean ± SEM, $n = 2$). Thus, HAMs freshly removed from the alveolar space also respond to SP-A in a manner that leads to enhanced adherence of *M. tuberculosis*.

Discussion

Aerosol droplet nuclei containing small numbers of *M. tuberculosis* reach the alveolus of the lung and are phagocytosed by alveolar macrophages during primary infection. Macrophages that migrate to this tissue site differentiate in the presence of local tissue factors unique to the alveolus, resulting in altered cell physiology, including phagocytosis (30). Thus, identification of the local factors that influence phagocytosis of *M. tuberculosis* by macrophages is critical in furthering our knowledge of disease pathogenesis. The present study demonstrates that SP-A, a major surfactant-associated protein produced uniquely in the lung, mediates enhanced phagocytosis of the virulent Erdman strain of *M.tuberculosis* by adherent human macrophages through a direct interaction with macrophages. Furthermore, this work demonstrates a critical role for the *N*-linked carbohydrates of SP-A in this interaction; and finally, it provides a mechanism for the enhanced phagocytosis of bacteria, i.e., up-regulation of macrophage mannose receptor activity.

The alveolar type II cell synthesizes and secretes the major components of surfactant, which include disaturated and unsaturated phosphatidylcholine, lesser amounts of other phospholipids, and four surfactant-specific proteins: SP-A, SP-B, SP-C, and SP-D (1,

31). SP-A belongs to the mammalian C-type lectin superfamily. The protein is further classified to the collectin subgroup of C type lectins, which are characterized by having spatially distinct collagen like-domains and CRDs. Other collectins include SP-D, mannose-binding protein, complement protein Clq, and conglutinin (6, 32, 33). The primary structure of SP-A is highly conserved among several species (34). SP-A (human and rat) assembles into oligomers composed of subunits of identical or nearly identical primary structure resembling a "basket of tulips" (35). The post-translational modifications to nascent SP-A include cleavage of the signal peptide, proline hydroxylation, and *N*-linked glycosylation (36, 37). Studies have demonstrated specific interactions between human SP-A and rat phagocytic and nonphagocytic cells, suggesting that the binding to host cells and some functional characteristics of human and rat SP-A may be similar (38).

In our studies, SP-A was found to enhance phagocytosis of *M. tuberculosis* by macrophages through a direct interaction between SP-A and the macrophage. This was demonstrated in several different types of experiments. In the first type, SP-A in solution was washed from macrophage monolayers before adding bacteria, and the resultant enhanced level of phagocytosis was found to be equivalent to the condition in which SP-A was maintained throughout the assay. Immunofluorescence microscopy studies demonstrated that APP SP-A in solution was readily internalized into macrophages during the preincubation period rather than bound to the cell surface. Lastly, macrophages plated on a substrate of covalently-bound SP-A demonstrated enhanced phagocytosis of *M. tuberculosis* on their apical surface. Our electron microscopy studies confirmed that the bacteria counted by light microscopy were indeed cell associated rather than bound directly to the substrate. These electron microscopy studies also demonstrated that macrophages exposed to SP-A contain increased numbers of ingested (phagocytosed) bacteria rather than only an increase in attached bacteria.

Our data provide strong evidence that the *N*-linked carbohydrate moieties of the protein play a major role in the interaction between SP-A and the macrophage, which leads to enhanced phagocytosis of *M. tuberculosis*. By enzymatically removing the carbohydrate moieties of APP SP-A or preventing glycosylation of recombinant SP-A with tunicamycin or site-directed mutagenesis, the enhanced phagocytosis of *M. tuberculosis* seen with intact proteins was abolished. Radiolabeled deglycosylated APP SP-A bound to the substrate at least as well as the intact protein. Thus, the difference observed between APP SP-A and deglycosylated APP SP-A could not be explained by a difference in the amount of protein on the coverslip. Furthermore, APP SP-A and APP SP-A boiled to denature the CRD and collagen-like domains provided similar levels of enhancement of *M. tuberculosis* phagocytosis. This latter observation provides support for a direct interaction between SP-A's carbohydrates and the macrophage. However, our studies do not exclude the possibility that peptides near the carbohydrate linkage sites are important in presenting the carbohydrates or that other domains of SP-A also participate in the binding interaction under normal conditions. This latter point may be more important when SP-A is placed in solution throughout the assay, a condition in which SP-A may also serve as a bacterial opsonin (39–41). Refolding of SP-A after heat denaturation within the time frame of the assay is also possible, although the protein was immediately covalently linked to the substrate after heating to minimize this possibility. Other functions for the carbohydrates of SP-A remain poorly understood, although a role in lipid aggregation has been proposed (2–3). Mutant recombinant SP-A proteins devoid of carbohydrate have recently been shown to retain this function, however (13). In addition, the carbohydrate moieties of SP-A have been shown to mediate binding of SP-A to herpes simplex virus type 1-infected cells (4).

Specific binding interactions between SP-A and mononuclear phagocytes have been described. Wintergerst et al. (28) studied the binding and uptake of human recombinant SP-A-coated gold particles by human monocytes and MDMs using electron microscopy. Uptake was markedly enhanced on MDMs compared with monocytes and was inhibited by mannose-BSA in a fashion comparable to mannose-BSA-coated gold particles. These studies indicated that either SP-A binds to α-D-mannosyl residues on the phagocyte via its CRD or the *N*-linked carbohydrates of SP-A act as a ligand for mannose-specific receptors on macrophages. Similar results were obtained with rat alveolar macrophages, in which the CRD was implicated (29). Other studies provide support for the importance of the collagen-like domain of SP-A in the binding interaction (42–45). These studies collectively indicate that there is likely more than one binding site for SP-A on phagocytes. The cellular response to engagement of one binding site or another may differ and is in part dependent upon the species of phagocyte studied (38, 46).

In our assay, APP SP-A enhanced the adherence of *M. tuberculosis* to macrophages to a greater extent than SP-A from healthy volunteers. This functional difference represents new and potentially important finding in our study. A difference in the structure between these proteins has been described previously. On SDS-PAGE, under reducing conditions, APP SP-A migrates as both a monomer and a dimer, whereas SP-A from healthy volunteers is predominantly a monomer (47). The nature of the major nonreducible dimeric form of APP-SP-A is unknown, but conceivably it may enhance the interaction of SP-A with macrophages (48). Alternatively, the composition of APP SP-A's carbohydrate moieties may be unique. To date, the carbohydrate composition of APP SP-A, but not other forms of native and recombinant SP-A proteins, has been studied and includes sialic acid, mannose, galactose, fucose, and glucosamine (49). Terminal carbohydrates include sialic acid, galactose, and fucose (50). Recombinant proteins produced in baculovirus-infected invertebrate cells are often glycosylated in a simple mannose rich manner. Recombinant SP-A enhanced the adherence of *M. tuberculosis* to macrophages in this study. Thus, it is possible that this effect is not dependent on a high level of complexity of the attached carbohydrate, but rather is dependent on the presentation of the carbohydrate or the branching structure.

Overall, there are three potential mechanisms for SP-A to modulate the *M. tuberculosis*-macrophage interaction: it may act as a bacterial opsonin, it may act as a "bridge molecule" to enhance the interaction between *M. tuberculosis* and the macrophage, and/or it may alter macrophage function to enhance phagocytosis of *M. tuberculosis*. Since in this study we have elected to focus only on the latter mechanism, we believe it is premature to draw firm conclusions on how functional differences between APP SP-A and SP-A from healthy volunteers relate to disease pathogenesis in normal hosts or in patients with alveolar proteinosis. That SP-A may act as an opsonin for *M. tuberculosis* adherence to murine alveolar macrophages has been described recently (51). However, a direct effect of SP-A on macrophages was not ruled out in that study.

In the present study, when SP-A proteins in solution were incubated with MDMs, APP SP-A and recombinant SP-A significantly enhanced *M. tuberculosis* adherence, whereas SP-A from healthy volunteers and rat SP-A did not. When MDMs were plated on a substrate of SP-A, however, all of the proteins with a full complement of attached carbohydrate significantly enhanced adherence of *M. tuberculosis* to macrophages. These data are reminiscent of earlier studies with fibronectin in which substrate-bound

protein enhanced the phagocytic capacity of cells to a greater extent than protein in solution (52), or more recent studies with substrate-bound mannose-binding protein (53). Functional differences between fluid phase and surface-bound SP-A proteins have been described (54). Our data suggest that the density of SP-A on the substrate or the orientation of covalently bound protein enhanced the interaction with macrophages in our assay. Since under normal conditions SP-A is primarily lipid associated as part of the alveolar lining material, it is likely that, in vivo, alveolar macrophages are presented with SP-A as a component of a lipoprotein complex. Studies with *Staphylococcus aureus* (38) have shown that lipid-bound SP-A can still enhance phagocytosis.

The ability of SP-A to enhance phagocytosis of *M. tuberculosis* by macrophages occurred rapidly (with 1–2 h). Since phagocytosis of *M. tuberculosis* is a receptor-mediated event (9), one mechanism for the effect of SP-A on macrophages may be to enhance the surface expression and/or function of preformed phagocyte receptors. In this regard, Tenner et al. (7) have demonstrated that phagocytosis of E-IgG by Fc receptors on monocytes and complement-coated erythrocytes by CR1 on macrophages is enhanced in the presence of SP-A. Thus, SP-A may function as a phagocyte "activation ligand" similar to fibronectin (52, 55), amyloid substance P (52), or laminin (56).

We have previously demonstrated that phagocytosis of Erdman *M. tuberculosis* by human macrophages is mediated by complement receptors and the mannose receptor (9). Thus, we reasoned that one mechanism for the enhanced phagocytosis of *M. tuberculosis* observed with SP-A may be to enhance expression and/or function of one or all of these receptors. Our studies demonstrate that the enhanced phagocytosis of *M. tuberculosis* by macrophages exposed to APP SP-A is completely inhibited by mannan and anti-mannose receptor Ab, agents that effectively and specifically block mannose receptor activity in our model (9). Thus, our studies indicate a novel immunoregulatory role for SP-A, i.e., up-regulation of macrophage mannose receptor activity. We are currently further evaluating the influence of other SP-A variant proteins on expression of this receptor.

Although SP-A has been postulated to function as a host defense molecule for the enhanced killing of extracellular pathogens in the lung (38), for host-adapted intracellular pathogens such as *M. tuberculosis*, SP-A may enhance entry of these pathogens into their host niche. Clinically, patients with alveolar proteinosis are predisposed to mycobacterial infections, including *M. tuberculosis*, as well as to infection with other intracellular pathogens (57). Thus, our in vitro model may provide one molecular mechanism for the higher propensity for *M. tuberculosis* infections in these patients.

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