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Binding of Pulmonary Surfactant Proteins A and D to *Aspergillus fumigatus* Conidia Enhances Phagocytosis and Killing by Human Neutrophils and Alveolar Macrophages

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To determine whether the lung surfactant proteins A (SP-A) and D (SP-D) are involved in the initial protective immunity against opportunistic pulmonary fungal infections caused by *Aspergillus fumigatus*, we performed a series of in vitro functional studies to see if SP-A and SP-D enhanced binding, phagocytosis, activation, and killing of *A. fumigatus* conidia by human alveolar macrophages and circulating neutrophils. Both SP-A and SP-D bound to carbohydrate structures on *A. fumigatus* conidia in a calcium-dependent manner. SP-A and SP-D were also chemoattractant and significantly enhanced agglutination and binding of conidia to alveolar macrophages and neutrophils. Furthermore, in the presence of SP-A and SP-D, the phagocytosis, oxidative burst, and killing of *A. fumigatus* conidia by neutrophils were significantly increased. These findings indicate that SP-A and SP-D may have an important immunological role in the early antifungal defense responses in the lung, through inhibiting infectivity of conidia by agglutination and by enhancing uptake and killing of *A. fumigatus* by phagocytic cells.

Aspergillus fumigatus is a major airborne fungus causing a wide spectrum of clinical conditions (allergic and invasive) depending on the host's immune status (2, 3). In the absence of adequate host defense, *A. fumigatus* disseminates to other tissues, such as bone, skin, and viscera, resulting in the invasive form of aspergillosis, which often proves fatal (2, 3). The treatment for the invasive form of aspergillosis, to provide a complete cure, is complicated by the chemotherapy of allergic patients with steroids and of chronically infected patients with aggressive antifungal drugs, which have highly adverse side effects, such as hepatotoxicity and nephrotoxicity. In most cases, the lung acts as a formidable barrier to the inhaled spores and prevents dissemination into other tissues. The immune mechanisms involved in the disease are not fully understood, although in the initial states of *A. fumigatus* infections, both humoral and cell-mediated immunity have shown little, if any, protective roles (17). Therefore, innate immunity, mediated by polymorphonuclear leukocytes and macrophages, forms the main line of defense against *A. fumigatus* infections (24), where the host can call upon not only resident pulmonary alveolar macrophages but also neutrophils and monocytes recruited from the circulation. The conidia of fungi, which are inhaled with air and infect the pulmonary tract, range from 2.5 to 3.0 μm in diameter. However, *A. fumigatus* conidia are resistant, to some degree, to reactive oxygen intermediates generated during stimulation of phagocytes (6). There has, therefore, been an intensive search for molecules present in the lung that can selectively enhance the contribution of the innate immunity mechanism of phagocytes against such infections.

There is increasing evidence to suggest that lung surfactant proteins A (SP-A) and D (SP-D), present in the lungs, have protective roles to play in the pulmonary defense against pathogens (22). SP-A and SP-D both belong to a group, of the family of C-type lectins, known as the collectins, which are believed to be involved in host defense (22). The amino-terminal end of each collectin polypeptide chain consists of a short, noncollagenous region, followed by a region of collagen-like sequence composed of repeating Gly-Xaa-Yaa triplets. The carboxy-terminal portion of the sequence is characterized by a globular structure, known as the carbohydrate recognition domain (CRD), which mediates calcium-dependent binding to carbohydrates on the surfaces of microorganisms. The collagenous regions of three polypeptide chains assemble to form a collagen-like triple helix, thus forming a subunit containing a triple-helical region, with three C-type lectin domains at its C-terminal end. Six of these triple helical subunits make up the overall structure of SP-A, while SP-D is composed of a cruciform-like structure, with four arms of equal length. The recent generation of mice lacking SP-A surprisingly showed no evidence of SP-A involvement in surfactant homeostasis and function in vivo (15). In contrast, SP-A binding to microorganisms, via its CRDs to the surface of the pathogens, appears to enhance recognition of the microorganisms by macrophages, leading to increased phagocytosis and respiratory burst activity by the cells (9, 26, 27). Human SP-D also binds, in a calcium-dependent manner, to carbohydrate structures on gram-negative bacteria, fungi, and viruses, bringing about their agglutination (10, 16, 25).

We therefore examined the possibility that SP-A and SP-D interact via their CRDs with carbohydrate structures on the surface of inhaled conidia of *A. fumigatus* and are involved in processing of the infectious forms in the respiratory tract. The interaction of SP-A and SP-D with phagocytes is thought to be mediated by specific cell surface receptors. Recently, putative receptors for SP-A and SP-D have been identified on alveolar macrophages (4, 13). The purpose of our study was to deter-

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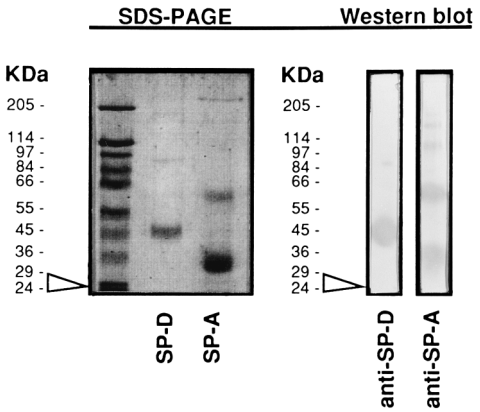

FIG. 1. SDS–10% PAGE of 5 μg of purified SP-D and SP-A examined after reduction of disulfide bonds. Lane 1, molecular weight markers. Western blots of the SP-A and SP-D preparations are shown. The pointers indicate the close proximities of the 29-kDa and 24-kDa markers.

mine whether interaction of infectious conidia stages of *A. fumigatus* with SP-A and SP-D influenced the antifungal activity of alveolar macrophages and neutrophils. We observed that SP-A and SP-D bound to *A. fumigatus* conidia in the presence of calcium, agglutinating them into large particles. Human SP-A and SP-D acted as potent chemoattractants for neutrophils. Furthermore, SP-A and SP-D enhanced the phagocytosis and respiratory burst response against the conidia, resulting in increased antifungal activity of adherent alveolar macrophages and neutrophils. These findings show that SP-A and SP-D contribute to the clearance and killing of *A. fumigatus* conidia by phagocytes and, therefore, contribute to our general understanding of these lung surfactant proteins in pulmonary defense mechanisms.

MATERIALS AND METHODS

Preparation and labelling of conidia. Spores and culture filtrate were obtained from the 285 strain of *A. fumigatus* isolated from the sputum of an allergic bronchopulmonary patient who had been diagnosed based on Rosenberg criteria (17). Cultures were maintained on Sabouraud dextrose agar slants. Conidia were allowed to swell in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} for 1 h before being used for binding, agglutination, phagocytosis, oxidative burst, and killing assays. Conidia were labelled by incubating 10^8 conidia with a 3-mg/ml stock of fluorescein isothiocyanate (FITC; Sigma Chemical Co.) in 0.5 M carbonate buffer, pH 9.0, for 18 h at 4°C in the dark and with mild agitation. The labelled conidia were washed twice. The conidia remained viable after labelling. A 1-hr preincubation, as previously reported by Schaffner et al. (24), was carried out to simulate the natural conditions of conidia since they are wetted by mucous secretions in the respiratory tract.

Isolation and preparation of neutrophils and alveolar macrophages. Human neutrophils were isolated from whole blood taken from normal donors by a one-step isolation procedure with Polymorphoprep (Nycomed Pharma, Birmingham, United Kingdom), as previously described (8). The isolated cells (>95% pure) were then suspended in PBS, pH 7.4, without calcium or magnesium, and maintained at 4°C until required. Alveolar macrophages were isolated from the bronchoalveolar lavage fluids of aspergillosis patients who had been diagnosed based on Rosenberg criteria (17). The cells were separated from the lavage fluid by centrifugation at 600 × *g* for 10 min at 4°C, washed, and maintained in Hanks balanced salt solution. The viability of the resulting cell preparations was higher than 95% as judged by trypan blue exclusion, and monocyte purity was assessed by modified Wright-Giemsa staining and/or nonspecific esterase staining. The macrophage cultures of at least 80% purity were used.

Preparation of native human SP-A and SP-D and antisera. Native human SP-A and SP-D were purified by affinity chromatography from bronchoalveolar lavage fluid obtained from alveolar proteinosis patients by modifications of previously described methods (11, 20). Both lungs were lavaged for therapeutic purposes, with a 1-week interval, by using a heat exchanger and a modified perfusion apparatus to improve the efficiency of the lavage. The bronchoalveolar lavage fluid was made 10 mM with respect to EDTA and centrifuged at 2,000 × *g* to remove aggregates, the resulting supernatant was recalcified by the addition of 35 mM $CaCl_2$, and the pH was adjusted to 7.4. Maltose agarose (Sigma) was packed into a column, washed, and then equilibrated with buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, and 0.02% (wt/vol) sodium azide. The SP-A- and SP-D-enriched supernatant was applied to the maltose agarose column. The column was washed with equilibrating buffer containing 1 M NaCl, and then SP-D was eluted with 20 mM Tris-HCl (pH 7.4)–100 mM $MnCl_2$. SP-A was eluted with an EDTA gradient (10 to 50 mM). Final purification was achieved by gel filtration on a Superose 12 column. Both preparations were judged to be pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Coomassie blue) and Western blotting (Fig. 1). The preparations of SP-A and SP-D were checked for contamination with human immunoglobulin G (IgG), IgM, and IgE antibodies by enzyme-linked immunosorbent assay (ELISA) with anti-human IgG–, anti-human IgM–, and anti-human IgE–peroxidase conjugates. No immunoglobulin contamination was detected. The anti-human SP-A and anti-SP-D antibodies were raised in rabbit against the purified human SP-A and SP-D. These antibodies were specific for SP-D and SP-A at the working dilutions, as assessed by ELISA and Western blotting.

Binding of human SP-A and SP-D to *A. fumigatus* conidia and to human neutrophils. *A. fumigatus* was grown on Sabouraud dextrose agar slants at 37°C. The conidia or spores were harvested after 72 h, washed twice in pyrogen-free

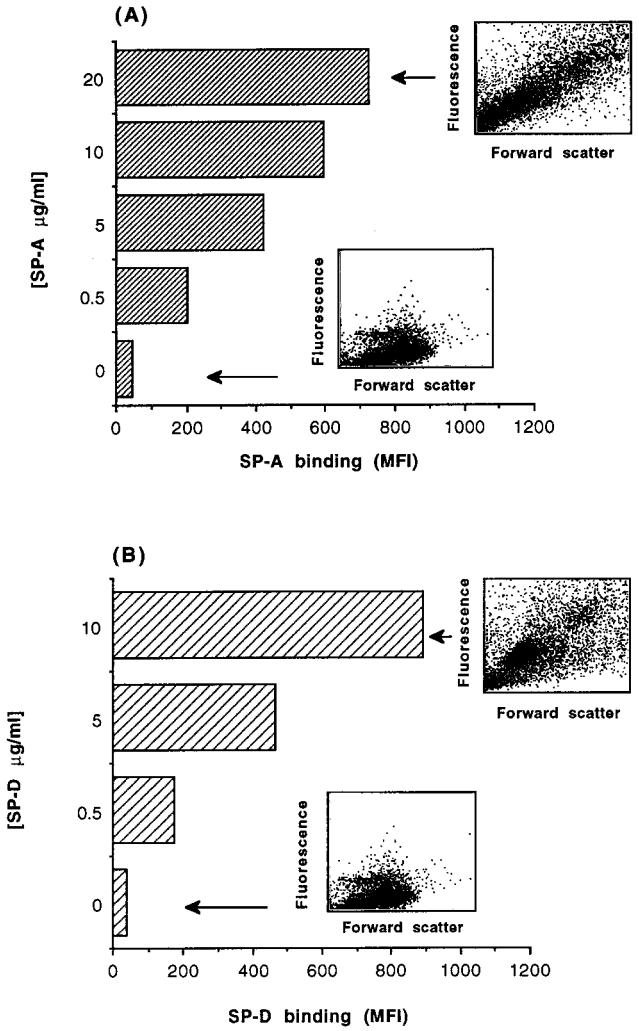

FIG. 2. Concentration-dependent binding of SP-A (A) and SP-D (B) to *A. fumigatus. A. fumigatus* conidia (10^6/ml) were incubated for 1 h at 37°C with various concentrations of SP-A (0, 0.5, 5, 10, and 20 μg/ml) or SP-D (0, 1, 5, and 10 μg/ml) in Ca^{2+}-containing buffer. Binding of the lectins to conidia was detected by flow cytometric analysis after incubation with anti-human SP-A or anti-SP-D followed by incubation with goat anti-rabbit IgG–FITC conjugate. Data are presented as MFI versus concentration of surfactant protein and represent the mean values of three separate experiments. The standard deviation of each concentration tested was within 5% of the mean value. The dot plots represent the analysis of fluorescence versus forward scatter (binding of SP-A or SP-D and agglutination properties, respectively) of lectin- and nonlectin-treated conidia.

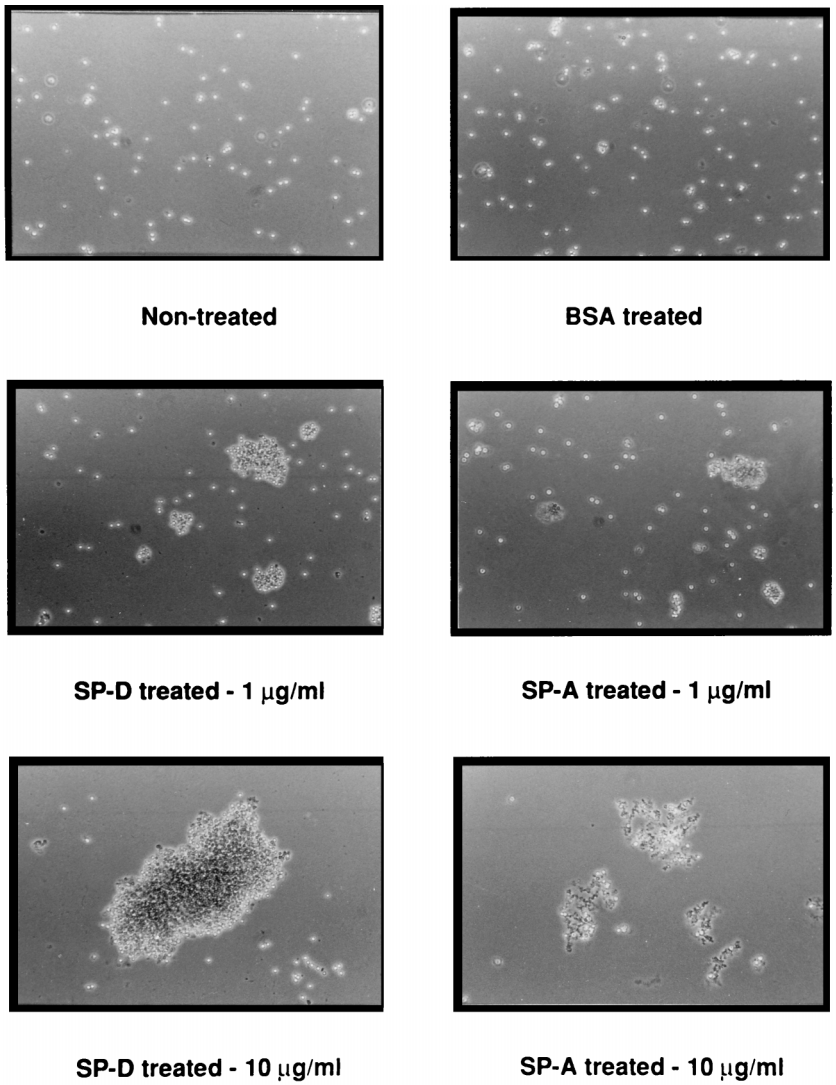

FIG. 3. Agglutination of *A. fumigatus* conidia. Conidia (100,000) were observed by light microscopy before and after incubation with either 1 or 10 μg of SP-A or SP-D per ml in a 5 mM $CaCl_2$ buffer for 60 min. Phase-contrast microscopy was performed on a Zeiss Axioskop transmitted-light microscope with a Zeiss MC 100 camera and TX 400 ASA black-and-white film (final magnification, ×400). The data shown are representative of three experiments.

saline, and stored in pyrogen-free saline. For interaction studies, conidia (10^6) were washed twice in filtered PBS containing 1% bovine serum albumin (BSA) and 5 mM $CaCl_2$ (PBS-BSA-Ca^{2+}) at pH 7.4 and centrifuged at 8,000 × *g* for 10 min at 4°C. The conidia were then suspended in 100 μl of PBS-BSA-Ca^{2+} and incubated with various concentrations of SP-A and SP-D for 1 h at 37°C. For inhibition studies, conidia were incubated with surfactant proteins in the presence of EDTA (10 mM), mannose (100 mM), or maltose (100 mM). The conidia-surfactant complexes were then washed twice with 200 μl of PBS-BSA-Ca^{2+} and incubated with 100 μl of rabbit anti-human SP-A or anti-human SP-D antisera (1:20 dilution) for 1 h at 4°C. After further washing as described above, the conidia were incubated with goat anti-rabbit Ig FITC-labelled antibody (1:50 dilution) for 1 h at 4°C and washed again. The conidia were fixed with 2% (wt/vol) paraformaldehyde in PBS. Conidia-associated fluorescence was analyzed by flow cytometry on a Becton Dickinson FACScan with Lysis II software. In the control preparations, conidia were incubated with rabbit preimmune sera and FITC-labelled antibody to assess background fluorescence.

The binding of SP-A and SP-D to the neutrophil cell surface was assessed by incubating SP-D or SP-A (concentration 1 μg/ml) with adherent neutrophils (10^5/well) at 37°C for 30 min. A fixed-cell ELISA method was used to detect the surfactant protein binding to adherent cells. Monolayers of adherent neutrophils were prepared by resuspending the cells in RPMI medium at a concentration of 10^6/ml, dispensing 100-μl aliquots into TC microwell 96 FSI flat-bottomed microtiter plates, and incubating the plates for 1 h at 37°C with 5% (vol/vol) CO_2 (Nunc, Roskilde, Denmark). The uniformity of cell adhesion in individual wells was determined by staining duplicate plates of cells with crystal violet as previously described (7). A standard curve was established by plating known concentrations of cells and staining with crystal violet. The cells were washed in Gey's buffer (Dulbecco's PBS with 1 mM Ca^{2+} and 1 mM Mg^{2+} prepared in endotoxin-free water [pH 7.4] supplemented with 2% [wt/vol] BSA and 0.1% [wt/vol] glucose) and fixed to the plates by adding 100 μl of 0.1% (vol/vol) glutaraldehyde per well and incubating for 5 min at 25°C. Nonspecific binding sites were blocked with Gey's solution for a further 30 min. Then, various concentrations of SP-A and SP-D were added to the neutrophils, and the cells were incubated for 30 min at 37°C. The cells were then washed three times with Gey's buffer followed by incubation with a 1:2,000 dilution of rabbit anti-human SP-A–anti-human SP-D antiserum for 30 min at 37°C. After three washings, the assessment of SP-A and SP-D binding to adherent neutrophils was achieved by incubating the cells with anti-rabbit IgG peroxidase conjugate for 30 min at 37°C, followed by incubation with 3,3′,5,5′-tetramethylbenzidine (TMB)-peroxidase substrate. Control preparations included cells with no primary or secondary antibody added and cells with antibodies but no SP-A or SP-D added. The blocking buffer prevented most nonspecific IgG binding; however, some nonspecific IgG binding to neutrophils was observed, and the values for this binding were subtracted from the final results.

Agglutination assay. *A. fumigatus* conidia were washed twice in PBS-BSA-Ca^{2+} buffer and centrifuged at 3,000 × *g* for 10 min at 4°C. The conidia (10^5 cells) were then resuspended in 100 μl of buffer containing various lectins (SP-A; SP-D; a control plant lectin, concanavalin A [ConA]; and mannan binding lectin [MBL]) at concentrations between 1 and 5 μg/ml in siliconized tubes (to avoid adherence to the tube walls), incubated at 37°C for 1 h, and observed by phase-

TABLE 1. Binding of human SP-A and SP-D to *A. fumigatus* spores and neutrophils in the presence of 5 mM Ca^{2+}, 10 mM EDTA, or competitive sugars[a]

Binding conditions	Binding to *A. fumigatus* (MFI ± SD)[b]		Binding to neutrophils (A_{405} ± SD)[c]	
	SP-A	SP-D	SP-A	SP-D
5 mM $CaCl_2$	950 ± 30	1,128 ± 36	0.43 ± 0.08	0.68 ± 0.03
10 mM EDTA	80 ± 5	48 ± 6	0.19 ± 0.04	0.16 ± 0.01
100 mM mannose and 5 mM $CaCl_2$	252 ± 15	590 ± 20	0.39 ± 0.07	ND
100 mM maltose and 5 mM $CaCl_2$	456 ± 20	120 ± 9	ND	0.68 ± 0.07

[a] *A. fumigatus* conidia (100 μl of 10^6 conidia/ml) were incubated with 10 μg of SP-A or SP-D per ml in the presence or absence of competitive sugars or EDTA. Binding was determined by flow cytometry. The data are the means ± standard deviations (SD) of three separate experiments. Binding of 100-μl aliquots of a 1-μg/ml concentration of SP-A or SP-D to adherent neutrophils under the same conditions as described above was assayed by ELISA. The results are the means ± SD for two subjects, each experiment performed in triplicate. ND, not determined.

[b] The MFI of conidia alone was ≤10.0.

[c] For neutrophils alone, A_{405} = 0.172 ± 0.002.

contrast microscopy. A Zeiss MC camera containing Kodak Tri-X Pan 400 ASA black-and-white film was used to photograph the agglutination of the spores in the presence of the collectins. The suspensions were also analyzed for particle size changes due to agglutination by dot plot analysis of cell forward and side scatter by employing a flow cytometer (data not shown).

Chemotaxis assay. Neutrophil cell migration was determined by a modified Neuroprobe 96-well microchemotaxis chamber assay. Briefly, neutrophil suspensions and native SP-D were suspended in Gey's buffer, and the proteins or assay buffer were placed in the lower compartments. These were then covered with 3-μm-pore-size, cellulose nitrate filters, and then 50 μl of the cell suspension (2×10^5 cells) was placed in the upper compartment. The chemotaxis chamber was then incubated for 1 h in a humidified incubator with 5% CO_2 and 95% air. After incubation, the plate was spun at 600 × *g* for 10 min in a bench centrifuge and the upper polycarbonate buffer was discarded. The test proteins and the buffer were removed, and the adherent cells were allowed to air dry in a 60°C oven for 15 min to permeabilize the cells. The number of cells that had traversed the filter were quantified by staining the cells with Hoechst dye (50 μg/100 ml of PBS containing 50 μl of 10% [wt/vol] SDS) and reading the fluorescence intensity 15 min later by employing a Cytofluor 2300 fluorimeter (Millipore) against the fluorescence reading for a known number of cells.

Phagocytosis and generation of reactive oxygen intermediates by blood neutrophils in the presence of SP-A- and SP-D-coated *A. fumigatus* conidia. Aliquots of whole blood (100 μl) were collected by finger prick into microfuge tubes containing 1 U of porcine heparin. The tubes were centrifuged at 600 × *g* for 5 min, and serum was aspirated. The red blood cells were lysed by 1 ml of 150 mM ammonium chloride lysis buffer (pH 7.4). The tubes were centrifuged at 600 × *g* for 5 min, and erythrocyte ghosts were removed by washing the leukocyte pellets three to four times with PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. For phagocytosis measurements, an optimal number of FITC-labelled conidia (10^5) were added to each tube along with different concentrations of SP-A and SP-D (0 to 5 μg/ml) and incubated at 37°C for the optimal time of 1 h with shaking. All the dilutions of conidia and proteins were made in PBS. Autologous serum was added to one of the tubes containing conidia and cells, which acted as a positive control. Cells incubated with 1 μg of SP-A or SP-D alone per ml without conidia were taken as negative controls. The pelleted cells were thoroughly washed three times. The cells were fixed in 2% (wt/vol) paraformaldehyde in PBS, and the fluorescence of the neutrophils was measured with Lysis II software, version 1.1 (Becton Dickinson).

In order to measure reactive oxygen intermediate production, aliquots of hydroethidine (100 ng/10 μl) were added to 10^5-leukocyte suspensions in 100 μl of PBS. Nonfluorescent hydroethidine diffuses within the leukocytes and is rapidly oxidized to red fluorescent ethidium bromide by the oxidative burst intermediates within the stimulated cells. Then, *A. fumigatus* conidia (10^6) were added to the tubes along with different concentrations of SP-A and SP-D diluted in PBS and were incubated at 37°C for an optimum time of 1 h with shaking. Autologous serum was added to one of the tubes containing spores and cells, and that tube was taken as a positive control. Cells with no conidia added were taken as negative controls. The tubes were centrifuged, and the pelleted cells were washed. The cells were fixed in 2% (wt/vol) paraformaldehyde in PBS, and the fluorescence of the neutrophils was measured with Lysis II software, version 1.1 (Becton Dickinson).

Killing activity of neutrophils and alveolar macrophages in the presence of SP-A and SP-D. The antifungal activities of neutrophils and alveolar macrophages in the presence of lung surfactant proteins were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide) colorimetric assay reported by Levitz and Diamond (19). The viable cells take up MTT and oxidize it to tetrazone in their mitochondria, whereas dead cells are not capable of oxidizing MTT. Alveolar macrophages or blood neutrophils (10^6/ml) (100 μl/well) in RPMI medium with 5% (vol/vol) fetal calf serum were plated onto 96-well tissue culture plates (Nunc) and were allowed to adhere in a humidified 5% (vol/vol) CO_2 incubator at 37°C. The supernatant medium was aspirated carefully without disturbing the monolayer. Initially a range of *A. fumigatus*-conidial-suspension-to-cell ratios were tested. Routinely, a stock suspension of conidia (10^7/ml) was prepared in PBS containing Mg^{2+} and Ca^{2+} for at least 1 h prior to use to induce swelling. Then, 100 μl of 10^6 conidia/ml for macrophages and 100 μl of 10^5 conidia/ml for neutrophils, in RPMI medium (without serum) containing different concentrations of SP-A and SP-D with and without polymyxin B (5, 50, and 500 U/ml to ensure that any enhancement observed in killing was not due to activation of cells by low levels of endotoxin), were added to each well, and the plates were incubated at 37°C for 1 h. The supernatants were discarded, and 100 μl of 0.5% deoxycholic acid per well in sterile PBS was added to lyse the macrophages and neutrophils. The plates were then washed three times with sterile water. Subsequently, 0.5 mg of MTT per ml in RPMI medium was added to each well, and the plates were incubated at 37°C for 3 h. The wells were then aspirated dry, and 200 μl of isopropanol was used to extract the dye in each well, volumes of 150 μl were transferred to another 96-well microtiter plate, and the absorbance was measured at 562 nm. Control wells containing macrophages or neutrophils alone were taken as negative controls, while wells containing conidia alone were taken as positive controls. Cells were examined with an inverted microscope. A Kodak Polaroid MicroSLR camera containing 331 Polaroid black-and-white film was used to photograph the killing of conidia.

RESULTS

Binding of SP-A and SP-D to *A. fumigatus* conidia and neutrophils. Primary infection of *A. fumigatus* occurs in the lungs following inhalation of conidia from the environment. An initial study was performed which examined the characteristics of binding of SP-A and SP-D to the conidia of *A. fumigatus*. Flow cytometric analysis revealed that both SP-A and SP-D bound to conidia in a dose-dependent manner in the presence of calcium (Fig. 2). Demonstrable binding of SP-A and SP-D was observed with concentrations as low as 1 μg/ml. Binding of SP-A to conidia appeared to reach saturation, whereas SP-D

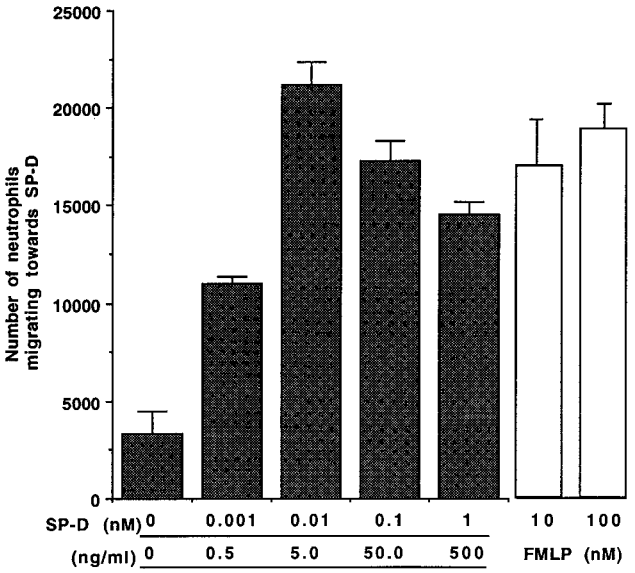

FIG. 4. Neutrophil migration towards SP-D. Cells (2×10^5/50-μl aliquot) were allowed to migrate toward various concentrations of highly purified human SP-D for 1 h at 37°C in the presence of 5% (vol/vol) CO_2. Numbers represent the means ± standard errors of the means of the total number of cells transversing a 3-μm-pore-size cellulose membrane towards SP-D above the number of cells observed with buffer alone for four subjects. The chemotactic response is compared with the mean response obtained for two concentrations of the potent chemoattractant FMLP for eight subjects (bars on right).

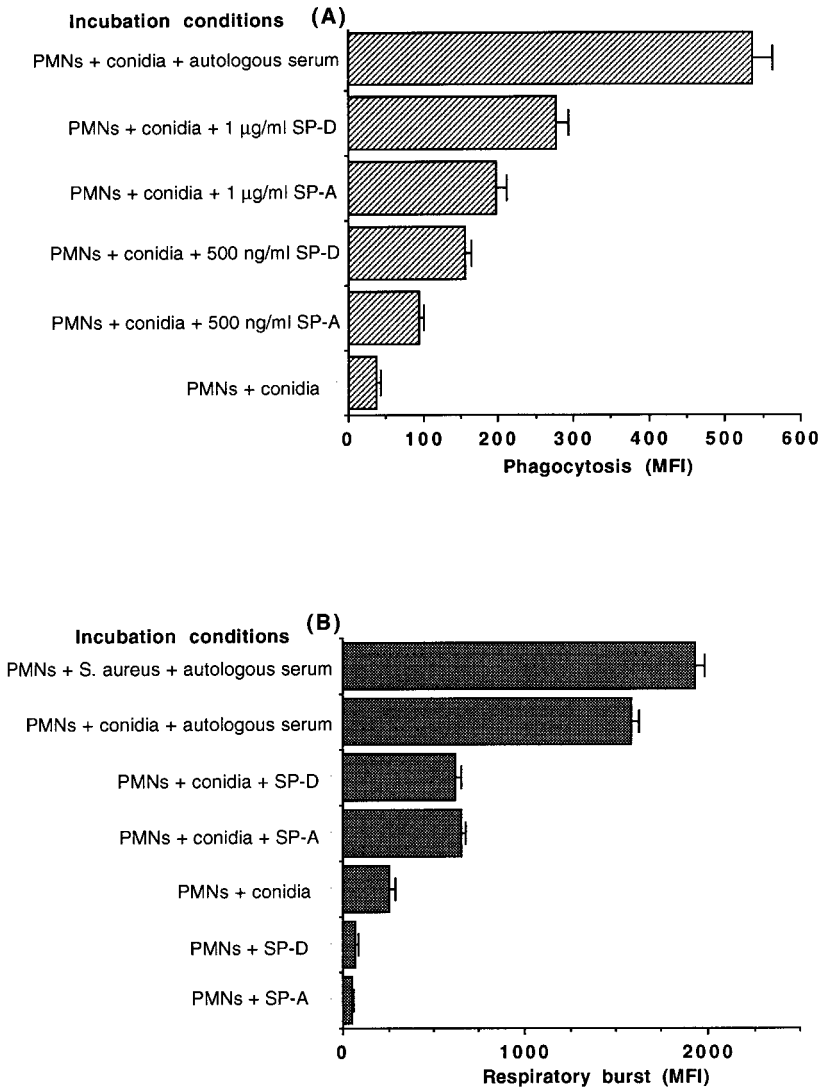

FIG. 5. (A) Phagocytosis of *A. fumigatus* conidia by neutrophils in the presence of autologous serum or SP-D or SP-A. FITC-labelled *A. fumigatus* conidia were added to washed-leukocyte preparations devoid of red cells and serum at a ratio of one conidium to one leukocyte, in the presence of autologous serum, SP-A, or SP-D and in the absence of opsonin. After 30 min of incubation at 37°C the leukocytes were washed and pelleted twice and fixed in 2% (vol/vol) paraformaldehyde, and the uptake of fluorescent conidia was assessed by flow cytometry. Dot plots of 10,000 leukocytes were gated for the neutrophil population and the mean ± standard deviation. The MFIs for three individuals are shown. (B) Stimulation of neutrophil oxidative burst by *A. fumigatus* in the presence of autologous serum or SP-D or SP-A. Washed leukocyte preparations were incubated in the presence of hydroethidine and *A. fumigatus* at a ratio of 10 conidia to one leukocyte, in the presence of 1-μg/ml SP-A, 1-μg/ml SP-D, autologous serum, or heat-killed *S. aureus* and in the absence of opsonin. Neutrophils producing an oxidative burst were assessed by flow cytometry. The experiments were performed three times. PMNs, polymorphonuclear leukocytes.

binding did not approach saturation binding with the concentrations used in this study. The data for a still-higher concentration of SP-D (20 μg/ml) was not plotted as it lies beyond the physiological range. Analysis of the dot plots of fluorescence versus forward scatter (a measure of size increase) suggested the conidia were agglutinating into larger particulate matter upon treatment with SP-A and SP-D.

To establish the physical effects brought about by the binding of SP-A, SP-D, and other C-type lectins to *A. fumigatus* conidia, different concentrations (0, 1, and 10 μg/ml) of SP-A, SP-D, MBL, collectin 43 (CL-43), and ConA were incubated with the conidia. Single conidia were approximately 2.5 μm in diameter. Within 30 min of incubation at 37°C with 1 μg of SP-A or SP-D per ml, visibly demonstrable agglutination of the conidia (Fig. 3) was evident and the sizes of the agglutinated clumps of conidia ranged from 8 to 30 μm in diameter, which confirmed the original increase in forward scatter observed during fluorescence-activated cell sorter analysis. Other collectins, such as MBL and CL-43, together with ConA, also induce some clumping of conidia, with sizes ranging from 5 to 15 μm in diameter, but to a lesser degree than that achieved with either SP-A or SP-D. The size of the agglutinated mass of conidia increased in proportion to the increase in SP-A and SP-D concentration. When conidia were incubated with 10 μg of SP-A or SP-D per ml, agglutination was further enhanced, with clumps of conidia having sizes ranging from 50 to 100 μm in diameter. Treatment of the conidia with nonlectin control proteins, such as BSA, did not induce agglutination. Both SP-A and SP-D on their own, at up to 10 μg/ml in 5 mM $CaCl_2$, showed no aggregation when viewed by light microscopy. These results show that SP-A and SP-D agglutinate and im-

mobilize fungal conidia in vitro, suggesting that in vivo this property would restrict conidia dissemination within the host.

SP-A and SP-D are both C-type lectins. We, therefore, examined if their binding to conidia and human neutrophils was calcium dependent. Binding of both SP-A and SP-D to conidia in the presence of 10 mM EDTA was reduced by approximately 90% (Table 1), confirming the calcium dependency of the binding event. Binding of both surfactant proteins to adherent neutrophils was also completely inhibited by EDTA but not by mannose or maltose (Table 1). SP-A and SP-D have previously been reported to have high affinity for immobilized mannose and maltose, respectively. Carbohydrate structures containing these sugars are potential targets for recognition, by SP-A and SP-D, on the surface of microorganisms. To assess the carbohydrate specificity of SP-A and SP-D binding to conidia, competition assays were performed in the presence or absence of 100 mM maltose or mannose. Specificity was confirmed, with 100 mM maltose significantly inhibiting binding of SP-D to *A. fumigatus* conidia (Table 1) ($P < 0.01$, Student's paired t test) while 100 mM mannose was more effective at inhibiting binding of SP-A (Table 1) ($P < 0.01$, Student's paired t test), suggesting that the CRD regions of SP-A and SP-D interact with carbohydrate structures on the conidia cell wall of *A. fumigatus*. In contrast, the finding that SP-A and SP-D binding to the surfaces of adherent neutrophils were not significantly inhibited by either maltose or mannose indicates that the carbohydrate binding properties of SP-A and SP-D are not involved in their binding to neutrophils (Table 1).

SP-A and SP-D enhancement of phagocyte recruitment, uptake, and killing of *A. fumigatus* conidia. In previous reports, human SP-A (29) and rat SP-D (5) have been found to be potent chemoattractants for human alveolar macrophages and neutrophils, respectively. In this study we sought to determine if human SP-A and SP-D enhanced human neutrophil migration. When SP-D was placed in the lower compartments of a 96-well chemotaxis microchamber (Neuroprobe, Cabin John, Md.), neutrophils migrated through the 3-μm-pore-size nitrocellulose filter barrier in a dose-dependent manner. A concentration of 10^{-11} M SP-D was optimal, inducing a migration of 20,785 ± 5,609 neutrophils (mean ± standard error of the mean; $n = 4$ subjects) (Fig. 4), which was comparable with the potent bacterial tripeptide chemoattractant-N-formyl-Met-Leu-Phe used at 10 to 100 nM ($n = 8$ subjects). Basal migration, measured as the number of cells migrating in the presence of Gey's buffer alone, averaged 1,113 ± 414 ($n = 10$ subjects). To ensure that the SP-D chemotaxis observed was directed migration and not simply random migration (chemokinesis), a checkerboard analysis in which SP-D concentrations varied about and below the filter was performed. When the concentration of SP-D was higher in the lower compartment compared with the upper chamber, neutrophil migration occurred along the SP-D concentration gradient, confirming that directional movement was taking place (data not shown). The chemotactic effect of SP-D could be blocked with rabbit anti-human SP-D polyclonal antiserum. When anti-SP-D at dilutions of 1:40,000, 1:4,000, and 1:400 were used, neutrophil migration was reduced by 66, 79, and 100%, respectively. Controls with irrelevant anti-human protein antisera, in the same molar ratios, did not impede migration. The migratory response of neutrophils from two subjects to SP-A was also studied. An SP-A concentration of 0.5 μg/ml led to the migration of an average of 19,422 cells above the control basal migration levels.

We then performed phagocytosis experiments by incubating FITC-labelled conidia with isolated leukocytes in the presence or absence of SP-D and SP-A and assessing uptake by flow cytometric analysis. The degree of phagocytosis was assessed for a number of conidium-to-leukocyte ratios and incubation times. An optimal uptake was obtained when a conidium-to-leukocyte ratio of 1:1 was employed. An increase of over two-fold in conidium uptake was observed in the presence of SP-A and in the presence of SP-D (Fig. 5A). Conidia incubated with washed leukocytes were ineffective at phagocytosis and yielded a background mean fluorescence intensity (MFI) of ≤25. As expected autologous serum proved to be the most effective opsonin.

Having established that SP-A and SP-D both acted as opsonins for the uptake of conidia by phagocytes, we wished to

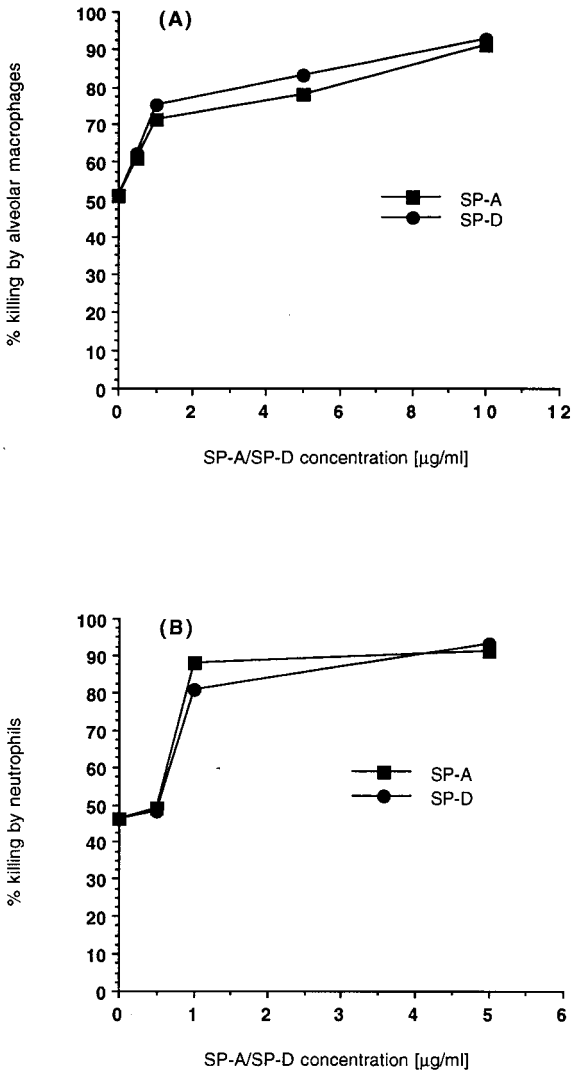

FIG. 6. Killing of *A. fumigatus* conidia by (A) alveolar macrophages (10^5/ml) or (B) adherent neutrophils (10^6/ml) after incubation with various concentrations of either SP-A or SP-D in a 5 mM $CaCl_2$ buffer for 60 min. Supernatant medium was removed and 100 μl of 0.5% (vol/vol) deoxycholic acid was added per well to lyse the macrophages or neutrophils. The plates were washed, and 0.5 mg of MTT per ml in RPMI medium was added to each well. After an additional incubation at 37°C for 3 h, the wells were then aspirated dry and 200 μl of isopropanol was used to extract the dye in each well, volumes of 150 μl were transferred to another microtiter plate, and the absorbance was measured at 562 nm. Control wells containing macrophages or neutrophils alone were taken as negative controls, while wells containing conidia alone were taken as positive controls. The results are the means ± standard deviations for triplicate determinations for between two and four individuals.

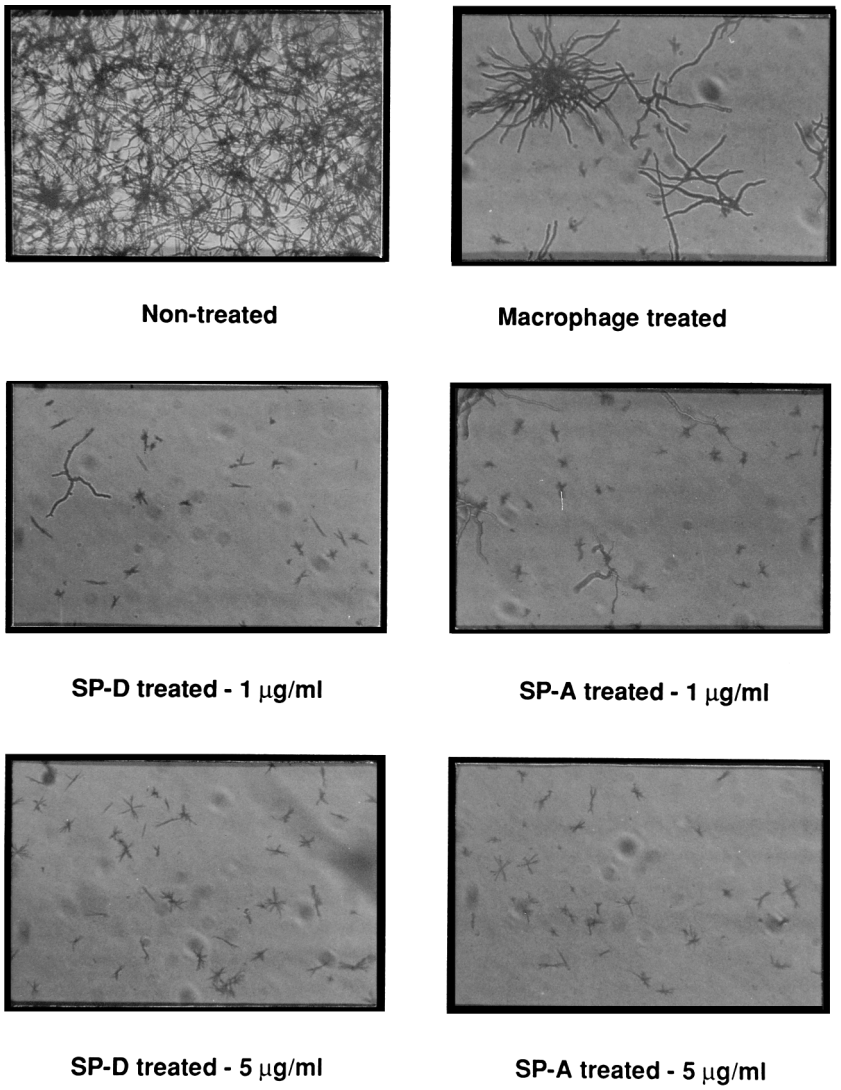

FIG. 7. Killing of *A. fumigatus* conidia by alveolar macrophages as observed by light microscopy after incubation of conidia with either 1 or 5 μg of SP-A or SP-D per ml in a 5 mM $CaCl_2$ buffer for 60 min. Hyphae germinated from resistant spores and conidia are shown. Light microscopy was performed on an inverted microscope, and the photographs were taken with a Kodak Polaroid MicroSLR camera with 331 black-and-white Polaroid film (final magnification, ×200).

ascertain the effect of SP-A and SP-D on the conidium-mediated respiratory burst in neutrophils. Washed leukocytes were incubated with the respiratory burst probe marker hydroethidine and incubated with *A. fumigatus* conidia in a ratio of 10:1 for 1 h at 37°C. Both SP-A and SP-D significantly enhanced the respiratory burst by neutrophils by twofold in the presence of *A. fumigatus* conidia (Fig. 5B), but similar doses of SP-A and SP-D alone had no effect on neutrophil respiratory burst.

We next examined whether the increase in respiratory burst induced in phagocytes correlated with increased killing of conidia by neutrophils and alveolar macrophages. Neutrophils and alveolar macrophages were incubated with increasing concentrations of SP-A and SP-D and a fixed concentration of conidia spores. Both proteins enhanced the antifungal effect of phagocytes in a dose-dependent manner (Fig. 6 and 7). Although neutrophils and macrophages alone appeared to be quite effective at killing conidia, the additional presence of SP-A or SP-D allowed killing of the more-resistant conidia. A visualization of the ELISA results (Fig. 7) shows the hyphae germinated from these resistant cultures. The presence of increasing doses of SP-A or SP-D led to the destruction of the germinating spores by the alveolar macrophages. The viability of phagocytes was not affected by the presence of the proteins. As little as 1 μg of SP-A and SP-D per ml significantly enhanced the killing of conidia by both alveolar macrophages and neutrophils (Fig. 6). Other soluble, C-type collectins, such as MBL, CL-43, and the plant lectin ConA, were also employed in these assays, but no significant effect on the killing of conidia by phagocytes was observed (killing $\leq$ 10%). To ensure that the killing was not due to activation of cells due to the presence of minor contamination by endotoxin, parallel killing experiments were performed in the presence of 50 to 500 U of the endotoxin deactivator polymyxin B per ml. There was no difference in the killing efficiency of the cells in the presence of polymyxin B, confirming that the enhanced killing was due to the effect of lectins and not to endotoxin. The ratio of alveolar macrophages to conidia also influenced the ability of the host cells to kill the conidia (Table 2). When the macrophages were presented with approximately 1,000 conidia per cell, the efficiency of killing fell by sixfold compared with the efficiency of

TABLE 2. Effect of macrophage-to-conidium ratio on killing of *A. fumigatus* by alveolar macrophages[a]

Cell-to-spore ratio	Conditions of incubation	OD_{562}		Mean % killing
		Patient 1	Patient 2	
1:1,000	Alone	0.319	0.312	9.0
	+5-μg/ml SP-A	0.183	0.186	55
	+5-μg/ml SP-D	0.178	0.175	58
1:100	Alone	0.279	0.282	21
	+5-μg/ml SP-A	0.143	0.156	67
	+5-μg/ml SP-D	0.136	0.145	70
1:10	Alone	0.240	0.238	36
	+5-μg/ml SP-A	0.076	0.088	91
	+5-μg/ml SP-D	0.082	0.084	90
1:5	Alone	0.202	0.218	46
	+5-μg/ml SP-A	0.061	0.068	96
	+5-μg/ml SP-D	0.053	0.052	100
1:1	Alone	0.182	0.176	56
	+5-μg/ml SP-A	0.052	0.056	99
	+5-μg/ml SP-D	0.046	0.049	100

[a] The effect of the adherent alveolar macrophage-to-conidium ratio on killing of a constant number of *A. fumigatus* conidia (100 μl of 10^6/ml) was studied in the presence or absence of 100 μl of 5 μg of SP-A or SP-D per ml, as detailed in Materials and Methods. The results are the means of triplicate experiments performed on cells isolated from two subjects. OD_{562}, optical density at 562 nm.

killing for a 1:1 ratio of cells to conidia. However, in the presence of 5 μg of either SP-A or SP-D per ml, the ability of the macrophages to kill was improved fivefold in experiments involving 1,000 conidia per macrophage (Table 2).

DISCUSSION

In this study, we investigated the binding of purified surfactant proteins to the infectious form of the fungal pathogen *A. fumigatus* and their effect on phagocyte-mediated host defense mechanisms. The conidia of *A. fumigatus* occur commonly in the air, especially during autumn and winter. They may cause sensitization in asthmatic subjects, colonize lung cavities or diseased airways, or occasionally invade the lung tissue, most commonly after lung infarction or in the immunosuppressed. The mechanisms by which *A. fumigatus* colonizes the respiratory mucosa are not known. Pathological conditions, such as bronchiectasis and cystic fibrosis, where mucociliary clearance defense mechanisms are already impaired, allow opportunistic *A. fumigatus* colonization to occur. This suggests that pulmonary defenses play an important role in prevention of establishment of *A. fumigatus* infections.

There is increasing evidence that the hydrophilic surfactant proteins SP-A and SP-D play a first-line-defense role in the alveolar airspace and interact with alveolar macrophages or phagocytic cells during the recognition and clearance of pathogens responsible for pulmonary inflammation. Binding of SP-A and SP-D to the cell surfaces of various pathogens and enhancement of phagocytosis via agglutination or receptor mediation has been well documented in the literature (9, 10, 25). Moreover, SP-A and SP-D have recently been found to bind to purified allergens from *Dermatophagoides pteronyssinus* mites and found to inhibit allergen-specific IgE binding to the mite extract *Der p I* and therefore may also be involved in modulation of allergen sensitization (28).

Located at the interface between host and pathogen cells, the fungal cell wall plays a major role during fungal invasion. It possesses several surface receptors involved in adhesion of the fungus to host protein and cells. Both SP-A and SP-D bound specifically to *A. fumigatus* conidia at concentrations comparable to their physiological concentrations in the host (21), as judged by bronchoalveolar lavage fluid concentrations of 1 to 10 μg/ml for SP-A and 0.3 to 0.6 μg/ml for SP-D. Concentrations of SP-A which agglutinate *A. fumigatus* conidia are consistent with the previous in vitro observations with *Staphylococcus aureus* (9). SP-D has been reported to agglutinate to the acapsular form of *Cryptococcus neoformans* at concentrations similar to those reported in the present study (25). Binding of both surfactant proteins to the conidia was inhibited in the presence of EDTA, consistent with earlier reports (16, 25). These results indicate the calcium-dependent binding of the C-type lectin domains present on the globular head of regions of SP-A and SP-D to carbohydrate structures present on the conidial surface.

Collectins can distinguish nonself carbohydrates on microorganisms from the self glycoproteins via their CRDs (14), and their specificity for various carbohydrates can be demonstrated by competitive binding assays with defined saccharides. The sugar competition data presented here show that binding of SP-A and SP-D to the conidia can be effectively inhibited by 100 mM mannose and 100 mM maltose, respectively. Similar carbohydrate selectivities of SP-A and SP-D have been observed in studies with *C. neoformans* of competitive inhibition by various monosaccharides (25). Little is known about the actual molecular architecture of the cell wall of *A. fumigatus*, but chemical analysis suggests that the cell wall contains predominantly glucan, chitin, and galactomannan (1). Hearn and Sietsma performed a detailed composition analysis of the mycelial cell wall and reported 71.5% of the dry weight as carbohydrate material whose monomeric composition, determined after complete hydrolysis, was glucose, glucosamine, galactose, and mannose (12). The *Aspergillus* cell wall contains most of the glycoprotein antigens secreted by the fungus during its active growth, which are the putative virulence factors (18). Further, the cell wall contains several receptor molecules involved in the adhesion of the fungus to host proteins and cells and in the colonization of the host tissues. Hence, the binding of lung surfactant proteins to different carbohydrate structures present on the conidial surface has potential to influence the *Aspergillus* infection, as it will not only restrict the fungal adhesions to various surfaces but may also inhibit the glycoprotein antigen virulence factors.

Another feature of SP-A and SP-D binding to *A. fumigatus* is the physical agglutination of the conidia in vitro. The agglutination properties of SP-A and SP-D have important implications in the binding of conidia to phagocytes resident within the lung. The formation of large aggregates of the organism may immobilize the pathogen in the lung so that alveolar macrophages and marginating neutrophils can detect and migrate to the foci of infection. In addition, both SP-A and SP-D were shown to be potent chemoattractants of neutrophils. Therefore, SP-A and SP-D possess functional characteristics which increase the interaction between pathogens and host phagocytes. The agglutination properties observed may reflect cross-linking between the globular head regions of the lectins and the conidia spores, creating a meshwork of surfactant protein and microorganisms. The CRDs of the globular head regions of SP-D have also been implicated to contain the active chemotactic phagocyte recognition sites. Indeed, recent studies have shown that in the presence of maltose, leukocyte chemotaxis towards SP-D is markedly inhibited (5). Once neutrophils and/or monocytes and macrophages have accumulated at the

site of lung infection, SP-A- and SP-D-bearing conidia adhere to receptors on the surface of the phagocytes. Two different proteins have recently been proposed as putative SP-A (4) and SP-D (13) receptors. The SP-D binding protein has a molecular mass of 340 kDa, is located on the surface of alveolar macrophages, and binds to SP-D in a Ca^{2+}-dependent manner, whilst the SP-A binding protein has a molecular mass of 210 kDa and is found on both type II cells and macrophages. The results presented here suggest that once the conidia coated with SP-A or SP-D are bound by alveolar macrophages or neutrophils, increased activation of the cellular respiratory burst occurs and killing of conidia is substantially increased by both cell types. As might be expected, the efficiency of killing is to some extent dependent upon the effector cell-to-conidium ratio (Table 2). Alveolar macrophages and neutrophils, isolated from different subjects, all showed enhanced killing of conidia in the presence of SP-A and SP-D. Our observations strongly suggest that both SP-A and SP-D have an important host defense role to play against fungal infections in the lung by enhancing uptake of the conidia, increasing activation of the cellular respiratory burst, and ultimately killing the infectious agents. In view of the relatively low amounts of SP-A and SP-D which may be required to enhance innate immunity in the lung, it could be expected that administration of purified native SP-A and SP-D, or recombinant forms of these proteins, might form a therapeutic means of treating some forms of pulmonary infection.

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