Surfactant protein A enhances alveolar macrophage phagocytosis of a live, mucoid strain of P. aeruginosa

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Mariencheck, William I., J ordan Savov, Qun Dong, Michael J ames Tino, and J o Rae Wright. Surfactant protein A enhances alveolar macrophage phagocytosis of a live, mucoid strain of P. aeruginosa. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L777–L786, 1999.—In this study, we investigate the interaction between surfactant protein A (SP-A) and a live, mucoid strain of Pseudomonas aeruginosa and identify a mechanism of clearance of this organism by alveolar macrophages. 125I-labeled SP-A bound live, but not heat-killed, P. aeruginosa organisms in a concentration-dependent manner. Unlabeled SP-A bound live bacteria, protein isolated from whole organisms, and specific proteins of the P. aeruginosa outer membrane. The binding of SP-A to P. aeruginosa and outer membrane components was inhibited by either EDTA or mannose. Phagocytosis assays with fluorescent microscopy demonstrated that the percentage of macrophages with internalized FITC-labeled P. aeruginosa was increased 1.8-fold (19 vs. 35%) by pretreating the live bacteria with SP-A. This finding was confirmed by direct visualization of ingested bacteria by electron microscopy. Adhering macrophages to SP-A-coated surfaces attenuated the increased uptake of P. aeruginosa pretreated with SP-A, suggesting that SP-A acts as an opsonin to stimulate macrophage phagocytosis of this strain of P. aeruginosa.

Pseudomonas aeruginosa; lung infection; innate immunity; cystic fibrosis

SURFACTANT PROTEIN (SP) A is the most abundant protein associated with pulmonary surfactant (12). SP-A is a multimeric protein composed of 28- to 36-kDa peptides with structural and functional similarities to other members of the collectin family including pulmonary SP-D, serum mannose binding protein, conglutinin, and CL-43 (14). These molecules contain an NH2-terminal collagenous domain and a COOH-terminal carbohydrate recognition domain capable of binding ligands via a calcium-dependent mechanism (7, 39).

A growing number of independent investigations have provided evidence that these proteins play an important role in host defense (reviewed in Refs. 4, 42). Specifically, SP-A-deficient mice have been shown to be more susceptible to pneumonia and sepsis from group B streptococcus than control mice (16). In vitro studies have demonstrated that SP-A binds to specific strains of several bacteria including Staphylococcus aureus, Streptococcus pneumoniae, group A streptococcus, Haemophilus influenzae, Escherichia coli, Klebsiella pneumoniae, bacillus Calmette-Guérin, and Mycobacterium tuberculosis. Furthermore, SP-A stimulates the phagocytosis of some of these organisms by alveolar macrophages (AMs) (15, 24, 30, 31, 40, 41).

Pseudomonas aeruginosa is an important pulmonary pathogen generally infecting humans with abnormalities of immune function or lung structure. Persons with cystic fibrosis (CF) are especially susceptible to chronic infection by mucoid strains of this organism contributing to the destruction of lung parenchyma and progressive loss of lung function in affected patients (34). Several lines of experiments suggested a role for SP-A in pulmonary host defense against this bacterium. Levine et al. (17) have recently demonstrated that intratracheal instillation of a mucoid strain of P. aeruginosa results in higher bacterial loads in the lungs of SP-A-deficient mice than in wild-type mice. Additionally, those investigators observed an earlier and more exuberant influx of neutrophils into the bronchoalveolar space, resulting in more severe and persistent pulmonary infiltrates in the SP-A-deficient lungs (17). Studies in humans have shown that the concentration of SP-A obtained by bronchoalveolar lavage is significantly reduced in patients with bacterial pneumonia (1, 18) as well as in persons with clinically stable CF compared with age-matched control subjects (11). Previous work (21) has also demonstrated that the association of another strain of P. aeruginosa organisms with AMs is significantly increased in the presence of SP-A in vitro, although this last finding did not involve a direct interaction between SP-A and that strain of P. aeruginosa.

This laboratory has previously reported (40) that SP-A does not affect AM phagocytosis of a mucoid strain of P. aeruginosa that had been heat killed. To further investigate the mechanism(s) of the above findings, we hypothesized that SP-A acts as an opsonin to increase phagocytosis of live P. aeruginosa organisms by macrophages. We report that SP-A bound to live, but not to heat-killed, P. aeruginosa organisms and interacted with specific components of the outer membrane of this bacterium. Preincubation of live, mucoid P. aeruginosa with SP-A resulted in increased phagocytosis of the bacteria by AMs. This effect was attenuated by adhering the macrophages to plates coated with SP-A before incubation with bacteria, consistent with a receptor-ligand-dependent interaction between complexes of SP-A and P. aeruginosa and AMs.

MATERIALS AND METHODS

Reagents and media. All chemicals except where noted were obtained from Sigma (St. Louis, MO). The bovine serum
albumin (BSA) used in these experiments was from fraction V, cell-culture tested, and fatty acid free, with reported endotoxin levels < 0.1 ng/mg. Dulbecco's phosphate-buffered saline (PBS) and RPMI 1640 medium were obtained from Gibco BRL (Life Technologies, Grand Island, NY).

Bacteria. A clinical isolate of a mucoid strain of P. aeruginosa from a patient with CF was a gift from Dr. Roy Hopfer (Medical Microbiology Laboratory, University of North Carolina at Chapel Hill Medical Center). Bacteria were suspended in 1× Luria-Bertani medium with 20% glycerol and frozen in aliquots at −80°C. Overnight cultures grown on nutrient agar (Difco, Detroit, MI) with 20% horse serum (GIBCO BRL) were suspended in PBS, pH 7.2, to quantify bacterial colony-forming units by optical density at 660 nm for use in subsequent assays. For studies with heat-killed bacteria, organisms were collected as above and then heated to 95°C for 10 min.

Labeling bacteria with fluorescent isothiocyanate. P. aeruginosa organisms from overnight cultures were collected by centrifugation at 5,000 g. This pellet was resuspended in 1 ml of 0.1 M sodium carbonate, pH 9.0, to optical density at 660 nm, equivalent to 10^8 colony-forming units/ml. Fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, OR) from a 10 mg/ml stock solution in dimethyl sulfoxide was added to a final concentration of 0.01 mg/ml. The suspension was protected from light and incubated for 1 h at room temperature with continuous shaking. Labeled bacteria were centrifuged and washed multiple times with PBS, pH 7.2, to remove unconjugated FITC and were stored in 100-µl aliquots in 15% glycerol at −80°C. Labeling did not affect viability of the bacteria as determined by colony-forming units produced by overnight plate incubation (data not shown).

SP-A isolation and purification. SP-A was purified from the bronchoalveolar lavage fluid of patients with alveolar proteinosis as previously described (43) and stored in 5 mM Tris, pH 7.4, at −20°C. SP-A preparations were treated with poly-myxgin agarose to reduce endotoxin contamination, dialyzed against 5 mM Tris, and centrifuged at 100,000 g for 30 min (22). Aliquots were tested for the presence of endotoxin by the Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD), and only samples containing <0.5 pg endotoxin/mg protein were used. Rat SP-A was purified from the lavage fluid of silica-treated rats by the nonbutanol method of Szwabe et al. (38).

Iodination of SP-A. SP-A purified as in SP-A isolation and purification was labeled with Na[125I] (DuPont, Boston, MA) with IODO-BEADS (Pierce, Rockford, IL) as previously described (40). Fractions with >85% trichloroacetic acid-precipitable counts were pooled and assayed for protein concentration by the bicinchoninic acid (BCA) assay (Pierce), and counts per minute per microgram of SP-A were determined by gamma counting. The specific activity of the [125I]-SP-A used ranged from 200,000 to 300,000 counts min⁻¹·µg⁻¹.

Isolation of rat AMs. Adult male rats (200–250 g) were obtained from Charles River (Raleigh, NC), anesthetized by intraperitoneal instillation of pentobarbital sodium, and killed by exsanguination. The tracheae were cannulated, and the lungs were lavaged to total lung capacity six times with PBS containing 0.2 mM EGTA, pH 7.4. The lavage fluid was centrifuged at 228 g, and the pellet was resuspended in PBS containing 1 mM CaCl₂. The cells were pelleted again at 228 g and resuspended in RPMI 1640 medium for immediate use.

Phagocytosis assay by fluorescence microscopy. AMs (3–3.5 × 10⁶) were adhered to Lab-Tek single-chamber slides that had been precoated with poly-D-lysine for 2 h at 37°C in 5% CO₂. FITC-labeled P. aeruginosa (1 × 10⁹) were incubated at 37°C with SP-A (25 µg/ml) or buffer alone in 200 µl of RPMI 1640 medium for 1 h at 37°C with rotation. For some experiments, bacteria were collected by centrifugation after incubation with SP-A, washed with 200 µl of RPMI 1640 medium, and centrifuged and washed again, and finally collected and resuspended in 200 µl of RPMI 1640 medium before addition to the AMs. The medium from the AMs was removed and replaced with the bacteria-protein mixture and incubated at 37°C in 5% CO2 for 1 h. Phagocytosis was terminated by washing the adhered macrophages with cold PBS. Extracellular fluorescence was quenched by the addition of 0.1% trypan blue in PBS for 15 min before fixation in 1% paraformaldehyde and staining of the macrophages with Evans blue. The slides were mounted with coverslips with 1,4-diiodoacyclclo(2.2.2)octane (Kodak, Rochester, NY), dissolved as a 25 µg/ml solution in 90% glycerol, 0.27 mM KCl, 0.15 mM KH₂PO₄, 13.7 mM NaCl, and 0.81 mM Na₂HPO₄, pH 8.6 (DABCO). The samples were analyzed with an epifluorescence microscope. One hundred to two hundred random macrophages were viewed for the presence of fluorescent particles, and the percentage of macrophages with any fluorescence was determined for each sample. For experiments comparing the effect of SP-A added as a soluble opsonin to the bacteria before incubation with macrophages adhered to lysine-coated surfaces, the absolute percentage of macrophages with internal fluorescence was reported as the mean ± SE from seven separate experiments. These data were analyzed for normal distribution, and statistical comparisons were made by two-sample, two-sided t-test, with α = 0.05 between the treatment group and the control group. For experiments comparing the effect of the substrate to which macrophages were adhered in addition to the pretreatment condition of the bacteria on the phagocytosis of P. aeruginosa organisms, the percentage of macrophages with internal fluorescence was determined in three separate experiments. The results of each experiment were normalized to the percent macrophages with internal fluorescence in the sample of macrophages adhered to lysine and incubated with bacteria without SP-A (control) for each experiment. The percent control response was analyzed with a multiplicative model that results in the relative percent of the control value, P₁, that is related to the treatment value P₂, by this factor that, when multiplied by 100, is equivalent to the percentage of the control response. This value, δ, was then analyzed with ANOVA and Student-Newman-Keuls t-test, with the null hypothesis that δ = 1 (100% control). Values of P ≤ 0.05 were considered significant.

Phagocytosis assay by electron microscopy. AMs (3–3.5 × 10⁶) were adhered to Lab-Tek single-chamber slides that had been precoated with poly-D-lysine for 2 h at 37°C in 5% CO₂. Live P. aeruginosa organisms (either 100:1 or 500:1 bacteria to macrophage final ratios) were incubated for 15 min in Dulbecco's PBS with 1 mM MgCl₂ in the presence and absence of SP-A (25 µg/ml). Medium of the adherent macrophages was replaced with PBS and 1 mM CaCl₂ to which the bacteria samples with and without SP-A were added to a final total volume of 4 ml. The slides were centrifuged at ~25 g for 3 min in a J ucan B3.11 tabletop centrifuge with microtiter plate holders and then placed at 70°C in 5% CO₂ for 1 h. Phagocytosis was terminated with 2% glutaraldehyde-paraformaldehyde in 0.085 M sodium cacodylate buffer. The cells were scraped from the slides and collected by centrifugation at 2,500 g for 8 min. The pellets were postfixed in 2% osmium tetroxide, stained with 2% uranyl acetate, dehydrated in a graded series of acetone, and then transferred and embedded in PolyBed 812 resin (Polysciences, Warrington, PA). Thin
sections were cut with a diamond knife, placed on Formvar-supported nickel grids (Ted Pella, Tustin, CA), and analyzed. Approximately 20 squares were scored for each sample, corresponding to 80–130 macrophages, and the percentage of macrophages with intracellular bacteria was quantified by a microscope who was blinded to the pretreatment condition of the bacteria.

Bacteria grown on six P-100 petri dishes as in P. aeruginosa by AMs, live P. aeruginosa were labeled with FITC and incubated with and without human alveolar proteinosis SP-A (25 µg/ml) for 1 h before being added to the macrophages adhered to a plastic slide coated with poly-o-lysine for 1 h in serum-free medium. After the chamber was washed with cold PBS and the extracellular fluorescence was quenched with trypan blue, the cells were fixed, stained with Evans blue, and visualized with fluorescent microscopy. The percentage of macrophages with internal fluorescence was determined by the BCA assay. Samples were used within 72 h of preparation because degradation was noted to occur with longer storage time.

RESULTS

SP-A increases the phagocytosis of P. aeruginosa by rat AMs. To examine the effect of SP-A on the uptake of P. aeruginosa by AMs, live P. aeruginosa were labeled with FITC and incubated with and without human alveolar proteinosis SP-A (25 µg/ml) for 1 h before being added to the macrophages adhered to a plastic slide coated with poly-o-lysine for 1 h in serum-free medium. After the chamber was washed with cold PBS and the extracellular fluorescence was quenched with trypan blue, the cells were fixed, stained with Evans blue, and visualized with fluorescent microscopy. The percentage of macrophages with internal fluorescence was determined and is expressed as percent phagocytosis (Fig. 1). Preincubating P. aeruginosa with SP-A resulted in an increase in the percentage of macrophages demonstrating phagocytosis of this organism from 19 ± 4 to 35 ± 5% (P < 0.03; n = 7 experiments). Further experiments were done in which the P. aeruginosa organisms were incubated in the presence and absence of SP-A isolated from the lavage fluid of silica-treated rats and then washed in three cycles of centrifugation and resuspension in RPMI 1640 medium before being...
added to the AMs. In experiments including this washing step, SP-A from the lavage fluid of silica-treated rats enhanced phagocytosis of *P. aeruginosa* by AMs compared with that in control bacteria (no added SP-A) by 191 ± 12% (*P*, 0.05; *n* = 6 experiments).

To confirm this finding, phagocytosis of *P. aeruginosa* organisms by AMs was directly visualized by electron microscopy. Live *P. aeruginosa* organisms were incubated with and without human alveolar proteinosis SP-A (25 µg/ml) before being added to the AMs. After processing, an electron microscopist blinded to the pretreatment condition of the bacteria determined the percentage of macrophages with internalized bacteria for each sample. Treatment of *P. aeruginosa* with SP-A before incubation with AMs resulted in an increase in the percentage of macrophages containing *P. aeruginosa* organisms from 13 to 32% (representative sections are shown in Figs. 2 and 3). With the magnification provided by fluorescent microscopy, we were unable to quantitate the number of bacteria internalized by a particular macrophage. However, with electron microscopy, we determined that AM phagocytosis of *P. aeruginosa* increases from 1.2 to 1.9 bacteria/AM (with internalized bacteria) by incubating the bacteria with SP-A before exposure to the AMs.

Adhering AMs to slides coated with SP-A attenuates the effect of pretreating *P. aeruginosa* with SP-A on phagocytosis. Adhering phagocytes to slides coated with ligand has been shown to cause localization of ligand-specific receptors to the adherent surface of the cell (45). Plating macrophages on plastic slides coated with human alveolar proteinosis SP-A (25 µg/ml) rather than with poly-d-lysine (or other controls without an exogenous coating surface) had no effect on macrophage phagocytosis of nonopsonized *P. aeruginosa* (Fig. 4). However, adherence of AMs to SP-A-coated surfaces attenuated the effect of pretreating *P. aeruginosa* with SP-A on the stimulation of phagocytosis. Adhering AMs to slides coated with SP-A results in rearrangement of the AM actin cytoskeleton (40a) and may sequester surface molecules of the macrophages that are required to facilitate internalization of *P. aeruginosa* via an SP-A-dependent process.

**Fig. 1.** Surfactant protein (SP) A increases phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. Live FITC-labeled *P. aeruginosa* organisms were incubated in presence and absence of SP-A (25 µg/ml) before being added to rat alveolar macrophages adhered to plastic slides precoated with lysine. After fluorescence of extracellular bacteria was quenched with trypan blue, percentage of macrophages exhibiting internal fluorescence was determined and is expressed as percent phagocytosis. Results are means ± SE of 7 experiments. *Pretreatment of *P. aeruginosa* organisms with SP-A resulted in a 1.8-fold increase in percent phagocytosis compared with control value, *P* < 0.05.

**Fig. 2.** Electron micrographs of alveolar macrophage phagocytosis of nonopsonized *P. aeruginosa*. Percentage of ~100 macrophages with internalized *P. aeruginosa* organisms was determined by an electron microscopist blinded to pretreatment condition of bacteria. A: several bacteria (arrowheads) are situated extracellularly in close proximity to macrophage plasma membrane. No ingested bacteria are seen. Original magnification, ×7,000. B: enlarged view of boxed region of A demonstrates a bacterium attached to pseudopodia of macrophage. Original magnification, ×5,000. Thirteen percent of macrophages incubated with live, nonopsonized *P. aeruginosa* were found to have internalized organisms.

**Fig. 3.** Immunoblot analysis demonstrates that SP-A binds to *P. aeruginosa* organisms in a concentration-dependent manner (Fig. 5A). Attempts to quantify this interaction were made by incubating 125I-SP-A with live *P. aeruginosa* organisms.
A previous study from this laboratory (40) did not demonstrate binding of 125I-SP-A to P. aeruginosa organisms. Initial experiments with similar concentrations of 125I-SP-A and live P. aeruginosa bacteria confirmed this finding. When this concentration range was increased to levels required for detection by immunostaining (above background retention of SP-A in BSA-coated microfuge tubes), however, a concentration-dependent increase in SP-A binding to live bacteria was observed (Fig. 5B). Detection of 125I-SP-A was significantly greater in tubes with live bacteria than with no bacteria when the concentration of SP-A was >10 µg/ml (P < 0.05).

The number of counts per minute in tubes containing bacteria were normalized to counts per 10^6 organisms, and the nanogram amount of SP-A bound to 10^6 organisms was calculated from the specific activity of the 125I-SP-A. For tubes that had no bacteria, the total number of counts was divided by the specific activity of the 125I-labeled SP-A to estimate the amount of background radiation accounted for by nonspecifically retained 125I-SP-A in the experiment. This value was consistently <10% of the counts per minute of the samples incubated with bacteria.

Binding assays were performed with 25 µg/ml of 125I-labeled SP-A in the presence and absence of 10 mM EDTA and 100 mM mannose as well as with heat-killed rather than with live P. aeruginosa organisms (Fig. 6). Chelation of calcium resulted in an ~75% reduction in binding of 125I-SP-A to live P. aeruginosa organisms. Similarly, 125I-SP-A binding to P. aeruginosa was reduced from 11.7 ± 4.4 to 3.2 ± 1.1 ng SP-A/10^6 bacteria in the presence of 100 mM mannose (P < 0.05). Binding of 125I-labeled SP-A to heat-killed P. aeruginosa was only 0.75 ± 0.34 ng SP-A/10^6 bacteria, suggesting that heat treatment of P. aeruginosa organisms alters bacterial ligands for SP-A.

To determine whether an outer membrane lipoprotein of P. aeruginosa is a ligand for SP-A binding, bacteria were treated with and without trypsin (0.1 mg/ml) and then with a trypsin inhibitor (10 mg/ml) and washed three times in TBS plus 2 mM CaCl_2 before being incubated with 125I-SP-A (25 µg/ml) in the same buffer (Fig. 7). Trypsin treatment of P. aeruginosa caused a marked decrease in 125I-SP-A binding to this organism (from 15.4 ± 1.3 ng SP-A bound/10^6 bacteria for control treated bacteria to 1.6 ± 0.5 ng SP-A bound/10^6 bacteria for trypsin-treated bacteria; P < 0.01; n = 4 experiments).

SP-A binds to specific components of the outer membrane of P. aeruginosa. Proteins were isolated from whole P. aeruginosa organisms and from the purified outer membrane of these bacteria, resolved by SDS-PAGE, and transferred to nitrocellulose. The membrane was incubated with unlabeled SP-A, and binding of SP-A to bacterial ligands was visualized by immunostaining. SP-A bound to three major bands (25, 22, and...
17 kDa) enriched in the outer membrane (1 µg of total protein) of P. aeruginosa (Fig. 8). These bands are faintly visualized in whole organism samples when a 20-fold excess of total protein (20 µg/well) was used from samples of whole organism protein. Additionally, SP-A bound to a 61-kDa protein present in lysis of the whole organisms but was not found in any of several outer membrane preparations. Control blots incubated without SP-A but with the polyclonal antibody to SP-A and goat anti-rabbit IgG conjugated to HRP followed by chemiluminescence do not demonstrate binding. Simi-

SP-A PHAGOCYTOSIS OF P. AERUGINOSA

10 mM EDTA
100 mM mannose

Bacteria
Live
Live
Live
Heat-killed

Fig. 5. A: SP-A binds to live P. aeruginosa organisms. Unlabeled SP-A was incubated in presence (+) and absence (−) of P. aeruginosa organisms [1 × 10^8 colony-forming units (cfu)] for 1 h at 37°C. Organisms were carried through 3 cycles of collection by centrifugation and washing in Tris-buffered saline (TBS) plus 2 mM CaCl_2. Final pellets were solubilized in sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The major immunoreactive product was the appropriate size of intact SP-A, and intensity of signal increased proportionately to amount of SP-A input into binding assay. [SP-A], SP-A concentration. Nos. at left, molecular mass in kDa.

B: 125I-labeled SP-A binds to live P. aeruginosa organisms. 125I-labeled SP-A was incubated in presence (+) and absence (−) of bacteria in TBS plus 2 mM CaCl_2 for 1 h at 37°C. Bacteria were pelleted, washed, and transferred to a new tube. Bacteria were again collected by centrifugation, and supernatant was aspirated. Radioactivity bound to resulting pellet was quantified with a gamma counter, and amount of SP-A bound/10^6 bacteria was calculated from specific activity of 125I-labeled SP-A. Data are means ± SE of 3 experiments done in triplicate. *Significantly greater binding with live organisms in absence of mannose or EDTA, P < 0.05.

Fig. 6. 125I-labeled SP-A binding to P. aeruginosa is inhibited by either mannose or chelation of calcium. 125I-labeled SP-A does not significantly bind heat-killed P. aeruginosa organisms. Live or heat-killed P. aeruginosa organisms (1 × 10^8 cfu) were incubated with 25 µg/ml of 125I-SP-A in presence and absence of mannose or EDTA. Bacteria were pelleted, washed, and transferred to a new tube. Bacteria were again collected by centrifugation, and supernatant was aspirated. Radioactivity bound to resulting pellet was quantified with a gamma counter, and amount of SP-A bound/10^6 bacteria was calculated from specific activity of 125I-labeled SP-A. Data are means ± SE of 3 experiments done in triplicate. *Significantly greater binding with live organisms in absence of mannose or EDTA, P < 0.05.

Fig. 7. Trypsin treatment of P. aeruginosa inhibits binding of 125I-labeled SP-A to this organism. Live P. aeruginosa were treated with and without trypsin for 30 min at 37°C, then collected by centrifugation and washed with 1% trypsin inhibitor in TBS plus 2 mM CaCl_2 before incubation with 125I-SP-A (25 µg/ml) as in Fig. 6. Data are means ± SE of 4 experiments. *SP-A binding was significantly inhibited by trypsin treatment of bacteria, P < 0.03.

Fig. 8. SP-A bands are faintly visualized in whole organism samples when a 20-fold excess of total protein (20 µg/well) was used from samples of whole organism protein. Additionally,
lar immunoassays were performed with unlabeled SP-A in the presence of 10 mM EDTA and 100 mM mannose. The presence of these substances resulted in no detectable binding of SP-A to P. aeruginosa outer membrane ligands (Fig. 8). Additionally, when bacteria were heat killed before isolation of protein, we observed no detectable binding of SP-A to bacterial components by this method (data not shown).

**DISCUSSION**

Summary. These studies demonstrate serum-independent binding of SP-A to a mucoid strain of P. aeruginosa and to specific components of the outer membrane of this organism. The binding of SP-A to live organisms and to specific P. aeruginosa ligands is inhibited by chelation of calcium or the presence of mannose. We further demonstrate that AM uptake of this live, mucoid strain of P. aeruginosa is increased by pretreating the bacteria with SP-A. This observation is in contrast to experiments done previously in this laboratory that did not demonstrate SP-A-dependent stimulation of macrophage phagocytosis of this strain of P. aeruginosa that had been killed by heating to 95°C for 10 min (40). This manipulation was done to avoid the confounding growth effects that bacteria could have on measures of binding and phagocytosis but may well have resulted in changes in the structure, availability, or number of bacterial ligands with which SP-A interacts. Our findings that SP-A binding to P. aeruginosa organisms and outer membrane components of these bacteria is inhibited by heat killing this organism are consistent with this hypothesis. Adhering macrophages to a surface coated with SP-A attenuates the stimulation of phagocytosis of P. aeruginosa, providing evidence that interactions between SP-A and these bacteria affect macrophage uptake of this organism.

Taken together, these observations suggest that SP-A acts as an opsonin to stimulate AM phagocytosis of this live, mucoid strain of P. aeruginosa.

Mechanisms of macrophage phagocytosis of P. aeruginosa. Recognition of structures present on the surface of microorganisms by phagocytic cell receptors that lead to ingestion of the bacteria in the absence of opsonins is termed nonopsonic phagocytosis (27). The mechanisms of nonopsonic phagocytosis of P. aeruginosa by macrophages and polymorphonuclear leukocytes have been examined in detail (20, 26, 35, 36). These studies have demonstrated that binding of P. aeruginosa to phagocytes is dependent on bacterial pili, flagella, and nonpilus adhesins; binding and subsequent phagocytosis of mutant strains lacking any of these structures is significantly reduced.

The effect of SP-A on nonopsonic phagocytosis of pathogens by AMs has not been evaluated in detail. One group (21, 28) found that preincubation of AMs in suspension with SP-A was sufficient to stimulate phagocytosis of nonopsonized strains of P. aeruginosa, E. coli, and S. aureus. Because the increased uptake of bacteria by these macrophages did not require demonstrable SP-A binding to the object of phagocytosis, SP-A was determined to be an activation ligand in those processes. To investigate whether SP-A could function as an activation ligand to stimulate nonopsonic phagocytosis of the strain of P. aeruginosa used in our study, macrophages were adhered to a surface coated with SP-A before phagocytosis of bacteria was assayed. Adhering cultured monocytes on surfaces coated with IgG or C3b causes localization of the corresponding receptor to the adherent surface of the cell, resulting in a loss of the activity of that specific receptor on the apical surface of the cell (45). If the effect of SP-A on increasing phagocytosis of P. aeruginosa was due to an
activating effect on the macrophage and independent of an opsonic effect of SP-A, this manipulation should be sufficient to stimulate the uptake of unopsonized P. aeruginosa. We observed no significant difference in the phagocytosis of P. aeruginosa between the macrophages adhered to SP-A compared with those adhered to IgY (Fig. 4). These data argue against a role of SP-A in affecting nonopsonic phagocytosis as an activation ligand in these studies, although our relatively small number of experiments do not entirely exclude this possibility.

Adhering AMs to a surface coated with SP-A attenuated the stimulation of phagocytosis of P. aeruginosa caused by pretreating the bacteria with SP-A. Adhering phagocytic cells to surfaces coated with SP-A has been reported to significantly reduce the effect of pretreating bacteria with SP-A on the association of S. aureus with monocytes (8) and phagocytosis of H. influenzae by AMs (40). Of note, SP-A was shown to bind to the strains of S. aureus and H. influenzae used in those studies.

Finally, SP-A pretreatment of P. aeruginosa was sufficient to enhance AM phagocytosis of this organism even after several washing cycles of the SP-A-bacteria complexes before addition to the AMs. Our findings are consistent with a receptor-mediated mechanism of uptake of SP-A-P. aeruginosa complexes by AMs, suggesting a role for SP-A as an opsonin rather than as an activation ligand in the phagocytosis of this live, mucoid strain of P. aeruginosa by AMs.

Targets for SP-A opsonization of P. aeruginosa. We have identified several components of the P. aeruginosa outer membrane that bind SP-A on ligand blots. Whether the interactions between SP-A and these specific P. aeruginosa outer membrane components are responsible for the binding of SP-A to intact organisms and enhancement of AM phagocytosis is not certain. Similarities between the inhibition of SP-A binding to both the live bacteria and outer membrane components provide indirect evidence that the carbohydrate-binding domain of SP-A is involved in the interactions between SP-A and this mucoid strain of P. aeruginosa, but other mechanisms must be considered. The P. aeruginosa pilus has been reported to have a molecular mass of 18 kDa (29), similar to one of the ligands SP-A bound in the ligand blot assay. The pilus protein binds to sialic acid, N-acetylgalactosamine, galactose \( (1,3) \) N-acetylgalactosamine, and mannose moieties (32) contained by several corneal epithelial cell glycoproteins, but the pilus of the strain of P. aeruginosa used in those experiments lacks carbohydrate itself (29). Because SP-A contains sialic acid, mannose, and N-acetylgalactosamine (2), it is possible that P. aeruginosa binds SP-A via interactions with carbohydrates on SP-A.

Candidates for the targets of SP-A binding to higher-molecular-mass P. aeruginosa outer membrane proteins include adhesins, which range in molecular mass from 22 to 48 kDa and have been shown to interact with mucin glycopeptides (33). The identity of the 61-kDa protein of P. aeruginosa lysate to which SP-A binds is not presently known. Whether this protein is available for interaction with SP-A on the surface of the bacteria is of obvious functional significance. Future studies are needed to examine these questions in more detail.

Effects of heat treatment of P. aeruginosa on measures of binding and phagocytosis. A previous study (36) has demonstrated that if P. aeruginosa are heat killed (56°C for 30 min) or treated with Formalin, neither binding nor ingestion of P. aeruginosa by AMs was observed. Another group of investigators (6) has reported that phagocytosis of a strain of P. aeruginosa (as well as strains of E. coli and S. albus) organisms by polymorphonuclear leukocytes in the presence of serum is significantly reduced when the bacteria are first treated with heat (immersion in a boiling water bath for 20 min) compared with that of live bacteria. Heat treating P. aeruginosa by boiling results in dramatic ultrastructural changes in the organism (data not shown). Using electron-microscopic techniques, we confirmed previous results that heat treatment of P. aeruginosa inhibits the enhancement of AM phagocytosis of this organism resulting from pretreating the bacteria with SP-A (40). We now show that this decrease in macrophage uptake is associated with a loss in demonstrable binding of SP-A to heat-killed P. aeruginosa. Several outer membrane proteins of P. aeruginosa have been shown to be modified by heat treatment, resulting in different mobility when resolved by SDS-PAGE (13, 25). These experiments suggest that heat treating P. aeruginosa results in changes in bacterial ligands that alter interactions between the organism and SP-A or macrophage surface molecules, which are required for phagocytosis of this organism. Precedent for this observation can be found with other molecules that bind bacteria. For example, C-reactive protein, an acute-phase reactant that functions in host defense as an opsonin, binds to and enhances the phagocytosis of several types of S. pneumoniae that have been killed by heat (70°C for 1 h) but not of live organisms (5).

Implications for pulmonary host defense against P. aeruginosa. There are many steps in the sequence from colonization of the lung with these bacteria to its ultimate clearance by the host immune system with or without accompanying destruction of lung and loss of pulmonary function (9, 10). Quantitative and/or local deficiencies in the concentration of SP-A [as in persons with CF (11)] may facilitate P. aeruginosa colonization of the lung. By stimulating macrophage uptake of this organism, SP-A may limit the proliferation of P. aeruginosa and damage to the host caused by both the bacteria and the host response to infection. Additionally, by binding to live P. aeruginosa organisms, SP-A may inhibit binding of P. aeruginosa to lung epithelial cells and facilitate the physical removal of this pathogen via mucociliary clearance. SP-A has been shown to be a potent chemotactant for AMs (44) and neutrophils (19), and a 210-kDa receptor on the surface of AMs has been shown to have a significant role in the uptake of complexes of SP-A-bound bacillus Calmette-Guérin by AMs (3, 41). Complexes of SP-A-P. aeruginosa may affect similar responses from host defense cells. Recent studies by LeVine et al. (17) have shown that mice produced by homologous recombination to
lack SP-A do not clear intratracheally instilled P. aeruginosa organisms (from the same host strain of bacteria used in this study) as effectively as wild-type mice. SP-A-deficient mice infected in this manner were found to have higher loads of bacteria and more severe neutrophil infiltrates in their lungs than wild-type control mice (17). Although the magnitude of SP-A binding to P. aeruginosa and its enhancement of AM phagocytosis appear quantitatively less in this study than the results reported for SP-A interactions with other bacteria, these novel findings suggest a role for SP-A in the host response to this important pathogen. Future work in this field is needed to further characterize the interactions between SP-A and P. aeruginosa as well as to examine the mechanisms by which P. aeruginosa may limit the innate immune response and may suggest strategies for therapeutic intervention in the management of pulmonary infection caused by this important pathogen.

We thank Dr. Claude Plantadosi for providing lavage fluid from alveolar proteinosis patients and Jillie Taylor for purifying surfactant protein A from these samples and conducting endotoxin assays on the resulting product. This work was supported by National Heart, Lung, and Blood Institute Grant HL-51134 (to J. R. Wright), the Lucille P. Markey Foundation Four Schools Program (W. I. Mariencheck), and the Veterans Affairs Medical Research Fund (J. Savov).

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Received 15 April 1999; accepted in final form 27 May 1999.

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