Surfactant Protein A Binds Mycoplasma pneumoniae with High Affinity and Attenuates Its Growth by Recognition of Disaturated Phosphatidylglycerols*

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Surfactant Protein A (SP-A) is an abundant, multifunctional lectin that resides within the bronchoalveolar compartment of the lung and plays an important role in the innate immunity of the organ. Mycoplasma pneumoniae is a human pathogen that resides in the same compartment as SP-A, and we examined the interaction between the two. Preparations of human and rat SP-A recognized the mycoplasma with high affinity in the presence of Ca\(^{2+}\), exhibiting apparent K_d values in the nanomolar range. Membranes prepared from the microbe also bound human and rat SP-A with similar characteristics and affinity to the intact cells. The ligand for SP-A was insensitive to proteolysis. Lipid extracts prepared from the mycoplasma, bound SP-A with high affinity when examined by ligand blot analysis. These lipid extracts were also potent competitive inhibitors (IC_{50} = 0.2 nM) of human SP-A binding to mycoplasma membranes. The major lipid ligands for the protein identified by mass spectrometry are a group of disaturated phosphatidylglycerols. The addition of SP-A to cultures of M. pneumoniae markedly attenuated the growth of the organism assessed by colony formation, metabolic activity, and DNA replication. The bacteriostatic effects of SP-A were reversed by dipalmitoylphosphatidylglycerol. These findings demonstrate that human SP-A can play a direct role in antibody-independent immunity to M. pneumoniae by interacting with lipid ligands expressed on the surface of the organism and implicate SP-A in the immediate host response to the bacteria.

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Pulmonary surfactant protein A (SP-A) is a prominent member of the collectin superfamily that also includes the closely related collectins surfactant protein D (SP-D), serum mannose-binding protein, bovine serum conglutinin, and CL-43 (1). SP-A is an abundant component of pulmonary surfactant, a complex mixture of lipids and proteins that reduces surface tension in the alveoli during expiration (2). A growing body of evidence demonstrates that SP-A plays an important role regulating the innate host defense system within the lung (3). Functional deletion of the SP-A gene from the mouse genome provides important evidence establishing the regulatory role of the protein in innate immunity in vivo (4–6). The interaction of SP-A with immune cells can occur through ligation of a number of cell surface receptors that include the lipopolysaccharide-binding protein, CD14 (7), a 210-kDa protein, SPR 210 (7), a complement binding protein, C1qR (8), CD91/calreticulin (9), and SIRPα (10). In addition to recognition of immune cells, the protein can bind a variety of pulmonary pathogens, including Pseudomonas, Escherichia, Mycoplasma, Candida, Aspergillus, Histoplasma, and Influenza species (summarized in Ref. 3). For some organisms, SP-A functions as an opsonin and enhances phagocytosis (11, 12). The interactions of SP-A with microorganisms and host cells are complex and incompletely understood. In some cases the ligation of microbes (or derived ligands) and immune effector cells leads to amplification of inflammatory responses (13, 14), whereas in others the inflammatory responses are markedly attenuated (7, 15, 16). The emerging picture is consistent with the nature of the foreign ligand and the predisposed state of the responding immune cell, both playing important roles in dictating the final inflammatory response of the host. In addition to modulating the actions of inflammatory cells SP-A and SP-D can also exert a direct antimicrobial effect upon several Gram-negative bacteria (17, 18) and Histoplasma capsulatum (18) by increasing membrane permeability. The effects upon Gram-negative cells appear to be mediated via surface lipopolysaccharide binding. The surface-binding molecule for Histoplasma is not known but could be a glycoconjugate.

In this report we have focused on the interactions of SP-A with Mycoplasma pneumoniae. Unlike Gram-negative bacteria, the mycoplasma lack lipopolysaccharides and are likely to have novel ligands for SP-A. M. pneumoniae is an important human pathogen that causes primary atypical pneumonia and other airway diseases, including tracheobronchitis and pharyngitis. Infection by the organism can also seriously exacerbate asthma (19, 20). Recent evidence also demonstrates the presence of the organism in a subset of chronic asthmatics suggesting that it may have a contributing role in the etiology of the disease (21). Several studies have previously implicated SP-A as a modulator of macrophage-dependent killing of the mouse pathogen M. pulmonis (22–24), but little current information is available regarding the direct interactions of SP-A with the human pathogen M. pneumoniae. The purpose of this study was to

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The abbreviations used are: SP-A, surfactant protein A; SP-D, surfactant protein D; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; 16:0/16:0-PtdCho, dipalmitoyl phosphatidylcholine; PtdGro, phosphatidylglycerol.
elucidate the interaction between SP-A and *M. pneumoniae*, 2) determine if membranes derived from the organism could also interact with SP-A, 3) characterize the types of mycoplasma ligands recognized by SP-A, and 4) evaluate the direct effects of the protein on mycoplasma growth. Our findings demonstrate high affinity interactions between SP-A and *M. pneumoniae*, dependent upon disaturated phosphatidylglycerols present in the membrane. In addition, SP-A directly inhibits the growth of the bacteria, and this action is reversed by disaturated phosphatidylglycerols. These findings demonstrate that SP-A plays an important role in controlling the antibody-independent host response to mycoplasma.

**EXPERIMENTAL PROCEDURES**

**Purification of SP-A and Antibodies**—Human SP-A was purified from alveolar proteinosis fluid as described previously (25). Rat SP-A was purified from CHO-K1 cells or Sf9 cells expressing the recombinant protein, by published methods (26). Polyclonal antibodies to the human and rat proteins were independently generated in rabbits, and the IgG fractions were isolated using protein A-Sepharose. For ELISA, the primary antibodies were conjugated to hors eradish peroxidase.

**Purification of *M. pneumoniae***—*M. pneumoniae* (10^7 g/ml) was grown in SP-4 medium (27) at 37 °C in an atmosphere of 95% air, 5% CO_2. The viability of the cells assessed by colony counting was essentially identical, because large amounts of protein precipitate co-sediment with mycoplasma in the initial centrifugation step and interfere with binding measurements and quantification of microbial protein.

**M. pneumoniae Membrane Preparation**—Mycoplasma membranes were prepared by mixing 1 ml of gradient-purified bacteria resuspended in SP-4 medium (27) at 37 °C in an atmosphere of 95% air, 5% CO_2. The changing color of SP-4 medium due to acidification was measured by spectrophotometry at a wavelength of 550 nm. In the acidification assay, varying concentrations of SP-A were incubated with 10^4 cells/ml of *M. pneumoniae* in microtiter wells, for 5 days at 37 °C with 5% CO_2. The bound SP-A was detected by 10 μg/ml HRP-conjugated antibody against either human or rat SP-A and quantified by ELISA with 1 mg/ml orthophenylediamine as the color-developing agent.

**Binding of SP-A to Lipids Extracted from *M. pneumoniae* Membranes Separated by Two-Dimensional Thin Layer Chromatography**—Lipids extracted from *M. pneumoniae* were separated by two-dimensional thin layer chromatography on SIL G plates (Macherey Nagel). Total lipids (equivalent to 40 nmol of lipid phosphorus) were applied to the plate and separated in a first dimension consisting of chloroform:methanol:water:acetic acid (50:25:2:5) and dried overnight. Lipid components were visualized by either 0.2% w/v anilino-1-naphthalenesulfonic acid (ANS) staining or orcinol staining for glycolipids. To determine binding of SP-A to the lipid, purified protein (10 μg) was suspended in 1 ml of buffer A containing 2% bovine serum albumin and 5 mM CaCl_2, and added to the plate and incubated for 1 h at room temperature. The plate was washed with buffer A containing 0.1% bovine serum albumin and 5 mM CaCl_2, three times. The bound SP-A was detected by using 10 μg/ml HRP-conjugated primary antibody against the protein and 1 mg/ml 3,3′-diaminobenzidine as the color development reagent.

**Lipid Competition for SP-A Binding to Solid Phase Mycoplasma Membranes**—Lipids extracted from *M. pneumoniae* membranes and dipalmitoylphosphatidylcholine (16:0/16:0-POPC, Avanti Pol Lipids, Alabaster, AL) were hydrated in 10 mM CaCl_2, pH 7.4, at 37 °C for 30 min. The plate was washed with buffer A containing 2% bovine serum albumin and 5 mM CaCl_2, three times. The SP-A bound to membranes was detected with 10 μg/ml HRP-conjugated primary antibody and quantified by ELISA.

**Inhibition of *M. pneumoniae* Growth by SP-A**—We evaluated the effect of SP-A on *M. pneumoniae* growth by colony counting, culture acidification, and [3H]thymidine incorporation. The colony counting assay, 1–10^7 g/ml SP-A was incubated with 10^4 cells/ml *M. pneumoniae* in SP-4 medium (27) at 37 °C for 1–5 days. For each day, an aliquot of the culture was spread on a pleuropneumonia-like organism (PPLO) plate. The PPLO plates were further incubated for another 7 days at 37 °C, and the colonies were counted. In the acidification assay, varying concentrations of SP-A were incubated with 10^4 cells/ml of *M. pneumoniae* in SP-4 medium, in microtiter wells, for 5 days at 37 °C with 5% CO_2. The changing color of SP-4 medium due to acidification was measured by spectrophotometry at a wavelength of 550 nm. In the radioactivity assay, cells (10^4 or 10^5 cells/ml) were inoculated into SP-4 medium supplemented with [3H]thymidine (1 μCi/ml) and incubated 5 or 6 days. The cultures were harvested by centrifugation at 10,000 × g × 10 min and washed by recentrifugation twice in PBS containing 1% BSA. The final pellets were resuspended in PBS with 1% Triton X-100, and the radioactivity was quantified by liquid scintillation spectrometry. We confirmed that the [3H]thymidine was incorporated into macromolecular DNA by precipitation with 5% trichloroacetic acid. The Acid treatment of mycoplasma revealed that >90% of the [3H]thymidine was precipitable. The antagonism of the SP-A effect upon mycoplasma was measured by lipid extract from the bacteria or 50 nmol of dipalmitoylphosphatidylglycerol to the cultures and measuring the effects upon [3H]thymidine incorporation.

**HPLC and Mass Spectrometry of Mycoplasma Lipids**—The spot identified on TLC as the major ligand for SP-A was scraped from plates and extracted from the silica gel by the method of Bligh and Dyer (29). The resultant preparation was subjected to reversed phase HPLC to purify...
phospholipid molecular species and was carried out on a 150 × 2.0-mm HPLC analysis used two solvents. Solvent A contained methanol/water (95:5, v/v) with 1 mM ammonium acetate. Solvent B was methanol made to 1 mM ammonium acetate. The gradient was developed from 20% to 100% solvent B in 35 min and remained at 100% solvent B for 15 min. The HPLC effluent was monitored at 220 nm using a photodiode array detector. 1-min fractions were collected from the HPLC and subjected to ligand blot analysis by TLC and direct mass spectrometric analysis. The individual HPLC fractions were analyzed by electrospray ionization in the negative ion mode by infusion of each sample into an API-III tandem quadrupole mass spectrometer (Applied Biosystems, Thornhill, Ontario, Canada). Tandem mass spectrometry was carried out using argon as the collision gas at a thickness of 235 × 10^3 molecules/cm^2. Nitrogen was used as the curtain gas with a collisional offset potential of 40 eV for product ion formation.

**RESULTS**

**SP-A Binds with High Affinity to M. pneumoniae Cells and Membranes**—Our initial studies examined the interaction of SP-A with the human pathogen *M. pneumoniae*. We devised a binding assay that utilized purification of the organisms through an albumin step gradient and detection of the bound protein by ELISA. The results presented in Fig 1A demonstrate that human SP-A binds the mycoplasma in a concentration-dependent and saturable manner. The binding shows an absolute requirement for Ca^{2+} and is completely inhibited by EGTA. Scatchard analysis of the binding data gave an estimated 31 × 10^3 binding sites per cell with an apparent *Kd* value of 7.8 nM. Similar findings were obtained with respect to the Ca^{2+} requirement and saturability when the rat protein was examined for interaction with the mycoplasma (Fig. 1B). The rat protein bound with an apparent *Kd* value of 8 nM and an estimated 52 × 10^3 binding sites per cell.

To more fully evaluate the binding of SP-A to mycoplasma we developed a solid phase binding assay using membranes adsorbed to microtiter wells. The binding of the human SP-A to the solid phase membranes also showed dependence upon Ca^{2+} and inhibition by EGTA as demonstrated in Fig. 2. The apparent affinity of the binding was higher using the membrane system, than that found for the binding of intact organisms in solution. For human SP-A the apparent *Kd* value is 0.3 nM. In these experiments we also compared the binding of SP-A derived from normal human subjects to that from alveolar proteinosis patients. There was no significant difference in the binding activity of the SP-A from either source. Examination of the binding of the rat SP-A produced in either CHO-K1 cells or S9 cells gave comparable results as shown in Fig. 3. The estimated *Kd* value for rat SP-A is 0.9 nM. From these data we conclude that SP-A from both human and rat sources binds *M. pneumoniae* membranes with high affinity and provides a useful cell free system for biochemical dissection of the process.

**The Ligand for Human SP-A Is a Lipid**—The high affinity interactions of SP-A with mycoplasma membranes provided a versatile system to elucidate the nature of the ligand expressed on the organism. In these experiments we first examined the protease sensitivity of the binding. The membranes were exposed to 1 mg/ml trypsin or 1 mg/ml proteinase K for 1 h at 37 °C. The resultant membranes were treated to inactivate the proteases and washed by centrifugation and adsorbed onto microtiter wells and examined for retention or loss of binding activity. The proteolysis of the membrane proteins was confirmed by gel electrophoresis and staining. We also determined that the levels of proteases added to the reactions were sufficient to completely digest amounts of BSA comparable to the amount of mycoplasma membrane protein present. The amount of mycoplasma membrane in both untreated and protease-treated samples was made equivalent with respect to their phospholipid content. The results shown in Fig. 4 demonstrate that proteolysis was without any significant effect upon either human or rat SP-A binding to the membranes. These findings indicate that the mycoplasma ligand is either a lipid or an extremely protease resistant protein.

To test the possibility that the ligand for SP-A is a lipid, the mycoplasma membranes were subjected to extraction with organic solvents, and the lipid fraction was examined for binding activity. The lipids were first separated by two-dimensional thin layer chromatography in parallel on four thin layer plates. Following the separation, the plates were stained for either total lipid (ANSA stain) or glycolipid (orcinol stain), and immunostained for human SP-A binding. The results of the experiment are shown in Fig. 5. The mycoplasma contains at least eight well resolved polar lipids and unresolved neutral lipids, which migrate close to the solvent front as shown in Fig. 5A. The identifiable polar lipids co-migrate with sphingomyelin (L1), phosphatidylcholine (L3), and phosphatidylglycerol (L6). At least five of the polar lipids give a strong reaction with the glycolipid detecting reagent used in Fig. 5C. The identifiable
glycolipids include dihexosyldiacylglycerol (G6) and monohexosyldiacylglycerol (G7). The G3/L4 spot has the expected migration of trihexosyldiacylglycerol. Although the G5 spot co-migrates with phosphatidylglycerol, independent experiments demonstrate that phosphatidylglycerol does not react with orcinol. Thus, the G5/L6 spot contains multiple components. The G1 spot migrates with complex glycosphingolipids. The plate incubated with human SP-A and antibody (Fig. 5C) shows one major and one minor lipid ligand. The major lipid ligand co-migrates with phosphatidylglycerol and an unknown glycolipid component. The control plate is shown in Panel D and demonstrates there is no antibody reactivity without the addition of SP-A. These data clearly indicate that one molecular category of high affinity ligand for SP-A in *M. pneumoniae* consists of lipids.

We next tested whether the lipid ligand for SP-A derived from mycoplasma was capable of inhibiting the binding of the protein to the solid phase membrane preparation. In experiments shown in Fig. 6, varying concentrations of the mycoplasma total lipid fraction, in the form of unilamellar liposomes, were added as competitor for the membrane binding reaction. The lipid fraction was able to completely inhibit the binding of SP-A to the solid phase membranes. The IC<sub>50</sub> for inhibition was 0.2 nmol based upon lipid phosphorus. In the same experiment we also determined the competition for membrane binding by the surfactant lipid, 16:0/16:0-PtdCho, which is known to be a high affinity lipid ligand for SP-A (30). Quite remarkably, the mycoplasma high affinity ligand was a more potent inhibitor of SP-A binding to the solid phase membranes than 16:0/16:0-PtdCho. The apparent IC<sub>50</sub> of the mycoplasma ligand exhibited more than 10-fold greater affinity for SP-A than 16:0/16:0-PtdCho in the competitive membrane binding assay. These results clearly indicate that *M. pneumoniae* expresses a lipid on its surface that has extremely high affinity for human SP-A.

**Fig. 2.** Normal and alveolar proteinosis-derived SP-A bind mycoplasma membranes on solid phase. Mycoplasma membranes (250 ng of protein) were coated onto microtiter wells at 4 °C, overnight. The indicated concentrations of normal and alveolar proteinosis (App)-derived proteins were incubated in the wells at 37 °C for 1 h in the presence of 5 mM CaCl<sub>2</sub> ( ), or 5 mM EGTA ( ). Control incubations without bacterial membranes were also performed ( ). After the incubation the wells were washed and the binding of the proteins to the membranes was detected with HRP-conjugated polyclonal antibody to human SP-A. Values are the means ± S.E. from three experiments.

**Fig. 3.** Recombinant rat SP-A binds mycoplasma membranes on solid phase. Mycoplasma membranes were coated onto microtiter wells at 4 °C, overnight. The indicated concentrations of recombinant rat SP-A produced in mammalian cells (CHO, ), or insect cells (Sf9, ) and ( ) were examined in the presence of 5 mM CaCl<sub>2</sub> or 5 mM EGTA ( , , and ) as indicated. The incubations were performed at 37 °C for 1 h. Control incubations without bacterial membranes were also performed ( ). Values are the means ± S.E. from three experiments.

**Fig. 4.** The membrane ligands for human and rat SP-A are protease resistant. Mycoplasma membranes were digested with 1 mg/ml of either trypsin or proteinase K and tested in solid phase binding assays for SP-A binding. The amount of membrane added to each well corresponded to 1 nmol of lipid phosphorus and the coating was performed at 4 °C, overnight. The binding reactions containing 630 ng of human SP-A and 5 mM CaCl<sub>2</sub> or 5 mM EGTA were incubated at 37 °C for 1 h. Values are the means ± S.E. from three experiments.
naphthalenesulfonic acid (ANS A). The components are unknown. In  and glycerol, respectively.

FIG. 5. SP-A recognizes lipid components present in mycoplasma membranes. Lipids were extracted from mycoplasma membranes and separated by two-dimensional thin layer chromatography on 4 Merck Sil 60 plates. The first dimension was developed in chloroform:methanol:NH₄OH (65:35:8), and the second dimension was developed in chloroform:methanol:acetic acid:water (50:25:8:5) as indicated by the arrows. In A, polar lipids were detected with 0.2% 8-anilino-1-naphthalenesulfonic acid (ANS A). The L₁, L₃, and L₆ components correspond to sphingomyelin, phosphatidylcholine, and phosphatidylglycerol, respectively. L₂ co-migrates with complex glycosphingolipids, and L₄ co-migrates with trihexosyldiacylglycerol. The identities of L₅, L₇, and L₈ are not known. In B, glycolipids were detected with orcinol. G₅, G₆, and G₇ co-migrate with phosphatidylglycerol, dihexosyl-, and monohexosyldiacylglycerol, respectively. The G₃ component migrates at the expected position for trihexosyldiacylglycerol. The G₁ component migrates at the position of complex glycosphingolipids. The G₂ and G₄ components are unknown. In C, 10 µg/ml human SP-A was added to the plate in the presence of a blocking buffer, and the mixture was incubated for 1 h at room temperature. The bound human SP-A was detected with HRP-conjugated polyclonal antibody to SP-A. The minor reactive ligand designated A₁ co-migrates with sphingomyelin (L₁). The major reactive ligand, designated A₂, co-migrates with phosphatidylglycerol (L₆) and a hexose containing glycolipid (G₅). In D, the control plate was treated identically to the SP-A immunoblot plate except that the SP-A was omitted.

FIG. 6. Lipids isolated from mycoplasma are competitive inhibitors of SP-A binding to solid phase membranes. Total lipid extracts (□) prepared from mycoplasma membranes, and 16:0/16:0-PtdCho (■) were sonicated to form unilamellar vesicles. The lipid vesicles at the concentrations indicated were added to 32.5 ng of human SP-A and incubated at 25 °C for 30 min. The SP-A solution was next transferred to wells precoated with mycoplasma membranes (250 ng of protein) and incubated at 37 °C for 1 h. Unbound SP-A was removed by washing the wells, and the bound fraction was quantified by ELISA. Values are the means ± S.E. from three experiments.

FIG. 7. Human SP-A decreases the metabolic rate of M. pneumoniae. Mycoplasma were inoculated into liquid medium at 10⁴ cells/ml in the absence or presence of the indicated concentrations of human SP-A. The cells were grown at 37 °C for 5 days. The medium was harvested, and the A₅₅₀ was used to measure its acidification. Values are the means ± S.E. from three experiments. Asterisks indicate p < 0.05 when compared with cultures without SP-A.

Therefore next examined the effect of SP-A on the metabolism of the cells by quantifying the ΔpH of the cultures with time. These experiments, shown in Fig. 7, demonstrate that SP-A has a concentration dependent effect upon the metabolism of the cells. At 25 µg/ml SP-A, there was a 4-fold difference in the concentration of protons produced by the cultures after 5 days of growth. Heat denaturation of the SP-A completely eliminates the inhibitory effect upon microbial growth.

The effect of SP-A on cell growth is dependent not only upon the concentration of the protein, but also on the ratio of the protein to the cell number of the culture. The preceding experiments used starting inocula of 10⁴ cells/ml. In Fig. 8 we show the results of using a starting cell number of 10⁵/ml and different concentrations of human and rat SP-A. For these studies we used [³H]thymidine incorporation into the macromolecular pool to evaluate the effects of the protein on replication. In these experiments the cells were grown for 6 days, which corresponds to mid log phase for the control cultures. The human SP-A markedly reduced the rate of [³H]thymidine incorporation into DNA by the mycoplasma in a concentration-dependent manner. At 10 µg/ml the human SP-A reduced the incorporation of radiolabel into DNA by more than 90%. In this same series of experiments we also demonstrated that rat SP-A produced in either CHO-K1 cells or baculovirus/Sf9 cells was capable of reducing the growth of the organism albeit to a lesser extent. These studies of M. pneumoniae growth clearly establish that SP-A reduces the cell number, culture metabolic rate, and replication of the organism. From the above findings we conclude that the high affinity interactions between SP-A and M. pneumoniae have potent antibacterial consequences.

The Major Lipid Ligands for SP-A in M. pneumoniae Membranes Are Disaturated PtdGros—We next sought to identify the major lipid ligands for SP-A present in mycoplasma membranes. Lipids were extracted from purified bacterial membranes and separated by TLC. The region of the plate corresponding to the major SP-A reactive component identified by

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ligand blot analysis (designated A2 in Fig. 5) was scraped and extracted to isolate the lipids. The recovered lipid extract was next subjected to HPLC and mass spectrometry as shown in Fig. 9. Individual fractions from the HPLC were subjected to both TLC and mass spectrometry. The SP-A-reactive material in each fraction was detected on TLC by the ligand blot method. As shown in Fig. 9A only three fractions contained material that reacted with SP-A, and they were present in HPLC fractions 26, 29, and 32. These fractions contained different molecular species of phosphatidylglycerol (PtdGro). Mass spectrometry shown in Fig. 9B revealed that the unique component present in fraction 26 had (M – H)− at m/z 721.5 corresponding to 32:0-PtdGro. Tandem mass spectrometry of the 32:0-PtdGro demonstrated that the major fatty acid had a carboxylate anion at m/z 255.2 corresponding to a 16:0 species. This analysis demonstrates that the SP-A-reactive lipid in fraction 26 is di-C16:0-PtdGro. Similar analyses were performed on fraction 29 and revealed that the unique component had (M – H)− at m/z 749.5 corresponding to 34:0-PtdGro. The tandem mass spectrometry of the 34:0-PtdGro demonstrated two prominent fatty acids with carboxylate anions at m/z 255.2 and 283.2 corresponding to 16:0 and 18:0 species, respectively. Repetition of these methods with fraction 32 revealed (M – H)− at m/z 777.5 of the unique component, consistent with a 36:0-PtdGro. There was insufficient material in fraction 32 for tandem mass spectrometry.

To confirm the nature of the major ligands for SP-A identified by mass spectrometry we performed a reverse analysis by subjecting purified lipids to ligand blot reactions. In these experiments shown in Fig. 10, disaturated lipids (16:0/16:0-PtdGro, 16:0/18:0-PtdGro, and 18:0/18:0-PtdGro) and monounsaturated lipids (16:0/18:1-PtdGro and 18:0/18:1-PtdGro) were separated by TLC and subjected to the SP-A ligand blotting procedure. The disaturated PtdGros corresponding to those identified by mass spectrometry were strongly reactive with SP-A, whereas the monounsaturated-PtdGros were very weakly reactive. The more highly unsaturated 18:1/18:1-PtdGro did not bind SP-A nor did PtdGros derived from egg PtdCho synthesized by transphosphatidylation. These results confirm the findings from the HPLC/mass spectrometry analyses that disaturated PtdGros constitute novel high affinity ligands for SP-A.

In an additional series of experiments we tested the biological relevance of disaturated PtdGros as receptors for SP-A. We utilized the growth arrest of mycoplasma by SP-A (see Fig. 8) as a measure of its biological function and tested the ability of di-C16:0-PtdGro to antagonize this effect. The results of these studies are presented in Fig. 11. M. pneumoniae cultures were grown under standard conditions and treated with either lipids derived from the bacteria, di-C16:0-PtdGro, or SP-A in various combinations. The data show that inclusion of either total bacterial lipid or di-C16:0-PtdGro has no effect on the growth of mycoplasma. In contrast, the addition of SP-A significantly attenuates the growth of the organism. Addition of either the total bacterial lipid preparation or di-C16:0-PtdGro reverses the inhibition of growth effected by SP-A. These data clearly demonstrate that saturated PtdGros provided in the medium can antagonize the binding and activity of SP-A as an antimicrobial agent. Collectively these data provide strong evidence that saturated-PtdGros present in mycoplasma membranes act as receptors for SP-A.

**DISCUSSION**

*M. pneumoniae* is an important human pathogen, and the full details of the host interaction and response to the organism are not well understood. In this report we sought to elucidate the interactions between SP-A and the bacteria. SP-A is an abundant protein within the bronchoalveolar environment inhabited by *M. pneumoniae*, and it interacts with bacteria, fungi, and viruses as a specialized pulmonary component of the innate immune system (3).

These studies provide clear evidence that SP-A from human and rat sources binds *M. pneumoniae* with high affinity. The SP-A binding shows an absolute requirement for Ca2+, which is typical of the interactions of the protein for most carbohydrate and lipid ligands. The apparent *Kd* values for human SP-A and mycoplasma and its isolated membranes are 7.6 and 0.3 nM, respectively. The apparent *Kd* values for rat SP-A and mycoplasma and its isolated membranes are 8.0 and 0.9 nM, respectively. We believe that the differences in apparent *Kd* values for intact organisms and membranes are due to the different assay systems employed. For intact organisms, both the processing time and the sample dilution are much greater than those used for the isolated membranes. It is likely, under the conditions employed for the intact cells, that greater dissociation of the SP-A ligand complex will occur during processing after the reaction is terminated. In this respect, the membrane binding measurements probably best estimate the true affinity of the SP-A ligand complex, because they can be executed relatively quickly.

Using isolated membranes, we demonstrated that human SP-A derived from either normal individuals or alveolar proteinosis patients behave identically in the binding reactions. The lung lavage material from alveolar proteinosis patients is a convenient source of human SP-A, but the protein displays some covalent cross-linking anomalies evident as a non-reducible dimeric form of the protein observed by gel electrophoresis under denaturing conditions (31). This finding validates the use of the alveolar proteinosis protein in these studies. We also compared the binding properties of recombinant rat SP-A produced in CHO-K1 cells and baculovirus/Sf9 cells and found that the proteins behaved similarly to each other and to the human protein. This indicates that the large collection of mutant forms of SP-A produced with the baculovirus/Sf9 system should be useful for examining structure-function relationships for interactions between SP-A and the microbial ligand.

The development of a binding assay using solid phase mem-
Fig. 9. The major *M. pneumoniae* ligands for SP-A are saturated PtdGroRs. A, lipids identified as the major reactive spot for SP-A by ligand blot analysis on TLC plates were recovered from the silica gel and applied to a reversed-phase HPLC column. The lipids were eluted using solvents A (methanol/water, 20:80, v/v, containing 1 mM ammonium acetate) and B (methanol made to 1 mM ammonium acetate) and a gradient that progressed from 20% B to 100% B over 35 min, followed by continued elution at 100% B for 15 min. The lipid eluting from the column was detected by A$_{220}$ nm B, analysis of fraction 26 by negative ion mass spectrometry and collision-induced decomposition (inset) of m/z 721.5. C, analysis of fraction 29 by negative ion mass spectrometry and collision-induced decomposition (inset) of m/z 749.5. D, analysis of fraction 32 using negative ion mass spectrometry.

**SP-A Binds M. pneumoniae via PtdGro and Inhibits Growth**

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that the mycoplasma lipid fraction contains one abundant class of high affinity ligand for SP-A as well as a ligand that appears less abundant. We have previously reported similar interactions between SP-D, a protein closely related to SP-A, and mycoplasma membranes and lipids (32). The spectrum of lipids recognized by SP-D is distinctly different from that recognized by SP-A, but there is some overlap. The major SP-A ligand co-migrates with the most abundant polar lipid class of the organism, which is PtdGro, as well as a prominent glycolipid that co-migrates in the same location. Previous studies with rat SP-A indicate that egg-PtdGro is not a ligand for the protein. We also tested the direct binding of human SP-A to egg-PtdGro by ligand blot analysis, and it is also not a significant ligand for this protein. However, more detailed analysis of the major SP-A ligand by combined TLC, HPLC, and mass spectrometry now reveals that the critical lipids for high affinity interaction are disaturated PtdGros. The 16:0/16:0-PtdGro, 16:0/18:0-PtdGro, and 18:0/18:0-PtdGro species were identified as the bacterial ligands. The high affinity interaction was further confirmed by demonstrating that commercially available disaturated PtdGros are high affinity ligands for human SP-A. Among the disaturated PtdGros present in mycoplasma the 16:0/18:0-PtdGro appears to be the most abundant molecular species.

The ligation of *M. pneumoniae* by SP-A is likely to have important consequences for the organism and its human host. Our binding studies reveal that SP-A attenuates the growth of the mycoplasma. The reduced growth was confirmed by colony counting, general metabolic activity, and DNA replication. The action of SP-A must be highly specific and effective insofar as the medium that mycoplasma is grown in is very complex and contains numerous sources of potential competitive inhibitors. Some of these potential inhibitors include 15% bovine serum, yeast extract, yeastolate, and protein hydrolysates. Both the concentration and stoichiometry of SP-A and mycoplasma also appear as important factors for the attenuation of growth. The effectiveness of SP-A is progressively reduced as the bacterial inoculum is increased. Our findings demonstrate that SP-A causes cell stasis rather than cell death. This effect is specific for SP-A, because the other prominent pulmonary collectin, SP-D, fails to alter the growth of mycoplasma.

Although SP-A has been reported to bind to many microorganisms, there are only a few reports of a direct effect of the protein upon microbial growth. van Rozendaal et al. (33) have shown that SP-D can cause aggregation and reduce the growth rate of *Candida albicans*. Wu et al. (17) demonstrated enhanced permeability and killing of selected Gram-negative bacteria by SP-A and SP-D via ligation of lipopolysaccharides. McCormack et al. (18) demonstrated killing of *H. capsulatum* by SP-A and SP-D also by increasing cell permeability through
unknown receptors. Our data now provide evidence that mycoplasma is also a target for the proteins, which recognize disaturated phospholipids on the cell surface. However, we observe bacteriostatic activity rather than bacteriocidal activity by SP-A. It seems likely that the direct antimicrobial effects of SP-A will be relevant for other organisms and serve as an additional function of the protein in conjunction with its opsonizing activity and modulation of inflammatory mediator production (3).

In summary, we have demonstrated that rat and human SP-A bind M. pneumoniae with high affinity. Bacterial lipids constitute the major molecular class of surface ligands for SP-A, and they bind with higher affinity than the major surfactant lipid. Human SP-A markedly attenuates the growth of mycoplasma and is likely to play an important role in controlling the antibody independent immunity to the bacteria in vivo.

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