Surfactant Protein D-Mediated Aggregation of *Pneumocystis carinii* Impairs Phagocytosis by Alveolar Macrophages

Suk-Joong Yong,1,2 Zvezdana Vuk-Pavlovic,1 Joseph E. Standing,1 Erika C. Crouch,3 and Andrew H. Limper1*

Thoracic Diseases Research Unit, Division of Pulmonary, Critical Care, and Internal Medicine, Mayo Clinic, Rochester, Minnesota 55905; Division of Pulmonary Medicine, Wonju College of Medicine, Yonsei University, Wonju, Korea; and Department of Pathology, Washington University, St. Louis, Missouri 63110

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*Pneumocystis carinii* pneumonia remains an important and potentially fatal cause of opportunistic pneumonia. Animal studies reveal that substantial quantities of surfactant protein D (SP-D) accumulate in the airspaces during *P. carinii* pneumonia and are particularly abundant in aggregates of organisms. Due to the multimeric structure of SP-D, we hypothesized that SP-D mediates aggregation of the organism. From previous clinical studies it is known that aggregated organisms are conspicuous in sections of lung tissue and bronchoalveolar lavage (BAL) fluids of humans with active *P. carinii* pneumonia. Herein, we observe that SP-D levels increased at least fourfold in BAL fluids of patients with *P. carinii* pneumonia. Next, a spectrophotometric sedimentation assay was developed to assess the aggregation of *P. carinii* in vitro by SP-D. *P. carinii* organisms were first stripped with glutathione to remove bound SP-D and subsequently incubated in the presence of SP-D and 2 mM calcium. *P. carinii* incubated with natural SP-D (10 μg/ml) containing dodecamers and higher-order forms exhibited aggregation and enhanced sedimentation compared to that of glutathione-stripped *P. carinii*. Aggregation was also enhanced by the concentrated supernatant of rat BAL fluid, and this effect was abolished by the selective removal of SP-D from the lavage fluid. *P. carinii* aggregation was reduced by maltose, mannose, and EDTA, consistent with the role of the SP-D C-type lectin domain (CRD) in the aggregation event. Comparisons of different molecular forms of SP-D showed that dodecamers—but not trimeric subunits—mediate optimal aggregation of *P. carinii*. Aggregation of *P. carinii* by SP-D was shown to be responsible for the impaired phagocytosis of the organisms by alveolar macrophages. Thus, SP-D-mediated aggregation of *P. carinii* may represent one means by which the organism avoids elimination by the host.

Recent investigations indicate that SP-D exerts various functions in innate immunity during infection of the respiratory tract. For instance, SP-D has been demonstrated to bind lipo-
polysaccharide present on the surface of *Escherichia coli* and to mediate agglutination of the organism (28). SP-D has also been reported to exert protective activities against influenza A virus (17, 19). In addition, SP-D participates in the interactions of *Mycobacterium tuberculosis* with alveolar macrophages (15).

It has previously been found that SP-D accumulates during *P. carinii* pneumonia in a rodent model and binds to the organizing thromboplastin via the CRD of SP-D (42). More recently, increased accumulation and expression of SP-D have been demonstrated in SCID mice with *P. carinii* pneumonia (3). The SP-D CRD interacts with both glycoprotein A (gpA), a major surface antigen of trophic and cyst forms of *P. carinii*, and β-glucan components of the cyst wall (2, 55, 56). Interestingly, the binding of SP-D to *P. carinii* results in enhanced attachment of organisms to rat alveolar macrophages, though macrophage uptake of *P. carinii* is reduced in the presence of SP-D (42). This impairment of macrophage uptake may be related to the formation of large aggregates of the organisms. The cruciform shape of dodecameric SP-D provides a structure potentially capable of aggregating the organism into such large agglomerates.

The following investigations were therefore undertaken to test the general hypothesis that SP-D induces aggregation of *P. carinii*. Studies were first performed to confirm for human *P. carinii* pneumonia the accumulation of SP-D that had previously been observed only in animal models of infection. Secondly, a spectrophotometric sedimentation assay was designed to evaluate the role of SP-D in *P. carinii* aggregation. Additional investigations were performed to evaluate the role of the CRD and the structure of SP-D in mediating these effects. Finally, the effects of SP-D-induced aggregation on *P. carinii* uptake by macrophages were measured.

**MATERIALS AND METHODS**

**Materials.** General reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Pittsburgh, Pa.) unless otherwise specified. *Pneumocystis carinii* L. sp. *carinii* was originally obtained through the American Type Culture Collection (Manassas, Va.) and was propagated in rats as reported previously (33). Ciprofloxacin was the kind gift of Barbara Painter of Miles Pharmaceuticals, Inc. (West Haven, Conn.).

**Measurement of SP-D in human BAL fluid.** Prior studies with rodent models of *P. carinii* pneumonia indicate that SP-D accumulates during the course of infection (3, 42). To determine the extent to which SP-D accumulates in the lower respiratory tract during *P. carinii* infection in humans, bronchoalveolar lavage (BAL) fluids were obtained from 11 patients with *P. carinii* pneumonia and concurrently from 11 immunocompromised patients without *P. carinii* pneumonia who were undergoing lavage for clinical evaluation of pulmonary infiltrates. The clinical features of these patients and the methods of flexible fiber optic bronchoscopy, lavage, and separation of the recovered BAL specimens into cellular and fluid components have been described previously (35, 36, 59). All lavage fluids were stored at −20°C until assay. SP-D concentrations in the BAL fluids were determined in duplicate by using a competitive enzyme-linked immunosorbent assay (ELISA) modified from the method of Neese et al. (40). Ninety-six-well ELISA plates were coated with human SP-D (5 μg/ml) in 100 mM NaHCO3 by overnight incubation at 37°C. Plates were then washed with Tris–HCl balanced salt solution (TBS) containing 1 mg of heat-denatured bovine serum albumin/ml, 1 mM CaCl2, and 1 mM MgCl2 and were further incubated for an additional hour with TBS containing 3 mg of heat-denatured bovine serum albumin/ml, 1 mM CaCl2, and 1 mM MgCl2 to block nonspecific protein binding sites. In separate test tubes, standard concentrations of SP-D and samples were incubated with a rabbit polyclonal antibody generated against the SP-D CRD (1 μg/ml) for 1 h at 37°C prior to plating onto the SP-D-coated wells (55). The plates were then incubated for an additional hour and washed, and a horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution, 1:5,000; ICN, Costa Mesa, Calif.) was added to each well and incubated for an additional hour. After a wash, o-phenylenediamine dihydrochloride (OPD) substrate was placed in each well. After development, colorimetric reactions were stopped with 1 N H2SO4 and the absorbances were read at 450 nm. A standard curve was generated and used to derive SP-D concentrations in BAL fluids.

**Preparation of SP-D from *P. carinii*-infected rats.** *P. carinii* pneumonia was induced in rats by immunosuppression with dexamethasone (4, 34). Specific-pathogen-free rats (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were provided with drinking water containing dexamethasone (2 mg/liter), tetracycline hydrochloride (500 mg/liter), and nystatin (200,000 U/liter). On a weekly basis, the animals also received oral ciprofloxacin (0.45 g/liter) for two consecutive days to further reduce the risk of bacterial infection (55). After 5 days, rats were intratracheally inoculated with *P. carinii* (500,000 organisms). Following an additional 6 weeks of immunosuppression, rats were exsanguinated and the lungs were perfused with Na Ca HEPES buffer (150 mM NaCl, 1.8 mM CaCl2, 25 mM HEPES [pH 7.4, 329 mosM]). The lungs were excised, minced in Na Ca HEPES, and homogenized in a stomacher laboratory blender for 5 min at room temperature. The remaining large tissue pieces were removed by filtration through gauze. The suspension was centrifuged (at 1,000 × g for 10 min) and treated with 0.85% NH4Cl, pH 6.8, for 5 min to lyse host cells. Following two washes with Na Ca HEPES, the material was sequentially filtered, first through polycarbonate membranes with 10-μm pores and finally through filters with 5-μm pores (Poretics Corp., Livermore, Calif.) (4, 48, 50). *P. carinii* isolates were quantified by counting *P. carinii* nuclei as described previously (33). *P. carinii* trophic forms represented more than 99% of the material on Diff-Quick-stained smears (24). In order to remove surface molecules from *P. carinii*, specified preparations were also treated with glutathione (0.5%) and EDTA (10 mM) and were washed again prior to use (22). This procedure has been previously documented to remove >99.5% of SP-A from the surfaces of *P. carinii* organisms (22).

**Generation of concentrated BAL proteins from *P. carinii*-infected rats.** After 6 weeks of immunosuppression, rats were exsanguinated, and whole-lung lavage was performed with 50 ml of Hank's balanced salt solution (HBSS) in sequential 10-ml aliquots. After centrifugation at 1,000 × g for 10 min, the lavage supernatant was concentrated 30-fold (Centriprep-3 concentrator; cutoff, 3,000 Da; Amicon, Inc., Beverly, Mass.). To remove SP-D from the BAL proteins, the concentrated solution was divided and a portion was treated with Toyopearl-amino-maltose resin (TosoHaas, Inc., Montgomeryville, Pa.) as prepared as previously reported (55). One-fourth volume of packed Toyopearl-amino-maltose resin was added to the concentrated BAL proteins and adsorbed with rocking over 3 h. BAL proteins were recovered by centrifugation and stored at −70°C until use.

**Spectrophotometric sedimentation assay.** To remove EDTA and glutathione prior to the assay, *P. carinii* suspensions were twice centrifuged at 1,000 × g for 10 min at 4°C, resuspended with TBS (50 mM Tris HCl–150 mM NaCl [pH 7.4]), and adjusted to a concentration of 7.5 × 107 *P. carinii* organisms per ml. The subsequent aggregation of *P. carinii* was quantified by using a modification of the spectrophotometric sedimentation assay of Ericson and coworkers (14, 28). The time course of macroscopic *P. carinii* agglutination was monitored using a spectrophotometer (model DU-74; Beckman Coulter Inc., Fullerton, Calif.) at a 700-nm wavelength (measuring optical density at 700 nm [OD700]). The baseline spectrophotometric reading was set at an OD100 of zero by using reference TBS solutions without organisms. Two milliliters of a suspension containing 15 × 106 *P. carinii* organisms was inserted into a 4.5-ml cuvette and equilibrated for 15 min at 37°C. Test solutions of SP-D (1 μl each) were prepared separately and added to the suspension at time zero in order to yield a final suspension concentration of 10 × 106 *P. carinii* organisms, 10 μg of SP-D/ml, 100 mM sugar as specified, and 2 mM calcium chloride. Some experiments also tested SP-D (10 μg/ml) in the presence of identical buffers without SP-D served as controls. The OD100 was monitored over the subsequent 6 h at 37°C. Estimates of percent aggregation were derived from the sedimentation assay data by taking the OD700 at 5 min after addition and mixing of the test solutions as 0% aggregation, and taking an OD100 of zero as 100% aggregation.

The conditions selected for these sedimentation assays were supported by our previous kinetic assays of binding of 125I-labeled SP-D to *P. carinii* organisms in the presence of 2 mM calcium. Binding of 125I-labeled SP-D to whole *P. carinii* organisms was rapid, achieving equilibrium binding as early as 15 min of incubation at 37°C. *P. carinii* possesses multiple binding epitopes for SP-D, including the mammone-rich gpA surface complex and cell wall β-glucans (54–56). Competitive binding assays further indicated that more than 65% of 125I-labeled SP-D binding to *P. carinii* was competitively inhibited by a 100-fold excess of cold SP-D (39). Furthermore, EDTA (1 mM) inhibited 125I-labeled SP-D binding to *P. carinii* by >90%. Saturable binding of SP-D to *P. carinii* occurred at concentrations higher than 1.25 μg/ml.
SP-D preparations. Natural rat SP-D was isolated from the 10,000 × g supernatant of BAL fluids obtained from rats with silica-induced alveolar lipoproteinosis as previously described (55). SP-D was purified by affinity chromatography on maltoyl-agarose (15, 18). The purity of SP-D preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Natural rat SP-D preparations demonstrated a single 43-kDa band under reducing conditions. The majority of natural SP-D obtained from these silica-treated rats was in the dodecameric form, with 4.0% of the material representing higher-order multimers of dodecameric forms (55). To evaluate the role of multiple CRD motifs in facilitating aggregation of P. carinii, two recombinant rat SP-D proteins were tested. A full-length recombinant rat SP-D (rSP-D) was generated and purified as previously described (12). The rSP-D protein comigrated with natural rat SP-D on SDS-PAGE gels under reducing and nonreducing conditions, bound efficiently to maltoyl-agarose, and cocultured predominantly with natural rat SP-D dodecamers in nondenaturing gel filtration over 4% agarose (12). In addition, mutant SP-D monomers which assemble into homotrimers (single arm) but do not form dodecameres were studied. This mutant (RrSP-Dser15,20) represents a full-length peptide containing serine substitutions for the two amino-terminal cysteines, which mediate disulfide cross-linking in the generation of dodecamers (7). Previous studies have shown that the mutant is fully active as a lectin but is defective in mediating bridging interaction between particulate ligands (7).

Uptake of P. carinii by alveolar macrophages. To address the functional consequences of P. carinii aggregation, we measured macrophage uptake of gluthathione-stripped P. carinii before and following SP-D aggregation. Uptake of P. carinii by macrophages was assayed by 31Cr labeling of the organisms (33, 34, 46). P. carinii organisms were isolated from rats as described above in the presence of EDTA (10 mM) and glutathione (0.5%) to strip SP-D from the surface. The organisms were radiolabeled by incubation for 5 h at 37°C in 2 ml of Dulbecco’s modified Eagle medium containing 20% fetal calf serum and 200 μCi of sodium 31Cr(OM) (New England Nuclear; Boston, Mass.) and were then washed in HBSS with 0.5 mM EDTA to remove unincorporated label and to again disaggregate the organisms. Rat alveolar macrophages, obtained by BAL of healthy animals, were plated in tissue culture plates (105 cells/well) that had been precoated with normal immunoglobulin G (IgG) (100 μg/ml for 60 min) in order to ensure firm adherence of the macrophages (9). After 1 h, the macrophages were gently washed with HBSS to remove nonadherent cells. 31Cr-labeled P. carinii organisms were aggregated either with SP-D or mutant homotrimeric (single-arm) RrSP-Dser15,20 (10 μg/ml) for 3 h. The P. carinii organisms were gently rinsed three times in HBSS containing 2 mM calcium, added to the macrophages (200 P. carinii organisms per macrophage), and incubated for six additional hours. Additional control P. carinii organisms were cultured with the macrophages in the presence of EDTA (10 mM). To measure the combined number of bound and internalized P. carinii organisms, the macrophages were incubated at 37°C. Parallel cultures were also incubated for 6 h at 4°C to measure only the number of organisms bound to the macrophage surfaces, but not those internalized. After the incubations, nonadherent P. carinii organisms were removed by gentle washing. The macrophage monolayers containing associated P. carinii organisms were solubilized in 1 N NaOH and quantified.

Phagocytosis of P. carinii was measured as the difference between the counts of organisms both bound and internalized (measured at 37°C) and the counts of organisms only bound to the macrophage surfaces (measured at 4°C).

Statistical analyses. Data are expressed as means ± standard errors of the means (SEM). Differences between experimental and control data groups were determined by using two-tailed Student’s t tests for normally distributed variables. Statistical testing was performed with the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.). Statistical differences between groups were considered significant if P values were <0.05.

RESULTS

SP-D accumulates in the lower respiratory tract during human P. carinii pneumonia. Because SP-D is known to interact with both P. carinii organisms and alveolar macrophages, we quantified this surfactant-associated protein in the lower respiratory tracts of patients with P. carinii pneumonia. BAL fluids were obtained from 11 patients with P. carinii pneumonia and concurrently from 11 immunocompromised patients without P. carinii infection, all of whom underwent bronchoscopy for clinical evaluation of diffuse pulmonary infiltration. SP-D concentra-

trations in BAL supernatants were determined in duplicate by competitive ELISA (Fig. 1). BAL fluids from patients with P. carinii pneumonia contained 1.260 ± 0.512 μg of SP-D/ml versus 0.264 ± 0.162 μg/ml in BAL fluids from patients without P. carinii pneumonia (P = 0.0432). Thus, as observed in prior studies with rat and murine models of infection, SP-D is present in significantly increased amounts in the lower respiratory tract during P. carinii pneumonia in humans.

SP-D promotes the aggregation of P. carinii. Our prior studies have shown that SP-D is associated with clusters of P. carinii in the rodent lung and in lavage fluid, suggesting that it could be responsible for aggregation of the organisms. To test this hypothesis, P. carinii organisms were freshly isolated and stripped with gluthathione and EDTA, which effectively remove surfactant proteins associated with the surfaces of the organisms (22). We next quantified the aggregation of P. carinii in the presence and absence of SP-D by using a spectrophotometric sedimentation assay as previously described (28). The initial P. carinii suspension appeared turbid and displayed an OD of 0.220 ± 0.022. Over time, however, as more P. carinii organisms aggregated and settled out of suspension, the OD700 decreased. Addition of SP-D to the sedimentation assay incubation markedly enhanced aggregate formation and sedimentation (Fig. 2). There was also macroscopic aggregation and precipitation of P. carinii in the presence of SP-D. The diameters of some SP-D-induced P. carinii aggregates increased to as much as 1.5 mm over the ensuing 4 to 5 h. In contrast, P. carinii incubated with SP-D in the presence of EDTA did not show this accelerated sedimentation rate. It should be noted that P. carinii organisms stripped of surface-associated host proteins still exhibited a low rate of autoaggregation. P. carinii cultured in the presence of EDTA exhibited minimal sedimentation, similar to that of P. carinii cultured in the presence of both SP-D and EDTA. After 240 min, P. carinii incubated in the presence of SP-D exhibited 81.8% ± 1.9% aggregation while P. carinii incubated in the absence of SP-D exhibited only 28.8% ± 3.1% aggregation (P = 0.0001; n = 3 experiments). After 5 h, P. carinii incubated with SP-D demonstrated 85.0% ± 0.6% aggregation compared to only 43.2% ± 4.0% aggregation by P. carinii alone (P = 0.0046; n = 3 experiments).

FIG. 1. SP-D is present in increased quantities in BAL fluids of patients with P. carinii pneumonia. BAL fluids from 11 patients with P. carinii pneumonia (PCP) and 11 immunocompromised patients without P. carinii who were sampled concurrently were quantified by competitive ELISA. * P = 0.0432 for comparison between these two patient groups. ICH, immunocompromised host.
Taken together, these data indicate that SP-D markedly enhances the aggregation of *P. carinii* organisms, in a manner requiring divalent cation-mediated binding of the collectin with the organism.

**BAL proteins also enhance *P. carinii* aggregation.** To next determine whether proteins present within BAL fluid also promoted aggregation of the organisms, we evaluated the sedimentation rate of glutathione-stripped *P. carinii* incubated in the presence of concentrated BAL protein (net effective concentration, 10-fold) (Fig. 3). For comparison, we evaluated identically concentrated lavage proteins that had been treated with immobilized maltose resin, which efficiently removes SP-D, but not SP-A, from the BAL protein. Though not as potent as purified SP-D (10 μg/ml), the 10-fold-concentrated BAL proteins promoted aggregation, and this effect was eliminated when the BAL proteins were treated with immobilized maltosyl resin. After 6 h, *P. carinii* incubated in the presence of total concentrated BAL proteins exhibited 43.2% ± 2.0% aggregation, compared to only 12.8% ± 0.9% aggregation for *P. carinii* exposed to concentrated BAL proteins treated with immobilized maltose to remove SP-D (*P* = 0.0001; *n* = 3 experiments). These observations indicate that BAL fluid contains proteins capable of mediating aggregation of *P. carinii*, and they indicate that SP-D, or a lectin with a similar specificity, could promote the aggregation of *P. carinii* in vivo.

**SP-D mediates *P. carinii* aggregation through its CRD.** Our prior studies have indicated that SP-D binds *P. carinii* epitopes, including the gpA major surface glycoprotein complex and *P. carinii* cell wall β-glucans, through its CRD. We therefore hypothesized that SP-D similarly mediates the aggregation of *P. carinii* via its CRD. To test this, we compared the SP-D-induced sedimentation of *P. carinii* organisms in the presence of maltose, mannose, and lactose (100 mM each). Calcium chloride (2 mM) was present to facilitate CRD binding (Fig. 4). For comparison, EDTA (5 mM), which completely inhibits *P. carinii* aggregation, was tested in parallel. *P. carinii* aggregation was inhibited by these sugars in the following order of potency, from highest to lowest: maltose, mannose, and lactose (Fig. 4). Once again, divalent cations were required for *P. carinii* aggregation. This pattern of sugar inhibitory capacity is compatible with prior observations of SP-D binding to whole organisms and gpA (55). Taken together, these observations indicate that SP-D promotes *P. carinii* aggregation through interaction of its CRD with the organism surface.

**Dodecameric forms of SP-D mediate *P. carinii* aggregation.** Based on our prior observations that SP-D interacts with *P. carinii* through its CRD, we further proposed that aggregation of the organism requires multimeric SP-D. Native SP-D is present largely as dodecameric structural forms (10, 55). These dodecamers are assembled from trimeric arms, each contain-
ing three chains composed of a triple helical collagenous domain, a neck region, and three CRDs at its carboxy terminus. The three CRDs of mannose binding lectin, a related collectin, have been shown to interact with ligands in a planar fashion (11). The amino-terminal association and cross-linking of four such trimeric arms result in the formation of the typical cruciform dodecameric structure (10). We hypothesized that single-arm trimeric SP-D would similarly bind organisms only in a planar fashion, would not effectively cross-link multiple organisms, and hence would have less effect on organism aggregation. To test this, we compared the abilities of recombinant rat SP-D dodecamers (rSP-D) and trimeric subunits of rSP-D (RrSP-Dser15,20) to aggregate P. carinii (Fig. 5). As predicted, the trimeric single-arm full-length SP-D exhibited minimal ability to promote the aggregation and sedimentation of P. carinii compared to dodecameric SP-D. After 240 min, the trimeric RrSP-Dser15,20 showed only 36.3% aggregation, only slightly more than the negative control. By contrast, the rSP-D dodecamers showed 62.1% aggregation (P = 0.005; n = 4 experiments). Thus, efficient aggregation of P. carinii by SP-D requires dodecameric or higher-order forms to most effectively cross-link and agglutinate adjacent organisms.

SP-D-induced aggregation of P. carinii impairs phagocytosis by alveolar macrophages. Lastly, we sought to determine the functional significance of SP-D-induced aggregation of P. carinii during interactions with alveolar macrophages, the principal cells responsible for phagocytosis of this organism (33). To first address the relative sizes of P. carinii aggregates and alveolar macrophages, microscopy was performed on glutathione stripped P. carinii following treatment with SP-D, EDTA, and inhibitory sugars (Fig. 6). Microscopic analysis revealed that SP-D-induced aggregates of P. carinii were quite large and amorphous (Fig. 6B). SP-D-mediated P. carinii aggregates ranged in size from 50 μm to 1.5 mm in the longest dimension, with most aggregates in the size range of 200 to 500 μm. While Pneumocystis organisms stripped of surface proteins with glutathione and EDTA appeared mainly as individual dispersed organisms (Fig. 6A), incubation with SP-D resulted in the development of dense aggregates of P. carinii organisms. Aggregation was effectively impaired by incubation of P. carinii with SP-D in the presence of either EDTA or maltose, but not in the presence of lactose, consistent with our earlier observations. For size comparison, we observed SP-D-aggregated P. carinii in the presence of alveolar macrophages (Fig. 6F). The SP-D-induced P. carinii aggregates were substantially larger than the typical alveolar macrophages, which range from 13 to 20 μm in diameter (27), supporting our hypothesis that SP-D-induced P. carinii aggregates are too large for effective alveolar macrophage uptake. The overwhelming majority of the P. carinii organisms were incorporated within the amorphous aggregates. The exact number of organisms in the aggregates was
impossible to quantify, because aggregated organisms are tightly packed on top of each other. Nonetheless, by visual impression most (~75%) of the *P. carinii* organisms treated with SP-D appeared incorporated into these large aggregates.

Prior studies had indicated that SP-D does facilitate binding of *P. carinii* to alveolar macrophages (42). However, uptake of the organisms was not enhanced by SP-D in those studies (42). To further address the effect of SP-D-induced aggregation of the organisms, we measured the phagocytosis of glutathione-stripped *P. carinii* by alveolar macrophages following 3 h of SP-D aggregation compared to phagocytosis of nonaggregated control *P. carinii* (Fig. 7). Macrophages cultured with control *P. carinii* in the presence of 2 mM calcium exhibited significantly greater uptake than macrophages cultured with *P. carinii* in the absence of calcium (with EDTA; \(P = 0.004\)). Approximately 35.1% ± 3.5% of the radiolabeled control *P. carinii* organisms were internalized by the macrophages over the 6-h incubation period. Furthermore, aggregation of *P. carinii* with SP-D (in the presence of calcium) also resulted in a dramatic reduction of organism uptake by alveolar macrophages (\(P = 0.0326\) for comparison to control *P. carinii*). In contrast, treatment of *P. carinii* with mutant homotrimeric (single arm) RrSP-Dser15,20 in the presence of calcium, a reagent that causes minimal aggregation of *P. carinii*, resulted in no significant alteration in phagocytosis of the organisms by alveolar macrophages. Thus, aggregation of *P. carinii* induced by dodecameric SP-D (but not trimeric SP-D) was associated with impaired uptake of the organism by macrophages.

**DISCUSSION**

Prior studies in immune-suppressed rodents demonstrated that SP-D accumulates in the lower respiratory tract during *P. carinii* pneumonia and that SP-D interacts with the mannose-rich glycoprotein gpA complex and also with cell surface β-glucans on the organisms (3, 42, 55, 56). The interactions of SP-D with *P. carinii* ligands are largely mediated through its CRD and are further enhanced by the multimeric structure of natural SP-D, predominantly composed of cruciform dodecameric forms (55). The present study demonstrates for the first time that SP-D levels are also higher in the lower respiratory tracts of patients with *P. carinii* pneumonia than in those of other immunocompromised patients without this infection. These studies also suggest that airspace SP-D is an important factor contributing to the observed agglutination of *P. carinii* trophic forms and cysts in the infected lung. Our investigations further reveal that the dodecameric structure of SP-D facilitates aggregation of the organisms, again through interaction of CRDs present on dodecameric SP-D with glycosylated ligands on the surface of the organism.

The data further indicate that the aggregation of *P. carinii* by SP-D impairs phagocytic uptake of the organism by alveolar macrophages. The present observations obtained using gluta-
SP-D dodecamers induce greater aggregation than identical concentrations of trimeric SP-D subunits. P. carinii organisms were stripped of associated host proteins by use of glutathione and EDTA, and aggregation was monitored in the presence of recombinant dodecameric rat SP-D or mutant RsSP-Dser15,20, which assembles as trimeric single-arm structures (10 µg/ml each). EDTA (5 mM) was studied in parallel. P. carinii incubated in the dodecameric SP-D exhibited significantly greater aggregation than P. carinii incubated in equal concentrations of RsSP-Dser15,20. Shown are results of an experiment representative of four experimental runs. For statistical comparisons of aggregation conditions, see Results.

SP-D induces aggregation by lung proteins recovered by BAL. In particular, adsorption of the BAL proteins with immobilized maltosyl supports, which are quite selective for SP-D, consistent with its known saccharide specificities, reversed almost all of the aggregating properties of BAL proteins. These findings are consistent with other studies that have shown that SP-D is a much more effective agglutinin of certain organisms such as influenza A virus (18). This has been attributed to much longer half-lives of SP-D as compared to SP-A, which makes SP-D a much more stable component of surfactant (1).

A number of mechanisms likely contribute to the accumulation of SP-D in the alveolar spaces during P. carinii pneumonia. The well-characterized binding activity of P. carinii suggests that the organisms themselves may act as affinity substrates to trap secreted SP-D within the alveoli (42). Furthermore, SP-D gene expression has also been shown to increase dramatically during P. carinii pneumonia (3). Other studies suggest that type II cell function is also impaired during this infection (49, 51). Thus, it is possible that abnormal surfactant protein clearance contributes to the accumulation of SP-D during infection. Interestingly, in certain immune suppression conditions, such as chronic steroid use, the accumulation of SP-D may indeed precede the development of infection (42). Therefore, it is possible that the accumulation of SP-D itself contributes to the development of P. carinii pneumonia.

Under normal conditions, alveolar epithelial lining fluid contains significant levels of the reducing agent glutathione, which
FIG. 6. SP-D induces the formation of amorphous aggregates of *P. carinii*. (A) *P. carinii* organisms were isolated and stripped of host proteins by use of glutathione and EDTA and were incubated in TBS buffer for 4 h in the absence of SP-D. Organisms were recovered by centrifugation, spotted onto glass slides, and stained with modified Wright-Giemsa stain (Diff-Quik). This stains *P. carinii* nuclei dark blue. *P. carinii* handled in this manner appeared as dispersed individual organisms or small aggregates containing several organisms. (B) In contrast, incubation of *P. carinii* in the presence of SP-D (10 μg/ml) for 4 h resulted in formation of large amorphous aggregates containing dozens of organisms. (C) Incubation of *P. carinii* with SP-D in the presence of EDTA inhibited formation of aggregates. (D) Similarly, maltose (100 mM) also inhibited aggregation of *P. carinii*. (E) In contrast, incubation with lactose (100 mM) continued to permit SP-D-mediated aggregation of the organisms. (F) For size comparison, alveolar macrophages incubated with aggregated *P. carinii* are shown. The alveolar macrophages bind the periphery of the aggregated organisms but are much smaller than SP-D-aggregated *P. carinii* (arrows). Magnification, ×630.
may under basal conditions reduce the extent of cross-linking of SP-D in the lung (8). However, during Pneumocystis pneumonia the lower respiratory tract is subjected to an excess oxidant burden, which limits the availability of such reducing agents (32). Indeed, our group actually has recovered increased fractions of dodecameric and higher-order SP-D aggregates associated with organisms in the setting of P. carinii pneumonia, further indicating that multimeric SP-D is present in the lungs during pneumonia to facilitate the aggregation of the organism (55).

Available information indicates that SP-D interacts with at least two prominent components on P. carinii, namely, the gpA surface complex and cell wall β-glucans, through interaction of its CRDs (42, 55, 56). Accumulating evidence demonstrates that P. carinii also binds a diverse array of other host proteins including fibronectin, vitronectin, laminin, collagen 1, and immunoglobulin (32, 37, 38, 58). The binding of SP-D through cell wall-associated ligands promotes aggregation of the organism. Optimal aggregation required dodecameric forms of SP-D. It should be noted, however, that P. carinii stripped of host proteins also exhibits a low level of autoaggregation that was abolished by EDTA chelation of divalent cations. Thus, the organism itself may contain additional lectins or molecules capable of self-association.

The net impact of SP-D interaction with P. carinii on the overall course of infection is not yet known. Investigations of P. carinii pneumonia in SP-D knockout mice are complicated by the accumulation of lipids and other surfactant components in the lungs as these animals mature (6, 16). The present study, however, suggests that the accumulation of SP-D is of potential benefit to the organism through impairment of macrophage uptake and subsequent modulation of inflammatory responses associated with P. carinii infection (40).

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