Inhibition of surfactant activity by *Pneumocystis carinii* organisms and components in vitro

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Wang, Zhengdong, Adam Foye, Yusuo Chang, Patricia R. Chess, Terry W. Wright, Samir Bhagwat, Francis Gigliotti, and Robert H. Notter. Inhibition of surfactant activity by *Pneumocystis carinii* (Pc) organisms and components in vitro. *Am J Physiol Lung Cell Mol Physiol* 288:L1124–L1131, 2005; doi:10.1152/ajplung.00453.2004.—This study examines the direct inhibitory effects of *Pneumocystis carinii* (Pc) organisms and chemical components on the surface activity and composition of whole calf lung surfactant (WLS) and calf lung surfactant extract (CLSE) in vitro. Incubation of WLS suspensions with intact Pc organisms (10⁷ per milligram of surfactant phospholipid) did not significantly alter total phospholipid levels or surfactant protein A content. Incubation with intact Pc organisms also did not impair dynamic surface tension lowering in suspensions of WLS or centrifuged large surfactant aggregates on a bubble surfactometer (37°C, 20 cycles/min, 0.5 and 2.5 mg phospholipid/ml). However, exposure of WLS or CLSE to disrupted (sonicated) Pc organisms led to severe detriments in activity, with minimum surface tensions of 17–19 mN/m vs. <1 mN/m for surfactants alone. Extracted hydrophobic chemical components from Pc (98.8% lipids, 0.1 mM) reduced the surface activity of WLS and CLSE similarly to sonicated Pc organisms, whereas extracted hydrophilic chemical components from Pc (primarily proteins) had only minor effects on surface tension lowering. These results indicate that in addition to surfactant dysfunction induced by inflammatory lung injury and edema-derived inhibitors in Pc pneumonia, disrupted Pc organisms in the alveolar lumen also have the potential to directly inhibit endogenous and exogenous lung surfactants in affected patients.

Lung surfactant; surfactant dysfunction; lung injury

*Pneumocystis carinii* (Pc) is an opportunistic microorganism that is widely disseminated in the general population (10, 24, 29, 47). Although benign under normal circumstances, Pc can cause life-threatening pneumonia in immunocompromised hosts. Pc pneumonia (Pcp) is a major presenting complaint in patients with acquired immune deficiency syndrome (10, 24, 25, 29, 47). Pc is also common in immunosuppressed patients undergoing organ transplant or bone marrow transplant or receiving therapy for hematological or other malignancies (7, 10, 41). Mortality rates for Pcp in immune-compromised patients in intensive care units are substantial, ranging from 8 to 60% depending on the patient groups involved (7, 10, 24, 25, 29, 41, 47). Despite the prevalence and medical importance of Pcp, many of the pathophysiological mechanisms by which Pc produces clinical signs and symptoms remain relatively poorly understood. Inflammation and edema are important contributors to the pathology of Pc, but the inflammatory mediators and pathways of injury involved in this condition have not been fully defined. The potential for direct Pc-induced surfactant dysfunction in Pcp is addressed in the present study.

Pulmonary surfactant is a complex mixture of phospholipids and apoproteins produced by type II pneumocytes in the alveolar lining (30). Lung surfactant is required for normal breathing and is deficient in premature infants with respiratory distress syndrome (RDS). Surfactant dysfunction also contributes to the pathophysiology of many forms of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (5, 30, 31, 34, 44). Reductions in lung surfactant activity have been documented in several animal models of Pcp, including steroid-treated rats (48) and severe combined immunodeficient (SCID) mice (2, 56). Our prior work (56) has linked surfactant dysfunction in Pcp-infected CD4⁺-depleted wild-type mice to CD8⁺-mediated pulmonary inflammation, edema, and increases in lavaged protein levels. Surfactant dysfunction in inflammatory lung injury is known to include interactions with inhibitory plasma proteins and other host-derived inhibitors in the alveoli as well as reductions in the content and activity of large surfactant aggregates (Refs. 30, 31, and 34 for review). An added mechanism investigated here involves the direct effects of Pc organisms themselves on surfactant activity or composition. Pc organisms and their surface component glycopolypeptide (gp) A (also called major surface glycoprotein or gp120) are known to bind surfactant proteins (SP)-A and SP-D (26, 28, 36, 58), but assessments of direct Pc-induced changes in lung surfactant surface activity or composition have not been done.

Experiments in this paper investigate alterations in surface activity for lavaged bovine whole lung surfactant (WLS) and a clinically relevant calf lung surfactant extract (CLSE) exposed in vitro to intact and disrupted (sonicated) Pc organisms. Subfractions of hydrophobic and hydrophilic chemical constituents extracted from Pc are also studied for their ability to impair the surface activity of WLS and CLSE. The primary hypothesis tested is that chemical constituents in disrupted Pc organisms have the potential to directly reduce surfactant activity in Pcp in addition to known detrimental effects on surfactant function from plasma proteins and other endogenous inhibitors present in the lungs in this condition as a result of inflammatory injury. To complement surface activity studies, experiments also assess whether incubation with Pc organisms in vitro substantially alters the phospholipid concentration or SP-A content of WLS dispersions.
MATERIALS AND METHODS

WLS and CLSE. WLS was obtained by bronchoalveolar lavage from the intact lungs of freshly killed calves (Gold Medal Packing, Oriskany, NY). Lavage was done with 2–3 l of 0.15 M NaCl per set of lungs, given in three divided doses. CLSE, which contains all the lipid and hydrophobic protein components in endogenous surfactant, was prepared by chloroform:methanol extraction of large aggregates pelleted from WLS by centrifugation at 12,500 g for 30 min (32, 51, 52). CLSE is the substance of the clinical exogenous lung surfactant Infasur (ONY, Amherst, NY).

Isolation of intact Pc organisms. C.B-17 SCID mice infected with a heavy Pc burden were killed by a lethal dose of intraperitoneal pentobarbital sodium in accordance with procedures approved by the University Committee on Animal Resources at Rochester. The pulmonary vasculature was perfused with 5 ml of sterile 0.15 M NaCl through the right ventricle, and the lungs were removed aseptically and homogenized with a sterile Tenbroeck tissue grinder in 10 ml of isolation buffer (1 × Hanks’ balanced salt solution, 0.5% glutathione, 20 mM HEPES buffer, pH 7.2). The crude homogenate was passed through a 26-gauge needle, and 10 ml of isolation buffer was added, followed by centrifugation at 50 g for 3 min to pellet tissue particles and large host cells while retaining Pc. The supernatant was passed twice through a 22-gauge needle and once through a 26-gauge needle and centrifuged at 1,500 g for 18 min to pellet Pc organisms. The pellet was resuspended in 2 ml of sterile distilled nonpyrogenic water for 35 s to lyse host erythrocytes, followed by 2 ml of sterile 2x phosphate-buffered saline to return the suspension to isotonicity. An additional 5.5 ml of isolation buffer and Dnase (Sigma, St. Louis, MO) were added to a final concentration of 10 μg/ml, and the suspension was incubated at 37°C in a water bath for 30 min. The Dnase reaction was halted by removal to ice, and the Pc suspension aspirated through a 26-gauge needle five times followed by centrifugation at 50 g for 3 min. The supernatant was collected, filtered through nylon mesh (5 μm), and centrifuged at 1,500 g for 18 min. The Pc pellet was resuspended in serum-free DMEM-Ham’s F-12 media (Sigma), and the suspension aspirated three times through a 26-gauge needle. To deplete any remaining host leukocytes, the Pc suspension was added to 100–ml tissue culture dishes pretreated with 0.3 μg/ml each of RB6, CD45, and CD16/32 antibodies (BD Pharmingen, San Diego, CA) and incubated at 37°C with 5% CO₂ for 1.5 h (57). After incubation, the Pc suspension was recollected and centrifuged at 1,500 g for 18 min. The pellet was resuspended in serum-free media and aspirated three times through a 26-gauge needle. Final preparations of intact Pc had no bacterial contamination and minimal host cell contamination when examined by light microscopy on slides stained with Diff-Quik (Dade, Duding, Switzerland). Pc numbers were defined by ammoniacal silver staining of cysts and by real-time PCR (57). PCR assessments utilized a primer/probe set specific for a 96-nucleotide region of the mouse Pc Kexin gene that was designed using the Primer Express software (Applied Biosystems) (22). Quantitation was determined by extrapolation against standard curves constructed from serial dilutions of known copy numbers of plasmid DNA containing the target Kexin sequences. Data were analyzed using the ABI Prism 7000 SDS version 1.0 software (Applied Biosystems) (57).

Sonicated (disrupted) Pc organisms. For a subset of biophysical studies, purified whole Pc organisms were sonicated on ice using four 30-s bursts (model W-220F probe sonicator, power setting 5; Heat Systems-Ultrasonic). The protein content of sonicated Pc was 4.53 mg per 10⁷ intact organisms based on the assay of Lowry et al. (23) modified by the addition of 15% by weight of SDS. Sonicated Pc organisms in biophysical studies were used at a uniform phospholipid concentration of 0.1 mM based on the assay of Ames (1).

Removal of Pc organisms from solution by antibody-coated magnetic beads. For experiments ruling out inhibition from non-Pc cellular material, intact Pc organisms (see Isolation of intact Pc organisms) were incubated for 30 min at room temperature in serum-free DMEM-Ham’s F-12 media containing 1.4 ml of seven pooled mouse anti-Pc IgG monoclonal antibodies shown previously to bind to the surface of Pc (22, 57). The suspension was centrifuged at 1,600 g for 20 min at 4°C, and the pellet was resuspended in 12 ml of serum-free DMEM-Ham’s F-12 containing 5 mg of Biomag goat anti-mouse IgG-coated beads (Qiagen) previously washed three times in the same media. The organism/bead mixture was incubated at 4°C for 30 min, placed against a magnet for 20 min to remove Pc, decanted to a fresh tube, and centrifuged at 1,600 g for 20 min at 4°C to pellet any remaining cellular material. This pellet was then resuspended in DMEM-Ham’s F-12 at an equivalent volume to the original Pc suspension (i.e., a volume originally containing enough organisms to generate a phospholipid concentration of 0.1 mM) and sonicated on ice using four 30-s bursts with a W-220F probe sonicator for use in in vivo activity studies with WLS and CLSE. The Pc-depleted pellet had a reduction in Pc organisms of 94–99% based on the concentration of cysts assessed by ammoniacal silver staining and Pc DNA content by PCR (22, 57).

Extracted hydrophobic and hydrophilic Pc components. Chemical components from purified Pc were extracted into chloroform:methanol by the method of Bligh and Dyer (6). The upper (methanol:water) phase of the extract contained primarily hydrophobic proteins (4.46 mg of protein per 10⁷ Pc organisms). The lower chloroform phase contained the hydrophobic components of Pc. The overall composition by weight of Pc hydrophobic components was 51.5% phospholipids, 47.3% neutral lipids, and 1.2% protein based on the dry weights of chloroform-extracted total lipids and acetone-extracted neutral lipids. For dry weight measurements, solvent-dispersed samples were evaporated under N₂ and incubated in an oven at 100°C with weighing every 24 h until three constant values were obtained. Hydrophobic Pc components had a phospholipid content of 0.098 μmol of phospholipid per 10⁷ organisms based on the assay of Ames (1) and an assumed average molecular weight of 750 g/mol. The molar phospholipid class distribution by thin-layer chromatography with solvent system C of Touchstone et al. (50) was 42.5% phosphatidylcholine, 7.4% lysophosphatidylcholine, 9.8% sphingomyelin, 10.7% phosphatidylinositol, 23.4% phosphatidylethanolamine, 3.9% phosphatidylglycerol, and 2.3% others. The neutral lipid fraction of Pc hydrophobic components contained 57.5% by weight cholesterol and cholesterol esters (43), 17.3% triglycerides, and 25.2% others. Triglyceride measurements were done with Infinity Reagent enzyme-based assay kit (Sigma Chemical) with 15% SDS added before final absorbance measurements to remove interference from phospholipids. For surface activity studies, hydrophobic Pc components were used at a fixed phospholipid concentration of 0.1 mM (approximately equivalent to 10⁷ organisms/ml).

Pc binding (sequestration) of phospholipid in dispersions of WLS. Intact Pc organisms in sterile 0.15 M NaCl plus 2.0 mM CaCl₂ were mixed in a round centrifuge tube with WLS (0.5 mg of phospholipid/ml in the same solvent) at a concentration of 10⁷ organisms per ml in the presence of surfactant phospholipid. Control samples containing Pc alone and WLS alone were also studied. Mixtures of WLS plus Pc, WLS, and Pc were incubated at 37°C for 2 h while the air-water interface was compressed and expanded at a rate of 20 cycles/min with a hemolitic agent (Red Rocker, model PR 50; Hoefer Scientific Instruments, San Francisco, CA). This procedure emulated cyclic interfacial expansion and contraction in the lungs in vivo and also facilitated physical contact between Pc and WLS during incubation. After 2 h, mixtures were centrifuged at 1,000 g for 10 min to pellet the majority of free Pc organisms away from WLS. The supernatant was then centrifuged at 12,500 g for 30 min to pellet large surfactant aggregates plus any remaining Pc organisms. The method of Ames (1) was used to measure phospholipid amounts, and percent recoveries following the different centrifugation steps were calculated relative to total initial starting phospholipid.
SP-A content of WLS in dispersion. Aliquots of WLS and WLS plus Pc from the binding studies above were separated by 12% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane for Western blot analysis. Equal loading and transfer was confirmed with Ponceau S staining (Sigma). The membrane was blocked with 5% casein and 0.1% Tween in Tris-buffered saline (blocking solution) for 1 h at room temperature. The membrane was then incubated with goat anti-SP-A antibody N-19 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:400 dilution in blocking solution. The membrane was washed three times in 0.1% Tween in TBS (TBS-T), incubated for 1 h in horseradish peroxidase-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology) at 1:5,000 dilution in blocking solution, and washed three times in TBS-T. For detection, the membrane was incubated in Supersignal (Pierce, Rockford, IL) for 5 min and exposed to Biomax MR X-ray film (Kodak, Rochester, NY). After confirmation of specificity of primary antibody using Western blotting, WLS and WLS plus Pc samples from the binding studies were directly loaded on a PVDF membrane using a mini blot dot blot apparatus (Schleicher & Schuell, Keene, NH). The membrane was blocked and probed for SP-A as above, and densitometric analysis was performed using Imagequant (Molecular Dynamics, Sunnyvale, CA).

Surface activity studies: pulsating bubble surfactometer. The surface tension lowering of surfactant dispersions was measured at 20 cycles/min at 37 ± 0.5°C on a pulsating bubble surfactometer (General Transco, Largo, FL). On the basis of the original design of Enhorning (11), this instrument gives a physiologically relevant assessment of overall surface activity that includes both dynamic film behavior and adsorption at physical conditions similar to those in the pulmonary alveoli (30). An air bubble, communicating with ambient air, was formed in a 40-μl sample of dispersed surfactant held in a sample chamber. The bubble was pulsated between maximum and minimum radii of 0.55 and 0.4 mm, and surface tension at minimum bubble radius (minimum surface tension, 50% area compression) was calculated as a function of time of pulsation from the measured pressure drop across the bubble interface using the Laplace equation for a spherical interface (11, 15). Surfactant concentrations studied were 0.5 or 2.5 mg of phospholipid/ml. Uncentrifuged WLS was studied on the bubble in 0.15 M NaCl, and centrifuged large aggregates and CLSE were studied following resuspension in 0.15 M NaCl plus 2.0 mM CaCl2.

Statistical analyses. Experimental data are reported as means ± SE. Statistical analyses used Student’s t-test for comparisons of discrete data points, and functional data were analyzed by one-way ANOVA with Scheffé’s procedure identifying points of significant difference. Differences were considered statistically significant if the probability of the null hypothesis (P) was <0.05.

RESULTS

Effects of Pc organisms on phospholipid content of WLS suspensions. Measurements of total phospholipid and calculated percent recoveries in centrifuged and uncleaved samples of WLS and WLS plus Pc indicated that intact organisms did not deplete the phospholipid content of lung surfactant in dispersion (Table 1). Only 11% of Pc phospholipid remained in the supernatant following centrifugation at 1,000 g (Table 1, Pc alone). Thus ~89% of Pc organisms were pelleted by this initial low-speed centrifugation. In contrast, suspensions of WLS and WLS plus Pc had equivalent high percentages of total phospholipid remaining in the supernatant after centrifugation at 1,000 g (89 ± 2% and 86 ± 2%, respectively, for WLS and WLS + Pc). In addition, WLS and WLS plus Pc had similar percentages of phospholipid that pelleted when the supernatants from the 1,000-g spin were subjected to further centrifugation at 12,500 g (Table 1). These results indicate that incubation with intact Pc organisms did not sequester surfactant phospholipid and alter its levels in dispersion.

Effects of intact Pc organisms on SP-A content of WLS suspensions. The possibility that Pc organisms might bind and deplete SP-A in suspensions of WLS was also specifically addressed (Fig. 1). SP-A in surfactant dispersions was identified by a goat anti-SP-A antibody shown by Western blot analysis to recognize bovine SP-A in WLS and WLS plus Pc without nonspecific binding artifacts. Quantitation of SP-A was done by densitometry on samples of WLS and WLS plus Pc (10′ organisms/mg of surfactant phospholipid) incubated in vitro and then centrifuged at 1,000 and 12,500 g as in the phospholipid experiments above. Pc organisms alone had a negligible amount of SP-A by dot blot analysis (Fig. 1). There was a trend toward increased levels of SP-A in the pellet from the 12,500-g centrifugation for WLS plus Pc compared with WLS alone (P = 0.054, Fig. 1). However, SP-A content was unchanged for WLS and WLS plus Pc in the pellet following centrifugation at 1,000 g (Fig. 1), which contained 89% of Pc-associated phospholipid based on the results in Table 1.

Effects of intact Pc organisms on surface activity of WLS and large surfactant aggregates. Overall surface tension lowering was assessed by measuring minimum surface tension as a function of time of bubble pulsation at low (0.5 mg/ml) and high (2.5 mg/ml) surfactant phospholipid concentrations. The time course of surface tension lowering of WLS and centrifuged large aggregates was affected only slightly by exposure to intact Pc organisms in vitro (Fig. 2). At 0.5 mg of surfactant phospholipid/ml suspensions of WLS reached minimum surface tensions of <1 mN/m after 20 min of bubble pulsation in the presence of intact Pc organisms, compared with 15 min of pulsation for WLS alone (Fig. 2A). Large aggregates pelleted from WLS by centrifugation at 12,500 g reached minimum surface tensions <1 mN/m after 10 min of pulsation with or without Pc organisms present (Fig. 2A). At the higher surfactant phospholipid concentration of 2.5 mg/ml, the time scale of surface tension lowering was more rapid for all mixtures studied (Fig. 2B). However, incubation with intact Pc organisms again did not have a detrimental effect on surface activity for either WLS or centrifuged large aggregates.

Effects of disrupted (sonicated) Pc organisms on surface activity of WLS and CLSE. In contrast to intact Pc, exposure to organisms disrupted by sonication had significant detrimental effects on the surface activity of WLS and CLSE (Fig. 3). The surface activity of both surfactants was impaired similarly by
exposure to sonicated Pc organisms. WLS reached minimum surface tensions of 1 mN/m after 15 min of pulsation compared with 0.2 mN/m after 20 min of pulsation in the presence of sonicated Pc (Fig. 3A). A similar inhibitory effect was found for CLSE plus sonicated Pc, with minimum surface tensions of 0.2 mN/m after 20 min of pulsation at 0.5 mg/ml (Fig. 3A). At a higher surfactant phospholipid concentration of 2.5 mg/ml, sonicated Pc also reduced the surface tension lowering ability of WLS and CLSE (Fig. 3B), although the absolute magnitude of the impairment was less than at low surfactant concentration. At 2.5 mg/ml, CLSE and WLS reached minimum surface tensions of 1 mN/m after 0.5 and 1 min, respectively, compared with 10 min in the presence of sonicated Pc (Fig. 3B). In contrast to sonicated Pc organisms, incubation with sonicated material prepared after depletion of Pc with antibody-coated magnetic beads did not impair the surface activity of CLSE or WLS at either concentration studied (Fig. 3). These latter results show that non-Pc material in suspension was not responsible for the activity detriments observed when sonicated Pc organisms were studied.

Effects of extracted hydrophobic and hydrophilic Pc chemical components on surface activity of WLS and CLSE. To assess the importance of different types of chemical constituents in Pc in impairing surface tension lowering, fractions of WLS and CLSE suspensions were disrupted and the surface activity was compared with that of uncentrifuged Pc alone. After centrifugation at 12,500 g for 30 min, the supernatant was carefully removed and the pellet was resuspended in aliquots of the original WLS or CLSE. After incubation at 37°C for 2 h, sonicated Pc organisms were added and the surfactant activity was measured as a function of time during pulsation on a bubble surfactometer (20 cycles/min, 50% area compression, 37°C). Data are means ± SE for n = 4–6.
containing extracted hydrophobic and hydrophilic components were studied (Figs. 4 and 5). At a low surfactant phospholipid concentration of 0.5 mg/ml, extracted hydrophobic Pc components had a significant detrimental effect on the surface activity of both CLSE and WLS (Fig. 4A). Minimum surface tension values after 20 min of pulsation in the presence of extracted hydrophobic Pc components were only 16.4 ± 0.5 mN/m for CLSE and 16.3 ± 0.3 mN/m for WLS (Fig. 4A). At a higher surfactant phospholipid concentration of 2.5 mg/ml, the inhibitory effects of hydrophobic Pc components were less pronounced but still apparent (Fig. 4B). At 2.5 mg/ml of surfactant phospholipid, mixtures of WLS plus hydrophobic Pc components reached minimum surface tensions <1 mN/m after 20 min of pulsation compared with only 1 min for WLS alone. Also, mixtures of CLSE plus hydrophobic Pc components reached minimum surface tensions <1 mN/m at 5 min of pulsation compared with 0.5 min for CLSE alone at this concentration (Fig. 4B). In terms of overall pattern, the inhibitory effects of extracted hydrophobic Pc components on the surface tension lowering of WLS and CLSE were similar to those of sonicated Pc organisms (Fig. 4 vs. Fig. 3).

The effects of extracted hydrophilic Pc components on the surface tension lowering of CLSE and WLS were also assessed on the pulsating bubble surfactometer (Fig. 5). At a low surfactant phospholipid concentration of 0.5 mg/ml, addition of hydrophilic Pc chemical components led to a small increase in the time course of surface tension lowering for WLS and CLSE (Fig. 5A). Compared with extracted hydrophobic Pc chemical components, however, the observed detrimental to surface activity were much less pronounced (compare Figs. 5A and 4A). At a higher surfactant concentration of 2.5 mg of phospholipid/ml, there was almost no detrimental effect from Pc hydrophilic components on the surface tension lowering ability of WLS or CLSE (Fig. 5B).

**DISCUSSION**

Pathogenic microorganisms induce a variety of inflammatory, immune, and injury responses in lungs in vivo and have the potential to affect surfactant activity and/or composition by direct and indirect mechanisms. Prior research has shown that surfactant dysfunction in acute inflammatory lung injury is caused by biophysical interactions with inhibitory host proteins or lipids in edema, from chemical degradation by lytic enzymes or reactive oxygen/nitrogen species, and from injury-induced changes that deplete or alter active large surfactant aggregates (30, 34, 44). Surfactant dysfunction from several of these injury-associated mechanisms is known to be important in the pathology of murine PcP (2, 56). However, as shown here, a potential added contributor to surfactant dysfunction in Pc-infected lungs is direct interactions between surfactant and chemical components of these microorganisms. Exposure to disrupted (sonicated) Pc organisms or extracted hydrophobic chemical components in vitro was found to significantly reduce

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*Fig. 4. Effect of extracted hydrophobic Pc chemical components on the dynamic surface tension lowering of WLS and CLSE. A: 0.5 mg of surfactant phospholipid/ml. B: 2.5 mg of surfactant phospholipid/ml. Surfactant suspensions were studied on the bubble surfactometer (20 cycles/min, 50% area compression. 37°C) in the presence or absence of hydrophobic chemical components extracted from Pc (0.1 mM phospholipid). Data are means ± SE for n = 4–6.*

*Fig. 5. Effect of extracted hydrophilic Pc chemical components on the surface tension lowering of CLSE and WLS. A: 0.5 mg of surfactant phospholipid/ml. B: 2.5 mg of surfactant phospholipid/ml. Surfactants were studied with and without added hydrophilic components from Pc (4.46 mg of protein/ml). Other details are as in Fig. 4 legend. Data are means ± SE for n = 4–6.*
the surface activity of lung surfactant (Figs. 3 and 4). In contrast, intact Pc organisms did not measurably impair lung surfactant activity (Fig. 2) or alter the phospholipid or SP-A content of surfactant dispersions in vitro (Fig. 1, Table 1).

Two forms of bovine lung surfactant, lavaged WLS and extracted CLSE, were utilized in experiments here. WLS contained the full spectrum of lipids and biophysically functional apoproteins (SP-A, -B, and -C) in endogenous surfactant in vivo. CLSE is equivalent to whole surfactant in its content of surfactant lipids and SP-B/SP-C (30, 35), but hydrophilic SP-A is not present. CLSE is the substance of the active clinical exogenous lung surfactant Infasurf, which is used to treat premature infants with RDS (30), and has also been shown to be beneficial in treating term infants and children with ALI/ARDS (3, 54, 55). Experiments with both WLS and CLSE showed similar conceptual patterns of surface activity inhibition from exposure to sonicated Pc organisms and extracted hydrophobic chemical constituents (Figs. 3 and 4).

The present study did not investigate the specific inhibitory effects of individual compounds within the broad subtraction of Pc hydrophobic components. The hydrophobic components of Pc comprise a distribution of lipid classes (see MATERIALS AND METHODS plus Refs. 13, 14, 21, 37, and 38). Several of these lipid classes are capable of inhibiting lung surfactant activity through direct biophysical interactions, including lysophosphatidylcholines (8, 9, 20, 52, 53), unsaturated membrane lipids (19, 52), cholesterol (33, 49), and fluid-free fatty acids (16, 17, 52). Phosphatidylethanolamine, a lipid class present at high levels in Pc, is also known to decrease the surface tension lowering ability of films of dipalmitoyl phosphatidylcholine and other disaturated phosphatidylcholine compounds found in lung surfactant (30). A major mechanism of action of these lipid inhibitors involves mixing into the interfacial film and compromising its ability to reach low surface tensions during dynamic compression (20, 30). A wide variety of bacteria and fungi contain a distribution of hydrophobic lipids similar to those in Pc, and these organisms would be expected to have analogous detrimental effects on surfactant activity if they were degraded or disrupted in vivo. Disrupted (lysed) mammalian cells, which also contain a broad distribution of membrane and other lipids, are known to be able to inhibit the surface activity of lung surfactant (19, 30).

In the sonication studies here, the possibility that non-Pc material (molecules or cells) in purified preparations of these microorganisms may have contributed to surface activity detriments was ruled out by several methods. Purification methods for Pc organisms were performed aseptically and contained multiple steps to remove blood cells and other host cell contamination. Purified preparations of intact Pc organisms had no visible bacterial contamination and contained minimal numbers of host cells on microscopic examination. In addition, exposure of WLS to intact purified Pc organisms for 2 h during in vitro cycling did not have a detrimental effect on surface activity (Fig. 2), indicating that any free lung-derived chemicals in solution were not inhibitory at the concentrations studied. Finally, material obtained by centrifugation following depletion of Pc organisms by magnetic beads coated with specific antibodies did not detrimentally affect surface activity when it was sonicated and added to WLS or CLSE (Fig. 3).

In terms of significance for PcP in vivo, our experiments utilized physiologically relevant surfactant concentrations and numbers of Pc organisms. A large number of studies on surfactant dysfunction in animal models of ALI/ARDS have used surfactant concentrations in the same range as those here (0.5–2.5 mg of phospholipid/ml on the pulsating bubble) and have found direct correspondence between observed reductions in surface activity and physiological deficits in gas exchange and lung pressure-volume mechanics (see Ref. 30 for review). Similarly, the numbers of Pc organisms in our inhibition studies (10^7 organisms per milligram of surfactant phospholipid) are reasonable for animal lungs infected with this microorganism. Although our findings show that disrupted Pc organisms have the potential to inhibit surfactant activity in vivo, it is difficult to predict the actual magnitude of such effects in PcP relative to surfactant dysfunction from other aspects of inflammatory injury. As discussed earlier, it is very likely that a major factor in surfactant dysfunction in PcP involves interactions with host-derived inhibitors (e.g., plasma proteins, cellular lipids) that are present in the lungs as a result of inflammatory injury (56). However, inhibitory lipids from disrupted Pc organisms could exacerbate surfactant dysfunction. Recent studies by McAllister et al. (27) have demonstrated that CD8^+ T cells are important in the killing of Pc in vivo, and the presence of CD8^+-mediated inflammation has previously been shown to be associated with increased surfactant dysfunction in mice with PcP (56).

In addition to changes in surface activity, the composition of lung surfactant can also be altered in acute pulmonary injury (ALI/ARDS). However, although Pc and gpA have been shown to bind surfactant components (26, 28, 36, 58), our study did not find appreciable changes in total phospholipid or SP-A content in surfactant dispersions incubated with intact Pc in vitro (Table 1, Fig. 1). Incubation of WLS with Pc resulted in a trend toward increased SP-A levels in the pellet from centrifugation at 12,500 g compared with WLS alone (Fig. 1) but had no effect on SP-A in the pellet from centrifugation at 1,000 g where 89% of Pc-associated phospholipid was obtained (Table 1). The finding that Pc organisms did not substantially reduce the SP-A content of dispersed WLS (10^7 organisms/mg PEl phospholipid) is consistent with physical reasoning. Assuming an SP-A content of 5% by weight in WLS relative to phospholipid and a molecular weight of 35,000 for the monomer protein (30), there were ~8.6 × 10^14 monomers of SP-A per milligram of phospholipid present in dispersion. Even if each Pc organism bound 10^6 monomers of SP-A, exposure to 10^7 organisms per milligram of surfactant phospholipid would reduce SP-A content by only 1.16%.

Although direct binding by Pc organisms is unlikely to substantially reduce SP-A levels in alveolar surfactant, changes in surfactant components do occur in PcC-infected lungs. Surfactant phospholipid levels have been reported to be decreased in lavage from rats (45) and humans (12, 18, 42) with PcP, whereas SP-A concentrations in lavage are increased (39, 40, 46). The expression of SP-A and SP-D has also been shown to increase in murine PcP (2), whereas the expression of SP-B can be decreased (2, 4) or unchanged (56) depending on the time of injury. However, these compositional alterations in surfactant in animals or humans with PcP are associated with the overall pathophysiology of inflammatory lung injury and are presumptively generated by processes other than direct interactions between Pc organisms and alveolar surfactant. Such processes could include both physiological and pathological responses
involving the regulation of synthesis or degradation pathways for surfactant proteins and/or phospholipids in type II cells in response to cytokines, chemokines, leukocytic cells, or other aspects of inflammatory lung injury.

In summary, this in vitro study has shown that disrupted (sonicated) Pc organisms and extracted hydrophobic chemical components can significantly impair the surface activity of WLS and a clinically relevant lung surfactant extract, CLSE. Freshly isolated intact Pc organisms did not significantly impair the surface activity of WLS and did not appreciably alter total phospholipid or SP-A concentrations in WLS suspensions. However, WLS and CLSE exposed to sonicated Pc organisms had greatly reduced surface activity, reaching minimum surface tensions of only 17–19 mN/m during prolonged cycling on a pulsating bubble surfactometer (37°C, 50% area compression, 0.5 mg of surfactant phospholipid/ml). WLS and CLSE had similar surface activity detriments when mixed with hydrophobic chemical components extracted from Pc. Although a substantial portion of surfactant dysfunction in PcP likely occurs in association with acute inflammatory injury and interactions with edema-derived inhibitors, the present study shows that exposure to disrupted Pc organisms or hydrophobic chemical components has the potential to directly induce surface activity detriments in endogenous and exogenous lung surfactants in the alveoli of patients with this disease.

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