Phospholipid Profile of *Pneumocystis carinii* and Its Interaction with Alveolar Type II Epithelial Cells

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*Pneumocystis carinii* is an obligate parasite of mammalian lungs, attaching to but not invading the alveolar epithelium. The alveolar air spaces are rich in phospholipids, which are secreted by steroid-responsive alveolar type II epithelial cells. *P. carinii* isolated from rat lungs was found to contain the expected structural phospholipids as well as a large amount of firmly attached disaturated phosphatidylcholine, the characteristic phospholipid of alveolar surfactant. In vitro, *P. carinii* cells synthesized phospholipids from simple radiolabeled precursors; disaturated phosphatidylcholine was not formed. However, washed *P. carinii* cells avidly adsorbed radiolabeled rat surfactant, a process that appeared to be saturable, not dependent on viability of the organisms, and abolished by incubation at 4°C. The surfactant was neither harmful nor beneficial to in vitro survival of the organisms. With the exception of high concentrations of arachidonic acid, fatty acids found in rat alveolar lining material were also toxic. In addition, cultures consisting primarily of rat type II alveolar epithelial cells were toxic to *P. carinii* when the organisms were added to monolayers of type II cells at ≤10:1 multiplicity. At higher multiplicities, the parasite survived (but did not increase in numbers), and the type II cells deteriorated. This mechanism for this effect has not been determined.

Although *Pneumocystis carinii* has become a pathogen of increasing importance due to the recent spread of the acquired immunodeficiency syndrome, it is still a poorly understood parasite. It is an obligate parasite that tightly adheres to but does not invade type I alveolar epithelial cells (15). Whereas infection with *P. carinii* appears to be common in humans and other mammals, disease due to *P. carinii* is seen only in the setting of profound immunodeficiency or chronic administration of high doses of glucocorticoids.

Normal alveoli are lined with alveolar surfactant, a phospholipid containing a large proportion of disaturated phosphatidylcholine (26, 30). Alveolar surfactant synthesis and release by alveolar type II epithelial cells is one of many nonimmunological biochemical processes that is responsive to glucocorticoid administration (7, 8, 24, 26, 30). The steroid sensitivity of surfactant production provides the rationale for the use of glucocorticoids in the prevention of neonatal respiratory distress syndrome (14, 26). In vivo, although adherent to alveolar epithelial cells, *P. carinii* cells appear to be included within the layer of alveolar surfactant which covers alveolar epithelial cells (15). The in vitro interaction of *P. carinii* with constituents of normal alveoli has not been investigated. Because alveolar type I cells cannot be cultured in vitro, attention was focused upon alveolar type II cells, a pluripotential cell type that can differentiate in vivo into type I cells, and upon the lipid constituents of alveolar lining fluid. Alveolar phospholipids may provide compounds necessary for growth of *P. carinii*, may contribute to host defenses against *P. carinii*, or both. I had expected that alveolar type II cells would serve as a substrate for growth of the parasite, an expectation that was not substantiated by the experiments described below.

**MATERIALS AND METHODS**

*P. carinii* cells were obtained by lavage from the lungs of Harlan Sprague-Dawley rats maintained on prednisolone and tetracycline in drinking water and an 8% protein diet for periods ranging from 3 to 6 months (23). Only animals that are in respiratory distress from overwhelming *P. carinii* infection are suitable for obtaining organisms in sufficient quantity and purity for these assays. All rats which survive for at least 6 weeks have readily demonstrable infections with *P. carinii*. However, most (>95%) animals originally started on the induction regimen do not yield *P. carinii* preparations that are suitable for in vitro studies. A number of factors, including epidemic superinfections in the colony (e.g., *Aspergillus* sp. during periods of nearby construction), dry air, or too rapid air exchange, interfere with induction of pneumocystosis. When lavage fluid contains >10⁶ polymorphonuclear leukocytes, it has not been possible to obtain large numbers of organisms free of polymorphonuclear leukocyte contamination. Most, but not all, of the animals with such inflammatory lavage fluids have evidence of bacterial superinfections, with >10⁶ bacteria isolated. The use of gradients (sucrose, Ficoll-Hypaque, Percoll), filtration through Nuclepore filters, or incubation of the preparations with carboxyl iron have all been tried with little success. Additionally, the ability of *P. carinii* (see below) from such preparations to survive in vitro manipulation is very poor. Use of laminar-flow hoods to prevent superinfections with other organisms allows the animals to tolerate better their intense steroid administration. However, whereas all animals so maintained eventually develop pulmonary pneumocystosis and there is less infection with other, more virulent organisms, only rare animals develop extensive disease. The best results are obtained when animals are maintained separate from other animals in a clean, small room with limited air exchange and with the relative humidity kept at 70% or greater.

Animals with respiratory distress were sacrificed by intraperitoneal injection of barbiturate and the lungs and trachea removed en bloc. With gentle massage, the excised lungs were lavaged with 0.05 M phosphate-buffered saline, pH 7.4 (PBS) (50 to 100 ml in 6-ml portions). After repeated washing in PBS, *P. carinii* cells were disaggregated by forced passage through 25- to 27-gauge needles with a pressurized hand pump. This also served to destroy many contaminating macrophages without effectively diminishing...
polymorphonuclear leukocyte numbers. If necessary, residual macrophages were removed by adherence to glass in medium 199 with 10% newborn calf serum and antibiotics in an atmosphere of air and 5% CO₂. In all studies reported here, P. carinii organisms, as estimated by examination of smears stained with Diff-Quik, outnumbered residual host cells by ≥1,000:1, and bacteria when present were at concentrations of ≤1,000 CFU/ml.

Healthy rats, not treated with prednisolone or tetracycline, were sacrificed, and their were lungs lavaged for the isolation of alveolar surfactant (13). Briefly, pooled lavage fluids from groups of 12 rats were sedimented at 200 × g to remove cells and aggregates. The cell-free fluid was centrifuged at 100,000 × g for 4 h at 4°C, and the resultant pellets of surfactant were pooled and suspended in a solution of NaCl with a specific gravity of 1.100. Ultracentrifugation under the same conditions then caused the lipids to float and the mucoproteins to pellet. The procedure was repeated at least six times, until material no longer pelleted during centrifugation of the high-density saline suspensions.

Lipids were extracted with chloroform-methanol by the method of Folch et al. (5) from P. carinii cells, normal rat lung tissue, and normal rat alveolar macrophages that were ground in glass tissue grinders. After separation from the aqueous layer, the extracted lipid was evaporated to dryness under a stream of N₂ and dissolved in chloroform for thin-layer chromatography (TLC) (11, 29). Several solvent systems were used to differentiate among the major phospholipids in P. carinii preparations by using TLC on silica gel or silicic acid (6). The majority of the experiments were conducted with chloroform-methanol-acetic acid-water (50:35:4:2, solvent CM). Because several components migrated together in this solvent, further distinction among major phospholipids was obtained by use of chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) or of tetrahydrofuran-methylalcohol-water (50:40:10:5.5). The use of these solvents allows resolution of most major phospholipid components (6). The fraction of the phosphatidylcholine that was disaturated (the major component of alveolar surfactant and a minor component of cellular lecithin) was determined by two-dimensional TLC in solvent CM with osmication of the material after migration in the first dimension (17). TLC plates were stained with iodine vapors and stained with Sudan black B to distinguish surfactant phospholipids in particular (11, 29). Quantitation of components was performed by determination of phosphorus in spots scraped from TLC plates by the method of Rouser et al. (27).

In other experiments, the ability of P. carinii to utilize exogenous phospholipids and precursors was examined. Utilization of radiolabeled [³H]choline, [¹⁴C]ethanolamine, or [¹⁴C]acetate was determined by allowing overnight incorporation of the labeled compounds by P. carinii in Earle salts containing 0.1% albumin, penicillin, and gentamicin in an atmosphere of air and 5% CO₂. The organisms were sedimented at 500 × g, rinsed six or more times with iced PBS, and suspended in 0.5 ml of PBS, and the lipids were extracted as described above. After separation of the phospholipids on TLC, the plates were divided into 0.5-cm² sections, based on migration of standards. The silica gel was then scraped into vials, and the incorporated radiolabel was quantitated by liquid scintillation counting.

Interaction of P. carinii with preformed alveolar surfactant was evaluated in several ways. The influence of alveolar surfactant added in concentrations from 10 μg/ml to 2 mg/ml, or fatty acids known to be present in alveolar lavage fluid (2) at concentrations of up to 200 μg/ml, on overnight survival of P. carinii was determined by using published methodology; the ability of P. carinii to convert [¹⁴C]glucose to [¹⁴CO₂] was used as the index of viability (23). For these experiments, since the substances are essentially insoluble in water, stable suspensions were produced by sonication at 50 W for 10 to 30 min at 4°C with 0.5% fatty-acid-free bovine serum albumin as a carrier for the fatty acids. In addition, the ability of P. carinii to adsorb or interiorize alveolar surfactant was studied by use of purified rat alveolar surfactant radiolabeled by sonication (50 W for 30 min) with [¹⁴C]phosphatidylcholine. P. carinii cells were added to sonicated suspensions of [¹⁴C]phosphatidylcholine-labeled surfactant in 2 ml of Earle salts and incubated at 4, 20, or 37°C on a tilt table for periods of time from 1 min to 1 h. In other experiments, the incubation was carried out for 18 h in medium 199 plus antibiotics. At time of harvest, the reaction mixtures were poured into 20 ml of iced PBS, and P. carinii cells were separated from unbound surfactant by multiple rinses and centrifugation at 100 × g. Incorporation of radiolabeled surfactant by P. carinii was determined by liquid scintillation counts of the multiplex washed pellets.

Because type II alveolar epithelial cells both are the source of secreted alveolar surfactant and serve as a stem cell to produce alveolar type I and type II cells after lung injury, the interaction of P. carinii with type II alveolar cell-enriched monolayers was also determined. The lungs of normal rats were lavaged with PBS to deplete macrophages, and the lungs were removed and dissected free of major airways. The lungs were minced and then further dissociated with collagenase (type II; Sigma Chemical Co.) and passed through nylon mesh (105 μm followed by 30 μm) to remove large aggregates. The cells were suspended in complete medium, and additional macrophages were removed by 1 h of adherence to glass. Nonadherent cells were transferred to Corning tissue culture flasks (approximately 5 × 10⁵ cells per flask) and left undisturbed for 18 h. The monolayers were then rinsed with PBS, and fresh complete medium was added. Such monolayers contained a monotonous cell population of nonphagocytic mononuclear cells with abundant cytoplasmic-phase lucent granules. Cell numbers remained stable for at least 3 days. These are characteristics of type II cell cultures (16, 18, 25). Additional confirmation of type II activity was sought by determining that [³H]choline was incorporated into disaturated phosphatidylcholine. P. carinii cells (10⁶ to 10⁷ per flask) were added to the monolayers, and viability after overnight incubation was assessed by phase-contrast microscopy and fluorescent microscopic determinations of acridine orange uptake (20). To allow comparison with their behavior on other cell types, P. carinii cells were also inoculated onto monolayers of lavaged macrophages, which can kill the organism (31), and of A549 lung cells, which can support limited replication (3, 4).

RESULTS

As determined by TLC, phospholipids of P. carinii were comparable to those expected in eucaryotic cells in general (Table 1). However, a large amount of disaturated phosphatidylcholine remained associated with the organisms despite extensive rinsing. In these experiments (n = 5), 25 ± 5% of the phospholipid isolated from P. carinii migrated on TLC after treatment with osmium tetroxide as disaturated phosphatidylcholine. This phospholipid, which is the primary component of mammalian alveolar surfactant but is only a minor component of other lecithins, could have been adsorbed by P. carinii in lungs or made by the organisms. To
test these possibilities, the ability of *P. carinii* to adsorb additional alveolar surfactant and the capacity of the organisms to synthesize phospholipids from radiolabeled precursors were evaluated.

Although phosphatidylcholine extracted from *P. carinii* obtained from rat lungs was composed of almost equal proportion of saturated and partially unsaturated phosphatidylcholine (Table 1), that synthesized in vitro from either \(^{3}H\)choline or \(^{14}C\)acetate contained only a small proportion of disaturated (<5% in three experiments with each substrate) phosphatidylcholine. Although, as expected, the \(^{3}H\)choline present in lipid extracts of *P. carinii* migrated on TLC only as phosphatidylcholine, all phospholipids, as well as a number of lipids which did not contain phosphorus, were formed from \(^{14}C\)acetate. Although the utilization of choline was consistent from batch to batch, that of acetate was highly variable, with some batches of *P. carinii* exhibiting active utilization and others exhibiting only slight uptake. This variability could not be correlated with apparent viability of the preparations or with contamination of the preparations with either host cells or culturable bacteria and remains unexplained. The only other precursor evaluated was \(^{14}C\)ethanolamine, which was incorporated only into phosphatidylethanolamine. Thus, *P. carinii* can synthesize a variety of lipids from simple precursors but does not appear capable of synthesizing the disaturated phosphatidylcholine found in preparations obtained from rat lungs.

*P. carinii* cells incubated in tumbling culture with \(^{14}C\)phosphatidylcholine-labeled normal rat surfactant rapidly removed the surfactant from the medium (Fig. 1). This removal was dependent on time, temperature, and numbers of organisms added to the incubation mixture. The uptake of radiolabel was diminished by the addition of excess unlabelled alveolar surfactant. Most of the uptake was completed within 15 min. After removal of the organisms from the medium containing radiolabeled alveolar surfactant and transfer to fresh medium, the radiolabel remained associated with the organisms for 24 h (the longest time measured) (83 ± 6% of 1-h counts present at 24 h; *n* = 3). Although incubation at room temperature (20°C) did not inhibit the accumulation of labeled surfactant to *P. carinii*, the process was abolished by incubation at 4°C.

Because *P. carinii* lives in a phospholipid-rich environment, the influence of alveolar surfactant and fatty acids known to be present in rat alveolar lining material on viability of the parasite was assessed. The addition of sonicated alveolar surfactant (100 µg/ml to 2 mg/ml wet

![Graph](http://iai.asm.org/)
organisms into long strands was observed in some experiments. The effect appeared to require some participation of the P. carinii cells, since P. carinii cells killed by incubation at 56°C for 30 min were not eliminated from medium and, at high inocula, were not toxic to type II cells.

These findings differed from experiments in which P. carinii cells were added to monolayers of either rat alveolar macrophages or A549 cells. Although rat alveolar macrophages can ingest and degrade P. carinii in vitro (31), they do not efficiently extract P. carinii cells from overlying medium; when $1 \times 10^5$ to $5 \times 10^6$ P. carinii cells were added to monolayers of approximately $5 \times 10^5$ macrophages, the numbers remaining uningested and viable after 24 h by acridine orange staining were $80 \pm 9\%$ of starting values ($n = 3$). On the other hand, A549 cells, which can support limited growth of P. carinii (4), in my hands, resulted in no change in numbers of P. carinii (95 ± 17%) present and viable in the medium at 24 h, when inoculating conditions were as described above. In two additional experiments, the addition of $10^8$ P. carinii cells to A549 cells resulted in the destruction of the A549 cell monolayer, again without apparent effect on the numbers of P. carinii cells.

**DISCUSSION**

These data further expand understanding of the biochemical capabilities of P. carinii and provide additional information that may be relevant to understanding the mechanisms involved in host defenses against the parasite. Available data suggest that P. carinii is capable of utilizing simple substrates in energy production and synthesis of proteins (23).

Metabolism of [14C]glucose to 14CO2 appears to be a major route of energy production (23), although the steps in the pathway remain obscure. The parasite resists destruction by antibody with or without complement (20) but is ingested and killed by alveolar macrophages (31), a process that is enhanced by presence of specific antiserum (19). P. carinii is susceptible to killing by reactive oxygen derivatives and by an acidic environment (21), both of which may contribute to the lethal activity of normal macrophages against the parasite as well as to the apparent elimination of the parasite during the course of acute Pseudomonas aeruginosa pneumonia (22).

In the alveoli, P. carinii grows as a surface parasite on the type I (or gas-transporting) cells, which have not yet been cultured in vitro, intimately associated with and perhaps totally enveloped by alveolar surfactant (15). Type II alveolar epithelial cells have at least two major roles in the lung. The produce alveolar surfactant and, after lung injury, dedifferentiate, replicate, migrate, and differentiate into type I epithelial cells (28). Because P. carinii grows in an environment rich in phospholipids, the current work concerning the interactions between P. carinii and lipids likely to be present in the alveolar was undertaken.

The data indicate that P. carinii, when obtained from the lungs of rats with steroid induced infections, is intimately associated with surfactant phospholipids and is capable of further adsorption of surfactant. Whether this is nonspecific adsorption or is mediated by specific binding of phospholipid or of surfactant apoprotein (26, 30) cannot be determined at this time. Similarly, the means for determining in an in vitro system the role of this absorbed surfactant is protecting the P. carinii from recognition or attack by host inflammatory cells are not evident. Other parasites appear to evade host defense effectors by acquisition of host molecules (1). Although it is plausible that surfactant serves that function for

**Fig. 2. P. carinii** cells inoculated on monolayers consisting primarily of type II alveolar epithelial cells did not replicate but at low inocula were eliminated from the cultures and at high inocula persisted with destruction of the monolayer of type II cells. Each dashed line represents the results seen in an individual flask. The experiments were conducted with organisms obtained at different days from five rats with steroid-induced pneumocystis.
P. carinii, direct proof cannot be obtained at the present time. However, it is clear from these data that the desaturated phosphatidylcholine used as a marker for surfactant was not made by P. carinii, since phospholipids synthesized by P. carinii contain little desaturated lecithin. With the relatively crude methodology of TLC and focusing specifically on phospholipids, no other phospholipids appear to be present in unusual amounts. The parasite does not appear to be dependent upon the host for production of structural phospholipids from simple precursors and appears capable of synthesizing a variety of such phospholipids.

Alveolar surfactant appears to function to stabilize alveoli against collapse; deficiency of surfactant is associated with respiratory insufficiency, most striking in the respiratory distress syndrome of newborns. It is possible that the adsorption of surfactant by P. carinii contributes to the hypoxia which is characteristic of the P. carinii pneumonia. Rats with steroid-induced pneumocystosis have been found to have a decreased amount of surfactant in cell-free bronchoalveolar lavage fluid at a stage of the disease which was less advanced than that of the animals used in these experiments (12). In addition, Kernbaum et al. (12) found increased levels of phospholipase activity in the lavage fluid of rats with pneumocystosis. Thus, it appears that the amount of surfactant may be reduced by adsorption and enzymatic degradation during pneumocystosis, and it is reasonable to assume that this deficiency of surface-active material contributes to the profound hypoxemia in the disease.

Since surfactant was rapidly adsorbed to P. carinii and the organisms are intimately associated with it in vivo, it seemed possible that it would be beneficial to in vitro growth of the organisms. If this were the case, it would support the suggestion that induction of pneumocystosis by glucocorticoids was partially mediated by nonimmunological biochemical alterations induced by the drugs. However, no improvement in growth of P. carinii on A549 cells or in vitro survival in tissue culture medium resulted from the addition of surfactant to the medium or by pretreating P. carinii cells with surfactant before incubation. Type II cells, rather than serving as substrates for growth (as I had hoped) or at least not affecting in vitro survival of P. carinii (as I had expected), caused elimination of the parasites from culture when the inoculum of the parasite was low. Attempts to delineate the mechanisms involved have not been successful.

The effect requires that viable P. carinii cells be coincubated with viable monolayers containing predominantly type II cells. Macrophage monolayers did not reproduce the phenomenon; although phagocytosis and intracellular killing of P. carinii did occur, elimination of the parasite from the medium within 24 h was not seen. Dead P. carinii cells did not disappear from the medium, and medium conditioned by prior culture with type II cells was not harmful to P. carinii. Although the analogy may be tenuous, the effect appears superficially similar to that documented for lysis of bacteria by penicillin, in which autolytic enzymes are activated by a low-molecular-weight compound (10). A variety of fatty acids known to be present in rat alveolar lining material (2) and which may be secreted by type II epithelial cells were tested; with the exception of arachidonic acid at concentrations that appear to be too high to be biologically relevant, none of the fatty acids which are lethal for pneumococci was detrimental to P. carinii survival. It remains possible that the interaction of P. carinii with alveolar lipids is more complex, requiring the presence of both lipids and lipases, as may be the case with Giardia lamblia (9).

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LITERATURE CITED


