Interaction of rat Pneumocystis carinii and rat alveolar epithelial cells in vitro

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Recent studies have identified specific molecules that mediate the binding of *P. carinii* to AECs (18). These studies have produced useful information concerning the roles of fibronectin (23–25, 27), vitronectin (17), and mannose (16) in adherence of *P. carinii* to epithelial cell lines. These studies have been limited in several respects. Because of the difficulties inherent in obtaining pure populations of AECs and in propagation of AECs in vitro, these studies were performed with cell lines that may differ in important ways from AECs in vivo. Furthermore, the presence of rat host molecules attached to *P. carinii* may introduce heterologous proteins that influence the behavior of human-derived epithelial cell lines. Recently, however, Pottratz and Weir (26) demonstrated that rat-derived *P. carinii* organisms adhere to rat-derived AECs in vitro. However, no studies have yet defined the direct influence of *P. carinii* on AEC function.

It is clear that adherence of *P. carinii* organisms to AECs alone is insufficient to produce clinically apparent pneumonia. Individuals may carry a significant burden of *P. carinii* without major gas-exchange abnormality. However, at some point in the course of infection, functional changes must occur in AECs, resulting in impaired barrier function and alveolar flooding. During the development of *P. carinii* pneumonia in corticosteroid-immunosuppressed rats, extensive endothelial leakage and an undermining of the AEC basement membrane. In mice specifically depleted of CD4+ T cells, an increasing burden of *P. carinii* is tolerated until inflammatory responses are triggered and severe pneumonia occurs (4). Taken together, these data indicate that increasing *P. carinii* burden may be present before the development of AEC functional derangement and clinical pneumonia.

Despite advances in understanding molecules important in adhesion, the effects of *P. carinii* on function of AECs are unknown. In the present study, we developed a homologous model system with rat-derived *P. carinii* and rat AECs in primary culture to determine whether the interaction of *P. carinii* and AECs results in alterations of AEC structural, metabolic, or barrier function.

**METHODS**

Rats. To provide a source of AECs in primary culture, specific pathogen-free male Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). To provide a source of *P. carinii* organisms, male Sprague-
Dawley rats were purchased from Hilltop Laboratories (Scottsdale, PA). All animal experimentation was performed in accordance with the policies of the Department of Veterans Affairs and the University of Michigan Committee on Use and Care of Animals.

Epithelial cell isolation. Primary cultures of rat AECs were prepared from the lungs of 150-g rats as described previously (9, 21). AECs were released from the basement membrane by digestion with porcine pancreatic elastase (Worthington, Freehold, NJ) administered via the trachea. Macrophages and lymphocytes were removed from the cell suspension bypanning on plates coated with immunoglobulin G (Calbiochem, San Diego, CA). Cells were plated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum, penicillin (5,000 units/ml) and streptomycin (5 mg/ml; all from Gibco, Gaithersburg, MD). In a typical harvest, the yield of AECs was 4 × 10^7 cells per rat, of which >95% were viable by trypsin blue exclusion. As demonstrated previously, 92–95% of the adherent cells are epithelial cells, as defined by staining with anti-cytokeratin antibodies. By ultrastructural criteria, >90% of these cells are AECs (20). Experiments were performed using AECs that had been in culture for 3 days, at which point the cells had spread and expressed a number of the surface characteristics of type II cells in vivo (21, 22).

P. carinii isolation. P. carinii organisms were isolated from the lungs of rats as previously described (12, 13). Briefly, rats weighing 150–200 g were immunosuppressed by the administration of dexamethasone (2 mg/kg) in their drinking water. Bacterial superinfection was prevented with the addition of tetracycline and ampicillin (0.5 mg/ml) to the drinking water. After 6–8 wk of immunosuppression, rats were killed. The lungs were removed aseptically, and touch preparations were stained with modified Giemsa stain (Diff-Quick, AHS del Caribe, Aguada, PR), gram stain, and crystal violet stain to identify and exclude any preparations containing bacteria or fungi. The lungs were minced, filtered through sterile wire mesh, and rinsed with minimal essential medium containing 20% fetal bovine serum (MEM-FBS). After centrifugation at 1,100 g for 10 min, the supernatant was discarded and the pelleted was resuspended in MEM-FBS containing 100 mM dithiothreitol (Sigma, St. Louis, MO). After repeat centrifugation (1,100 g for 10 min), the pellet was suspended in 15 ml of 0.85% NH₄Cl and incubated at 37°C for 15 min, and the insoluble material (including P. carinii) was collected by centrifugation (1,100 g for 10 min). This pellet was resuspended in phosphate-buffered saline (PBS), filtered through an 8-μm polycarbonate filter (Millipore, Marlborough, MA) and washed with PBS. The filtrate was again centrifuged at 1,100 g for 10 min, and the pellet was resuspended in MEM-FBS. Samples were stained with crystal violet stain. We have found that enumeration of P. carinii cysts is more reproducible, with less interobserver variation, than when counting trophic forms (3). Therefore, the P. carinii preparations were evaluated by microscopic counting of the P. carinii cysts, and the number of organisms in each experiment is presented in terms of the number of cysts. The number of trophic forms exceeds the number of cysts in the rat lung by a factor of at least 50:1 (30).

Measurement of P. carinii adherence to AECs. To fluorescently label P. carinii, a stock solution of 1 mg/ml of [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR) was prepared in DMSO. P. carinii organisms (equivalent to 60 × 10⁶ cysts) were suspended in 10 ml of PBS with 1% BSA. The carboxyfluorescein stock (10 μl) was added to the P. carinii, mixed by inversion, and incubated for 45 min at 23°C. The mixture was then centrifuged for 8 min at 400 g at 20°C. The pellet was washed three times with PBS containing 1% BSA. The organisms were resuspended in 13.5 ml of DMEM with 10% newborn calf serum.

To quantitate adherence of P. carinii organisms to AECs, AECs were cultured for 3 days in 96-well microtiter plates at a density of 1 × 10⁶ cells per well. Labeled P. carinii organisms were then added in increasing concentrations, and the cocultures were incubated for 24 h at 37°C. After the wells were vigorously washed with PBS, the remaining adherent P. carinii organisms were quantitated by fluorimetry (Cytofluor 2300, Millipore, Bedford, MA). Comparison of fluorimetric data with morphologic counts of the P. carinii organisms demonstrated a linear relationship between the fluorimetric intensity and number of enumerated organisms (data not shown). To confirm attachment of P. carinii organisms, scanning and transmission electron microscopy were performed, using cocultures fixed with Karnovsky’s fixative, pH 7.2.

Measurement of AEC protein synthesis. To determine whether P. carinii modulated the metabolic activity of AECs, AECs were placed in culture in 24-well plates (4 × 10⁵ cells per well) for 3 days, followed by addition of increasing numbers of P. carinii organisms. Control wells included AECs alone and P. carinii alone. After 3 days, the medium was changed to DMEM + 10% FBS to which l-[^35]S)methionine (–1,000 Ci/mmol, Amersham, Arlington Heights, IL) had been added to a final concentration of 100 μCi/ml. After 16 h, the medium was removed, and the cells were washed with cold PBS, exposed to 0.3 N NaOH at 23°C for 15 min, and then precipitated with 10% trichloroacetic acid at 4°C. The insoluble material was collected on nitrocellulose filters and counted by liquid scintillation counting (Beckman, Schaumburg, IL).

Measurement of P. carinii metabolic activity. Because P. carinii, but not mammalian cells, are capable of de novo folate synthesis, we utilized metabolism of p-aminobenzoic acid (PABA) to folates as a specific measurement of P. carinii metabolic activity. AECs were placed into 24-well tissue culture plates for 3 days. P. carinii organisms were added at a concentration of 10⁶–10⁷ per well for 2 days. As a negative control, duplicate AEC wells received P. carinii that had been boiled for 10 min. Metabolism of labeled PABA, to folates by P. carinii was measured as previously described (12). Briefly, 0.2 μCi of [14]H)PABA was added to the cocultures at 37°C for 24 h. The cocultures were harvested and were collected by centrifugation. The pellet was resuspended in 200 μl of Tris-HCl buffer (pH 8.0) containing 3% β-mercaptoethanol, and cells and organisms were lysed by sonication. The membranes and debris were discarded, and 1 ml of 1 M citrate-phosphate buffer (pH 3.8) was added to the cytosol. This aqueous suspension was extracted four times with diethyl ether, which removes >99% of unreacted PABA (12). The remaining radioactivity in the aqueous layer was then determined by liquid scintillation counting (Beckman). Previous work has demonstrated that the ether extraction method correlates well with measurement of folate metabolites by high-performance liquid chromatography (12).

Immunofluorescence microscopy of AEC cytoskeletal and functional proteins. To determine whether P. carinii altered AEC expression of structural and cytoskeletal proteins, AECs were cultured in wells on tissue culture-treated slides (LabTek, Nunc, Naperville, IL) for 2 days. P. carinii organisms were then added for 16 h. At the conclusion of the experiments, the cells were washed with cold PBS, then fixed in precooled methanol for 20 min at –20°C. The slides were dipped in acetone (–20°C) three times and allowed to dry. For immunofluorescence staining, antibodies were diluted in PBS
with 1% BSA. Specimens were exposed successively to 10% FBS and primary antibodies to evaluate prototypic cytoskeletal and junctional protein expression: murine anti-cytokeratin 8 (Boehringer Mannheim, Indianapolis, IN), murine anti-E-cadherin (Transduction Laboratories, Lexington KY), and rabbit anti-occludin (Zymed Laboratories, South San Francisco, CA). After exposure to primary antibodies for 30 min at 37°C, slides were stained with tetramethylrhodamine isothiocyanate-conjugated goat anti-murine IgG (cytokeratin 8 and E-cadherin) or FITC-conjugated goat anti-rabbit IgG (occludin; all from Sigma) for 30 min at 37°C. Specimens were viewed on a Nikon Labophot-2 microscope equipped with epifluorescence and photographed using Tri-X film (Kodak, Rochester, NY). For each condition, specimens identically stained with an irrelevant antibody demonstrated no staining.

Measurement of AEC transepithelial resistance. To determine whether P. carinii modulated the barrier function of AEC monolayers, AECs were plated at 1 × 10⁶ cells per well in the upper chamber of Transwell culture dishes on tissue culture-treated polycarbonate membranes (0.4-μm pore size, 6.5-mm diameter; Corning Costar, Cambridge, MA) for 3 days. The resistance across the monolayer was determined using a Millicell device (Millipore) with one sterile electrode in the medium in the upper chamber and one sterile electrode in the medium in the lower chamber. Thereafter, P. carinii organisms were added to the upper chamber, and resistance measurements were performed on a daily basis without disturbing the cells and organisms adherent to the membrane. To determine whether the viability of the P. carinii preparations was necessary to effect resistance, transepithelial resistance was measured in AEC wells incubated with previously boiled P. carinii. To exclude a possible confounding effect caused by residual donor rat proteins in the P. carinii preparation, transepithelial resistance was measured in AEC wells incubated with a sham lung preparation prepared from the lungs of uninfected donor rats.

Statistics. Data are presented as means ± SE. Experimental conditions were compared by analysis of variance with Newman-Keuls follow-up testing (37). Statistical significance was accepted for P values < 0.05.

RESULTS

P. carinii adherence to AEC. To confirm that rat-derived P. carinii organisms adhered to rat AECs in vitro, P. carinii organisms and AECs were cocultured. Light microscopy demonstrated that P. carinii organisms adhered to AECs (Fig. 1A). To quantitate adherence, P. carinii organisms were fluorescently labeled (Fig. 1B). P. carinii adhered to AEC monolayers in a dose-dependent manner (Fig. 2). At the maximum number of P. carinii organisms added (5 × 10⁶ cysts/well), 17.8 ± 1.8% of the labeled organisms adhered to the AECs after vigorous washing. Both scanning and electron microscopy demonstrated that P. carinii trophic forms were adherent to the AEC (data not shown).

AEC junctional and cytoskeletal proteins. To determine whether the structural integrity of the epithelial cell monolayer was compromised by adherence of P. carinii to the AECs, immunofluorescence microscopy was performed to assess the expression and distribution of important epithelial cell structural proteins. The distribution of cytokeratin 8, an intermediate filament protein involved in the structural integrity of the epithelial cell monolayer, was not altered by AEC interaction with P. carinii (Fig. 3, A and B). Similarly, the distribution of E-cadherin, a molecule important in adherens junctions, was also unaffected by the presence of P. carinii (Fig. 3, C and D). Occludin is a junctional protein that is a constituent of tight junctions responsible for epithelial cell barrier function (1). When AEC monolayers that had been in culture with P. carinii for 3 days were immunostained with an antibody specific for occludin, there was no difference in expression or distribution of this junctional protein compared with cells cultured in the absence of organisms (Fig. 3, E and F). Thus, in these experiments, the AEC monolayer remained intact, with no change in expression of these important epithelial cell structural proteins following the adherence of P. carinii.

AEC protein synthesis. In preliminary studies, we found that AECs cultured with P. carinii continued to exclude trypan blue, as did AECs cultured alone (data not shown). To determine whether AECs remained...
metabolically active during coculture with P. carinii, incorporation of labeled methionine by AECs was measured. In the presence of P. carinii, the AECs continued to synthesize new proteins (Fig. 4). The rate of new protein synthesis was not diminished by culture with the P. carinii compared with AECs cultured alone. The methionine incorporation was not due to metabolism by P. carinii organisms, as incorporation of [35S]methionine into acid-insoluble material by the maximum number of P. carinii organisms when cultured alone (counted as $4 \times 10^6$ cysts) resulted in counts $< 10 \times 10^3$ counts/min (cpm). Furthermore, the incorporation of labeled methionine did not increase with the addition of increasing numbers of P. carinii organisms, further indicating that methionine uptake by the organisms did not contribute to any large extent to that attributed to the AECs. Thus the interaction of AECs with P. carinii in vitro did not impair protein synthesis by AECs.

P. carinii metabolic activity. To determine whether P. carinii remained active in the coculture system, we measured P. carinii metabolism of PABA to folates. AECs were cultured in 24-well plates for 3 days before the addition of P. carinii organisms for 2 days. [3H]PABA was added to the cultures to measure P. carinii metabolism of PABA to folates. Cocultures of AECs and live P. carinii produced 80 fmol folates (0.63 pmol/mg protein). In contrast, cocultures of AECs and boiled P. carinii or AECs alone contained 4 fmol folates. As expected, AECs alone were unable to metabolize PABA to folates. Boiling of P. carinii before its addition to the AECs abrogated folate metabolism.

AEC transepithelial resistance. Because the pathogenesis of P. carinii pneumonia in vivo involves disruption of AEC barrier function, we performed experiments to measure transepithelial resistance across AEC monolayers during interaction with P. carinii. Transepithelial resistance increased in a dose-dependent manner over 3 days in culture (Fig. 5). In these experiments, the number of P. carinii trophic forms placed in culture with the AECs in these experiments, based on the ratio of trophic forms to cysts, exceeded the number that induced growth inhibition of human cell lines in previous studies (12). When transepithelial resistance measurements were performed daily, P. carinii increased transepithelial resistance over 3 days of coculture (Fig. 6). The transepithelial resistance of AECs cultured with P. carinii remained greater than that of control wells with AECs alone through 7 days of coculture (data not shown). To determine whether coculture with viable P. carinii was required for this effect, AECs were cocultured with boiled P. carinii and with sham lung preparations. Neither boiled P. carinii nor the sham lung preparations modulated transepithelial resistance. When Evans blue dye was placed in the lower chamber of the Transwell apparatus, transit of the dye across the monolayer into the upper chamber was undetectable in the presence or absence of P. carinii (data not shown). Thus the interaction with P. carinii did not directly impair AEC barrier function.

**DISCUSSION**

These data demonstrate that the direct interaction of P. carinii with AECs does not disrupt the structural integrity of AECs in vitro and preserves the metabolic activity of the P. carinii organisms. Using a homologous in vitro culture system with primarily isolated AECs, we demonstrate that P. carinii adheres to the AECs in a dose-dependent manner, that the AECs and P. carinii remain metabolically active, that expression of AEC junctional and cytoskeletal proteins is preserved, and that transepithelial resistance across AEC monolayers is maintained during interaction with P. carinii organisms. Thus, although P. carinii organisms adhere closely to AECs in the lung, these studies suggest that the impaired alveolar barrier function seen in patients with severe P. carinii pneumonia is not simply a direct result of the interaction of the organism with the alveolar epithelium.

Several significant characteristics of the model system that we have used for these studies are noteworthy. In our studies, we developed a homologous system in which both P. carinii and AECs were obtained from rats. P. carinii strains are species specific, and so organisms capable of infecting one species will not produce disease in other species (10). Additionally, glycoproteins differ on P. carinii organisms obtained from different species (11), and host material remains adherent to harvested P. carinii organisms. A homologous system more closely models in vivo interactions and avoids these important methodological considerations. Next, these studies used AECs in primary culture rather than transformed cell lines. In the lung, P. carinii interact closely with type I cells. However, methodological barriers limit the ability to culture type I cells. When rat type II cells are placed in culture under the conditions of these experiments, however,
they express many of the characteristics that are associated with the type I cell phenotype in vivo, including the expression of cell surface molecules such as intracellular adhesion molecule 1 (6), T1α (8), and 2BVIII (7). We chose to plate AECs in medium containing serum and at relatively low density to promote cell spreading (21). These culture conditions yield relatively low measured resistances across the monolayers. The transepithelial resistances in these experiments remain significantly greater than in injured or disrupted monolayers. Thus this model more closely simulates the interactions of *P. carinii* with lung epithelial cells in vivo than do studies using transformed cell lines. Finally, these experiments for the first time allow the measurement in vitro of the effect of *P. carinii* on a biologically important function of AECs, maintenance of a tight barrier (29).

The particular AEC parameters that were evaluated were chosen because they represent important features that are central to the function of these cells in the alveolar space. AECs form the most important barrier against alveolar flooding (27). For this role, it is necessary that the cells remain viable, metabolically active, and able to maintain their intercellular junctions. Adherens junctions, of which E-cadherin is an important component, promote the physical interaction between cells so that they are not pulled apart. Tight junctions form the barrier to the passage of solutes between the interstitium and the alveolar space and serve to segregate the apical and basolateral plasma membranes. Finally, whereas occludin staining provides morphological evidence of an epithelial cell barrier, the measurements of transepithelial resistance provide direct functional confirmation of the ability of the epithelial cell monolayer to block the passage of solutes across the monolayer and thus to preserve the integrity of the alveolar space. Therefore, in these studies we have examined the effects of interaction with *P. carinii* on AEC characteristics that are essential for normal lung function.
Previous work demonstrates that lung injury is a component of the pathogenesis of *P. carinii* pneumonia. Studies of bronchoalveolar lavage (BAL) fluid indicate that patients with severe pneumonia due to *P. carinii* have increased alveolar-capillary permeability, with leakage of serum proteins into the alveolar space (28). Furthermore, biopsies of patients with severe *P. carinii* pneumonia demonstrate the histologic pattern of diffuse alveolar damage with interstitial edema, type I cell erosion, and type II cell proliferation (5, 31). However, experience with animal models and with human patients suggests that the direct interaction of *P. carinii* with AECs need not itself lead to AEC injury. Corticosteroid-treated rats (35) and mice depleted of CD4+ lymphocytes (4) can live with relatively little respiratory compromise despite carrying very high concentrations of *P. carinii* within the alveolar space. Similarly, humans infected with HIV often demonstrate an indolent course of *P. carinii* infection, with little gas-exchange abnormality despite large numbers of organisms, when investigated by BAL (31). Thus, our observation that the interaction with *P. carinii* does not diminish transepithelial resistance provides an explanation for the ability of these patients and rodents to tolerate *P. carinii* infection, at least for a period, without developing increased permeability pulmonary edema.

Nonetheless, patients who enter the hospital with *P. carinii* pneumonia may proceed to respiratory failure. It is likely that additional signals lead to impaired alveolar epithelial barrier function and to type I cell loss. Of note, the interaction of rat-derived *P. carinii* organisms with a human epithelial cell line results in growth inhibition of the cell line (12) but does not induce cell death (2, 15). This important observation implies that additional factors are required for AEC damage. Inflammatory mediators such as tumor necrosis factor-α (TNF-α) are present in abundance in patients with severe *P. carinii* pneumonia (14). Sustained exposure to high levels of TNF-α leads to loss of AEC barrier function in vitro (32). The benefits of early...
corticosteroid therapy for patients with moderately severe infection are consistent with a role for the host inflammatory response in the development of diffuse alveolar damage in the context of P. carinii pneumonia. Our observation that transepithelial resistance actually increases modestly in the presence of P. carinii indicates that the epithelial cells are not unaffected by this interaction, although the mechanisms and ultimate consequences of this change in resistance have not yet been defined. Similarly, the effects of more prolonged exposure of AECs to P. carinii in vivo await further study. However, the ability of immunosuppressed rodents to recover from P. carinii pneumonia with large numbers of organisms when immunosuppression has been removed leads us to speculate that other features, such as host inflammatory mediators, are likely the triggers leading to increased alveolar capillary permeability and respiratory failure in this infection.

In summary, we have used a homologous model system to examine the interaction of rat-derived P. carinii and rat AECs in vitro. Our investigations demonstrate that adherence of metabolically active P. carinii to AECs does not result in impaired metabolic activity, altered structural protein expression, or loss of alveolar epithelial barrier function. These findings suggest that additional factors, such as the host inflammatory response, play a major role in the progression to respiratory failure in severe P. carinii pneumonia. This model system can provide important new insights into the cellular events leading to physiological compromise in the context of infection caused by this poorly understood pathogen.

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