Rapid Separation of *Pneumocystis carinii* from Lung Lavage Fluids

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A simple, rapid procedure to separate *Pneumocystis carinii* obtained by lavage of lungs of steroid-treated rats from rat leukocytes is described. Commercially available monoclonal antibody to rat common leukocyte determinants is used to sediment the leukocytes, resulting in supernatant fluid containing *P. carinii* cells virtually free of intact rat cells.

Pneumocystis carinii has become a common cause of pneumonia in patients with the acquired immunodeficiency syndrome. In vitro studies of *P. carinii* are hampered by the difficulty in separating the organism from host cells, which may be much more metabolically active than the parasite. Diagnosis of infection relies upon identification of the organism in stained specimens of airway secretions or lung sections. While there are several stains that adequately stain the parasite, rapid stains, such as the Diff-Quik stain (Dade Diagnostics Inc., Aguada, P.R.), are difficult to interpret in a sample that has a large number of leukocytes.

A variety of methods, including differential centrifugation on gradients (6), passage through membrane filters (4), fluorescence-activated cell sorting (1), and removal of phagocytes by mechanical disruption and adherence to glass or plastic (3, 5), have been used to enrich samples for *P. carinii*. All of these procedures are more or less successful. Those which yield fractions of very high purity (e.g., gradient centrifugation) result in a substantial loss of organisms. Others which allow more complete recovery of *P. carinii* (e.g., glass adherence) require long incubations of *P. carinii* with the cells. All these procedures are time-consuming. None of them has any potential for application in a diagnostic laboratory.

Recently, the use of monoclonal antibody against rat common leukocyte determinants to enhance separation of rat alveolar epithelial cells was described (7, 8). We have successfully used this reagent to quickly purify *P. carinii* from rat lung lavage samples that were too heavily contaminated with host cells to otherwise be useful for in vitro studies, as well as to rapidly remove host cells from preparations that would have been satisfactory after purification by other methods.

P. carinii pneumonia was induced in Sprague-Dawley rats by chronic administration of prednisolone and tetracycline (3). The rats were also maintained on low-protein chow. Since these rats were used to provide *P. carinii* for in vitro studies, the disease was allowed to progress until the rats become overtly ill. They were then sacrificed by intraperitoneal injection of pentobarbital sodium (Nembutal). The heart and lungs were removed en bloc, and the alveolar contents were lavaged with phosphate-buffered saline with gentle massage (3).

The lavage fluid from a single rat was sedimented at $3,000 \times g$ for 15 min at 4°C , and the entire pellet was suspended in 35 to 40 ml of Dulbecco modified Eagle medium-5% calf serum-cefotaxime (100 $\mu\text{g/ml}$)-amikacin (100 $\mu\text{g/ml}$) (tissue

culture medium; TCM). Murine ascites fluid containing monoclonal antibody to the rat common leukocyte antigen (anti-CLA; 50 μl) (catalog no. 15708-1; Pelfreeze Biologicals, Rogers, Ark.) was added, and the suspension was gently mixed. After incubation on a tilt table, the material was sedimented at $1,500 \times g$ at 4°C for 10 min. The pellet was suspended in fresh TCM and vigorously vortexed, and centrifugation at $1,500 \times g$ was repeated. At each step, the aggregated macrophages and polymorphonuclear leukocytes were pelleted, while *P. carinii* was sedimented much less efficiently. When the aim of the procedure was recovery of as many *P. carinii* cells as possible, this was repeated two to three times, and the recovery of *P. carinii* in the supernatant fluid was monitored by light microscopy. After each centrifugation at $1,500 \times g$, the supernatant fluid containing *P. carinii* was decanted, to be subsequently centrifuged at $3,000 \times g$ for 15 min at 4°C to pellet the organisms. In additional experiments, we evaluated the relative efficiencies of antibody alone, antibody plus rat complement (Sigma Chemical Co., St. Louis, Mo.), and incubation at 37 and 4°C on separation of *P. carinii* from host cells, in an effort to define the least cumbersome method of separation.

The results of this method of separation of *P. carinii* from cellular elements of lung lavage material are shown in Fig. 1. The lavage fluid from the animal contained $>2 \times 10^6$ polymorphonuclear leukocytes per ml (45-ml total volume), secondary to superinfection with gram-negative bacteria before treatment with anti-CLA (Fig. 1A). After exposure to anti-CLA and centrifugation (Fig. 1B), <200 polymorphonuclear leukocytes per ml remained suspended (40-ml total volume). *P. carinii* is identified only with difficulty in the untreated lavage fluid (Fig. 1A), with most fields containing no recognizable *P. carinii* cells. Before testing anti-CLA, we had found no satisfactory method to isolate *P. carinii* from lavage fluid samples containing $>10^6$ polymorphonuclear leukocytes. After treatment of the fluid with anti-CLA, no intact host cells were present and *P. carinii* trophozoites and presumably encysted forms were readily recognizable (Fig. 1B).

With other samples similarly contaminated with host cells, we compared various concentrations of anti-CLA and the effects of complement and incubation temperatures. As assessed by the purity of *P. carinii* and volume of pelleted *P. carinii* from the supernatant, the use of 50 μl of anti-CLA per 40-ml sample was as good as the use of 250 μl , the addition of rat complement was not beneficial, and the incubation temperature was not critical. Separation at 4°C was equivalent to that at 37°C , a finding that is important when the goal of the separation is obtaining *P. carinii* cells damaged as little

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FIG. 1. Samples of bronchoalveolar lavage material taken from rat with steroid-induced *P. carinii* pneumonia before (A) and after (B) treatment of the fluid with anti-CLA. For these photomicrographs, the samples were brought to equal volumes (45 ml), and 10- μ l samples were spotted onto acetone-cleaned glass slides, allowed to air dry, and stained with Diff-Quik stain. Before treatment of the lavage fluid with anti-CLA (A), *P. carinii* cells were detected only with difficulty, with the organisms shown in this micrograph representing some of the most distinct examples. In such preparations containing large numbers of intact and degenerated phagocytes, identification of *P. carinii* is difficult unless the encysted forms (◄) can be found. Trophozoites (►), although present, are difficult to distinguish from inflammatory cell structures. After treatment of the fluid with anti-CLA (B), the supernatant fluid contains no intact phagocytes, and numerous trophozoites (►) (only five have been marked; most of the material in the field, at different planes of focus, is also *P. carinii*), as well as encysted forms (◄), are visible. The magnification for both micrographs is identical; the bar at the lower right corner of panel A represents 10 μ m. The $\times$ 100 objective was used.

as possible by exposure to toxic secretory products of phagocytes (2).

Although these experiments were conducted as a part of an ongoing effort to study rat *P. carinii*, it seems likely that, with slight modification, similar reagents (e.g., monoclonal antibody to human myeloid-histiocyte antigen; Dako Corp., Santa Barbara, Calif.) could be useful in studies of human *P. carinii* pneumonia. Concentration of *P. carinii* to small volumes after elimination of most host cells from the sample could allow diagnostic evaluation of a bronchoalveolar lavage sample to be performed rapidly with simple, rapid stains, such as the Diff-Quik stain, which allow recognition of all phases of *P. carinii*. This method may also permit more in vitro studies of human *P. carinii*.

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