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Techniques for Examining *Pneumocystis carinii* in Fresh Specimens

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***Pneumocystis carinii* was examined in fresh preparations of infected rat lung homogenates and tissue culture supernatants by a variety of light microscope techniques, vital dyes, and histologic stains. Phase-contrast microscopy, Nomarski interference-contrast microscopy, and bright-field microscopy with oblique illumination provided excellent views of *P. carinii*. Erythrosin B, and to a lesser extent trypan blue, were helpful in assessing organism viability. The use of Triton X-100–Giemsa stain permitted differentiation of the developmental stages in the *P. carinii* life cycle. The techniques developed here are easily adaptable to the microbiology laboratory and thus should have important clinical and research applications.**

Pneumocystis carinii is well recognized as a major opportunistic pulmonary pathogen, but little is known about its life cycle, metabolism, or antigenic constituents. In recent years, interest in *P. carinii* has increased markedly because of its importance as a cause of morbidity and mortality in the acquired immune deficiency syndrome (AIDS). Most of these reports have focused on the clinical aspects of *P. carinii* pneumonia in AIDS or on methods of diagnosis (8, 14, 23, 24, 31). There is little published information on the morphologic features of *P. carinii* in fresh specimens or on guidelines for determining organism viability. These data are not only important to the investigator who wishes to begin working with *P. carinii* in the laboratory but may also have practical significance in assessing the results of therapy. *P. carinii* frequently persists for long periods of time in AIDS patients despite clinically effective treatment (30).

We have been interested in animal models of *P. carinii* (34, 35) and, more recently, in in vitro cultivation (4a, 5, 6). In the course of these studies we have developed microscope and staining techniques to examine *P. carinii* in fresh lung homogenates, tissue culture supernatants, and fixed specimens. In the current study, we present a detailed description of these techniques and discuss their possible application to work with *P. carinii* in research and in clinical settings.

MATERIALS AND METHODS

Preparation of *P. carinii*. *P. carinii* obtained from the lungs of Sprague-Dawley rats treated with corticosteroids for 6 to 8 weeks was prepared, quantitated, and cultured as described previously (4a, 6).

The lungs were removed en bloc, minced, and homogenized in a Stomacher blender (Tekmar Co., Cincinnati, Ohio). The erythrocytes present in the homogenate were lysed with a 0.85% ammonium chloride solution. The lysed inoculum was serially diluted for quantitation of *P. carinii* nuclei per milliliter. Homogenates were either examined directly or inoculated into tissue cultures for observation after selected periods of culture. The in vitro system consisted of 48-h-old confluent cell monolayers of either A549 (ATCC CCL 185) or WI-38 VA13 subline 2RA (ATCC CCL 75.1) cell lines grown in 25-cm^2 flasks or 24-well plates (Becton-Dickinson and Co., Oxnard, Calif.). Cultures contained media recommended by American Type Culture Collection: Dulbecco modified eagle medium or minimal essential medium (Hanks salts) and 20% fetal bovine serum (MA Bioproducts, Walkersville, Md.). The following antimicrobial supplements were used: penicillin (200 U/ml), streptomycin (200 μg/ml), and amphotericin B (0.5 μg/ml) (GIBCO Laboratories, Grand Island, N.Y.); or gentamicin (100 μg/ml) (Schering Corp., Kenilworth, N.J.) with amphotericin B (0.5 μg/ml) (GIBCO). *P. carinii* was obtained from cultures by removal of a small volume of the tissue culture fluid where the organism was found growing in clusters.

Microscopy. Different kinds of *P. carinii* preparations (lung homogenate or culture supernatant, fresh or stained) were viewed by a variety of light microscope techniques: phase-contrast, Nomarski interference-contrast (NIC), bright-field, and bright-field with oblique illumination. The last method involves shifting a bright-field condenser off-center to give an oblique angle of illumination. The image is formed by both direct and narrowly scattered light and has a degree of interference contrast. Additional control of the amount and quality of image contrast is given by the stage diaphragm (stopped down or open) and by the use of filters (neutral density, diffusing, or polarizing). Oblique bright-field illumination can be obtained on most standard bright-field microscopes and is very useful for viewing *P. carinii* preparations stained with Triton X-100–Giemsa (described below).

Viability tests by dye exclusion. Aqueous stock solutions of 0.4% trypan blue or erythrosin B were prepared according to standard methods (26). One part of stock solution was added to nine parts of the *P. carinii* sample. Approximately 5 μl of the resulting solution was placed on each well of a three-well microscope slide (Carlson Scientific, Peotone, Ill.) with a no. 1 cover slip (22 by 50 mm) and examined under oil immersion with the different microscope techniques listed above. Specimens were examined within 5 min of dye addition unless otherwise stated. In separate viability experiments (data not shown), erythrosin B was excluded by cysts and trophic forms over a 24-h period when kept at 4°C. However, specimens which were allowed to remain at room temperature showed some loss of viability, reflected by uptake of the stain by the various forms of *P. carinii*. It was important then to perform viability assays within 5 min of the addition of stain.

Triton X-100–Giemsa stain. A stock solution of 1.2% (wt/vol) Giemsa stain was made by adding 1 g of Giemsa to a mixture of 66 ml of methanol and 66 ml of glycerol. A stock

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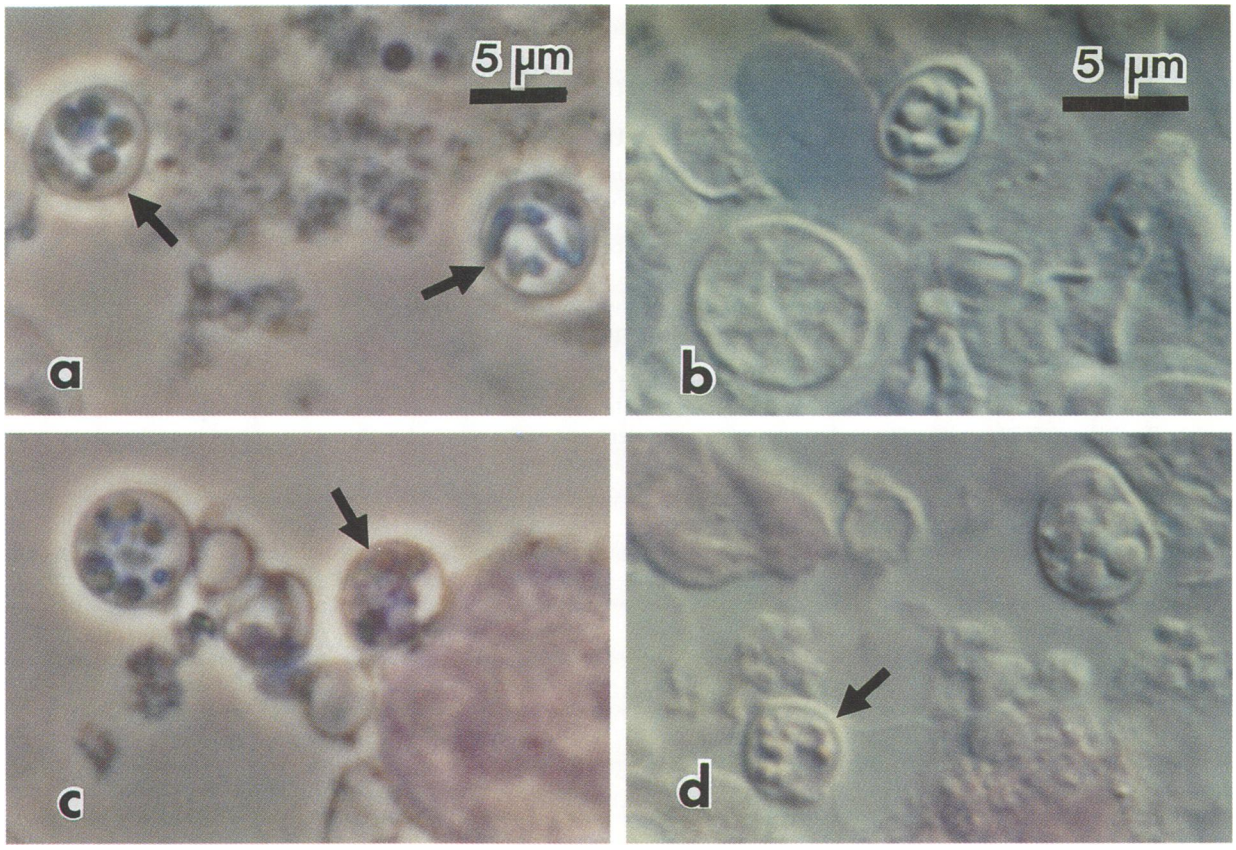

FIG. 1. Fresh preparations of *P. carinii* from a lung homogenate. (a) Unstained preparation viewed by phase-contrast microscopy, showing two cysts (arrows). The blueish cast of the intracystic bodies is an optical artifact. (b) Preparation containing trypan blue added as an indicator of viability viewed by NIC. A cyst (upper middle) and a host cell (lower left) exclude the dye, which has been taken up by another host cell nucleus between them (upper left). (c) Preparation kept in erythrosin B for 21 h at room temperature and viewed by phase-contrast microscopy. A dead cyst (arrow) has taken up the stain, while the nearby cyst (upper left) has excluded it. The host cell adjacent to the nonviable cyst is a useful color guide because other material in the cyst partially obscures the pink-staining intracystic bodies. Magnification scale as shown in panel a. (d) Preparation containing erythrosin B viewed by NIC, showing one cyst that has taken up the stain (arrow) and one that has excluded the dye (upper right). Magnification scale as shown in panel b.

solution of 0.5% Triton X-100–0.9% Giemsa stain was made by mixing 1 part of 2% (vol/vol) Triton X-100 in distilled water with 3 parts of Giemsa stock.

To stain a preparation of *P. carinii*, 100 μl of Triton X-100–Giemsa stain was added to 500 μl of the organism preparation to give a 1/6 dilution and a final concentration of 0.08% (vol/vol) Triton X-100–0.15% (wt/vol) Giemsa. About 5 to 8 μl of stained material was placed on a clean glass slide and covered with a 22-mm^2 cover slip.

RESULTS

The appearances of *P. carinii* organisms in fresh lung homogenates and tissue culture supernatants were identical. The cyst forms were easily identified by their characteristic morphology and intracystic bodies when viewed by phase-contrast microscopy (Fig. 1a) and NIC microscopy (Fig. 1b). Viable cysts could be distinguished from nonviable cysts in these preparations by the exclusion of erythrosin B and trypan blue dyes (Fig. 1b, c, and d). Erythrosin B provided better contrast than did trypan blue and hence made interpretation easier. The cystic form of *P. carinii* was used to illustrate the effects of vital staining because this stage is immediately recognizable to investigators and could be most helpful in a diagnostic situation. However, viable intermediate and trophic forms excluded the stains, and nonviable forms incorporated the dyes when examined within 5 min of dye addition.

Trophic forms in fresh preparations appeared as rounded, grapelike clusters by NIC microscopy (Fig. 2a). When this technique was applied to fixed and stained smears, the organisms had a more flattened and amorphous appearance (Fig. 2b). NIC microscopy also gave a clear-cut distinction between cysts with intracystic bodies and empty cysts in fresh preparations (Fig. 2c). On fixed and stained smears, the typical morphologic characteristics of cysts (e.g., oval shape) were still discernable (Fig. 2d).

In the course of these studies, a variety of developmental forms in the life cycle of *P. carinii* were observed in fresh preparations. Specific identification and quantitation of these stages were difficult by phase-contrast or NIC microscopy even when vital dyes were used. Application of stains such as Giemsa or aqueous methyl green was unsatisfactory because these reagents did not stain the cyst forms well. This problem was overcome by combining the stain with the detergent Triton X-100. When examined by bright-field microscopy with oblique illumination, different stages of *P. carinii* in fresh preparations stained with Triton X-100–Giemsa could be clearly differentiated (Fig. 2e). It was

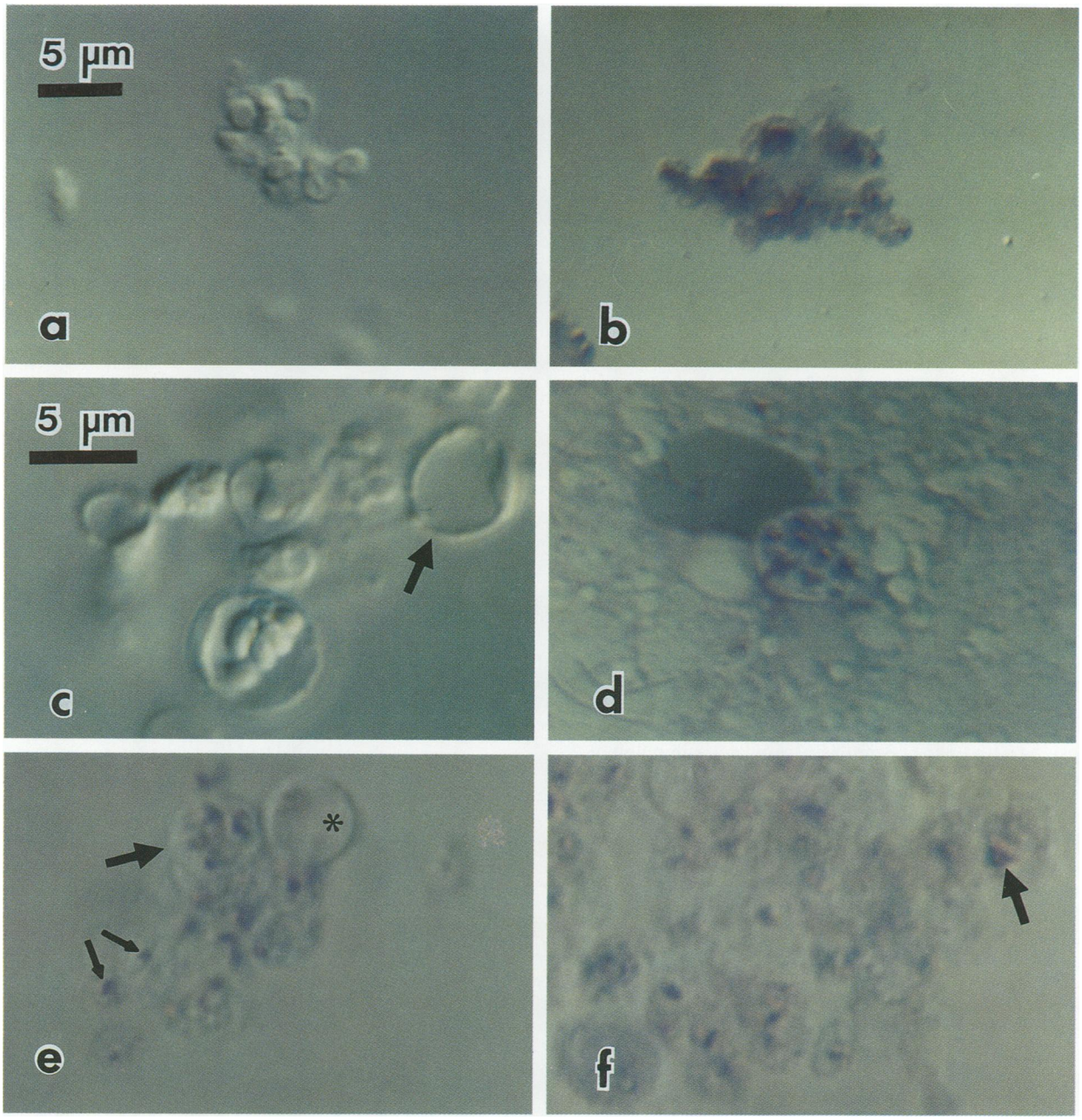

FIG. 2. Unstained and stained preparations of *P. carinii*. (a) Unstained trophic forms from a culture supernatant viewed by NIC. (b) Diff-Quik-stained smear of trophic forms from a culture supernatant viewed by NIC. Magnification scale as shown in panel a. (c) Unstained preparation of culture supernatant viewed by NIC, showing a mature cyst with intracystic bodies and an empty cyst (arrow). (d) Diff-Quik-stained smear of a cyst from a lung homogenate viewed by NIC. Magnification scale as shown in panel c. (e) Lung homogenate preparation stained with Triton X-100–Giemsa and viewed by oblique bright-field illumination by using a polarizing filter and by having the stage diaphragm partly stopped down. A mature cyst (large arrow), empty cyst (asterisk), and trophic forms (small arrows) can be clearly distinguished by this method. Magnification scale as shown in panel a. (f) Same preparation as described in panel e showing a clump of trophic forms, including a large trophic form (arrow). Magnification scale as shown in panel a.

also possible to count the trophic forms within clumps by focusing up and down on the field (Fig. 2f).

DISCUSSION

Clinical and investigative interest in *P. carinii* has long focused on the morphological and staining properties of the organism. The most common type of study has involved staining of lung imprint smears or tissue sections to improve the rapidity of diagnosis (10, 19, 21, 28). Stains (e.g., methenamine silver, toluidine blue O, cresyl echt violet, and Gram Weigert) which selectively stain the wall of the cyst have been popular because they provide easy identification of this form of the organism (3, 4, 29). Giemsa, polychrome methylene blue, Diff-Quik, Gram and Wright stains all stain the internal contents of the cystic and the trophic forms but require experience for proper interpretation (7, 9, 12, 18).

Scanning, freeze-fracture, and transmission electron microscopy have been used to examine the *P. carinii* life cycle, taxonomy, and interaction with host cells (2, 22, 37). Immunologic techniques used to evaluate the antigenic characteristics of *P. carinii* have included immunofluorescence and immunoperoxidase (15–17, 36). In general, these studies have used fixed specimens.

The present study has focused on fresh specimens and has employed three light microscope techniques. Phase-contrast microscopy, which is widely available in clinical microbiology laboratories, provides rapid identification of *P. carinii*.We have used phase-contrast microscopy to monitor the course of *P. carinii* growth in tissue cultures (4a). When combined with a vital dye, phase-contrast microscopy provides an excellent means to assess organism viability. Phase-contrast microscopy has been used by several authors in investigative work with *P. carinii* (20, 27, 33, 38) but has not gained widespread application. One disadvantage is a halolike effect around objects (especially cysts) which limits detailed examination of their morphology.

NIC microscopy is a well-established technique, but there is little published information about its use in the study of *P. carinii*. It shows more detailed features of organism morphology than does phase-contrast microscopy and is not hampered by optical artifacts such as the halo effect. However, NIC microscopy requires specialized equipment which may limit its application.

Bright-field microscopy with oblique illumination provides a view of *P. carinii* similar to that obtained with NIC microscopy. Since no special equipment is needed, bright-field microscopy with oblique illumination should be easily adaptable to most laboratories.

Working with fresh specimens permits determination of *P. carinii* viability. Exclusion of vital dyes such as erythrosin B or trypan blue has been a popular technique because these agents are easy to use (1, 6). We have favored erythrosin B, but the selection of a specific vital dye is largely a matter of personal preference. Uptake of dyes such as acridine orange and neutral red has been used by some authors (25, 32), but we encountered difficulties in interpreting our results with these methods. Recently, fluorescein diacetate and ethidium bromide have been successfully used to determine the viability of *Leishmania* spp. and other protozoa (13); a preliminary report suggests that they might also be applied to *P. carinii* (M. S. Bartlett, M. Durkin, J. Piskura, and J. W. Smith, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 619, 1984). Studies such as these require fluorescence microscopy to detect color changes and phase-contrast microscopy to confirm that the structures taking up the dye are the organisms in question. This may be difficult with lung homogenates which contain considerable tissue debris.

Fresh specimens of *P. carinii* can be used to provide an accurate measurement of organism growth. We have developed a method of quantitating *P. carinii* in lung homogenates and tissue culture supernatants which is based on counting organism nuclei in fixed and Diff-Quick- or Giemsa-stained preparations (4a, 6). While this system provides a reliable overall assessment of *P. carinii* replication, it does not provide data about the specific stages in the life cycle of the organism. Such information can only be obtained by direct microscopic examination of fresh specimens. The use of the Triton X-100–Giemsa stain facilitates this process, and preliminary data in our laboratory indicate that differential counting of the developmental stages of *P. carinii* is possible.

Since rat *P. carinii* is morphologically indistinguishable from human *P. carinii*, the techniques developed in this study are useful for clinical applications. Several reports have shown that *P. carinii* can be demonstrated by histologic stains in the bronchoalveolar lavage fluid in the vast majority of AIDS patients with pneumocystis pneumonia (23, 24, 31). However, problems in processing, staining, or interpreting the specimens might occur which would result in delays in establishing the diagnosis. The ability to examine lavage fluid by one of the light microscopy techniques presented here may be helpful as an adjunct method when controversy still exists after established diagnostic methods have been used.

Treatment of *P. carinii* pneumonia in AIDS patients is characterized by a slow response, a high rate of recurrence, and the continued presence of organisms in bronchoalveolar lavage fluid even after several weeks of drug administration (8, 11, 14). Since the clinical significance of these persisting organisms is unclear, it has been difficult to formulate precise guidelines for the duration of treatment. A determination of which forms of *P. carinii* are persisting and of their viability would be a helpful first step in addressing this problem.

In conclusion, the high frequency of *P. carinii* pneumonia has presented new clinical challenges and opportunities for laboratory investigation. Traditional methods have mainly employed fixed and stained preparations. The analysis of fresh specimens as described in this report can provide important information about the presence, viability, growth, and life cycle stages of *P. carinii* for both clinical and research applications.

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