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Quantification and Assessment of Viability of *Pneumocystis carinii* Organisms by Flow Cytometry

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Analysis of drug efficacy in animal models of *Pneumocystis carinii* pneumonia requires an accurate method of quantification of organisms, as well as a means of assessing viability. Lung homogenates were prepared from a colony of athymic nude F344 rats experiencing a spontaneous outbreak of *P. carinii* pneumonia. With the fluorescent nucleic acid stain propidium iodide, flow cytometric analysis was able to quantify *P. carinii* cysts and trophozoites reproducibly. As this stain is excluded by living cells, this method was also used to assess the viability of organisms. Application of this technique to analysis of bronchoalveolar lavage specimens was demonstrated.

Pneumocystis carinii is a common opportunistic pathogen in patients with AIDS, as well as other immune defects, and in patients receiving immunosuppressive therapy (12, 16). Progress in studying this organism has been hampered by the difficulties encountered in its in vitro culture (6). A rapid, accurate method for quantification of *P. carinii* in lung homogenates and tissue culture specimens would be of considerable scientific and clinical value. In vitro and in vivo investigations of chemotherapeutic agents and studies of the life cycle and growth kinetics of the organism would be significantly facilitated (3, 16). Currently, quantification is achieved by manual counting of cysts (13, 17), trophozoites (1), or nuclei (10). Although these techniques enable changes in the number of organism to be documented, they are time consuming, require experienced personnel, and are inaccurate for quantification of aggregated organisms. In addition, they are insufficiently sensitive to exclude definitively the presence of residual organisms in animals which have undergone successful chemotherapy of *P. carinii* pneumonia (10). The most effective method for assessment of viability of *P. carinii* is by erythrosin B dye exclusion (7). This method appears to be most accurate when applied to the cyst form but considerably less so for trophozoites, particularly when clumping occurs.

Identification of *P. carinii* cysts by flow cytometry was originally described by Libertin and colleagues in 1984 (11), but the technique appears to have found little application in pneumocystis research (8, 11). This may relate to the small size of the organisms compared with that of eukaryotic cells and their tendency to aggregate in suspension. In the present study, we attempted to overcome these difficulties by assessing size and nucleic acid content on logarithmic scales and by developing a method of quantification which analyses individual as well as aggregated organisms. On the other hand, the flow cytometer has the significant advantage of rapid, quantitative analysis of large numbers of cells in a highly reproducible manner. The ability to evaluate multiple physical and biological properties of individual cells by means of various fluorescent stains and monoclonal antibodies may enhance flow cytometric analysis of *P. carinii* with monoclonal antibodies that have been developed (12).

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MATERIALS AND METHODS

Experimental animals. *P. carinii* organisms were obtained from the lungs of athymic nude rats (F344 *nu/nu*) from a colony experiencing a spontaneous outbreak of *P. carinii* pneumonia. The rats were maintained ad libitum on a normal diet and received no drug therapy. After the rats were killed by halothane anesthesia overdose, the lungs were used either for extraction of *P. carinii* or to obtain bronchoalveolar lavage (BAL) fluid. The presence of *P. carinii* infection was confirmed in each animal by microscopic examination of impression smears of lung tissue stained with RapiDiff (Clinical Sciences Diagnostics, Booysens, South Africa). Control animals were hairy F344 rats lacking the mutation which showed no evidence of *P. carinii* pneumonia.

Preparation of *P. carinii* specimens. Purified rat lung homogenates and BAL fluid specimens were prepared for analysis by flow cytometry.

(i) Lung homogenates. Two methods of processing lung tissue were used. Initial studies to develop a flow cytometer program capable of identifying the organism required a highly purified preparation. A method based on that described by Walzer and coworkers was used (18). The lungs were homogenized in 20 ml of phosphate-buffered saline in an Ultra-turrax homogenizer (Janke and Kunkel, Staufen, Federal Republic of Germany) for 30 s and passed through sterile gauze. After centrifugation at 1,000 × *g* for 15 min, the pellet was suspended in an ammonium chloride solution (0.16 M ammonium chloride, 0.1 M sodium bicarbonate, 0.1 mM EDTA) to lyse erythrocytes. Enzymatic digestion of the lung tissue with 0.1% collagenase (Sigma Chemical Co., St. Louis, Mo.) and 0.1% hyaluronidase (Sigma) for 60 min at 37°C was followed by several washes in Hanks balanced salt solution containing 1 mM EDTA. The suspension was then filtered through a 10-μm-pore-size Mitex filter (Millipore Corp., Bedford, Mass.).

Partial separation of cysts and trophozoites was achieved by density gradient centrifugation in a discontinuous gradient of 2, 6, and 10% Ficoll (Sigma) in 16% Hypaque made up in Hanks balanced salt solution with 1 mM EDTA. Centrifugation was at 600 × *g* for 30 min. A trophozoite-enriched suspension was obtained from the upper (2%) layer, and a cyst-enriched specimen was obtained from the interface between the 6 and 10% phases. This was verified by micros-

copy of appropriately stained samples. These specimens were used only to confirm that both cysts and trophozoites were being counted on the flow cytometer, and quantitative assessment of the separation procedure was not performed.

Once the flow cytometer parameters had been set and the *P. carinii* population had been identified, a more rapid method for preparation of lung homogenates was used for quantitative studies. Homogenization and ammonium chloride lysis of erythrocytes were performed as described above, but the enzyme digestion step was eliminated. This was followed by hypotonic lysis of host cells by incubation in a solution of 40 mM KCl–25 mM NaCl. The homogenate was then washed several times in Hanks balanced salt solution and passed through a 90-μm-pore-size nylon mesh and then a 10-μm-pore-size Mitex filter.

(ii) BAL. BAL fluid was obtained from rats by tracheostomy via an anterior neck incision. A 16-gauge plastic cannula was inserted in the trachea, and the lungs were lavaged with four 5-ml volumes of normal saline. Approximately 15 ml of lavage fluid was recovered from each rat. This was passed through a 90-μm-pore-size mesh prior to flow cytometry.

(iii) Microscopy. All homogenates and lavages were assessed for the presence of *P. carinii* by microscopy after staining with RapiDiff, a Wright-Giemsa variant commonly used for staining trophozoites (2, 15), and with a modified toluidine blue O stain for cysts (5).

(iv) Fixation and fluorescent staining. Specimens for quantification were fixed with 70% methanol for 30 min or 1/20 of a volume of buffered Formol-saline for 12 h, whereas viability studies were performed on unfixed specimens.

The organisms were stained in a phosphate-buffered saline suspension with propidium iodide at a concentration of 20 μg/ml 15 to 120 min prior to flow cytometry. Propidium iodide produces red fluorescence on excitation at 488 nm, the intensity of which correlates with the nucleic acid content of the cell (14).

Flow cytometry. All samples were analyzed by using an Epics Profile flow cytometer (Coulter Electronics, Hialeah, Fla.) with an argon laser tuned to 488 nm. The instrument was calibrated by using Immuno-Check polystyrene fluorospheres (Epics Division, Coulter Electronics) prior to each study. The same log amplification and photomultiplier tube voltage values were used throughout. The sample volume of each specimen analyzed on the flow cytometer was 50 μl. To confirm the reproducibility of the volume of each sample aspirated and to ensure adequate mixing of the suspension, 40 μl of Immuno-Check polystyrene fluorospheres per ml was added to each specimen and counted simultaneously with the *P. carinii*.

Because of the small dimensions of the organisms studied, a logarithmic scale of forward scatter (LFS) was used in size assessment. Red fluorescence was also presented on a logarithmic scale (LFL), and the photomultiplier tube voltage was set so that the fluorescence of the Immuno-Check polystyrene fluorospheres fell within the last channel of the fluorescence scale. This voltage was higher than that used for eukaryotic cells but allowed the most sensitive assessment of *P. carinii* organisms. All data were stored electronically for subsequent analysis.

RESULTS

Identification of *P. carinii* organisms. The organisms were identified by means of propidium iodide fluorescence on a flow cytometer histogram of LFS (size) against LFL (nucleic acid content). To identify the regions on the histogram representing *P. carinii* organisms and host (rat) cells, the following samples were analyzed: (i) lung homogenates from a *P. carinii*-infected rat; (ii) lung homogenates from an uninfected rat; (iii) the supernatant after centrifugation of a *P. carinii* suspension; (iv) rat peripheral blood mononuclear cells prepared by Ficoll-Hypaque centrifugation (4).

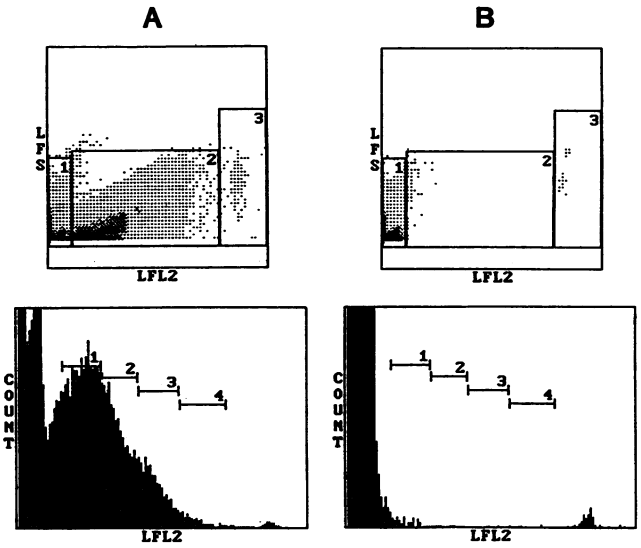

FIG. 1. Flow cytometric histograms of rat lung homogenates. Purified lung homogenates from a *P. carinii*-infected F344 *nu/nu* rat (A) and an uninfected rat (B) were stained with propidium iodide and analyzed by flow cytometry. The upper histograms plot LFS, representing size, against LFL (LFL2), indicating nucleic acid content. Windows were set to demarcate electronic interference (window 1) from *P. carinii* organisms (window 2) and host cells (window 3). The lower histograms plot particle counts against LFL. Gates 1 to 4 were used to quantitate the *P. carinii* load. The fluorescent population in panel A represents *P. carinii* trophozoites, aggregates, and cysts. The only fluorescent particles observed in specimens from normal rat lung tissue (B) are compatible in nucleic acid content with host (rat) cells. The high number of counts on the extreme left of each histogram represents electronic interference.

The borders of these regions were defined on the flow cytometer program by creating three partitioning windows on the LFS-LFL histogram (Fig. 1). The signals detected were accounted for by background electronic interference (window 1), *P. carinii* organisms (window 2), and rat cells (window 3). The histogram enabled quick visual assessment of the presence of *P. carinii*. The diffuse, upwardly sloping nature of the *P. carinii* population on the LFS-LFL histograms suggests clumping of the organisms (Fig. 1A).

Although the trophozoite-enriched specimen tended to have less forward scatter and fluorescence than the cyst-enriched suspension, distinct cell populations could not be distinguished. It is noteworthy, however, that both trophozoites and cysts were counted.

Flow cytometry of BAL specimens demonstrated that *P. carinii* organisms could be readily identified, although they occurred in smaller quantities (Fig. 2A). The window 3 cells observed on the analysis of BAL specimens from both *P. carinii*-infected and normal rats, with sizes and nucleic acid contents similar to those of rat peripheral blood mononuclear cells, almost certainly represent a population of alveolar macrophages and pneumocytes (Fig. 2A and B).

Quantification of *P. carinii* organisms. Quantification by

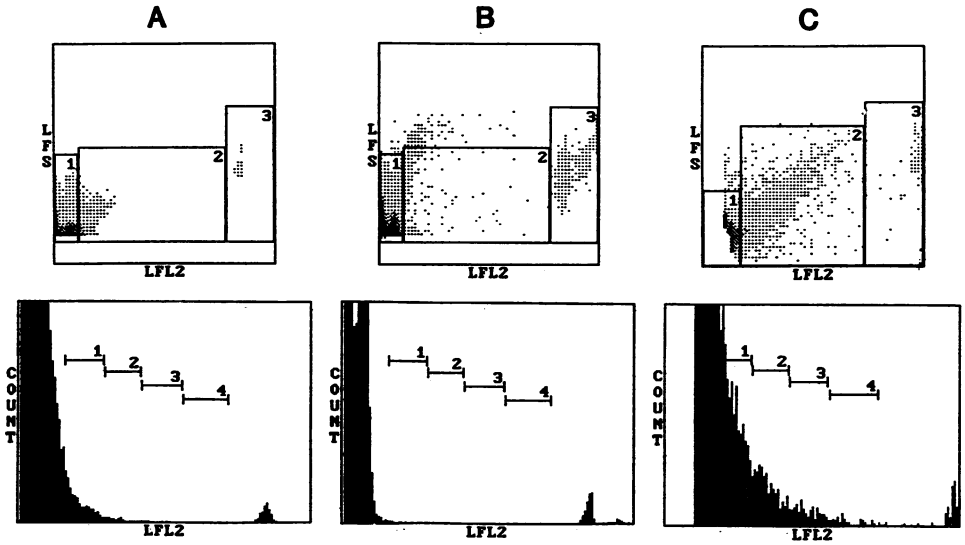

FIG. 2. Flow cytometry of propidium iodide-stained BAL specimens. The upper histograms represent LFS (size) in relation to LFL (nucleic acid content; LFL2). The lower histograms plot particle counts against nucleic acid contents. Windows and gates were set as described in the legend to Fig. 1. (A) BAL from a rat with *P. carinii* infection. (B) BAL from a normal rat with no evidence of *P. carinii* infection. (C) BAL obtained from a human subject with proven *P. carinii* infection. During diagnostic bronchoscopy, BAL was performed with 100 ml of normal saline and a sample was processed for flow cytometry as described for rat BAL specimens. The particles with high fluorescence on the extreme right of the histogram represent host (rat or human) alveolar cells, with human cells demonstrating greater nucleic acid content than rat cells.

fluorescent particle counting of window 2 cells on the LFS-LFL histogram resulted in a wide degree of variation of results on repeated analysis of the same sample. This was presumed to relate to the degree of aggregation and the inability to distinguish individual trophozoites from aggregates of trophozoites or cysts accurately.

To obtain a value corresponding to the total *P. carinii* organism count, a weighting system was developed to correct for single particles with high nucleic acid contents, viz., aggregated trophozoites or cysts. A flow cytometer histogram plotting counts versus LFL (count-LFL histogram) was used. Four discrete ranges of intensity of fluorescence were defined on the basis of minor peaks of counts noted on several count-LFL histograms obtained from specimens of *P. carinii*. Gates were demarcated on the flow cytometer program, allowing the counts within each separate range of fluorescence to be analyzed (Fig. 1 and 2, lower histograms). Empirically, these levels of fluorescence were designated to represent single trophozoites, doublets, and aggregates of four and eight fluorescent *P. carinii* nuclei, respectively. Particle counts for each gate were multiplied by the appropriate factor (1, 2, 4, or 8), and the sum was used to represent the total *P. carinii* load, which would include all forms of the organisms, thereby obtaining a value expressed as nuclei per milliliter. As rat lung specimens were weighed before homogenization and purified in standardized volumes, the *P. carinii* load could be expressed as nuclei per gram (wet weight) of lung tissue.

A comparison of the reproducibility of quantification by simple particle counts and the weighted correction method is given in Table 1. *P. carinii* samples were analyzed both before and after procedures to diminish clumping of trophozoites, namely, (i) addition of EDTA (1 mM) and (ii) syringing through a fine-bore needle. The weighted correction method was superior, and with standardization of the procedures to reduce aggregation, excellent reproducibility was achieved. This was confirmed by analysis of a *P. carinii* suspension in different dilutions (Fig. 3).

Viability. Propidium iodide is a positively charged fluorescent stain which does not enter intact cells. This feature has resulted in its use in the assessment of membrane integrity of cells, which correlates to a large extent with viability (14). The alteration of cell membrane proteins by fixation of cells allows entry of this stain.

Three samples of a fresh *P. carinii* specimen were analyzed (Fig. 4). (i) As a control for the concentration of organisms in the homogenate, a sample was fixed with 1/20 of a volume of buffered Formol-saline for 60 min prior to staining with propidium iodide. Quantification by the method described above demonstrated that this specimen contained

TABLE 1. Reproducibility of flow cytometric methods of quantification of *P. carinii*

Quantification method[a]	Mean count	Coefficient of variation (%)
Particle counting	3.27×10^5	22.9
Particle counting with standardized syringing through a fine-bore needle and addition of EDTA	4.13×10^5	9.5
Particle counting with correction for higher-fluorescence particles	9.84×10^5	14.4
Particle counting with correction for higher-fluorescence particles and with standardized syringing through a fine-bore needle and addition of EDTA	1.09×10^6	4.4

[a] Five samples were tested by each method.

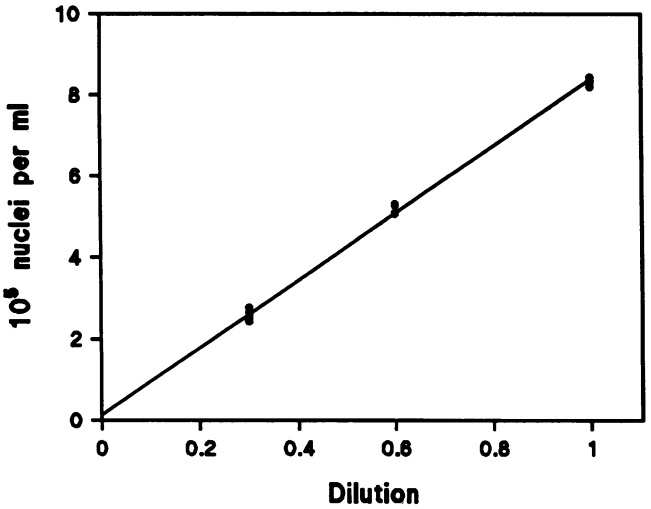

FIG. 3. Reproducibility of flow cytometric quantification of *P. carinii*. Organisms purified from lung homogenate were diluted, stained with propidium iodide, and quantified as described in the text. Four closely overlapping data points are shown for each dilution.

7.18×10^6 stained nuclei per ml. (ii) An unfixed specimen was stained with propidium iodide (20 μg/ml) to evaluate the quantity of nonviable organisms. Quantification demonstrated that 4.36×10^5 nuclei per ml (6.1% of the total concentration) took up the propidium iodide. This suggests that 93.9% of the organisms in the fresh specimen were viable. (iii) The third sample was exposed to an arbitrary heat inactivation regimen (75°C for 15 min) and then stained similarly. A decrease in viability to 78.8% was noted after this heat treatment.

DISCUSSION

This report describes a method for accurate quantification and assessment of viability of *P. carinii* cysts and trophozoites with a flow cytometer. Organisms from lung homogenates and BAL fluid were stained nonimmunologically with propidium iodide, a fluorescent intercalating stain for double-stranded nucleic acid. Identification of these *P. carinii* organisms on a flow cytometer was achieved by plotting particle size against fluorescence on a flow cytometer histogram and by using partitioning windows to delineate the region representing organisms. Negative controls, namely, a lung homogenate from an uninfected rat and the supernatant after centrifugation of a *P. carinii*-containing homogenate, were used to confirm the presence of *P. carinii*. Contaminating nucleated host cells were clearly differentiated from *P. carinii* by their greater nucleic acid content, while erythrocytes do not contain double-stranded nucleic acid and therefore do not fluoresce.

The intensity of fluorescence was used as a marker of the number of trophozoites in an aggregate passing the flow cytometer laser, enabling counting of all of the *P. carinii* organisms in the sample. Exclusion of the stain by living cells permits, in addition, a simple method for assessment of the viability of the organisms in a specimen. This provides an accurate, automated method for both quantification and viability assessment of *P. carinii* trophozoites and cysts.

We feel that the primary application of flow cytometry of *P. carinii* is in research and do not advocate its use in the routine diagnosis and management of *P. carinii* pneumonia. The initial use of the technique described may be in the study of lung homogenates from laboratory animals as part of trials of various antipneumocystis agents. Flow cytometric analysis could be compared with standard methods of quantification and viability testing. Currently available antipneumocystis chemotherapeutic agents do not appear to differ markedly in clinical efficacy (12), but a number of newer agents are under investigation. Assessment of viability, in addition to quantification of organisms in lung tissue, may be of great value in the more accurate evaluation of these new agents. In addition, the significance of residual organisms found in the lungs after apparently successful treatment of *P. carinii* pneumonia is uncertain (9) and assessment of the viability of these organisms may aid in the development of agents capable of inhibiting recurrent infections.

Analysis of BAL specimens may be useful for serial follow-up of treated human subjects, but further study and experience are necessary to overcome the greater tendency of human-derived *P. carinii* organisms to aggregate (3). Moreover, identification by flow cytometry of other organisms (e.g., *Candida albicans*) frequently found in immunocompromised patients will be necessary. Other potential research roles for flow cytometric analysis of *P. carinii* include analysis of the ploidy of *P. carinii* organisms and life cycle studies.

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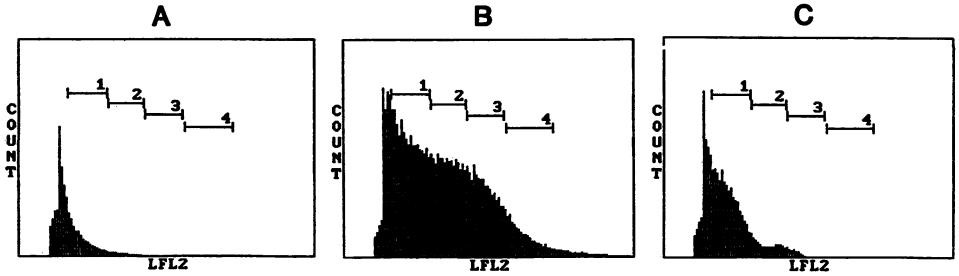

FIG. 4. Viability of *P. carinii* assessed by flow cytometry. A suspension of *P. carinii* was stained with propidium iodide, which is excluded by organisms with an intact cell membrane. Quantification of fluorescent organisms by the weighted correction method was performed on a fresh suspension of *P. carinii* demonstrating few fluorescent (nonviable) organisms, which yielded a viability of 93.9% (A); Formol-saline-fixed control specimen used to assess total organisms (B); and a sample exposed to 75°C for 15 min, demonstrating 78.8% viability (C).

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