Analysis of *Pneumocystis carinii* Cysts with a Fluorescence-Activated Cell Sorter

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Human sera from *Pneumocystis carinii*-infected patients and specific rabbit antisera have antibodies against the cyst form of the organism. Lung tissue concentrations from cortisone-treated C3H/HeN mice and six open lung biopsy-positive patients were centrifuged and suspended, and immunofluorescent staining was done. We utilized the fluorescence-activated cell sorter to analyze and sort *P. carinii* cysts from lung homogenates into a morphologically distinct population. A quantitative basis was used for the definition of the cyst population by displaying the frequency of cells as a function of parameter (fluorescence intensity and light scatter) expression.

In 14 of 15 histogram analyses, *P. carinii*-infected homogenates were differentiated from normal- and bacterial-control homogenates. The parameter range of light scatter (size) was 2 to 8 μm, and the fluorescence intensity was greater than a threshold based on the histogram profile.

The fluorescence-activated cell sorter (FACS) is a sophisticated, computerized laser illumination system that is used both for analysis and for separation of cells in suspension (1, 4, 6). It rapidly measures the size and fluorescence intensity of individual cells as they flow in a stream past a laser illumination system coupled with a highly sensitive detector. The measurements, collected and displayed, create an analytical profile of cell populations. Differential light scatter patterns have been used to identify bacteria (17) and virus-infected cells (2). A wide range of applications includes measurement of the DNA content of individual chromosomes for karyotyping (3) as well as the amount of surface immunoglobulin on B cells (9).

We utilized the FACS to analyze and sort *P. carinii* cysts from lung homogenates into a morphologically distinct population. A quantitative basis was used for the definition of a cyst population by displaying the frequency of cells as a function of parameter (fluorescence intensity and light scatter) expression.

This technique may provide a method of determining parasite counts in cell procedures as well as of isolating the organism for life cycle studies.

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MATERIALS AND METHODS

Experimental animals. C3H/HeN mice, weighing 90 to 120 g each, were used in all experiments. The animals were randomly assigned to control or treatment groups and maintained ad libitum on a low protein (8%) diet (ICN Nutritional Biochemical, Cleveland, Ohio). Cortisone acetate injections were given intraperitoneally (1 mg twice weekly) during weeks 3 through 9. Tetracycline (1 mg/ml) in drinking water was given to all groups. This protocol has been shown by ourselves and by others (15, 16) to induce *P. carinii* lung infections. Mice were sacrificed by cervical dislocation at week 9 and autopsied immediately. Each lung was removed, and impression smears were prepared on glass slides which were stained with methenamine silver to assure the presence of *P. carinii* cysts (5, 7).

Patients. Six patients at the Mayo Clinic during this time period had *P. carinii* cysts on impression smears from open lung biopsy (OLB) specimens. Tissue samples and sera were obtained from these individuals. In addition, six OLB patients with only bacterial infections and six with negative microbiological tests (i.e., negative cultures for virus, fungi, *Chlamydia* spp., *Mycoplasma* spp., and other bacteria) were used as control specimens.

Preparation of lung specimens. Each lung tissue was homogenized in 2 ml of nutrient broth by blending for 2 min in a stomacher (Tekmar Co., Cincinnati, Ohio) (12). The homogenate was centrifuged for 20 min at 800 × g. The concentrate was then incubated for 45 min at room temperature with the pooled human sera from *P. carinii*-infected patients or specific rabbit antisera against *P. carinii* obtained from Linda Pfizer, University of Tennessee, Memphis. Fluorescein-labeled goat anti-human or goat anti-rabbit immunoglobulin sera (Cappel Laboratories, Cochranville, Pa.) were incubated with the concentrate for 45 min followed by two washings with phosphate-buffered saline, pH 7.2. The suspension was immediately analyzed with the FACS.

Reagents. Sera were obtained from patients with OLB-confirmed *P. carinii* infection and from normal control humans. To determine the presence and titer of antibody to *P. carinii* in sera, an indirect immunofluorescence test was done. Touch impression smears of *P. carinii* were overlaid with dilutions of the sera to be tested. After incubation at room temperature for 30 min in a humid chamber and washings with phosphate buffered saline to remove nonbound antibodies, the smears were stained with fluorescein-labeled anti-human immunoglobulin G globulin (Cappel Laboratories). The titer of antibody in the serum was established as the reciprocal of the highest dilution which resulted in at least 2+ fluorescence of *P. carinii* cysts. The average titer of sera from OLB patients with *P. carinii* was 1:32, and that of sera from normal control humans was 1:8. All of the six normal human control sera demonstrated anti-*P. carinii* antibodies. Pfizer et al. (11) and Norman and Kagan (10) similarly demonstrated the presence of anti-*P. carinii* antibodies in normal human sera.

Fluorescein-conjugated reagents were stored at 4°C under...
sterile conditions to prevent formation of aggregated materials. Just before staining, all reagents were centrifuged at 100,000 × g for 20 min to remove any remaining aggregated material that could interfere with analysis.

**Instrument.** All samples were analyzed under the same conditions by a FACS IV cell sorter (Becton Dickinson and Co., Paramus, N.J.). The homogenate of cells was forced under pressure through a micronozzle, in which the cells were centered in the effluent jet by an outer coaxial flow of cell-free fluid. The stream, which was ca. 50 μm in diameter, was illuminated by an argon-ion laser (model 164: Spectra-Physics, Palo Alto, Calif.) immediately after leaving the nozzle. The laser beam was focused on the stream with a spherical lens having a focal length of 124 mm. The noise in the fluorescence channel was decreased with a dual-channel mode of operation. The fluorescence channel was activated only when a scatter signal indicated that the cell was present (scatter-gated fluorescence). The reciprocal operation, fluorescence-gated scatter, was performed so that the scatter signal was not accepted unless the fluorescence pulse was greater than a specified threshold of intensity.

Light for the scattering channel was collected at a forward angle of 0.5 to 12% from the incident beam. The fluorescence detector was a photomultiplier tube. The filters used in the fluorescence channel for fluorescein emission included two cut-on filters (520.0 and 530.0 nm; series D; Ditric Optics, Inc., Marlboro, Mass.). Fluorescein was excited by the 488-nm line of the laser, which was operated at a 400-mW output. Cells were analyzed at a rate of 500 to 1,000/s.

Before each experiment, the FACS was adjusted to give the same scatter and fluorescence profiles with a standard preparation of glutaraldehyde-fixed chicken erythrocytes. Parallel analyses of normal, bacterial-infected, and *P. carinii*-infected lung homogenates were done. For each homogenate, dot plots and histograms were obtained and compared with one another. Sorted cells were placed on microscope slides, fixed in methanol, and stained with methenamine silver stain for cytological examination.

**RESULTS**

Various reagents were reacted and assayed by indirect immunofluorescence techniques against five different lung homogenates to confirm antibody specificity (Table 1). Sera from *P. carinii*-infected patients and rabbit anti-*P. carinii* sera revealed significant immunofluorescence within the lung tissue. When stained with methenamine silver, the immunofluorescent organisms were recognized as typical *P. carinii* cysts. Pooled normal human sera demonstrated anti-*P. carinii* antibodies but at lower titers (1:8) than these antibodies were demonstrated in sera from *P. carinii*-infected patients (1:32) or in rabbit anti-*P. carinii* sera.

This serological reactivity in normal humans has been demonstrated by others (10, 11, 13) and is consistent with the omnipresent nature of this opportunistic pathogen.

The negative controls of normal mouse and normal human lungs failed to show immunofluorescence of the lung tissue. Bacteria-infected human lung homogenates were also used as a control to show that staining was specific and not secondary to an inflammatory cell response. These bacteria-infected lungs failed to demonstrate immunofluorescence in all experiments, indicating that the immunofluorescence in *P. carinii*-infected tissue was not due to other opportunistic pathogens, bacteria, or a nonspecific host reaction. The presence of specific fluorescent antibodies enabled us to separate these organisms into a distinct population of cells by the FACS.

### Table 1. Results of treatment of lung homogenates with various reagents and assay of these treated homogenates by indirect immunofluorescence

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>No. of positive samples/no. tested when treated with</th>
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<tbody>
<tr>
<td></td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Normal mouse lung</td>
<td>0/9</td>
</tr>
<tr>
<td><em>P. carinii</em>-infected mouse lung</td>
<td>0/9</td>
</tr>
<tr>
<td>Normal human lung</td>
<td>0/6</td>
</tr>
<tr>
<td>Bacteria-infected human lung</td>
<td>0/6</td>
</tr>
<tr>
<td><em>P. carinii</em>-infected human lung</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Sera obtained from six patients with *P. carinii* pneumonia diagnosed by OLB. Titer was 1:32.
* Sera obtained from normal control patients. Titer was 1:8.
* Antisera obtained from L. L. Pifer, University of Tennessee, Memphis.

To show that the FACS was capable of identifying a subpopulation of cells representing *P. carinii* in mouse and human lung homogenates, homogenates treated with the various reagents listed in Table 1 were analyzed, and data were presented in two manners, the dot plot and composite histograms.

Dot plots, representative of the light scatter parameters and fluorescence intensity measurement, were taken for each cell in each homogenate after 10^6 cells were analyzed (Fig. 1). Each dot plot represents a cell at a given level of fluorescence and light scatter (size). The upper left quadrant of the dot plot represents an area of highly fluorescent small cells. The lower one-third of the dot plot represents low-fluorescent background materials, which are not *P. carinii* cysts. The blanked area represents the location of a scatter (size) threshold (gate) which allowed for analysis of cells larger than 2 μm. The dot plots of normal mouse (data not shown) and normal human lung homogenates (Fig. 1A) showed no specific distinctive population of cells that could be identified. Similarly, the dot plots of bacteria-infected lung homogenates did not reveal such a population of highly fluorescent cells (Fig. 1B). However, the dot plots of *P. carinii*-infected human lung specimens showed a population of cells that were brightly fluorescent and small (Fig. 1C). All *P. carinii*-infected mouse lung homogenates demonstrated dot plots with a detectable population of fluorescing cells. These dot plots provided a semiquantitative indicator of a population density present in *P. carinii*-infected homogenates.

By combining dot plots from designated homogenates, we obtained composite histograms. Histograms display the distribution of cells as a function of linear increase in parameter (size and fluorescence) for each sample. A composite histogram is comprised of two linear graphs in each diagram representing a given homogenate. In each histogram, the graph line farthest to the left is the normal human homogenate. If no area between the two graph lines exists or the graph lines are superimposed on one another, no distinct population of cells exists between the compared homogenates. However, if a histogram reveals a significant area between the two graph lines, then a distinct subpopulation of
cells exists in that homogenate which is not present in the control. All fluorescence histograms with the FACS were scatter gated. Fluorescence-gated scatter was performed so that the scatter signal was not accepted unless the fluorescence pulse was greater than a specified threshold of intensity. Cells were analyzed at a rate of ca. 500 cells per s.

Composite fluorescence histograms of normal human homogenates were obtained and compared with one another. No difference among the graphs, which would represent a cell population unique to a given homogenate, was found with each analysis (Fig. 2A). Likewise, normal and bacteria-infected lung homogenates, when analyzed by composite histograms, demonstrated no significant differences in graphs (Fig. 2B). These data are consistent with their respective dot plots and demonstrated a population of cells devoid of *P. carinii* cysts.

Composite fluorescence histograms of *P. carinii*-infected human homogenates revealed significant differences when compared with those of normal human homogenates in all but 1 of 15 instances (*P < 0.01; range, 95%; confidence level, 68.1 to 99.8%). This exception was a patient who had undergone OLB in which routine methenamine silver staining of touch preparations of the tissue failed to show the presence of *P. carinii* cysts. However, utilization of a tissue concentration method (15) revealed the presence of a few organisms in 15 fields examined at \( \times 200 \) magnification. Many other variables including the quantity of organisms per homogenate could have contributed to failure to identify this small population of cysts in the homogenate. Nevertheless, composite histograms of lung homogenates showed a distinct cell population of *P. carinii* cysts in infected samples and not in normal or bacteria-infected homogenates. A comparison of fluorescence histograms of a normal human lung homogenate and of a *P. carinii*-infected human homogenate is shown in Fig. 2C. The difference between the two composite graphs represents the population of fluorescent cells, *P. carinii* cysts, that are unique to the *P. carinii*-infected homogenate. With the scatter-gate set, this population of cells was reanalyzed to obtain a second histogram on the vertical axis showing the fluorescence distribution of cells within the size range of 2 to 8 \( \mu \text{m} \).

Consequently, these fluorescence histograms provided a quantitative basis for the definition of the population of cysts by displaying the frequency of cells in the population as a function of parameter expression. Last, the histograms allowed visualization of cell clustering within our restricted parameter range and light-scattering size of 2 to 8 \( \mu \text{m} \), and fluorescence intensity was greater than a threshold band on the histogram profile. When cells within this restricted parameter range were sorted and stained with methenamine silver, cytological findings were consistent for *P. carinii* cysts.

DISCUSSION

This investigation demonstrated that the FACS was capable of analyzing and sorting *P. carinii* cysts from lung homogenates of infected mice and humans. A quantitative basis was used for the definition of the cyst population by displaying the frequency of cells as a function of parameter (fluorescence intensity and light scatter) expression. The parameter range of light scatter (size) was 2 to 8 \( \mu \text{m} \), and the fluorescence intensity was greater than a threshold based on a histogram profile.

Application of FACS technology to *P. carinii* research would be beneficial. Analysis and sorting of cysts from lung homogenates would provide a means of separation based on immunofluorescent staining but would not interfere with their biological function. Homogenate samples in microliter volumes potentially could be analyzed at rates to 5,000 cells per s, at purities to 99%, and at recoveries to 90% (6). This method of single-cell analysis offers an expedient, quantitative measurement of individual cells such as *P. carinii* cysts within cell populations and has very high statistical precision. For example, FACS methodology would provide a means of determining parasite counts in cell culture procedures.

With the FACS system, it is also possible to determine the frequency distribution of cells of different sizes, i.e., trophozoites (2 to 4 \( \mu \text{m} \)) and cysts (2 to 8 \( \mu \text{m} \)). In addition, two fluorochromes, i.e., fluorescein and rhodamine, may be...
detected simultaneously at different wavelengths with the same cell. Since the trophozoites stain with auramine-rhodamine (14) and cysts stain with fluorescein, the FACS could potentially analyze and sort cysts containing the trophozoites for life cycle studies.

In any field of science, a powerful new method permits a new series of questions to be asked and answered. Similarly, the powerful tool of the FACS system may assist in parasitology research.

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LITERATURE CITED