In Vitro Differentiation of Human-Derived *Pneumocystis carinii*†

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Two major impediments to the development of an in vitro cultivation system for *Pneumocystis carinii* are lack of an accurate means of quantitation and difficulty in determination of viability over time. Human-derived *P. carinii* exists as aggregates of cysts and trophozoites in bronchoalveolar lavage fluid. These aggregates persisted over time in an in vitro system consisting of a monolayer of radiated or nonradiated A549 cells, RPMI 1640, and 10% fetal calf serum. Parallel measurements at specified times after introduction into the in vitro system of the number of aggregates, total aggregate area, and total number of cysts varied and appeared to be a function of the number of aggregates initially added into the system. However, cyst density, the number of cysts per unit aggregate area, was independent of the total number of aggregates added into the system. Cyst density was determined by staining an aggregate with a cyst-specific stain, such as toluidine blue, and counterstaining with Diff-Quik, allowing simultaneous visualization of cysts and aggregate area. Preliminary experiments suggested an increase in cyst density over time. Cyst density may be a means of accurate in vitro quantitation of *P. carinii*.

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**Materials and Methods**

**Preparation and sampling of *P. carinii***. Bronchoalveolar lavage fluid containing *P. carinii* was obtained from patients with acquired immunodeficiency syndrome undergoing diagnostic bronchoscopy. The material was frozen at −20°C in 10% glycerol in phosphate-buffered saline (pH 7.4) within 1 h of bronchoscopy and subsequently stored at −70°C. When needed, lavage fluid was thawed in a 37°C water bath and washed three times in phosphate-buffered saline. A 5-μl sample of the sediment was stained with a rapid Giemsa (Diff-Quik; American Scientific Products, McGaw Park, Ill.) and examined microscopically. If morphologically intact aggregates of *P. carinii* were present, the sediment was considered appropriate for further culture studies. Samples (0.1 to 0.5 ml) of sediment were added to 5 ml of medium in 25-cm² flasks, or 5 to 10 μl of sediment was added to 1 ml of medium in chamber slides. In experiments in which flasks were used, at designated time points, the supernatant was sampled by the removal of a sample of supernatant or removal of the entire supernatant (6). When chamber slides were used, the medium was removed from the chamber, and the monolayer and the *P. carinii* organisms that had settled onto it were air dried. Sampling was done at 24-h intervals in triplicate. The monolayer was stained with Diff-Quik. In later experiments, the monolayer was first stained with toluidine blue and then counterstained with Diff-Quik. These two stains together allow visualization of cysts and delineation of the aggregate area containing cysts and trophozoites. The toluidine blue stain was done by the method of Gosey et al. (9). Trophozoite morphology, visible when Diff-Quik is used by itself, is lost by combining the two stains.

**Cell line and culture conditions.** The first series of experiments was done to determine the conditions under which human-derived *P. carinii* could be observed in vitro. *P. carinii* was added onto either A549 or Vero cell monolayers, with either RPMI 1640 or Dulbecco medium, under atmospheric conditions of either 5 or 10% CO₂, with or without 10% fetal calf serum. A549 or Vero cells were trypsinized and passaged into four-chamber tissue culture slides (Labtek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and passaged at 72-h intervals to maintain an active cell line. Following further trypsinization, the slides were rinsed six times with Hanks balanced salt solution and used immediately in experiments. The cultures were monitored for the presence of *P. carinii* by inspection of human body fluids containing the organism and by culture studies. *P. carinii* was isolated in a 100-mm petri dish in air-liquid interface culture medium (RPMI 1640 or Dulbecco medium plus 10% fetal calf serum), with or without added antibiotics, under atmospheric conditions of either 5 or 10% CO₂.

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VOL.

of these Il (15) A549 propagated in propagation of P. line has been extensively used and studied in the in vitro inson biochemical features human pulmonary sampling of the supernatant and the cell sphere of 5% changes. incubation radiation-induced changes of point. A mean densities of each chamber. The Wilcoxon mined by taking the mean value of the the first series of 10 experiments were determined. The means, standard densities of each chamber. The Wilcoxon morphologic of aggregates and aggregate area was determined for each time points. A mean cyst density for each time point was determined by taking the mean value of the individual mean cyst densities of each chamber. The Wilcoxon matched-pairs signed-ranks test was used to compare values (16).

RESULTS

When bronchoalveolar lavage sediments containing P. carinii were inoculated into the medium of A549 cells in 25-cm² flasks, sampling of the culture medium did not reveal the presence of P. carinii. Therefore, the system was permanently modified to allow visualization of activity on the monolayer with the use of four-chamber glass slides. The first series of 10 experiments consisted of the addition of bronchoalveolar lavage fluid sediment containing P. carinii onto either A549 or Vero cell monolayers under various conditions. At a designated time point, the medium within a Labtek chamber was removed, and the remaining monolayer with whatever had settled onto it was air dried, stained, and examined microscopically. Organisms were only rarely observed in the supernatant sediments. Therefore, supernatant sediments were not routinely examined in later experiments. The monolayers were scrutinized, and the numbers of aggregates and aggregate areas were determined. The numbers of aggregates and aggregate area were equivalent over time on the two different cellular monolayers. However, the morphologic integrity of individual trophozoites, and cysts making up the aggregates was best maintained with the A549 cell monolayer cultivated in the presence of RPMI 1640 with 10% fetal calf serum (J. R. Scientific, Inc., Woodland, Calif.), penicillin (200 U/ml), streptomycin (200 µg/ml), miconazole (0.5 µg/ml), 200 mM L-glutamine, and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). Bacterial and fungal contamination were significantly reduced after the addition of antibiotics. Host cells present in bronchoalveolar lavage fluid, such as alveolar macrophages and squamous and columnar epithelial cells, which were added into the medium along with the P. carinii organisms, did not survive in the culture system. They degenerated and within 24 h were no longer visible on the monolayer or in the supernatant.

The next series of five experiments established the utility of irradiation of the A549 cells prior to the addition of P. carinii. When bronchoalveolar lavage fluid containing aggregates of P. carinii was added to the medium of nonirradiated A549 cells, the A549 cells overgrew the aggregates (Fig. 1A). After 48 h, the morphologic integrity of individual cysts and trophozoites making up the aggregates was lost, and continued observation of the aggregates was not possible. Addition of P. carinii-containing lavage fluid to irradiated A549 cells allowed attachment of aggregates of organisms to the cells without crowding (Fig. 1B) or loss of morphologic integrity over time. Observation beyond 48 h was possible. Aggregates of P. carinii organisms were identifiable on the monolayer of irradiated A549 cells for up to 8 days (Fig. 2).

Following the establishment of conditions under which P. carinii could be added and followed over time, organisms from four patients were observed in vitro. Trophozoites predominated in culture at the 24- and 48-h sampling points (Fig. 2A). After 120 h, cysts predominated (Fig. 2B). Approximately 80% of cysts excluded erythrosin B, a vital dye (6), after 120 h in culture. There was no difference in the mean number of aggregates or mean aggregate area among different chambers sampled at the same time point or over time. The total number of aggregates and total number of cysts varied independently of time. There was no change in cyst density within the first 24 h. However, at 48 h on a nonirradiated cell monolayer, cyst density increased. The change was significant at the 0.02 level when compared with cyst density at time 0 and at the 0.01 level when compared with cyst density at 24 h. Cyst density increased after 72 h on a radiated cell monolayer. The change was significant at the 0.01 level compared with all earlier time points (Table 1).

DISCUSSION

Observations on the in vitro cultivation of human-derived P. carinii are reported. The system used is a modification of a previously reported system that supports the growth of rat-derived P. carinii (6). The major modifications are irradiation of the feeder A549 cell layer prior to the addition of the P. carinii and observation of activity on the monolayer. A549 cells, a neoplastic cell line, rapidly overgrow P. carinii. Irradiation of the A549 cells slowed down their growth considerably, allowing subsequently added P. carinii an opportunity to settle onto the monolayer and adhere to the cells. It is possible that the procedure may be simplified by using chemical cellular inhibitors to slow the growth of the A549 cells, eliminating the need for irradiation.

The source of P. carinii used in these experiments was bronchoalveolar lavage fluid from patients with acquired immunodeficiency syndrome and P. carinii pneumonia. Although evidence of in vitro growth, as defined by increased numbers of organisms over time, was not obtained, differentiation from one life-cycle form to another over time, as determined by an increase in cyst density, was observed. This differentiation implies ongoing metabolic activity of the
FIG. 1. Monolayer of A549 cells to which bronchoalveolar lavage fluid containing *P. carinii* was added after 24 h in culture and stained with Diff-Quik. (A) Nonradiated A549 cells are overgrowing the central cluster (arrow) of *P. carinii* organisms. (B) Radiated A549 cells do not overgrow the central cluster (arrow) of *P. carinii* organisms. Magnification, ×100.
FIG. 2. Cluster of *P. carinii* organisms on the irradiated monolayer after 48 (A) and 192 (B) h in culture and staining with Diff-Quik. (A) Trophozoites predominate at 48 h. Arrow indicates a single trophozoite. (B) By 192 h, cyst forms predominate. Arrow indicates a single cyst. Magnification, ×1,000.
**TABLE 1.** Cyst density as a function of time in culture*

<table>
<thead>
<tr>
<th>Cell monolayer and time (h) in culture</th>
<th>No. of cysts</th>
<th>Aggregate area (μm²)</th>
<th>No. of aggregates</th>
<th>Cyst density* (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonirradiated A549</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1,820</td>
<td>78.4</td>
<td>43</td>
<td>25.3</td>
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<td>24</td>
<td>1,414</td>
<td>55.7</td>
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<tr>
<td>48</td>
<td>1,241</td>
<td>32.7</td>
<td>38</td>
<td>41.7</td>
</tr>
<tr>
<td>Radiated A549</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>658</td>
<td>35.0</td>
<td>41</td>
<td>19.1</td>
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<td>24</td>
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<td>39</td>
</tr>
<tr>
<td>72</td>
<td>10,521</td>
<td>270</td>
<td>60</td>
<td>39</td>
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<tr>
<td>144</td>
<td>7,450</td>
<td>110</td>
<td>56</td>
<td>68</td>
</tr>
</tbody>
</table>

* Data show two separate experiments. Each experiment represents *P. carinii* organisms from one patient observed over time. Data are the totals of findings on monolayers of three chambers to each of which 5 μl of bronchoalveolar lavage fluid sediment was added at time 0.

*Cyst density equals the number of cysts per square millimeter of the aggregate area.

**P. carinii** in the culture system. Further modification of the system may define conditions that promote encystation, as well as encystation and growth.

In vitro culture studies of rat-derived *P. carinii* have quantified numbers of organisms present by counting individual cysts (13) or trophozoites (2, 6). Similar methods of quantitation have been used to evaluate antimicrobial susceptibility of rat-derived *P. carinii* in culture (1, 5, 14). These methods are not easily applicable to human-derived organisms. The tight clustering of human organisms makes distinction of individual trophozoites very difficult. The patchy distribution of aggregates over the monolayer obscures derivation of a mean organism number per oil immersion field.

In the current study, the use of the toluidine blue stain, with its ability to demarcate the cyst form of *P. carinii*, in conjunction with the rapid Giemsa counterstain, allowed simultaneous enumeration of cysts and visualization of the overall aggregate area. Thus, it was possible to quantitate the change in cyst numbers over time relative to overall *P. carinii* aggregate area. The resultant measure, cyst density, was independent of the number of aggregates in the system.

An increase in cysts per unit area over time might indicate differentiation of the trophozoite form to the cyst form as an adaptation to adverse conditions, or it might indicate growth. In this system, if growth of *P. carinii* were to occur, growth might be inferred by an increase in mean aggregate size over time. This might occur with an increase, no change, or decrease in cyst number. For instance, a decrease in cysts might accompany an increase in trophozoites. The measurement techniques used in this study were insufficiently sensitive to detect a minute increase in an aggregate such as that which might occur with growth. The total aggregate area reflects the number of aggregates added into the medium at time 0.

Much of the work that we have done to date has been concerned with the establishment of conditions under which human-derived *P. carinii* can be observed in vitro. In this study, we report the use of irradiated A549 cell monolayers for the observation of human-derived *P. carinii*. Subsequent to the observation that irradiation of A549 cell monolayers permits longer study of human-derived *P. carinii* in vitro, we have had the opportunity to study the addition of four bronchoalveolar lavage fluids containing *P. carinii* into the system under conditions that allowed observation over time. Since only four bronchoalveolar lavage fluids were studied under the most optimal conditions, it is not presently known whether the in vitro increase in cyst density is a phenomenon common to all human-derived *P. carinii*. It may be that as the system is further modified, conditions may be found that are more amenable to prolonged survival and growth. For instance, purification of the organisms from the bronchoalveolar lavage fluid by density gradient centrifugation, before adding the *P. carinii* into the in vitro system may be beneficial. Use of fresh material, instead of frozen bronchoalveolar lavage fluid, might result in better in vitro survival. Furthermore, because we did not correlate clinical features with numbers or morphology of *P. carinii* in lavage fluid, we do not know the impact of possibly relevant clinical features, such as extent of disease or therapy with anti-Pneumocystis agents before bronchoalveolar lavage on our results. Further study and experience with this system will be required to determine if the observed increase in cyst density over time that we observed is reproducible and whether it merely represents differentiation of the trophozoite to the cyst form as a response to adverse in vitro conditions or is a manifestation of growth.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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