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Growth and Serial Passage of *Pneumocystis carinii* in the A549 Cell Line

MELANIE T. CUSHION AND PETER D. WALZER*

Division of Infectious Diseases, Department of Internal Medicine, Cincinnati Veterans Administration Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

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Pneumocystis carinii obtained from infected rats and patients was cultured in the A549 cell line, a presumptive alveolar type 2 cell line derived from a human lung carcinoma. Standard criteria were established for organism sampling, quantitation, and growth. The trophozoite form of *P. carinii* was a more sensitive indicator of growth than was the cyst. Rat *P. carinii* increased 10-fold in primary culture and could be serially passed three additional times to new cultures; success in growing human *P. carinii* was limited and appeared to be related to the quality of the specimen received for culture. Growth pattern experiments suggested that close interaction of *P. carinii* with the cell monolayer is an important step in the life cycle of the organism. Thus, the A549 culture system should be useful for in vitro studies of the immunobiology of *P. carinii*.

Once considered little more than a medical curiosity, *Pneumocystis carinii* has emerged as an important cause of pneumonia in the immunocompromised host. Prime targets for the disease have been malnourished infants, children with immunodeficiency disorders, and patients of all ages receiving immunosuppressive therapy (26). Recently, *P. carinii* has gained recognition as an important and often lethal pathogen associated with the acquired immune deficiency syndrom (3, 5). Little is known about the basic biology or life cycle of *P. carinii* because of difficulty in growing the organism in vitro. Previous attempts at *P. carinii* culture have met with limited success (1, 9, 19, 21, 22). Problems with these studies have included a lack of reproducibility among laboratories, the absence of standardized techniques, a lack of long-term cultivation of the organism, and an inability to grow human *P. carinii*.

For the past several years we have explored a variety of systems as a possible growth matrix for *P. carinii* without success. These studies included the use of chicken embryonic lung epithelial culture (21), Vero cell culture (9), explant culture of peripheral lung (24), and fetal lung organotypic culture (4). More recently, we turned our attention to lung-derived cell lines available through the American Type Culture Collection. In these studies greater growth of *P. carinii* was observed in primary culture with the A549 cell line, an alveolar epithelial cell line derived from human lung carcinoma, than in other cell lines tested (M. T. Cushion and P. D. Walzer, J. Infect. Dis., in press).

The purpose of the present study was to present a description of the A549 cell line culture system with emphasis on the following: methods of sampling and quantitation of *P. carinii*, criteria for and patterns of growth, and serial passage.

MATERIALS AND METHODS

Cell line. The A549 cell line was obtained from the American Type Culture Collection (CCL no. 185) (6) and maintained in Dulbecco modified eagle medium (KC Biological, Inc.) with a supplement of 10% fetal bovine serum (M. A. Bioproducts, Inc.) at 37°C in a water jacketed incubator. Either 25-cm^2 or 75-cm^2 tissue culture flasks (Corning Glass Works) were used with caps tightly secured to ensure a closed system.

Source of *P. carinii*. (i) Rats. *P. carinii* infection was induced in Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) by either subcutaneous injection of 25 mg of cortisone acetate (Cortone; Merck Sharp & Dohme) twice weekly or by oral administration of 0.001 mg of dexamethasone (Hexadrol; Organon, Inc.) per ml incorporated in the drinking water for 6 to 8 weeks as previously described (27, 28). The severity of the infection was enhanced by using a low-protein diet (8%) (Bio-Serv, Inc.) in place of regular rat chow. Tetracycline hydrochloride (Polyotic; American Cyanamid Co.) (1 mg/ml) was added to all of the animals' drinking water to prevent secondary bacterial infection.

After sacrifice by halothane (Fluothane; Ayerst Laboratories, Inc.) anesthesia, the rat lungs were removed en bloc, immediately placed in sterile phosphate-buffered saline without magnesium or calcium, with 1 mM EDTA, 200 μg of streptomycin, 200 U of penicillin, and 0.5 μg of amphotericin B per ml (referred to as PBS+), and transferred to a laminar-flow hood. Lungs were separated from adherent blood and debris by surgical removal and sequential rinsing in PBS+. Impression smears were stained with Giemsa and Gram stains to check for *P. carinii* and the presence of bacterial or fungal contamination. The intensity of *P. carinii* infection on the smears was graded on a scale of 1+ or light (few organisms on scattered oil immersion fields) to 4+ or heavy (large masses of organisms). The number of rat lungs used in an experiment depended on the intensity of *P. carinii* infection in individual rats and the number of flasks to be inoculated. Approximately 20 25-cm^2 flasks or 10 75-cm^2 flasks could be inoculated with the lungs of a single rat. No differences in *P. carinii* growth were noted when individual or pooled rat lungs were used.

Lung pieces were minced with sharp scissors and then homogenized by using a Teflon pestle to grind the tissue through a stainless steel (60 mesh) screen. The homogenate was rinsed through the screen with PBS+ and then centrifuged at 1000 × *g* at 4°C for 15 min. After the supernatant was decanted, the pellet was suspended in PBS+ and

* Corresponding author.

centrifuged again. The supernatant was decanted, and the pellet was mixed with the volume of PBS+ needed for the experimental inoculum. Slides were made of the inoculum for enumeration of *P. carinii* cysts.

The cell lines were used as complete monolayers in all experiments. Immediately before infection with *P. carinii* lung homogenate, the tissue culture flasks had all medium removed, and fresh medium supplemented with the same antibiotics in PBS+ was added. To the 75-cm^2 flasks were added 9.0 ml of medium and 1.0 ml of inoculum, and to the 25-cm^2 flasks were added 4.5 ml of medium and 0.5 ml of inoculum.

(ii) Humans. Ten human specimens for culture were obtained from pateints with diagnosed *P. carinii* infection, either locally or shipped by mail in viral transport medium. Three of the samples were transbronchial biopsy-bronchoalveolar lavage fluid, six were open lung biopsies, and one was post mortem tissue. Lung tissue was prepared for culture in the manner described above for rat lung; the lavage fluid was simply washed with PBS+ and inoculated directly into the culture.

Staining and quantitation of *P. carinii*. Based on current concepts of life cycle, the forms of *P. carinii* include the following: (i) the cyst, defined as a large (5 to 10 μm), thick-walled stage of the organism that contains up to eight daughter forms called sporozoites or intracystic bodies; (ii) the trophozoite, a smaller (1 to 4 μm) form of the organism that is unicellular and pleomorphic, having a distinct nucleus and amorphous cystoplasm. Presumably, the sporozoites excyst and develop into trophozoites.

Identification of cysts was accomplished by use of the cresyl echt violet (CEV) stain (Roboz Surgical Instrument Co., Inc.), which selectively stains the cyst wall (2). Initially, the Giemsa stain was used to identify the trophozoite form of the organism, but later the Diff Quik (DQ) Differential stain (Harleco, Inc.) became the routine method of identification. The DQ staining method gives results similar to Wright-Giemsa and utilizes the following three dyes: triarylmethane, xanthene, and thiazine. Our investigations showed that the DQ strain was superior to Giemsa in terms of consistency of color and in a reduced staining time (15 to 20 s versus 30 to 60 min). Trophozoites stained with these methods showed characteristic reddish purple nuclei and light blue cytoplasm.

Cysts and trophozoites were quantitated by the Virginia Polytechnic Institute method (7) for counting anaerobic bacteria, as modified by Bartlett et al. for *P. carinii* (1). In this technique, two 0.01-ml drops were placed on a glass slide and stained with either CEV or DQ. Each drop covered an area of approximately 1 cm^2. Ten oil immersion fields in each drop were randomly scanned for a total of 20 oil immersion fields. The mean number of *P. carinii* trophozoites or cysts per oil immersion field was established, and the total count in 1.0 ml was calculated according to the following formula: (no. of *P. carinii* trophozoites per ml) = (mean no. of *P. carinii* trophozoites per oil immersion field) × (no. of oil immersion fields per cm^2) × (dilution) × 10^2.

Reproducibility of the quantitation system was determined by counting the same tissue culture supernatant 20 times with each stain. The mean ± standard error of the mean *P. carinii* counts were as follows: 15.1 ± 0.63 × 10^5 cysts (CEV); 25.7 ± 2.28 × 10^5 trophozoites (Giemsa); 31.4 ± 1.75 × 10^5 trophozoites (DQ).

To study variation among tissue culture flasks, 10 flasks were infected with the same inoculum and then sampled on day 3 of culture. The mean ± standard error of the mean *P. carinii* count for the 10 flasks was 17.3 ± 0.60 × 10^6 trophozoites per ml by the DQ stain. Samples from the 10 flasks were also pooled; the *P. carinii* count on this specimen was 18.5 × 10^6 trophozoites per ml.

Determination of growth. After *P. carinii* was inoculated into cell monolayer cultures, growth was determined by the following methods.

(i) Continuous culture method. Samples (0.5 or 1.0 ml) of supernatant fluid (corresponding to flask size of 25 cm^2 or 75 cm^2, respectively) were removed daily or at specified intervals from each of two or three flasks and replaced with an equal volume of fresh medium. The supernatant samples were pooled and sampled by preparing two slides with two 0.01-ml drops: one slide for cyst counts (CEV stain) and one for trophozoite counts (DQ stain). In some experiments where initial inoculum counts were low, the pooled supernatant samples were centrifuged at 1,000 × *g*, brought to 1/10 the starting volume with PBS+, and then sampled. The dilution was compensated for in the Virginia Polytechnic Institute formula.

(ii) Whole-flask method. Duplicate or triplicate culture flasks were harvested on the designated days of sampling. The total supernatant of each flask was removed, centrifuged, and then sampled. Cyst and trophozoite counts were expressed as the means of the individual flasks. Different methods of monolayer preparation were examined for the ability to dissociate cysts from the cell milieu: freeze-thaw, enzyme digestion, and sonication. Of the three, sonication provided the best results and was incorporated into the standard procedure. Individual monolayers were scraped into 10 ml of PBS+ and sonicated with a Branson 185 sonifier at setting 7 by using a microtip for three 15-s intervals. Cyst counts were expressed as the means of the individual monolayer counts.

Serial passage of *P. carinii*. Serial passage studies were performed by transferring on selected days 1.0 ml of rat *P. carinii*-infected culture supernatant to naive confluent monolayers with 4 ml of fresh medium. Cultures were sampled by the continuous method for assessment of growth. This procedure was repeated for each successive passage.

Culture repopulation with *P. carinii*. In these studies entire supernatants were removed from rat *P. carinii*-infected cuture flasks, and the monolayers were washed vigorously twice with 5 ml of PBS+. Fresh medium was then added to the cultures, and they were sampled over the next several days for evidence of *P. carinii* growth.

Viability of *P. carinii*. Different vital stains were compared for assessing the viability of *P. carinii*. With trypan blue or Evans blue *P. carinii* organisms could not be confidently distinguished from tissue culture artifacts by using oil phase-contrast microscopy. With erythrosin B both *P. carinii* cysts and trophozoites could be recognized; viable organisms excluded the stain, whereas dead organisms were stained red. The erythrosin B stain became our routine method of assessing organism viability in the culture system.

Controls. To validate the appearance of *P. carinii* growth in culture as a real occurrence and not simply persistence, a number of control cultures were included in the various experiments. Autoclaved, dry-heated (3 h, 90°C) frozen (−20°C), and formaldehyde-fixed organisms were inoculated under the same culture conditions and assayed in the same manner as live *P. carinii*. Also included in experiments were flasks that contained live inoculum in cell-free medium to substantiate the effects of the cells and their products on the organism's growth. Uninoculated cultures were examined for cell debris resembling either form of *P. carinii*.

The tissue culture system was monitored for bacterial and

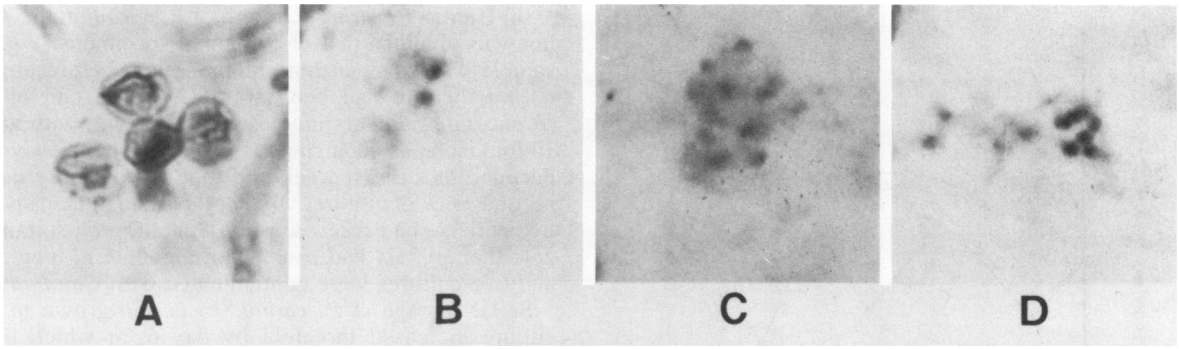

FIG. 1. Forms of rat *P. carinii* from A549 cell culture supernatant visualized by two stains, ×1,000. (A) *P. carinii* cysts stained by CEV. The collapsed appearance is typical of cyst wall morphology. (B) Two *P. carinii* trophozoites stained by the DQ stain. Note the dark nucleus and surrounding cytoplasm of each organism. (C) Large cluster of *P. carinii* trophozoites stained by DQ. Eleven organisms were identified, but some may not be discernible because they are out of the focal plane. (D) Form of *P. carinii* that looks like a mature cyst containing sporozoites. The lack of a definable border may make it difficult to distinguish from a group of trophozoites.

fungal contamination by microscopy and surveillance cultures.

Cytopathic effects. The culture system was examined daily for cytopathic effects from *P. carinii* by inverted phase microscopy.

RESULTS

Staining and quantitation of *P. carinii*. *P. carinii* was quantitated by enumerating its two principal forms. The cyst was easily identifiable with the CEV stain by its characteristic rose-violet color and distinctive morphology (Fig. 1A). Cysts in samples obtained from supernatant sources could be readily counted. When the cell monolayer was examined by the whole-flask method of sampling, cysts tended to adhere to host cells, sometimes in large aggregates. Sonication disrupted the monolayer cells, but left *P. carinii* morphologically intact. The organisms were more uniformly suspended with only occasional small clusters and could be easily quantitated.

Trophozoites in lung homogenates or cell monolayer sonicates could not be accurately quantitated because the DQ and Giemsa stains also stained host cellular material. However, the tissue culture supernatants were largely free from host cells, and the trophozoites could readily be identified and quantitated. The trophozoites appeared as reddish-purple nuclei surrounded by blue cytoplasm and occurred individually or in small groups (Fig. 1B) or in larger clusters (Fig. 1C).

Quantitation of other forms of *P. carinii* by the DQ stain posed different problems. Intermediate stages in the life cycle between the trophozoite and cyst were very pleomorphic. Forms that appeared to be mature cysts containing sporozoites sometimes lacked a definable border, making it difficult to distinguish them from a group of trophozoites (Fig. 1D). To simplify the quantitation process, we did not attempt to distinguish the various stages of *P. carinii*; rather, we counted as individual trophozoites all forms with reddish-purple nuclei and blue cytoplasm. Bacteria were easily distinguished from trophozoites by their characteristic morphology and lack of surrounding blue cytoplasm.

Growth of *P. carinii* in primary culture. (i) Rat *P. carinii*. The growth of rat *P. carinii* studied in the A549 cell line, monitored by daily sampling of supernatants with the continuous culture method, showed that the trophozoites increased approximately 10-fold from day 1 (the first day they were counted) to day 7 of culture (Fig. 2). Cysts showed a lesser increase in counts, with peak numbers on days 3 and 5.

Growth analysis of *P. carinii* in the same experiment by the whole-flask method revealed approximately equal numbers of cysts in the supernatant and monolayer compartments at the beginning of the culture (Fig. 3). Cyst counts increased mainly in the supernatant, reaching a peak on day 5. When the cyst counts from both compartments were combined, a 10-fold increase was realized.

Of 22 experiments performed, growth of rat *P. carinii* in primary culture occurred on 15 (68%) occasions. Certain characteristics of the A549 cell line influenced its success as a *P. carinii* culture matrix. Growth of *P. carinii* was usually better with lot no. F2530 than with lot no. F2343 of the cell line, with younger (<4 days) than with older (>7 days) cell

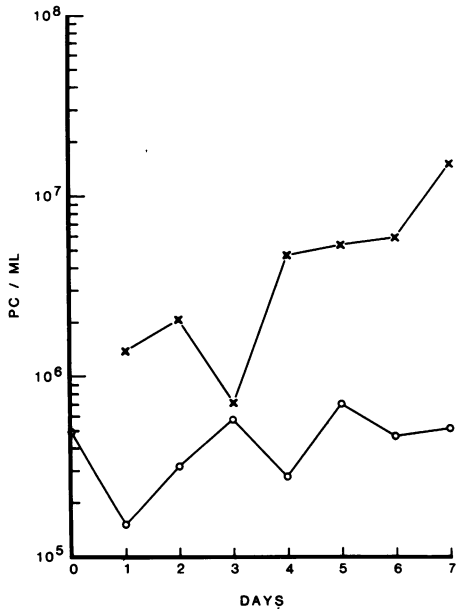

FIG. 2. Growth of rat *P. carinii* in the A549 cell line over 7 days assessed by the continuous culture method of sampling. (×) *P. carinii* trophozoites in culture supernatants stained by DQ; (○) *P. carinii* cysts in culture supernatant stained by CEV.

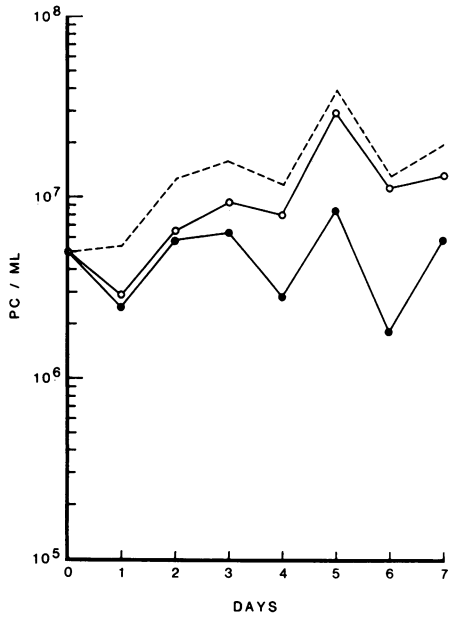

FIG. 3. Growth of rat *P. carinii* in the A549 cell line over 7 days assessed by the whole flask culture method of sampling. (●) *P. carinii* cysts associated with the cell monolayer stained by CEV; (○) *P. carinii* cysts in culture supernatant stained by CEV; (- - -) addition of monolayer and supernatant cyst counts.

cultures, and with cell line cultures which had been serially passaged <15 times than cell cultures passaged >15 times after receipt from the American Type Culture Collection. The older and more extensively passaged A549 cells sometimes exhibited morphological changes, but their specific relationship to the growth of *P. carinii* was unclear.

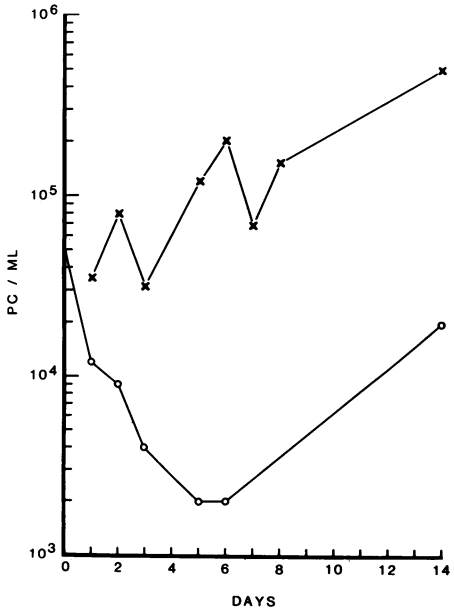

FIG. 4. Growth of human *P. carinii* in the A549 cell line over 14 days, assessed by the continuous culture method of sampling. (×) *P. carinii* trophozoites in culture supernatant stained by DQ; (○) *P. carinii* cysts in culture supernatant stained by CEV.

(ii) Human *P. carinii*. Growth of *P. carinii* in the A549 cell line was obtained in 1 of 10 human specimens (Fig. 4). The sample was an open lung biopsy taken before any anti-*P. carinii* therapy had been started. At the end of 1 week trophozoite numbers increased 6- to 8-fold, with an overall 10-fold increase occurring at the end of 14 days. Cyst counts declined in the first week and then rose slightly during the second week of culture. Of the other nine human specimens, five patients had received prior trimethoprim-sulfamethoxazole therapy, six had insufficient amounts of lung material, and two cultures were contaminated with *Candida* sp.

Serial passage of *P. carinii*. *P. carinii* grown in primary culture increased threefold by day 6, at which time new culture flasks were inoculated (Fig. 5). Trophozoites counts increased eightfold on the first passage and threefold over the inoculum on the next two passages.

Culture repopulation with *P. carinii*. *P. carinii* was grown in primary culture until day 6, when the supernatants were removed. The monolayers were then washed, and fresh medium was added (Fig. 6). By day 1 of the new culture organisms appeared in the supernatant and increased 20-fold over a 20-day period. These data suggest *P. carinii* maintains an intimate relationship with the monolayer cells at certain stages in its life cycle.

Viability of *P. carinii*. Viability of *P. carinii* as judged by the exclusion of erythrosin B stain was >90% in the lung inoculum and throughout the culture period. Organisms appeared to retain their viability and morphological features even during times when no replication occurred.

Controls. Autoclaving, dry-heating, freezing, and formaldehyde fixation resulted in a 10- to 100-fold reduction in the number of organisms compared with live inocula when sampled on day 1 of culture. *P. carinii* counts observed in the culture system over a 2-week period after these inactivation procedures either remained the same or declined further. Similarly, cultures of *P. carinii* in cell-free medium failed to exhibit growth. Organisms subjected to these procedures lost their viability and typical morphology.

Contamination of the tissue culture with other microorganisms occasionally occurred. The offending pathogens were usually *Flavobacterium meningosepticum*, *Pseudomonas* sp., and *Candida albicans*.

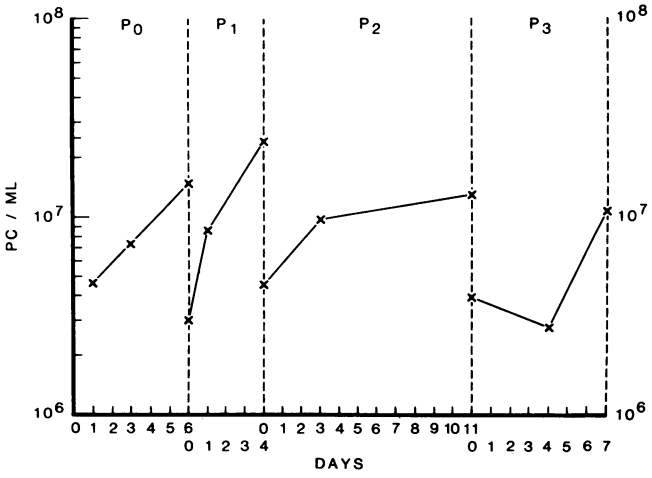

FIG. 5. Growth of rat *P. carinii* in the A549 cell line in primary (P_0) and serially passed cultures (P_1, P_2, P_3) as assessed by the continuous culture method of sampling. (×) *P. carinii* trophozoites in culture supernatant stained by DQ.

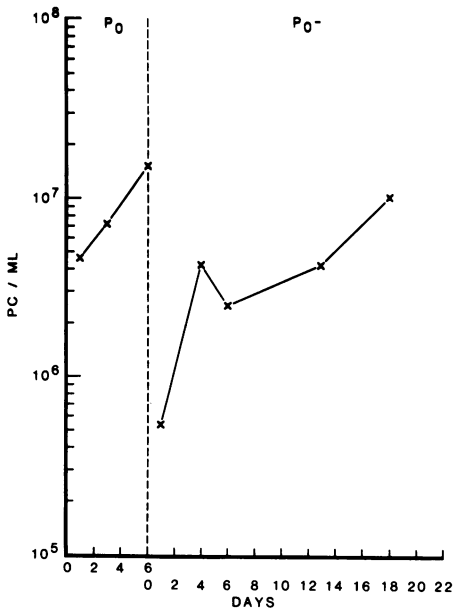

FIG. 6. Growth of rat *P. carinii* in the A549 cell line in primary culture (P_0) and in the same culture (P_0^-) after supernatant had been removed, the monolayer had been washed, and fresh medium had been added. (×) *P. carinii* trophozoites in culture supernatant stained by DQ.

Cytopathic effects. No specific cytopathic effects on the culture system could be attributed to *P. carinii*.

DISCUSSION

In vitro studies with *P. carinii* depend on adequate numbers of organisms free from contaminating host cells and other microorganisms. Much of the information currently available about *P. carinii* has been derived from in vivo observation or by use of uncultured organisms in tissue homogenates. In some studies *P. carinii* separated from lung tissue by enzyme digestion or differential centrifugation (or both) was used in immunofluorescence and immunoenzyme work (8, 10–13, 15–18, 29). *P. carinii* obtained from bronchoalveolar lavage fluid was used to investigate the organism's metabolism, sensitivity to antiparasitic drugs, or interaction with host alveolar macrophages (14, 19, 20, 25). The results of these studies have not been entirely satisfactory because of the technical problems resulting from contaminants in the crude preparations.

Successful in vitro cultivation of *P. carinii* has been reported by only a few investigators (Table 1). There was modest growth in primary culture and serial passage for up to a few cycles in subsequent cultures. These studies differed considerably with respect to cell line, form of *P. carinii* studied, histological stain, and methods of sampling and quantitation. Since successful cultivation of *P. carinii* achieved in one laboratory could often not be confirmed by other laboratories, no one system has gained widespread use. More importantly, no generally accepted standards of *P. carinii* growth have been developed so the results of one study can be meaningfully compared with those of another.

The present study was designed to address some of these problems. Attention was devoted to investigating both the cyst and trophozoite forms of *P. carinii*. CEV, the stain chosen for cyst enumeration, selectively stains the cyst wall and provides a reliable measurement of the number of cysts in host lungs (16, 28) and in tissue culture. Other cell wall stains (e.g., methenamine silver, toluidine blue) work as well as CEV, and thus the use of a particular stain is largely a matter of personal preference. Since CEV and other cell wall stains do not stain the internal structure of the cyst, they cannot be used to assess organism viability or to distinguish the different stages in the life cycle of *P. carinii*. We have found that the CEV stain provides little additional data about the growth of *P. carinii* that cannot be obtained from DQ or Giemsa stains, and hence we no longer routinely employ this stain in our culture system. CEV also stains fungi, which might possibly be confused with *P. carinii* if contamination of the culture system occurred.

The DQ and Giemsa stains, which stain *P. carinii* tropho-

TABLE 1. Studies of in vitro cultivation of *P. carinii*

Source of data	Cell lines supporting growth	Cell lines not supporting growth	Quantitation method	
			Form of PC	Stain
Pifer et al. (21, 22)	Chicken embryonic lung	WI-38 L-cells Rat lung	Cyst	Toluidine blue
	Vero	Secondary chicken embryo fibroblast Owl monkey kidney Baby hamster kidney AV-3 WI38	Cyst	Toluidine blue
Latorre et al. (9)	Vero Chang liver MRC-5	LLC-MK-2 FL McCoy	Microscopic observation of floating aggregates	
Bartlett et al. (1)	WI38 MRC-5		Trophozoite	Giemsa
Cushion and Walzer (in press)	A549	WI38 L2 4/4RM4 RFL-6	Trophozoite Cyst	DQ CEV

zoites, sporozoites within the cyst, and intermediate stages, provide a sensitive and reliable assessment of organism culture dynamics. The DQ stain offers advantages over the Giemsa stain in terms of rapidity and consistency of color results. The present study has confirmed and extended the observations of Bartlett et al. (1) that with these stains *P. carinii* can be counted in tissue culture supernatants with a reasonable degree of accuracy. Proper interpretation of the different forms of *P. carinii* requires at least some degree of experience, and we hope the definitions we have made here will be helpful in establishing uniform objective criteria for determining the growth of the organism. Quantitation of *P. carinii* by the DQ and Giemsa stains in lung homogenates or tissue culture monolayers is even more difficult because these stains also stain host cells.

Two sampling methods were used to monitor *P. carinii* in tissue culture in this study. The continuous culture technique, which involved sequential sampling the same flasks, was easy to perform and provided good data about growth patterns of *P. carinii*; this method became our routine method of monitoring the culture system. The continuous culture technique only sampled the tissue culture supernatant and provided an underestimate of the number of *P. carinii* organisms. Because 1/10 of the volume of the culture fluid was removed and replaced with fresh medium each time sampling was performed, a dilution factor was artifically introduced. The whole-flask method examined both compartments of the tissue culture system and provided a good check on the continuous culture method; however, the whole-flask method was cumbersome and inefficient because the entire contents of a flask had to be sacrificed to obtain a single point in time.

The present study has demonstrated that the A549 cell line can support the growth and serial passage of rat *P. carinii*. The levels of organism replication achieved here are similar to those reported for other tissue culture systems (1, 9, 21, 22). The A549 cell line is available from the American Type Culture Collection and is easy to maintain in the laboratory. Hopefully, this culture system can be developed to study *P. carinii*'s life cycle metabolism, antigenic characteristics, and sensitivity to antimicrobial drugs. The A549 cells are presumptive alveolar type II epithelial cells, and the close relationship of *P. carinii* to the A549 monolayer suggests the cell line will be helpful in examining the mechanisms and kinetics of organism attachment. Our previous studies in rats have demonstrated that the interaction of *P. carinii* with alveolar type I cells (which are derived from type II cells) occupies a central role in the pathogenesis of *P. carinii* pneumonia (30, 31).

Further studies in other laboratories will be needed to determine the popularity of the A549 cell line in *P. carinii* cultivation work. Enthusiasm for this system must also be tempered by the realization that the A549 cell line is a heterokaryon, and morphological changes may occur in the cells after many days in culture or extensive serial passage. Biochemical studies have revealed conflicting results concerning the products (e.g., phosphatidylcholine) secreted by the A549 cells (6, 23). Since older A549 cells did not appear to support the growth of *P. carinii* as well as did younger ones, the application of these techniques to our culture system might be helpful in elucidating the metabolic requirements of *P. carinii*.

Nine of the 10 specimens we received for culturing human *P. carinii* exhibited problems of low inoculum size, prior anti-*P. carinii* therapy, or contamination by other microorganisms. The only specimen deemed suitable for culture resulted in growth of *P. carinii*. We are encouraged by this modest success and believe that the A549 cell line deserves further evaluation as a possible growth matrix for human *P. carinii*.

ACKNOWLEDGMENTS

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