

Propagation of *Pneumocystis carinii* in Vero Cell Culture

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Pneumocystis carinii derived from infected murine lung was found to be capable of limited growth in Vero African green monkey kidney cell cultures. The observed increase in the number of cyst forms was influenced by the ratio of cysts to host cells in the inocula, duration of passage, and formulation of the cell culture media. Maximum growth was achieved by inoculating 1.3×10^5 cysts per 75-cm² flask containing about 2×10^7 Vero cells (cyst-to-cell ratio = 1:154) maintained on minimal essential medium supplemented with 2% fetal bovine serum. Under these conditions of culture, a 10.8-fold increase in cyst forms was observed during a 3-day passage interval, whereas only a 3.6-fold increase occurred during a 7-day passage in cell culture utilizing cyst-to-cell ratios ranging from 1:28 to 1:2,778.

Pneumocystis carinii pneumonitis was first recognized in the United States in 1956. Since that time, it has increased in prevalence, presumably because of the extended longevity of susceptible patients, i.e., those on immunosuppressive therapy and those with compromised immunity (2).

The in vitro cultivation of *P. carinii* (5) and the elucidation of its morphology and host-parasite interrelationships by scanning electron microscopy (4) have been achieved only recently. Highly purified organisms grown in primary cell cultures have been effectively utilized in diagnostic and epidemiological studies of *P. carinii* infection in both normal and immunosuppressed children (L. L. Pifer, W. T. Hughes, S. Stagno, and D. Woods, Pediatrics, in press). To date, *P. carinii* has been propagated in primary embryonic chick epithelial lung cell cultures (5), owl monkey kidney, baby hamster kidney, AV-3 (6), Vero, Chang liver, and MRC-5 cells (3).

The objective of this study was to quantitatively assess the growth rate of *P. carinii* in Vero cell cultures and to determine the potential usefulness of a permanent cell line in the production of purified *P. carinii* organisms for use in diagnostic and other investigative procedures.

MATERIALS AND METHODS

P. carinii organisms derived from the cortisone acetate-treated Sprague-Dawley rats (1) were prepared and quantitated as previously described (5). Twenty-four-hour-old monolayers of Vero cells or other cell lines (Microbiological Associates, Bethesda, Md.) at 80 to 90% confluency maintained on minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) with 2% fetal bovine serum (FBS) (K. C. Biological, Inc., Lenexa, Kans.) or on

medium 199 (Microbiological Associates) with 10% FBS were routinely used in these studies. Growth media were discarded, and inocula in the range of 1.3×10^5 to 8.5×10^5 cysts per culture were applied to the monolayers in a volume of 1 to 2 ml of media. Cells and inocula were tested for the presence of mycoplasma and found to contain none. The inocula were allowed to absorb for 2 h at 37°C, and the monolayers were then supplemented with fresh media and returned to the incubator. Ten microliters of media was removed from the cultures at daily intervals, and the cysts were quantified (5). Previously, this method of quantitation yielded coefficients of variation ranging from 0.7 to 1.6% for 1,459 and 2,047 cysts, respectively, in a volume of 10 μ l.

Monolayers were examined qualitatively on a daily basis for evidence of cytopathic effect. The cells were harvested by scraping with a rubber policeman at various times after infection, combined with the cysts in the supernatant, and concentrated by centrifugation before enumerating the total number of cysts per culture.

In one type of experiment utilizing Vero cultures, cysts in the culture supernatant were harvested by centrifugation and inoculated onto fresh monolayers every 24 h for 7 days. The parent monolayers in each instance were supplemented with fresh media, either MEM with 2% FBS or medium 199 with 10% FBS, and incubated for an additional 7 days. Ten-microliter samples were taken from each culture in each passage for 7 days, and the cysts were counted. This experiment was conducted to determine if fresh cells would enhance the yield of *P. carinii* from Vero cultures.

RESULTS

Table 1 illustrates the increase in cyst forms in the culture media at 24-h intervals for 72 h in Vero cultures with a cyst-to-cell ratio of 1:154 maintained on MEM with 2% FBS. The greatest single increase in a 24-h period (3.6-fold) occurred during the first 24 h in culture. The

overall increase from zero time to 72 h was 10.8-fold, after which time a diminishing yield was obtained. Cytopathic effect caused by patchy sloughing of the monolayers was visible to a moderate extent. Minimal essential medium with 2% FBS was superior to medium 199 with 10% FBS in supporting these cultures.

The values given in Table 2 represent cyst counts from four consecutive serial passages at 7-day intervals of inocula containing three dilutions of *P. carinii* cyst forms. Cyst-to-cell ratios were 1:28 in group A, 1:278 in group B, and 1:2,778 in group C. All cultures were maintained on MEM with 2% FBS.

Group A, which received the largest number of cysts, showed a 3.2-fold increase at the end of the first passage, followed by a small decline in cyst numbers at the end of the second passage to approximately the same level as that of the original inoculum. *P. carinii* in group C did not appear to grow at all, since the number of organisms in the inoculum was apparently not sufficient to establish productive infection. Cysts in group B increased 3.2-fold during the first passage, declined during the second, and increased slightly in the third and fourth passages by 1.5- and 1.8-fold, respectively. Cytopathic effect appeared to fluctuate accordingly.

Attempts to enhance growth by transferring the organisms in the culture supernatant at 24-h intervals to fresh Vero cell cultures were only minimally successful.

Very little growth (a threefold increase) of the organisms occurred in owl monkey kidney, baby hamster kidney, and AV-3 cell cultures, and no

growth was observed in WI-38 cells and secondary chicken embryo fibroblast cultures (6).

DISCUSSION

P. carinii is capable of limited growth in Vero cell cultures. The maximum increase in cyst forms (about 11-fold) occurred at 72 h postinoculation in cultures with an initial cyst-to-cell ratio of 1:154. This is in contrast to a 100-fold increase in cyst forms observed in the chick epithelial lung cell system utilizing a similar multiplicity of infection. In all instances, cysts were enumerated by direct count rather than by gross estimation.

"Blind" passage of the organisms maintained for 12 weeks with passage at weekly or 3-day intervals in an attempt to adapt *P. carinii* to Vero cells was not successful. In all cases, cyst forms increased in number until the third or fourth passages and then precipitously declined. The possibility that virtually all organisms in these cultures were in the trophozoite stage was examined by staining, but relatively few intact thin-walled forms were seen. It should be noted, however, that trophozoites are stained and identified only with considerable difficulty. Cyst forms were seen with decreasing frequency concomitantly with diminished cytopathic effect, which constitutes good evidence that no further replication, at least to an appreciable extent, was occurring.

In conclusion, primary embryonic chick epithelial lung cell cultures, though considerably more inconvenient to prepare, are superior to other cell types tested to date for the in vitro propagation of *P. carinii*. However, if a smaller yield of pure organisms is acceptable for the intended purpose, then quantity may be sacrificed for convenience. This may prove particularly useful in immunological studies in which relatively small numbers of pure, antibody-free cysts would be required.

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TABLE 1. Growth of *P. carinii* in Vero cell cultures during a 72-h interval

Time (h)	Cysts in culture supernatant ^a	Fold increase	
		Per 24-h interval	From zero time
0	1.3 × 10 ^{5b}		
24	4.7 × 10 ⁵	3.6	3.6
48	9.8 × 10 ⁵	2.1	7.5
72	1.4 × 10 ⁶	1.4	10.8

^a Average values derived from replicate cultures.

^b Cyst-to-cell ratio = 1:154.

TABLE 2. Passage of three dilutions of *P. carinii* at 7-day intervals on Vero monolayers

Group	Inoculum	Cyst/cell ratio	Total cysts/culture in passage ^a :			
			1	2	3	4
A	7.2 × 10 ⁵	1:28	2.3 × 10 ⁶	6.8 × 10 ⁵	6.2 × 10 ⁵	4.5 × 10 ⁵
B	7.2 × 10 ⁴	1:278	2.3 × 10 ⁵	5.4 × 10 ⁴	1.1 × 10 ⁵	1.3 × 10 ⁵
C	7.2 × 10 ³	1:2,778	NG	NG	NG	NG

^a "Passage" refers to incubation of infected cultures for 7 days before quantitative transfer of total cysts in culture to fresh monolayers. NG, No growth.

LITERATURE CITED

1. Frenkel, J. K., J. T. Good, and J. A. Schultz. 1966. Latent pneumocystis infection of rats, relapse and chemotherapy. *Lab. Invest.* **15**:1559-1577.
2. Hughes, W. T., H. Kim, R. A. Price, and C. Miller. 1973. Attempts at prophylaxis for murine *Pneumocystis carinii* pneumonitis. *Curr. Ther. Res.* **15**:581-587.
3. LaTorre, C. R., A. J. Sulzer, and L. G. Norman. 1977. Serial propagation of *Pneumocystis carinii* in cell line cultures. *Appl. Environ. Microbiol.* **33**:1204-1206.
4. Murphy, M. J., Jr., L. L. Pifer, and W. T. Hughes. 1977. *Pneumocystis carinii* in vitro: a study by scanning electron microscopy. *Am. J. Pathol.* **86**:387-402.
5. Pifer, L. L., W. T. Hughes, and M. J. Murphy, Jr. 1977. Propagation of *Pneumocystis carinii* in vitro. *Pediatr. Res.* **11**:305-316.
6. St. Jude Children's Research Hospital. 1973. Annual report, p. 20. St. Jude Children's Research Hospital, Memphis.