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Cultivation of *Pneumocystis carinii* with WI-38 Cells

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Pneumocystis carinii has been successfully cultured with WI-38 human embryonic lung fibroblasts. Inoculum was obtained from infected lungs of cortisone-treated Sprague-Dawley rats. Trophozoites reached peak numbers between days 4 and 8 and grew in two subcultures, but then proliferation ceased. If primary cultures were harvested and new medium were added, a second and sometimes a third harvest could be obtained. Cell monolayers were not destroyed. After growth of the initial inoculum had ceased, monolayers supported growth of a new inoculum. Harvested organisms had few contaminating tissue culture cells. Additional studies of growth requirements are needed. Although cultures cannot be used to diagnose *P. carinii* infection, cultured organisms should be useful for studies of biology and pathogenesis and for the development of immunodiagnostic techniques.

Pneumocystis carinii is a frequent cause of pneumonia in immunocompromised individuals (6). Diagnosis has required morphological demonstration of the organisms because culture methods for diagnosis are not developed and lack specificity and sensitivity. Cultivation of *P. carinii* allows the investigation of parasite biology and provides organisms for the study of host defense mechanisms and the development of serological methods for antigen and antibody detection. Recently, successful cultivation with MRC-5 cells was reported by Latorre et al. (3), and cultivation with embryonic chicken epithelial cells was reported by Pifer et al. (5). In the study with MRC-5 cells (3), numbers of organisms were determined by microscopic observation of floating clumps in the tissue culture flask, and numbers of trophozoite and cyst forms were not determined. In the study with the embryonic chicken epithelial cell culture, only cyst forms were enumerated. In this cell line there was a cytopathic effect directly related to the number of organisms present. Indefinite culture was not achieved. We report successful propagation of *P. carinii* with WI-38 human embryonic lung fibroblasts, a cell line commonly used for virus isolation. In this cell line there is no destruction of the cell monolayer. Counts of trophozoites allowed the construction of growth curves showing trophozoite proliferation, with peak numbers at between 4 and 8 days.

MATERIALS AND METHODS

Sprague-Dawley rats treated with cortisone acetate by the procedure of Frenkel et al. (1) spontaneously develop pneumocystis pneumonia. Lungs of 23 such rats were used to prepare culture inocula. Giemsa-stained impression smears of lungs were examined to determine the extent of infection. A piece of infected lung (ca. 1 cm^3) was rinsed in sterile Eagle medium containing 10% fetal calf serum, 50 μg of streptomycin sulfate per ml, 100 U of penicillin G potassium per ml, and 10 U of nystatin per ml (designated outgrowth medium). The lung was then placed in a Ten Broeck grinder in 5 ml of the outgrowth medium. After hand grinding, the supernatant was withdrawn and centrifuged for 10 min at 150 $\times$ *g*. One milliliter of the supernatant was used as the inoculum for one 250-ml-capacity Falcon flask containing a monolayer of WI-38 or MRC-5 cells. Culture flasks were incubated at 35°C without CO_2.

Monolayers were prepared with MRC-5 and WI-38 cell strains, both of which are human fetal lung fibroblast lines. Cells obtained as tube cultures from Microbiological Associates, Walkersville, Md., and Flow Laboratories, Inc., McLean, Va., were used to prepare monolayers in 250-ml flasks. After monolayers had formed, fresh outgrowth medium was added to the flasks that were used immediately. For the flasks held for a few days, medium containing 2% fetal calf serum, designated maintenance medium, was used.

Inoculated cultures were checked both by observing the monolayers microscopically and by calculating the number of organisms per milliliter for each culture supernatant. One milliliter of culture was removed from each flask, and new medium was added to replace that removed. After centrifugation at 500 $\times$ *g* to pack the trophozoites, the supernatant was decanted, and the organisms were resuspended in 0.1 ml of buffer. With a 10-μl Eppendorf pipette, a drop of culture was removed, transferred to a 1-cm square on a glass slide (1 by 3 in. [ca. 2.54 by 7.62 cm]), dried, fixed in methanol, and stained with Giemsa. Multiple fields were examined, and the organisms were counted. Using the formula for determining the size of a 100$\times$ oil immersion field (2), we calculated the number of or-

ganisms per milliliter. Counts were plotted for each culture flask from days 1 through 10.

Subcultures of initial cultures were made by transferring 1 ml of culture fluid from a 4- or 5-day culture to a new monolayer. The growth in subcultures was monitored as previously described.

When trophozoites were required for other experiments, cultures were harvested. All culture medium was removed from each flask and centrifuged at 500 × *g* for 10 min. After the supernatant was decanted, the organisms were resuspended in buffer free of calcium and magnesium ions. This buffer was used to reduce clumping of organisms. New outgrowth medium usually was added for a second or third growth of organisms.

Monolayers no longer yielding organisms upon the addition of new medium were termed used cells and were used for additional cultures with new rat lung inocula. The growth in all cultures was monitored as described previously.

RESULTS

There was some growth of *P. carinii* trophozoites in 20 of 23 rat lungs cultured. The amount of growth varied among rat lungs. A very heavily infected lung did not necessarily yield a large number of trophozoites in culture. Cultures were not always counted daily; as a result, peak numbers could be definitely determined for only some cultures. Some rat lungs contained bacteria or fungi which overgrew the *P. carinii* before peaks could be determined. In addition, organisms from some rat lungs grew so poorly or so abundantly that it was difficult to determine the numbers. Generally, cultures 1 or 2 days old contained a few single organisms (Fig. 1). By day 4 or 5, the cultures contained numerous clumps of organisms (Fig. 2). All of the cultures were relatively free of cellular debris or monolayer cells.

Growth usually peaked at between days 4 and 8. A typical growth curve of a culture (Fig. 3) shows rapid proliferation of trophozoites, with a peak at day 6. Growth curves differed somewhat for different rat cultures. The five culture growth curves shown in Fig. 4 illustrate rapid proliferation, with a peak at between 4 and 8 days for four of the five cultures. Rat J1 culture peaked late, as did the cultures for two other rats. Some of the cultures were not monitored after the apparent peak because trophozoites were harvested for use in other experiments.

Cultures from 11 rats inoculated on both WI-38 and MRC-5 cell lines had sufficient growth and enough sequential counts in both cell lines for evaluation. There were greater peak numbers

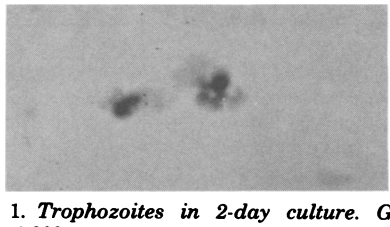

FIG. 1. *Trophozoites in 2-day culture. Giemsa stain, ×1,000.*

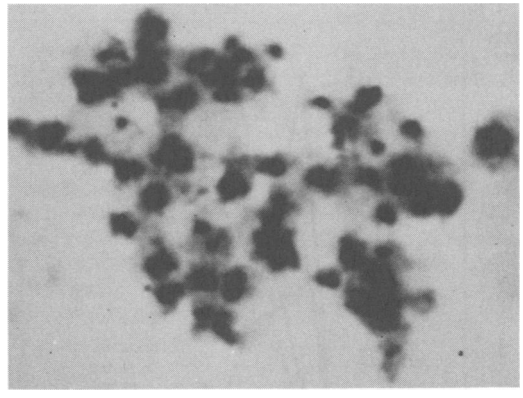

FIG. 2. *Trophozoites in 5-day culture. Giemsa stain, ×1,000.*

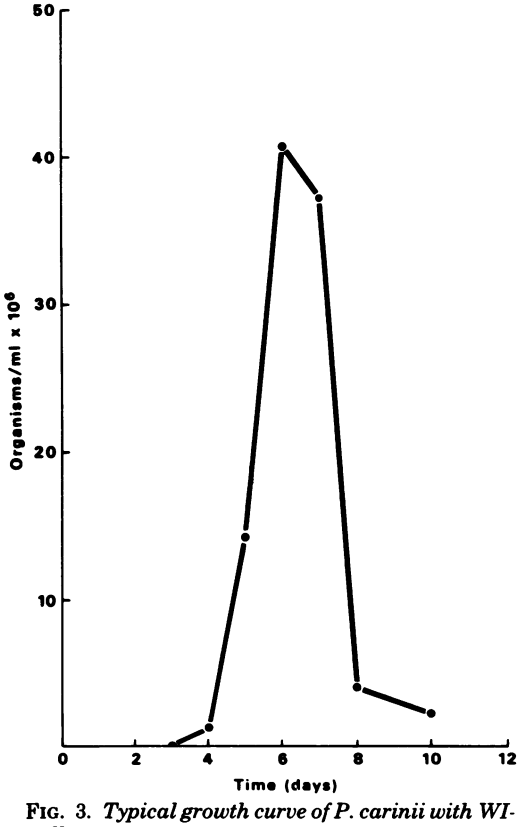

FIG. 3. *Typical growth curve of P. carinii with WI-38 cells.*

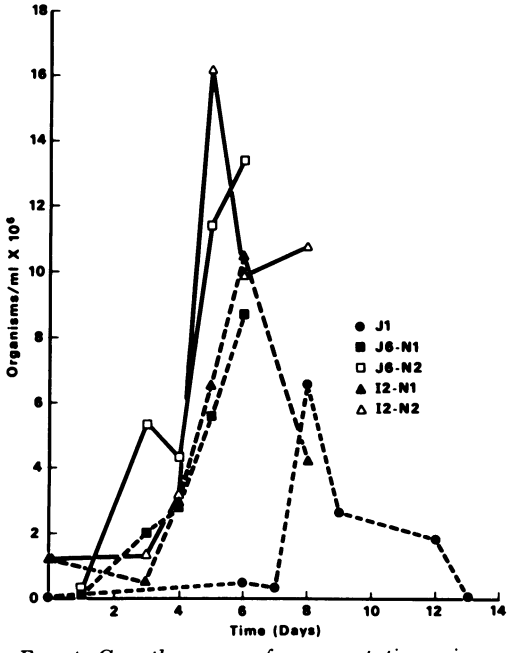

FIG. 4. *Growth curves of representative primary cultures. J6-N1 and J6-N2 are simultaneously inoculated cultures from lung of rat J6, and 12-N1 and 12-N2 are from rat 12.*

of trophozoites in at least four of the WI-38 cultures (Table 1). In one MRC-5 culture there were more organisms. In the other cultures the numbers were about the same.

There was a proliferation of trophozoites in subcultures, although peak counts were not as high as in primary cultures (Table 2). The amount of proliferation in subcultures was not necessarily proportional to the inoculum size. The subculture of rat 13 contained the greatest number of organisms, although the inoculum was smaller than that of three other rats. Subcultures could not be carried indefinitely. After two subcultures, there was no more proliferation.

Monolayers to which new medium was added after removal of the primary culture yielded a second growth which we termed second harvest. With three rat cultures, it was possible to obtain a third harvest. The second and third harvests never contained as many organisms as did the primary cultures. The peak numbers of organisms in second-harvest cultures correlated with the numbers of organisms in the primary cultures. Culture G4, with the greatest number of organisms in the primary culture, also had the greatest number in the second harvest (Table 3). Ten cultures on used cells supported growth of organisms after a repeat inoculation with infected rat lung. One monolayer was used for three different rat lung inocula over a period of 3 months. Cultures on used cells did not produce as high a yield as did primary cultures with seven rat cultures and yielded about the same numbers of organisms as new monolayers with two rat cultures (Table 4).

DISCUSSION

P. carinii trophozoites can be grown with WI-38, a readily available cell line, but a continuous culture has not been achieved. This cell line is equal or superior to the MRC-5 cell line. There is little contamination of the cultures with monolayer cells. The decreased growth in subcultures and second harvests suggests that the culture system does not supply all needed nutrients. The organisms apparently require the cells for growth but do not destroy them. The cells are not sufficiently damaged or altered to prevent their supporting the growth of additional inoc-

TABLE 1. *Comparison of cell lines*

Rat	No. of organisms × 10^6 on peak day	
	WI-38	MRC-5
J6	8.7	0.9
J6	13.4	2.8
J7	4.2	2.3
J1	6.8	6.7
H2	5.2	10.9
J7	4.4	3.3
J8	2.6	0.7
K4	1.8	1.8
G3	4.1	3.1
F5	14.2	16.1
I2	17.5	8.4

TABLE 2. *Subcultures*

Rat	No. of organisms × 10^6	
	Inoculum	Peak
J6	0.9	2.9
J1	0.4	3.8
I3	0.3	6.1
I2	0.1	1.2
H2	0.6	2.9

TABLE 3. *Primary versus second-harvest cultures*

Rat	No. of organisms × 10^6	
	Primary	Second harvest
I6	27.3	11.6
G4	117.9	12.5
M13	9.0	1.0
M15	8.6	1.4
J6	13.4	2.9
J7	4.2	2.0

TABLE 4. *Comparison of new and used cells*

Rat	No. of organisms × 10^6 on peak day	
	New	Used
H2	5.2	2.6
H2	10.9	7.3
J1	10.7	2.7
I2	14.9	5.6
I2	17.5	8.4
I1	8.6	1.1
J7	4.2	2.0
J7	1.2	1.1
K4	1.8	1.3

ula. The fact that growth decreases with subculture or second harvest suggests that some factor(s) in the initial inoculum becomes depleted. Additional studies of nutritional requirements which may provide an improved culture system are needed.

The role of the monolayer cells in the culture system is not clear. Other studies (4) show that the *P. carinii* trophozoites attach to the cells by fimbria, possibly to obtain essential nutrients. Three rat lung cultures did not grow, although impression smear stains contained large numbers of organisms. We speculate that the lack of growth may have been due to the immune or nutritional status of the rats. We observed that organisms from lungs of steroid-treated rats fed a low-protein diet did not grow as well as those from such rats fed a regular diet. Cysts did not appear in cultures until about days 15 to 17, and even then they were few in number and appeared to be immature. We have been unable to achieve complete encystment of cultures, but have not attempted extensive studies in this area.

Four attempts to culture infected human lung were unsuccessful. Two cultures had low numbers of organisms on days 4 through 8 but never enough organisms for reliable counts. At this time, culture with this system is not a worthwhile diagnostic procedure.

P. carinii can be cultured with WI-38 cells to provide trophozoites for studies of pathogenesis and immunology of infection and biology of the organism. We are using cultured organisms to study immunodiagnostic methods and to study phagocytosis and killing of *P. carinii* (R. S. Oseas, M. S. Bartlett, R. L. Baehner, L. A. Boxer, and J. W. Smith, Am. Assoc. Pathol., Dallas, Tex. [Fed. Proc. **38**:1002, 1979]).

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