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NOTES

Serial Propagation of *Pneumocystis carinii* in Cell Line Cultures

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Pneumocystis carinii was propagated on three cell lines routinely cultured in many laboratories; the method is practical and convenient. Organisms produced were found to be reactive to *Pneumocystis* antisera. Studies of antigenic relationships, life cycles, and diagnostic methods will be made easier by these cultures.

Pneumocystis carinii has become prominent among the agents causing intercurrent infections in immunocompromised patients, such as those who have leukemia or malignancies of other types or who have received organ transplants. A serious obstacle to study of this organism is its failure to grow in vitro (2). This has prevented the production of antigens in large enough quantities to develop serological diagnostic tests, inhibited drug studies, and hindered study of its morphology and life cycle. Pifer and Hughes have recently described the culture of *P. carinii* on primary chicken embryo lung monolayers (4), a very important step toward solving some of the problems mentioned. They have used these tissue cultures of organisms for in vitro drug studies; organisms from the cultures have been studied by phase-contrast and electron microscopy (L. L. Pifer, W. T. Hughes, and M. J. Murray, Jr., Pediatr. Res., in press).

Our primary interest in cultures of *Pneumocystis* was as a source of antigen, especially for use in an indirect immunofluorescence test (3). However, the difficulty of obtaining and maintaining chicken embryo lung suggested that a more routine culture method was desirable. We have succeeded in establishing *Pneumocystis* on three cell line cultures maintained routinely in many laboratories. The inoculum required is minimal, and the product is relatively free of substrate.

Sprague-Dawley rats were treated with cortisone acetate and hydrocortisone to induce spontaneous infection of *Pneumocystis* (1). For this purpose, rats of any age from young to old adults of either sex are suitable. Although rats of other strains will develop *Pneumocystis* infections when treated with cortisone, Sprague-Dawley rats seem particularly susceptible. The actual source of the infecting *Pneumocystis* is unknown. The organism is probably widely disseminated in the environment and opportunistically proliferates in immunocompromised hosts. *Pneumocystis* appeared in their lungs 7 to 12 weeks after inoculation. Lungs were removed aseptically and examined for *Pneumocystis* cysts or extracystic forms by impression smears stained with toluidine blue-O and Giemsa. The degree of infection was estimated by examining tissue sections stained with methenamine silver. About one-sixth of a lightly to moderately infected lung was rinsed gently with sterile Eagle maintenance medium to remove extraneous blood and debris. The tissue was placed in a Ten Broeck grinder with 5 ml of maintenance medium and homogenized by hand-grinding it slowly. The homogenate was centrifuged at $500 \times g$ for 10 to 15 min, and the supernatant was collected for use as inoculum. The sediment, still containing many cysts and extracystic bodies, was suspended again in maintenance medium. After recentrifugation, the supernatant was collected again. A third supernatant was obtained in the same manner. Each of the three supernatants was inoculated into a separate series of monolayers of cell line cultures in Falcon flasks.

Six cell line cultures were used as substrates: Vero (ATCC CL-81) (derived from African green monkey kidney), Chang liver (ATCC CL-13) (established from a human), MRC-5 (ATCC CL-171) (human embryo lung fibroblast), LLC-MK-2 (ATCC CL-7) (rhesus monkey kidney), FL (ATCC CL-62) (developed from human amnion cells), and McCoy (human synovial fluid). Incubation was at 37°C without CO_2.

Monolayers overlaid with Eagle essential

medium containing 10% fetal calf serum, penicillin, and streptomycin (growth medium) were inoculated with the supernatants in the proportion of 1 ml of supernatant per 5 ml of overlay. Two flask sizes were used, a 30-ml flask with 5 ml of overlay and a 150-flask with 20 ml of overlay. The cultures were incubated at 37°C. After about 4 days of incubation, clumps could be seen floating free in the overlay; some were apparently attached to the monolayer. That they were *Pneumocystis* was confirmed by staining with toluidine blue and Giemsa (Fig. 1 and 2).

Inocula for subculture were harvested in two ways, each equally successful. Either the supernatant containing free *Pneumocystis* was poured off, or about one-fourth of the monolayer was scraped free of the flask and included with the harvested material. The suspension in the harvested overlay was centrifuged at 500 × *g* for 15 min. The supernatant was removed, except for 1 ml left on the sediment.

After suspending the sediment in the residual supernatant, the suspension was diluted with growth medium to a sufficient volume for inoculation of fresh monolayers. The rate of inoculation was 1 ml of suspension to 5 ml of fresh overlay in the new flasks. The harvest from a small flask containing 5 ml of overlay sufficed to inoculate one 5-ml and one 20-ml overlay flask. Harvest from a large flask with 20 ml of overlay sufficed to inoculate two 20-ml and one 5-ml overlay flasks. Subculturing has been carried out weekly for about 4 months.

Cell lines Vero, Chang liver, and MRC-5 supported growth; lines LLC-MK-2, FL, and McCoy did not. Growth was best in lines inoculated from the first and second supernatants; the third supernatant grew *Pneumocystis* in only a few trials. After each passage, growth was allowed to proceed until the density of organisms, usually in the form of clumps, had attained the level of the culture from which the inoculum originated. Density was determined by visual observations of culture flasks under the microscope. As indicated above, material from each flask was inoculated into volumes of overlay slightly twice the volume of the overlay from which it was harvested. Conservatively, then, the increase was at least a factor of 2 for each passage. For the 16 passages reported here, the increase was at least 2^{16} or 65,536 times. Multiplication demonstrated that the organisms were viable.

Three rats, from which cultures were established, had been treated with 25 mg of cortisone every 2 days for at least 7 weeks, and all three had infections of *Pneumocystis*, as seen by

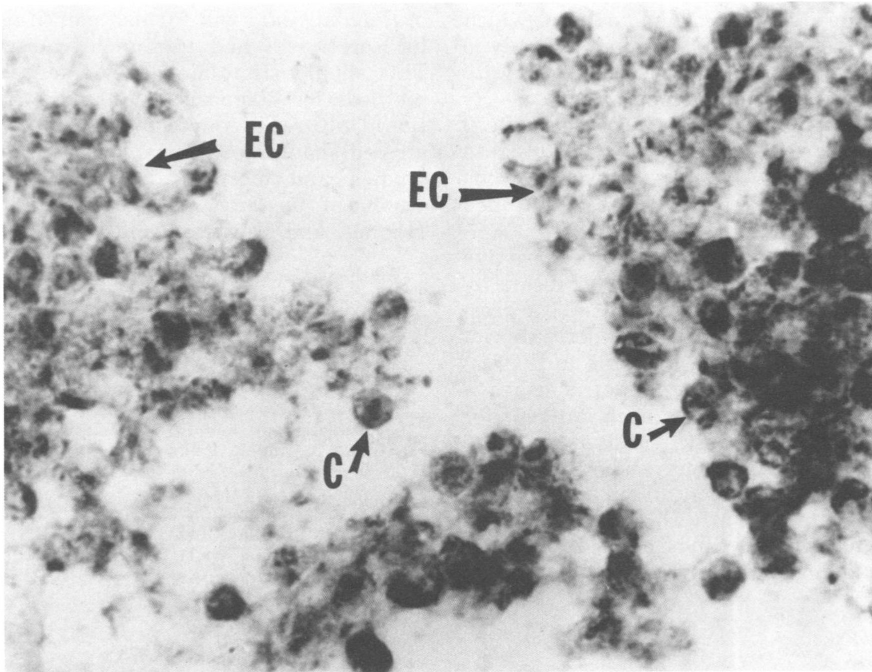

FIG. 1. *Clumps of Pneumocystis organisms as they appear in the supernatant of Vero cell lines. Masses of extracystic (trophozoite) forms (EC) contain many rounded cyst (C) forms. Giemsa stain, ×1,000.*

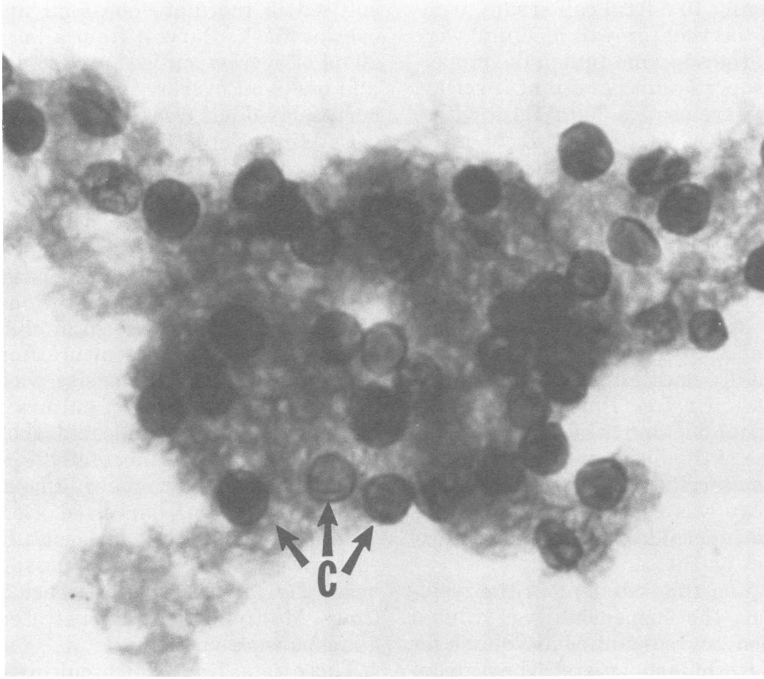

FIG. 2. *Toluidine blue preparation. Only cysts (C) remain with the more fragile extracystic forms removed by the staining procedure. ×1,000.*

stained impression smears of the lungs. *Pneumocystis* failed to grow from a fourth rat, which had been treated with cortisone for only 5 weeks, and very little evidence of *Pneumocystis* was seen on stained lung smears.

Free-floating organisms in Vero and MRC-5 cell lines were relatively free of cell substrate when harvested by decanting the overlay. Harvests from Chang liver cultures contained many substrate cells.

Organisms from all cultures proved reactive in preliminary tests of positive control sera by the indirect immunofluorescence test. The presence of substrate cells in material derived from Chang liver were not objectionable in fluorescence test antigens; indeed, they furnished a desirable contrast; we prefer such antigens for this test. For antigens in which tissue cells are objectionable, the organisms can be produced in relatively pure suspension directly on harvest by using Vero and MRC-5 substrates. The method described here can be carried out in any laboratory doing routine tissue culture.

The availability of relatively large amounts of *Pneumocystis* will furnish plentiful antigen for serological and immunological purposes. This should stimulate development of better methods for diagnosis of *Pneumocystis* infection. Cultures produced by the method we described should be applicable for in vitro drug studies and morphological and histological studies of *Pneumocystis* as described by (Pifer, Hughes, and Murray, Pediatr. Res., in press).

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