NOTES

Murine *Pneumocystis carinii* Adherence to Vertical Monolayers of Cultured Mink Lung Cells (MiCl1)

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We describe a method for adherence and culture of murine *Pneumocystis carinii* in mink lung cells (MiCl1) grown on vertical supports. The vertical cultures were infected with *P. carinii*; the surrounding medium and inoculum were stirred to ensure circulation and contact with MiCl1 cells. When compared with conventional horizontal culture, the vertical method offers a more suitable system for assessing *P. carinii* adherence. This approach has proved suitable for quantitative evaluation of *P. carinii* adherence to MiCl1 cells in the presence of inhibitors.

Because of the stationary nature of most in vitro culture systems for *Pneumocystis carinii*, natural adherence mechanisms may be difficult to evaluate. In a conventional culture system, the inoculum (*P. carinii* plus cellular debris) settles to the bottom of flat culture flasks; this debris subsequently obscures the detection of *P. carinii* and contributes to nonspecific background staining in immunochemical and fluorescence studies. With purified preparations of *P. carinii* that contain little or no lung debris, the inoculum is forced by gravity (in the absence of stirring) to grow in direct contact with the feeder cell layer. Even without specific ligand-receptor interaction, prolonged cell contact could promote adsorption between *P. carinii* and feeder cells through hydrophobic forces. The present study entails the utilization of vertical cell monolayers of mink lung pneumocytes (MiCl1; type I pneumocytes) in stirred culture medium. This culture method provides a cellular target that requires active adherence by *P. carinii*.

The source of murine *P. carinii* was lung tissue from mice with severe combined immunodeficiency obtained from Jackson Laboratory, Bar Harbor, Maine. These animals were maintained in germfree laminar flow hoods and given food and water ad libitum, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (6a). Infected animals were continuously provided by routinely cohousing six uninfected with two infected severe combined immunodeficient mice for 1 week. After 2 to 3 months of daily observations, the animals appeared moribund and were sacrificed. The animals were autopsied; the lungs were excised and stored at -70°C in RPMI growth medium for later use.

MiCl1 cells were obtained from the American Type Culture Collection, Rockville, Md. Coverslips coated with MiCl1 cells were prepared as follows. MiCl1 cells were grown on untreated glass coverslips in 2 ml of complete medium (RPMI 1640 medium plus 5% fetal calf serum, penicillin-streptomycin [250 U/ml], and 1% L-glutamine). After 24 h of growth at 37°C in a 4% CO_2 atmosphere, the

MiCl1-coated coverslips were removed from the original six-well plastic culture dishes (horizontal culture), and the cultured coverslips were placed into sterile 50-ml conical centrifuge tubes containing 12 ml of continuously stirred complete medium (vertical culture). The cultures were stirred with a Thermolyne/Sybron type 45600 Cell Grow Stirrer at a speed of 150 rpm with Teflon-coated stirring fleas (magnetic stir bars [10 by 3 mm]). The stirred vertical cultures were inoculated with 0.5 ml of infected lung homogenate from mice with severe combined immunodeficiency containing approximately 10^6 organisms (Fig. 1). The infected cultures were incubated for 1 to 4 days, after which the coverslips were fixed in absolute methanol.

Fluorescein-conjugated tomato lectin (TOL-FITC; 2 mg/ml) (Vector Laboratories, Burlingame, Calif.) is specific for chitin and chitin oligosaccharides and was used to demonstrate binding of *P. carinii* to MiCl1 cells in vitro. Previous investigations have shown that human and murine *P. carinii* are immunoreactive for chitin in all recognized life stages of *P. carinii* (4). Twenty microliters of TOL-FITC in 1 ml of phosphate-buffered saline (pH 7.4) (PBS) was applied to each coverslip for 1 h in the dark. The coverslips were then

FIG. 1. Schematic representation of vertical culture method. MiCl1 cells are grown on coverslips for 24 h and then placed in medium in a conical tube. Lung homogenate from a mouse with severe combined immunodeficiency (SCID) is then added. The culture is stirred with a stirring flea. PNC, *P. carinii*.

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FIG. 2. Two-day horizontal culture of MiCl1 cells and *P. carinii*. TOL-FITC binding of background debris obscures the fluorescence of *P. carinii* (arrow) (original magnification, $\times 400$).

rinsed in PBS, mounted on glass slides, and examined by fluorescence microscopy. During the 4-day period, cell debris accumulated and could be observed on MiCl1 monolayers in the horizontal cultures (Fig. 2 and 3). In contrast, during the same period, the debris was absent from the monolayers in the vertical cultures (Fig. 4 and 5).

Monolayers of mammalian cells have been used for the *in vitro* propagation of *P. carinii* (1, 3, 4, 7). Infected horizontal

monolayers, however, frequently contain a large amount of debris that interferes with the observation of the infectious process. Studies have been performed with purified *P. carinii* to assess the attachment of *P. carinii* to pneumocytes (8). Although the use of purified *P. carinii* eliminates interference by debris, it does not test the intrinsic ability of the organism to encounter and bind a target cell.

To evaluate a pathogen's ability to bind a target cell, the

FIG. 3. Four-day horizontal culture contains TOL-FITC-labeled *P. carinii* (arrows), but the large amount of debris obscures host-parasite interaction (original magnification, $\times 400$).

FIG. 4. Two-day vertical culture of MiCl1 cells and *P. carinii* (arrow) stained with TOL-FITC. Note the absence of background debris. Equivalent numbers of organisms were bound in both horizontal and vertical preparations (original magnification, $\times 400$).

process should proceed in a fully dynamic system. In the present study, a series of vertical and horizontal cultures were prepared that represented a timed course of growth. The use of unpurified lung homogenate as the inoculum to infect MiCl1 slide cultures with *P. carinii* may be desired in certain instances. Such an unmanipulated inoculum has not been altered by enzyme treatment or centrifugation, nor has it been

exposed to density gradient materials. Furthermore, it contains *P. carinii* in all stages of development. In horizontal cell cultures, however, the lung debris falls to the bottom, obscuring the cell layer and possibly interfering with *P. carinii*-MiCl1 cell contact. When the same concentration of inoculum is added to vertical cell cultures, there is no detectable accumulation of lung debris on the feeder cell layer.

FIG. 5. Four-day vertical culture of MiCl1 cells and *P. carinii*. *P. carinii* can still be readily identified with TOL-FITC (arrows) (original magnification, $\times 400$).

FIG. 6. Comparison of MiC11 cell cultures infected with 10^6 *P. carinii* (PNC) cysts in the presence of monosaccharides (10 and 100 μ g of D-mannose, D-glucose, and D-galactose). Gomori's methenamine silver-stained *P. carinii* were counted on the coverslips by using a 40 $\times$ objective. Six hundred and fifty 1- μ m² quadrants were sampled, and average counts were computed from multiple samples. Error bars show the standard error of the mean. The data given are representative of multiple experiments.

Using this procedure, a clear assessment of *P. carinii* binding can be made. For example, Fig. 6 illustrates the effectiveness of this technique in assessing inhibitors of adherence. Galactose, glucose, and mannose were added to vertical coverslip cultures for 4 days at concentrations of 10 and 100 μ g/ml. After 4 days, the coverslips were fixed in absolute methanol and stained with Gomori's methenamine silver stain. Both concentrations (10 and 100 μ g/ml) of mannose, a known inhibitor of *P. carinii* binding (5), blocked adherence of *P. carinii* to MiC11 monolayers during this incubation period. As noted in Fig. 6, the blockade of binding was nearly 100%.

The vertical coverslip method is applicable to both qualitative and quantitative investigations of host-parasite interaction. The procedure requires that binding occur as an active process since *P. carinii* does not fall and rest on the

target cells as a consequence of gravity. Instead, *P. carinii* contacts the target cell randomly. If binding does not occur, the organism is swept away in the stirred medium. Moreover, because of the vertical technique, whole-lung homogenate can be used as the inoculum. Although centrifugal methods are currently used for partial purification of *P. carinii* (6), there is no satisfactory procedure for completely separating *P. carinii* from lung debris. Filtration has also been used to purify *P. carinii* from lung homogenates (2). Filtering, however, removes clumped *P. carinii*, which may represent an important subfraction of the infectious population. Thus, vertical culture provides a means of eliminating artifactual binding of non-*P. carinii* material without altering the population of *P. carinii* present in lung homogenates.

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