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Biological Profile and Response to Anti-Pneumocystis Agents of *Pneumocystis carinii* in Cell Culture

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Although the growth characteristics of *Pneumocystis carinii* have been described in several cell culture systems, the response of this organism to the drugs of choice, trimethoprim-sulfamethoxazole and pentamidine isethionate, have not been described in vitro. The effect of various concentrations of drugs against *P. carinii* on the growth of this potentially hazardous opportunistic organism as well as the methodology for in vitro assay of these agents have been detailed. Fluorescence profiles illustrating size ranges of trophozoites and cysts derived from cell culture are described.

Although the clinical course and treatment of *Pneumocystis carinii* have been well defined (9, 11), only limited effort has been devoted to the investigation of the biological and biochemical properties of the organism. Other than the report by Pesanti et al. (18), no in vitro method has been reported for evaluating existing and potential therapeutic agents against *P. carinii*. The need for such a system has become increasingly apparent in view of recent reports of trimethoprim-sulfamethoxazole treatment failures (17) and of the increased incidence of *P. carinii* infections in homosexual males (8), individuals with acquired immunodeficiency syndrome and their intimate contacts (2), intravenous drug abusers (15), infants (3), hemophiliacs (16), and Haitians (4). The purpose of the present study, therefore, is to describe in vitro growth characteristics and an in vitro method for evaluation of drugs against *P. carinii*.

MATERIALS AND METHODS

Animals. *P. carinii* organisms were obtained, by methods previously described in detail (19), from the lungs of male Sprague-Dawley rats each weighing less than 200 g and treated with cortisone acetate to provoke infection (7). The animals were sacrificed with sodium pentothal, and the lungs were aseptically removed and shaken vigorously in 25 to 50 ml of cold calcium- and magnesium-free phosphate-buffered saline supplemented with penicillin (500 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), and gentamicin (50 μg/ml).

Inocula for cell culture. The organisms were decanted and centrifuged at 13,200 × *g* for 15 min in a Sorvall HB4 rotor at 4°C. The pellet was washed repeatedly with cold calcium- and magnesium-free phosphate-buffered saline, suspended in medium 199 (Microbiological Associates, Bethesda, Md.), and cultured for contaminating microorganisms before the addition of antibiotics. The number of *P. carinii* organisms harvested were quantified by a method involving direct counts of measured volumes of the inocula (19). Organisms were stained for the purpose of quantitation with toluidine blue O (5) and for Cytograf analysis with 10^{-4} M acridine orange.

Cell cultures and growth curves. Embryonic chicken epithelial lung cells were prepared from 14-day-old embryonated eggs by successive treatments with 0.25% pronase by a method designed to eliminate fibroblasts (6). Primary epithelial cell cultures were grown in 2-ml capacity Leighton tubes containing cover slips (2 by 11 cm) for 24 h before inoculation. The cultures were infected with *P. carinii* cysts ranging from 7.5×10^3 to 7.5×10^4 cysts per culture, or 3.8×10^3 to 3.8×10^4 cysts per ml. Control cultures were mock infected with normal rat lung washings. The inoculum was allowed to adsorb to the cell monolayers for 2 h, after which time they were supplemented with prewarmed medium 199 in a total volume of 2 ml per culture. Each culture was prepared in triplicate.

At various intervals postinoculation, organisms in the supernatants from Leighton tubes were harvested by centrifugation, suspended in 0.5 ml of medium 199, and counted, as described previously (19). An estimate of cell-associated organisms was made by counting 500 oil immersion fields on each cover slip. The separate counts of cell-associated cysts and those in the supernatant constitute the values plotted at each time interval unless otherwise indicated.

Drugs against *P. carinii*. Trimethoprim-sulfamethoxazole (TMP-SMZ) was a gift from Hoffmann-La Roche Inc., Nutley, N.J. It was prepared in phosphate-buffered saline, diluted in medium 199 immediately before use in concentrations of 1.8, 6.0, 18.0, and 54.0 μg/ml, and sterilized by filtration. These concentrations represented a 1:5 ratio (wt/wt) of TMP and SMZ, respectively; i.e., 1.8 μg/ml of TMP-SMZ contained 0.3 μg of TMP and 1.5 μg of SMZ. Pentamidine

isethionate (PI) was obtained from the Centers for Disease Control, Atlanta, Ga., and was prepared in like manner in concentrations of 0.3, 1.0, 3.0, and 9.0 μg/ml.

For the purpose of drug evaluation, cell cultures were established in Leighton tubes, and each 24-h-old monolayer received 5.4×10^4 cysts. After a 2-h adsorption period at 37°C, during which the organisms were applied to the monolayers in a volume of 0.2 ml of medium, drug-containing medium 199 was added. Control cultures (as described earlier) were mock infected, and equivalent concentrations of drugs were added to the monolayers to test for drug-induced cytopathic effect or toxicity. At the intervals indicated postinoculation, the organisms that were found associated with the cells on Leighton tube cover slips and those in the supernatant were quantified.

Cytographic analysis of *P. carinii* cysts and trophozoites. The UV absorption of *P. carinii* organisms in 10^{-4} M acridine orange was measured on a 100-channel Cytograf analyzer standardized with 2- and 7-μm latex beads. These analyses were conducted on washed organisms from the third cell culture passage. Organisms were suspended in isotonic buffer. To avoid contamination resulting from other cells and microorganisms normally present in rat lungs, only cell culture-grown organisms were used in these studies. Each preparation was stained with toluidine blue O and with Giemsa to determine cross-contamination. Each preparation of purified cysts and purified trophozoites contained about 5% trophozoites or cysts, respectively.

P. carinii cysts and trophozoites were separated by low-speed differential centrifugation at room temperature for 8 min at 2,500 rpm (1,800 × *g*) in phosphate-buffered saline. Trophozoites remained in the supernatant, whereas most of the cysts were in the pellet. Another useful technique involved treating the cyst and trophozoite preparations with sterile, distilled water supplemented overnight with antibiotics. Trophozoite "ghosts" in the supernatant were then removed by low-speed centrifugation that loosely pelleted the cysts.

RESULTS

Growth curves. Growth curves (Fig. 1 and 2) were plotted by counting a sample of cell-associated cysts and those concentrated by centrifugation from the culture supernatants at time zero and at 24, 48, 120, 144, 168, and 192 h postinoculation. Each value plotted represents the calculated (20) total number of cysts on the cover slips (Fig. 1) or in the supernatants (Fig. 2) of triplicate cultures. Sowar and Walzer (Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 22, 1982) have reported attempts to quantify the highly pleomorphic trophozoite phase of *P. carinii*; however, we concluded that such data derived from our primary cell culture system were not sufficiently accurate to report. Although monolayers were rinsed before inoculation to remove nonviable or unattached cells, the amount of debris present in primary cultures made this measurement impractical.

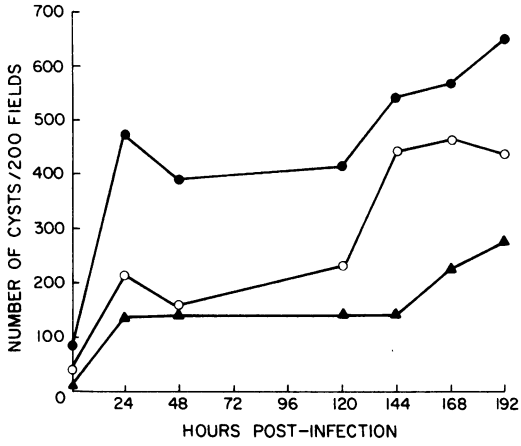

FIG. 1. Number of cell-associated *P. carinii* cysts per 200 oil immersion fields, as determined from triplicate cultures at various intervals postinoculation and employing three dilutions of inocula: ●, 7.5×10^4 cysts; ○, 3.8×10^4 cysts; ▲, 0.75×10^4 cysts.

Three concentrations of inocula were used to determine the optimal cyst-to-cell ratio for growth of the organisms. It was determined that inocula exceeding 8.5×10^4 cysts per 2.0×10^7 primary chicken epithelial lung cells resulted in toxicity and, consequently, a diminished yield of *P. carinii* organisms. With regard to organisms in inoculum A (7.5×10^4 cysts), during the first 24 h of culture, the number of cysts in the supernatants and those attached to the cell sheet increased rapidly, with the former exhibiting the

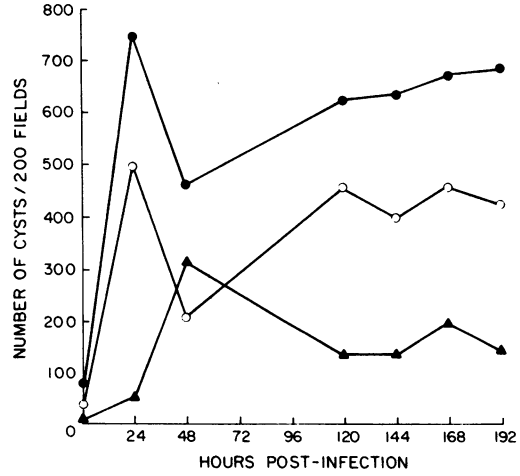

FIG. 2. Number of *P. carinii* cysts harvested from portions of culture supernatants sampled in triplicate at various intervals postinoculation and employing three dilutions of inocula: ●, 7.5×10^4 cysts; ○, 3.8×10^4 cysts; ▲, 0.75×10^4 cysts.

greater rise in titer (about two logs) per total supernatant. Those organisms associated with the monolayer receiving inoculum A showed a more modest increase in number, from 7.5×10^4 cysts in the original inoculum to about 4.9×10^6 at the end of 24 h. Peak numbers of cysts in this same series receiving inoculum A at 192 h were about 6.9×10^6 in the supernatant and 6.6×10^6 associated with the monolayer. Increases in other cultures receiving inocula ranging from 3.8×10^4 (inoculum B) to 0.75×10^4 (inoculum C) cysts were essentially proportional to those observed in inoculum A. In general, more cysts were detected in the supernatant, but not significantly more. It appeared possible that decreases in the numbers of cysts at certain intervals in the growth curve perhaps suggest a synchronous reproductive pattern. During this time, most organisms in the culture may have completed excystment, and trophozoite forms appeared to predominate. Gross observation supported this hypothesis, although quantitation was not possible.

In vitro drug studies. TMP-SMZ and PI were each tested at four concentrations against an inoculum of *P. carinii* consisting of 2.7×10^4 cysts per ml or 5.4×10^4 cysts per Leighton tube. Drug concentrations were prepared to approximate optimal blood levels of TMP-SMZ in human subjects (1:5); these consisted of 1.8, 6.0, 18.0, and 54.0 μg/ml. Concentrations of 0.3, 1.0, 3.0, and 9.0 μg of PI per ml were tested.

Both TMP-SMZ and PI appeared to exert an inhibitory effect on the growth of *P. carinii* cyst-phase organisms in primary chicken epithelial lung cell cultures. A dose response, although not one that is likely to be significant, was observed with TMP-SMZ. A similar response was not detected in the cultures containing PI. All tubes supplemented with PI contained cysts ranging from 6.4×10^4 to 6.8×10^4 at 72 h postinoculation. The control cultures at the same time interval contained 1.2×10^5 cysts. The 72-h count in tubes containing 1.8 μg of TMP-SMZ per ml was 5.8×10^4 and about 4.1×10^4 for those in cultures supplemented with 54 μg of TMP-SMZ per ml.

Although accurate quantitative enumeration of trophozoites was not possible in primary cultures, there were grossly fewer of these forms in cultures receiving any concentration of either TMP-SMZ or PI.

To determine if these agents exhibited a "cidal" or statical effect on *P. carinii* organisms in cell culture, the organisms were harvested and washed in phosphate-buffered saline and reinoculated into fresh cell cultures. No growth resulted in these tubes during 2 weeks of incubation. This suggests that, at least in cell culture, TMP-SMZ and PI both have a cidal effect on *P. carinii* under the conditions described. In recent years, TMP-SMZ has been shown to exert only a statical effect on *P. carinii* organisms in vivo (10).

Cytographic analysis. Cytographic analysis was carried out with acridine orange stain at 260 nm with two diameters of latex beads as particle size standards (Fig. 3 and 4). Purified cysts and trophozoites (Fig. 5 and 6) from cell culture were subjected to analysis, and it was determined that most trophozoites from cell culture fall in the average range of 2 μm in diameter with intermediate sizes, probably representing older trophozoites, in transition to the cyst phase. The cyst preparation peaked in the 7-μm range but also showed the presence of smaller transitional forms, as might be expected. These studies were done before biochemical characterization to assess the relative purity of cysts and trophozoites and to obtain a more specific value on the relative sizes of the two major forms from cell culture. As a result of these studies and also on the basis of microscopic examination, it was determined that the trophozoites contained an unacceptable amount of 2-μm-sized debris to yield reliable data for biochemical analysis. The

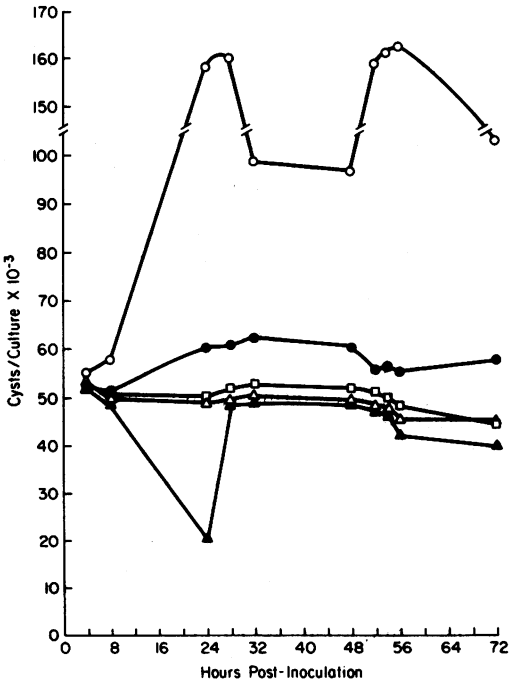

FIG. 3. Growth curve of *P. carinii* represented by cyst-phase organisms sampled in triplicate at various intervals postinoculation in the presence of four concentrations of TMP-SMZ. ○, Control. TMP-SMZ was used at the following concentrations (micrograms per milliliter): 1.8 (●), 6.0 (□), 18.0 (△), and 54.0 (▲). Inoculum, 5.4×10^4 cysts/tube.

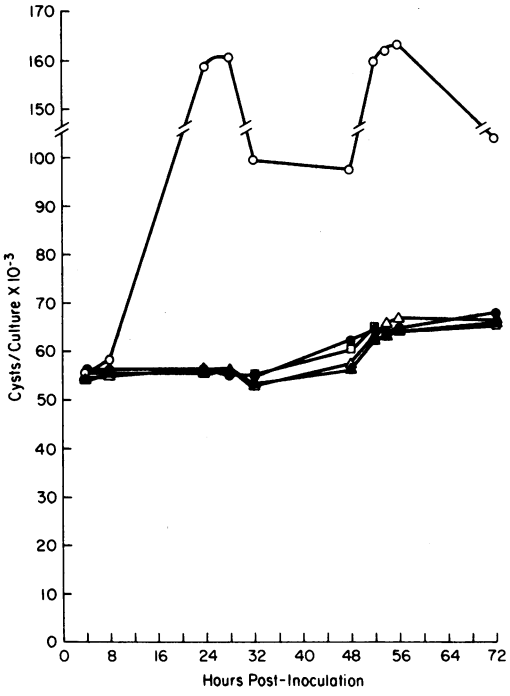

FIG. 4. Growth curve of *P. carinii* represented by cyst-phase organisms sampled in triplicate at various intervals postinoculation in the presence of four concentrations of PI. ○, Control. PI was used at the following concentrations (micrograms per milliliter): 0.3 (●), 1.0 (□), 3.0 (△), and 9.0 (▲).

cysts, however, were considerably more readily purified and consequently were subjected to preliminary biochemical analysis. In general, cytographic analysis showed that microscopic estimates of fixed and stained cysts and trophozoites are essentially accurate with regard to purity.

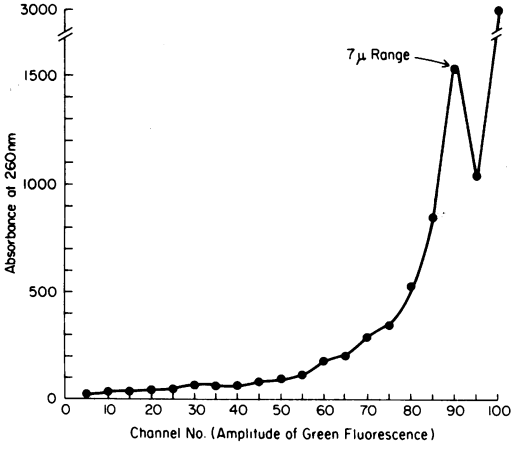

FIG. 5. Cytographic fluorescence profile of purified *P. carinii* cyst-phase organisms from cell culture.

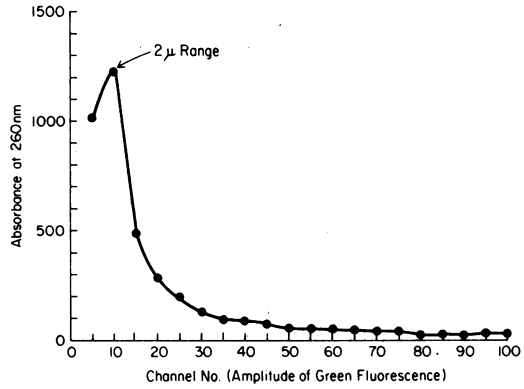

FIG. 6. Cytographic fluorescence profile of purified *P. carinii* trophozoite-phase organisms from cell culture.

DISCUSSION

Since the initial report of Pifer et al. (19), several attempts at cultivation of *P. carinii* have been described (1, 12, 21). The need for a reliable means of producing substantial numbers of *P. carinii* organisms for serological testing is obvious. The noninvasive counterimmunoelectrophoresis test for *P. carinii* antigenemia relies on these methodologies and appears very promising. This technique uses antibody prepared against cell culture-grown organisms, thus emphasizing the importance of pursuing better methods for cultivating *P. carinii* (20). Recently, researchers at the Centers for Disease Control have verified the specificity of the counterimmunoelectrophoresis test for detecting *P. carinii* antigenemia and have corroborated the concept presented in 1978 (20) that *P. carinii* may be present as a subclinical entity in human patients (13, 14). Immunofluorescence tests for *P. carinii* antibody in which culture-produced cysts are used appear to be more sensitive, perhaps because organisms grown in culture are not coated with antibody, as are those freshly isolated from lung (20). The cultivation of *P. carinii* in vitro, therefore, has found practical application in providing pure and immunologically unreacted organisms for these much-needed adjuncts to the more hazardous invasive methods of diagnosis. At present, it does not appear likely that direct culture without resort to invasive techniques will ever prove useful in confirming *P. carinii* infection. There is considerable room for improvement, however, in obtaining higher yields from any culture system yet reported.

The cell culture system may prove to be a practical means for assessing the efficacy of

drugs against *P. carinii*, particularly during the screening stages of evaluation. Multiple analyses may be conducted simultaneously by this convenient, miniaturized method, whereas a similar undertaking with the rat model would likely prove impractical. The cell culture system might be used most efficiently as a preliminary technique for selecting the most promising pharmaceutical candidates for trials in the animal model. Although for obvious reasons the latter is not likely to be replaced, results from cell culture are generally available after 1 week, whereas the less well-refined, more tedious and costly animal model usually does not yield definitive results for at least 8 to 12 weeks.

In conclusion, cell culture has provided (i) antibody-free cysts for use in immunofluorescence assays of antibody to *P. carinii*, (ii) a source of relatively pure antigen for use in antibody production, thus augmenting the development of specific, noninvasive immunological tests for *P. carinii* infection, (iii) biological data partially describing the replicative cycle of *P. carinii* and (iv) a system for evaluating the efficacy of potential drugs to be used in treating *P. carinii* infections.

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