Method of Testing the Susceptibility of *Pneumocystis carinii* to Antimicrobial Agents In Vitro

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Rat *Pneumocystis carinii* grown on lung-derived cell lines in tissue culture flasks and multiwell plates was tested for susceptibility to four antimicrobial agents currently being used in the treatment of human pneumocystosis. Standard criteria for organism quantitation, replication, viability, and inoculum size were established. Trimethoprim-sulfamethoxazole inhibited *P. carinii* growth at a concentration ratio of 1:19 µg/ml, and pentamidine isethionate was active at 0.1 µg/ml. α-Difluoromethylornithine, an inhibitor of polyamine biosynthesis, inhibited *P. carinii* at a concentration of 1 mM once erythrocytes (which are high in polyamine content) were removed from the inoculum; this effect could be overcome by the polyamine putrescine. Dapsone suppressed *P. carinii* replication at a dose of 0.1 µg/ml, but this effect was lost after 72 h in culture. Overall, the reduction in *P. carinii* numbers with these drugs was relatively modest (45 to 84%), which is consistent with their lack of lethal effects on the organism in vivo. Thus, the system presented here should be helpful in developing new anti-*P. carinii* agents and in elucidating their mechanism of action.

Over the past two decades, *Pneumocystis carinii* has been recognized as a leading cause of pneumonia in immunosuppressed patients. With the emergence of the acquired immune deficiency syndrome (AIDS), the number of cases of pneumocystosis has greatly increased (5). Not only is AIDS the major predisposing host factor in the development of pneumocystosis, but *P. carinii* is also the most frequent opportunistic infection and a leading cause of death in AIDS. Trimethoprim-sulfamethoxazole (TMP-SMX) and pentamidine isethionate have been the principal drugs used in the therapy of pneumocystosis (17, 34, 39). The high recurrence rate of pneumocystosis and high frequency of adverse reactions to TMP-SMX in patients with AIDS emphasize the need to develop new forms of treatment (12, 14, 21, 30).

The search for new drugs active against *P. carinii* in humans has rested primarily on naturally infected rodents. Rats administered corticosteroids for 8 weeks spontaneously develop pneumocystosis with histopathologic features identical to those in humans (9, 35). Although the system is time consuming and has permitted the testing of relatively few antimicrobial agents, there has been a high correlation between drug efficacies in rats and humans (9, 18, 20). The most recent application of this form of drug development is dapsone (19), which is currently undergoing clinical trials. An exception seems to be α-difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis; this agent has been ineffective in the rat model (19) but has shown promising activity in small numbers of *P. carinii* patients (11, 31). Thus, there is a need for alternative methods of drug evaluation.

One approach to this problem has involved in vitro systems. Several different techniques of cell culture have been used for the cultivation of *P. carinii*, but these studies have been limited by a lack of reproducibility among different laboratories (4, 22, 28). We have recently developed an in vitro cultivation system for *P. carinii* and proposed standard criteria for organism quantitation and growth (6, 7, 8). In the

present study, we used this system for the investigation of the susceptibility of *P. carinii* to antimicrobial agents.

MATERIALS AND METHODS

Organism source, culture, and quantitation. These procedures have been described in detail previously (6, 7, 8, 35). Briefly, *P. carinii* was induced in adult male Sprague-Dawley rats by corticosteroid administration, and the lungs were removed en bloc, minced, and homogenized in a Stomacher (Tekmar, Cincinnati, Ohio). Erythrocytes were lysed with an ammonium chloride solution, and the inoculum was quantitated for the number of *P. carinii* nuclei per ml. Viability was assessed by erythrosin B dye exclusion.

P. carinii was inoculated into 48-h-old confluent tissue culture cells grown in triplicate 25-cm² flasks or 24-well tissue culture plates (Beckton-Dickinson Labware, Oxnard, Calif.) and incubated in a water-jacketed incubator at 37°C in a 5% CO_2 atmosphere. The final numbers of *P. carinii* nuclei in culture were 10^7/ml unless otherwise indicated. Two human lung-derived tissue culture cell lines were used; A549 (CCL 185) and WI-38 VA13 subline 2RA (CCL 75.1) (American Type Culture Collection, Rockville, Md.). Medium optimized for *P. carinii* culture contained ATCC-recommended medium with 20% fetal bovine serum (M.A. Bioproducts, Walkersville, Md.), penicillin (200 U/ml), streptomycin (200 µg/ml), and amphotericin B (0.5 µg/ml; M.A. Bioproducts) or gentamicin (100 µg/ml; Schering Corp., Kenilworth, N.J.) and amphotericin B (0.5 µg/ml; GIBCO Laboratories, Grand Island, N.Y.). These drugs, which were routinely used in the tissue culture system, did not inhibit *P. carinii* growth or appear to have any interaction with the test antimicrobial agents added to the culture. In additional experiments, gentamicin and amphotericin B at doses of up to 200 and 2.5 µg/ml, respectively, did not impair *P. carinii* growth.

After inoculation, cultures were sampled on days 1, 3, 5, and 7 by removing 1/10 of the volume from at least three flasks or wells and pooling the supernatants. *P. carinii* nuclei were stained with Diff Quik (American Scientific Products,

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McGaw Park, Ill.), and three 0.01-ml drops were counted for a total of 30 oil immersion fields per data point; conversion to organism nuclei per ml was calculated by a standard formula (15). An equal amount of experimental medium was added to replace the amount withdrawn for sampling. The effects of pooling sample portions were evaluated at different times in the culture period by comparing organism counts from 10 separate wells with the counts after pooling those same samples.

Antimicrobial agents. TMP-SMX was obtained from Burroughs-Wellcome, Co., Research Triangle Park, N.C. A stock solution of TMP (16 μg/ml)-SMX (304 μg/ml) was dissolved in culture medium without serum, and the pH was adjusted to 7.0 with 1 N NaOH. Fetal bovine serum was added after the solution was sterilized with a 0.22-μm (pore size) filter. Serial dilutions were made with culture media for final concentrations of 8 μg of TMP with 152 μg of SMX per ml and 1 μg of TMP with 19 μg of SMX per ml.

Pentamidine isethionate (May and Baker Ltd., Dagenham, England) was obtained from the Centers for Disease Control, Atlanta, Ga., and prepared in a similar manner as TMP-SMX. A stock solution of 10 μg was prepared in medium without serum, filter sterilized, supplemented, and serially diluted for final concentrations of 1.0 and 0.1 μg/ml.

DFMO (Merrell Dow Research Institute, Cincinnati, Ohio) was prepared as described above with final concentrations of 1 (237 μg/ml), 5 (1,185 μg/ml), 10 (2,370 μg/ml), 25 (5,925 μg/ml), and 125 mM (29,625 μg/ml).

Putrescine (Sigma Chemical Co., St. Louis, Mo.) was added to medium without serum for a concentration of 0.5 mM (44 μg/ml) and filter sterilized, and fetal bovine serum was then added. The final concentration of putrescine was 0.1 mM (8.8 μg/ml).

Dapsone (Jacobus Pharmaceutical Co., Inc., Princeton, N.J.) was dissolved in dimethyl sulfoxide for a stock solution of 20 mg/ml. The stock solution was diluted in distilled water so that when added to culture medium at the proper concentrations, the percentage of dimethyl sulfoxide was never over 0.02%. Preliminary studies indicated that dimethyl

sulfoxide at this level did not inhibit *P. carinii* growth. The final concentrations of dapsone in medium were 10, 1.0, and 0.1 μg/ml.

RESULTS

Growth in plates and flasks. The techniques used for in vitro drug susceptibility testing in our laboratory originally used 25-cm^2 flasks for host cell and *P. carinii* growth. Later, 24-well plates were substituted for the flasks, because this allowed more drugs to be assayed and less reagents to be used. The growth of *P. carinii* was compared in the multi-well plates with the established flask method, and no difference in the support of growth was found (Table 1). With an inoculum of 10^7 organisms, *P. carinii* grew 5 to 6-fold, with peak numbers reached on day 3. As in previous experiments with flasks (6), the inoculum which had erythrocytes lysed with 0.85% ammonium chloride was compared with unlysed inoculum for *P. carinii* growth in multi-well plates; the data indicated that the presence or absence of erythrocytes had no effect on subsequent *P. carinii* growth (Table 1).

Experiments were then performed to evaluate different types of sampling and quantitation of *P. carinii* growth in multi-well plates. The culture was inoculated with 10^7 organisms. At different times over a 7-day period, samples from 10 wells were removed, counted individually, and expressed as the mean ($\pm$ the standard deviation) number of *P. carinii* nuclei per ml. The 10 samples were also pooled, and the number of organism nuclei per ml in this specimen was calculated. The results for individual and pooled specimens were as follows: day 1, 3.57 $\pm$ 0.17 $\times$ 10^7/ml versus 3.72 $\times$ 10^7/ml; day 3, 5.43 $\pm$ 0.34 $\times$ 10^7/ml versus 5.73 $\times$ 10^7/ml; day 5, 3.73 $\pm$ 0.33 $\times$ 10^7/ml versus 3.81 $\times$ 10^7/ml; day 7, 2.92 $\pm$ 0.12 $\times$ 10^7/ml versus 2.93 $\times$ 10^7/ml. As in our previous studies with flasks (6), there was close correlation between these two methods; thus, pooled specimens were used for the evaluations of antimicrobial agents.

TMP-SMX. Growth patterns of *P. carinii* in the presence of TMP-SMX were similar in flasks and multi-well plates with lysed and unlysed inocula (Table 1). TMP-SMX suppressed *P. carinii* growth by >50% at all three doses throughout the experiment. The extent of suppression for TMP-SMX and other antimicrobial agents has been expressed as the ratio of the peak organism counts in drug-treated cultures to the peak organism counts in control (untreated) cultures over the entire culture period. This was chosen rather than day 3 (when peak *P. carinii* counts usually occurred), because the day to reach the highest organism numbers sometimes varied among drug-treated and control cultures as well as among different experiments.

TMP-SMX was also used to evaluate the role of inoculum size on antimicrobial activity. Preliminary studies performed prior to the establishment of methodology to precisely quantitate inoculum size in the lung homogenate had clearly indicated that TMP-SMX suppressed *P. carinii* growth. The estimated inoculum size based on the growth kinetics of *P. carinii* was 10^6/ml. To examine this more formally, we performed an experiment with *P. carinii* inocula of 10^7, 2 $\times$ 10^6, and 4 $\times$ 10^5 and a fixed dose of 8 μg of TMP and 152 μg of SMX per ml (Table 2). In control cultures, the numbers of *P. carinii* increased 6- and 3.5-fold with inoculum doses of 10^7 and 2 $\times$ 10^6, respectively. TMP-SMX inhibited growth by >65% in both cultures. With an inoculum size of 4 $\times$ 10^5, growth of *P. carinii* was only twofold, and the effects of TMP-SMX were less apparent. Based on these data, we concluded that inoculum sizes of 10^6 to 10^7/ml could be reliably used to evaluate the effects of antimicrobial agents

TABLE 1. Effects of TMP-SMX on *P. carinii* growth

Experimental technique	TMP-SMX ratio (μg/ml)	No. of *P. carinii* ($\times10^7$)[a] per ml on day (after inoculation):				Peak ratio[b]
		1	3	5	7	
Flasks[c]	0:0	2.9	5.6	3.2	0.7	
	1:19	1.9	2.8	1.8	0.4	0.50
	8:152	1.8	2.6	1.8	0.5	0.46
	16:304	1.4	1.6	1.2	0.2	0.26
Multi-well plates[c]	0:0	3.4	5.7	3.1	1.7	
	1:19	2.2	2.6	2.0	1.0	0.46
	8:152	2.0	2.4	2.2	0.8	0.42
	16:304	1.8	2.2	1.9	0.4	0.38
Multi-well plates[d]	0:0	3.4	5.6	3.2	1.8	
	1:19	2.3	2.7	1.5	0.7	0.48
	8:152	2.1	2.5	2.2	0.9	0.45
	16:304	1.9	2.1	2.0	0.2	0.38

[a] Inoculum = 10^7 organisms. Each data point represents the number of *P. carinii* nuclei per ml of at least triplicate pooled samples in the A549 cell line.
[b] Ratio of peak organism numbers in treated cultures to peak organism numbers in control cultures.
[c] Erythrocyte-lysed inoculum.
[d] Unlysed inoculum.

TABLE 2. Effects of inoculum size and TMP-SMX on *P. carinii* growth

Inoculum (no. of *P. carinii* per ml)	TMP-SMX ratio (μg/ml)	No. of *P. carinii* per ml[a] on day (after inoculation):				Peak ratio[b]
		1	3	5	7	
10^7	0:0	1.3^c	6.2	3.6	2.5	
	8:152	0.8	1.7	1.3	0.7	0.27
2.0 × 10^6	0:0	2.7^d	7.0	1.9	<0.1^e	
	8:152	2.2	2.4	<0.1	<0.1	0.34
4.0 × 10^5	0:0	7.2^f	8.1	<1.4^e	<1.4	
	8:152	6.4	2.1	<1.4	<1.4	0.79

^a Each data point represents the number of *P. carinii* nuclei of at least triplicate pooled samples.
^b Ratio of peak organism numbers in treated cultures to peak organism numbers in control cultures.
^c ×10^7/ml.
^d ×10^6/ml.
^e Detection limit of quantitation system, 1.4 × 10^5 organisms per ml.
^f ×10^5/ml.

on *P. carinii* growth in culture. In the present study, 10^7 organisms per ml was chosen to ensure uniformity of results.

Pentamidine. The effects of pentamidine on *P. carinii* growth were tested with lysed and unlysed inocula (Table 3). In control cultures, *P. carinii* numbers increased sixfold, peaking on day 5. Once again, the presence or absence of erythrocytes had no effect on drug efficacy. At concentrations of 0.1 to 10 μg of pentamidine per ml usually inhibited *P. carinii* growth by >75%.

DFMO. The first two experiments with DFMO were performed prior to the development of the technique for quantitating inoculum size. With inocula containing erythrocytes, DFMO at concentrations of 1 to 5 mM did not impair *P. carinii* growth (Table 4). In fact, these drug levels seemed to augment growth of the organism. High concentrations of DFMO had inconsistent effects on *P. carinii* and sometimes appeared to be toxic to the cell monolayer.

Two experiments were then performed with lysed inocula (Table 4). In the first study, DFMO at low concentrations inhibited *P. carinii* growth by >40%. Since erythrocytes are known to be high in polyamine content (37), and since the mechanism of DFMO is to inhibit polyamine biosynthesis, it

TABLE 3. Effects of pentamidine isethionate (PI) on *P. carinii* growth

Inoculum	PI (μg/ml)	No. of *P. carinii* (×10^7)[a] per ml on day (after inoculation):			Peak ratio[b]
		1	3	5	
Lysed	0	2.1	4.1	6.1	
	0.1	1.6	0.6	0.6	0.27
	1	1.2	0.4	0.3	0.20
	10	1.1	0.3	0.3	0.18
Unlysed	0	2.5	4.8	6.4	
	0.1	1.5	0.6	0.5	0.23
	1	1.4	0.6	0.5	0.22
	10	1.1	0.4	0.3	0.17

^a Inoculum = 10^7 organisms. Each data point represents the number of *P. carinii* nuclei per ml of at least triplicate pooled samples.
^b Ratio of peak organism numbers in treated cultures to peak organism numbers in control cultures.

TABLE 4. Effects DFMO and putrescine on *P. carinii* growth

Inoculum	DFMO (mM)	Putrescine (mM)	No. of *P. carinii* (×10^7)[a] per ml on day (after inoculation):				Peak ratio[b]
			1	3	5	7	
Unlysed	0	0	0.3	1.9	1.8	ND^c	
	1	0	0.6	1.4	3.2	ND	1.68
	5	0	0.4	1.2	2.8	ND	1.47
	25	0	0.6	1.6	1.7	ND	0.89
Unlysed	0	0	0.5	0.6	1.3	1.0	
	25	0	0.4	0.9	0.7	0.5	0.69
	125	0	0.4	0.4	ND	0.1	0.31
Lysed	0	0	1.8	5.0	ND	6.2	
	1	0	1.3	1.8	ND	3.4	0.55
	5	0	1.6	2.1	ND	3.6	0.58
	25	0	2.0	2.3	ND	1.0	0.16
Lysed	0	0	4.2	4.9	3.7	2.1	
	0	0.1	3.7	4.5	3.5	2.4	0.92
	1	0.1	2.8	2.4	0.9	0.8	0.57
	1	0	1.1	1.2	0.8	0.7	0.24
	10	0.1	2.6	1.8	0.6	0.6	0.53
	10	0	1.0	0.8	0.6	0.5	0.20

^a Inoculum size in experiments with unlysed inocula could not be accurately determined; based on growth patterns, it was probably ≥10^6 organisms. Inoculum size of lysed inocula was 10^7 organisms. Each data point represents the number of *P. carinii* nuclei per ml of at least triplicate pooled samples.
^b Ratio of peak organism numbers in treated cultures to peak organism numbers in control cultures.
^c ND, Not done.

was postulated that erythrocytes might be supplying exogenous polyamines to *P. carinii* which would counteract the effects of DFMO. An experiment was designed to investigate this phenomenon, which included lysed *P. carinii* inoculum and 0.1 mM putrescine as an exogenous source of polyamines. The concentration of putrescine (0.1 mM) was lower than the doses used to reverse the effects of DFMO on other protozoa in vitro (10). The results indicated that DFMO at concentrations of 1 and 10mM inhibited *P. carinii* by 76 to 80%; in the presence of putrescine, DFMO only inhibited *P. carinii* growth by 43 to 57% (Table 4). Thus, putrescine reversed the effects of DFMO but did not itself have any effect on *P. carinii* growth.

Dapsone. Dapsone was tested at doses of 0.1, 1.0, and 10 μg/ml (Table 5). Control cultures exhibited a typical *P. carinii* growth pattern with a 7-fold increase by day 3. Dapsone at all three concentrations inhibited *P. carinii* growth by >75% during this period. However, the organism exhibited a definite trend toward overcoming the effects of the drug by day 5 of the study.

Viability. Viability of control cultures was ≥90% throughout the 7-day culture period, as determined by erythrosin B dye exclusion. In drug-treated cultures *P. carinii* was present in smaller numbers but maintained a viability of ≥90%.

DISCUSSION

There is little published information on the in vitro susceptibility of *P. carinii* to antimicrobial agents. Investigators who have been unable to grow the organism have examined the effects of different drugs on *P. carinii* viability and metabolism by the use of lysosomotropic vital dyes and radiolabeled substrates and precursors (25, 26). Workers who have succeeded in the culture of the organism have used cell monolayer systems to evaluate antimicrobial activity (3,

TABLE 5. Effects of dapsone on *P. carinii* growth

Dapsone concn (μg/ml)	No. of *P. carinii* (×10⁷)[a] per ml on day (after inoculation):				Peak ratio[b]
	1	3	5	7	
0	3.3	7.2	4.2	3.9	
0.1	0.9	1.6	3.1	3.6	0.22
1	0.8	1.4	2.7	3.0	0.19
10	0.7	1.3	2.5	2.2	0.18

[a] Lysed inoculum = 10^7 organisms. Each data point represents the number of *P. carinii* nuclei per ml of at least triplicate pooled samples.

[b] Ratio of peak organism numbers in treated cultures to peak organisms in control cultures during the first 3 days after inoculation.

29). These studies have provided valuable insights into susceptibility screening, but the results are difficult to compare; each system has differed in the cell line used, method of determining drug activity, and amount of drug introduced (Table 6).

Our laboratory has cultured *P. carinii* with two lung-derived cell lines, the A549 and WI38 VA13 subline 2RA (VA13) (6, 8). The A549 line was used in our susceptibility studies because alterations in medium supplements and environmental conditions have been shown to detrimentally affect the VA13 cell line. We also found that the VA13 cell line adapts poorly to growth in multi-well tissue culture plates. Improvement in quantitation techniques has allowed us to inoculate all cultures with a uniform inoculum. With such a standard, the results of studies from different laboratories, as well as different experiments from the same laboratory, can be compared. Susceptibility screening in multi-well plates allows for a larger number of agents to be tested with smaller amounts of drugs. This is an important consideration when experimental drugs which have not been mass produced are used.

Evaluation of antimicrobial agents in our tissue culture system was based on the growth characteristics of *P. carinii*. The organism grows in clusters which can be seen both in the supernatant and attached to the cell monolayer. An intracellular phase of *P. carinii* was not identified. Growth of *P. carinii* was based on sampling the supernatant and quantitating organism nuclei. Whereas this technique probably underestimates the extent of *P. carinii* replication, it has

TABLE 6. Different methods used to assay the effects of TMP-SMX and pentamidine isethionate (PI) on *P. carinii* growth in vitro

Investigators	Assay method	MIC (μg/ml) of:	
		TMP-SMX (ratio)	PI
Cushion et al. (6)	*P. carinii* nuclei per ml	1:19	0.1
Bartlett et al. (3)	Trophozoites per ml	50:200	0.5
Pifer et al. (29)	Cysts per ml	0.3:1.5	0.3
Pesanti and Cox (25)	Neutral red uptake	>200:200[a]	1.0
Pesanti and Cox (26)	$^{14}CO_2$ production from [U-^{14}C]glucose	100:200[b]	1.0

[a] No inhibition was achieved with 200 μg of TMP and 200 μg of SMX per ml alone or in combination.

[b] Decrease in viability only.

proven to be reliable and reproducible. *P. carinii* increases up to 10-fold in primary culture, but the day when peak organism counts occur may vary in different experiments.

In the present study, drug assessment was based on a comparison of *P. carinii* growth in treated and control cultures over the entire culture period (usually 7 days). As more agents are studied, it may be feasible to select a particular day (e.g., day 3) or level of growth inhibition (e.g., 50%) to screen for anti-*P. carinii* activity. It may also be possible to study the effects of drugs on *P. carinii* growth in subculture, since under optimal conditions, the organism can be serially passaged up to three times (8).

Although the antimicrobial agents used in this study have established or promising value in the treatment of human pneumocystosis, they resulted in a relatively modest (45 to 84%) inhibition of organism growth. Even at drug concentrations which greatly exceeded those that could be achieved clinically, large numbers of viable *P. carinii* organisms remained. Studies of AIDS patients have demonstrated that *P. carinii* may persist in bronchoalveolar lavage fluid after many weeks of treatment (30). In both patients and the rat model, TMP-SMX is successful in the chemoprophylaxis of *P. carinii* pneumonia only as long as it is being given (16, 38). Thus, it appears that current anti-*P. carinii* drugs are not lethal for the organism.

The reasons for this static effect are unclear, but they raise the possibility that some stages in the life cycle of *P. carinii* are resistant to antimicrobial drug activity. Previous studies (23), as well as data from our laboratory, have indicated that incubation of the thick-walled *P. carinii* cyst with drugs (e.g., pentamidine) results in no morphological changes. Recently, we have developed microscopic and staining techniques which should be helpful in studying the life cycle of *P. carinii* in tissue culture (J. J. Ruffolo, M. T. Cushion, and P. D. Walzer. J. Clin. Microbiol., in press). Targeting drug development to specific stages in the life cycle of *P. carinii* might improve the killing efficiency of currently available agents.

TMP-SMX and pentamidine are equally effective in the treatment of pneumocystosis, but TMP-SMX has been preferred in non-AIDS patients because of its lower toxicity (17, 39). Efficacy of TMP-SMX has been related to adequate levels in serum, which are 3 to 5 μg of TMP per ml in children and ≥5 μg of TMP per ml in adults with 100 to 150 μg of SMX per ml. The present study has shown anti-*P. carinii* activity of TMP-SMX in vitro at levels well below these concentrations. Other investigators have found widely variable effects of TMP-SMX at different dose levels (Table 6).

Although pentamidine has been available for many years, little is known about the pharmocokinetics of the drug, because serum assays have been difficult to perform (32, 33). The recent development of a simple bioassay should be helpful in alleviating this problem (4a). The data so far indicate that peak levels of pentamidine in serum after intramuscular or intravenous injection are low (0.5 to 3.4 μg/ml). Pentamidine in the present study inhibited *P. carinii* growth at concentrations well within this range; other investigators have reported similar findings (Table 6).

The activity of DFMO against *P. carinii* in our culture system was related to the presence in the inoculum of erythrocytes, which have high levels of polyamines (37). Presumably, the erythrocytes release their polyamine contents after the lung inoculum is homogenized and placed in tissue culture medium; removal of erythrocytes by ammonium chloride lysis prior to inoculation of the culture obvi-

ated this problem. The lysis procedure appeared to be specific for DFMO, because it did not interfere with *P. carinii* growth or the activity of other drugs and could be reversed by the polyamine putrescine. In vivo, the presence of intact erythrocytes has no effect on the antiparasitic activity of DFMO, as evidenced by its effectiveness in the treatment of the blood forms of African trypanosomiasis (2).

With the lysed inoculum, DFMO inhibited *P. carinii* growth at all concentrations tested (1 to 10 mM). These doses are similar to those which have been found to be active against other parasites (e.g., *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*) in vitro (24, 37). Pharmacokinetic studies in humans have indicated that DFMO reaches levels of 0.5 to 0.6 mM in serum (1, 13); with the much higher dosage regimens currently used in the treatment of pneumocystosis, inhibitory effects of DFMO against the organism should be well within achievable concentrations in serum.

The varying effects of DFMO on *P. carinii* in tissue culture, the rat model, and humans illustrate the complex problems which might be encountered in developing new treatment strategies for this organism. Although *P. carinii* organisms in humans, rats, and other animals are morphologically indistinguishable, animal challenge experiments and antigenic studies suggest that species or strain differences exist (9, 36). Whether such differences can be related to the activity of antimicrobial drugs is unknown, because human *P. carinii* has been difficult to culture and little information is available about the biochemical pathways of the organism. The lack of efficacy of DFMO in the rat model (19) might also be related to such factors as drug dose, pharmacokinetics, or metabolism. Clinical experience with DFMO has so far been limited (1, 31), and thus the precise role of the drug in the treatment of human pneumocystosis remains to be determined.

Dapsone administered orally achieves peak levels of 0.1 to 7.0 μg/ml in serum (27). In the present study, dapsone inhibited *P. carinii* growth in culture at concentrations well within these levels; however, the drug differed from other agents tested in that *P. carinii* overcame its antimicrobial activity after 72 h. Possible mechanisms for this effect include alterations in dapsone (e.g., conversion to the glucuronide or diacetyl derivative) or changes in *P. carinii* (e.g., the emergence of drug-resistant mutants or drug-induced changes in specific life cycle stages). Alterations in the culture medium or cell monolayer appear less likely, because this effect was observed in repeated experiments.

It is unknown whether the ability of *P. carinii* to overcome the inhibitory effect of dapsone has any relevance to the in vivo situation. Dapsone alone is effective in the treatment of *P. carinii* pneumonia in the rat model (19); clinical experience in patients has so far been limited.

The dapsone data reported here suggest that the culture system might be used to study drug interactions. It would be of interest to determine whether synergistic effects between sulfones or sulfonamides and inhibitors of dihydrofolate reductase can be demonstrated on *P. carinii*.

Drug treatment of pneumocystosis has largely been empiric, because of a lack of knowledge of the basic biology of the organism. We have developed an in vitro assay system for the evaluation of antimicrobial compounds on rat *P. carinii* replication which mirrors the in vivo effects of anti-pneumocystis agents in humans. This system should be helpful, not only in the development of new drugs, but also in the elucidation of their mechanism of action.

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