Development and Characterization of a Rapid Screening Assay for Identifying Antipneumocystis Agents

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We developed a rapid assay for screening of compounds with potential antipneumocystis activity on the basis of incorporation of [³⁵S]methionine into proteins newly synthesized by *Pneumocystis carinii*. Unambiguous evidence that *P. carinii* synthesizes proteins in vitro was provided by immunoprecipitation studies demonstrating the incorporation of [³⁵S]methionine into the major surface glycoprotein. Treatment with two clinically active antipneumocystis agents, atovaquone (10⁻⁴ M) or pentamidine (10⁻⁴ M), prevented this incorporation. Total [³⁵S]methionine incorporation paralleled incorporation into the major surface glycoprotein, permitting rapid assessment of anti-*P. carinii* activity by scintillation counting. Treatment with pentamidine (1 × 10⁻⁴ M), atovaquone, trimethoprim (1 × 10⁻⁴ M)-sulfamethoxazole (7.9 × 10⁻⁴ M), piritrexim (1 × 10⁻⁷ M), RO11-8958 (1 × 10⁻⁴ M), and amphotericin B (1 µg/ml) resulted in a greater than 67% inhibition (*P* < 0.05) of [³⁵S]methionine incorporation. No decrease in [³⁵S]methionine incorporation was seen with dapsone (10⁻⁵ M), trimethoprim (10⁻⁴ M), recombinant mouse tumor necrosis factor (500 ng/ml), or gamma interferon. This rapid in vitro assay should be a useful adjunct in the development of new antipneumocystis agents.

Pneumocystis carinii remains an important opportunistic pathogen in human immunodeficiency virus-infected patients, despite recent important advances in the management of this infection (17). New antipneumocystis agents that surpass the clinical efficacy and toxic-therapeutic margin of trimethoprim-sulfamethoxazole or pentamidine are needed.

Unfortunately, the development of safe, clinically effective agents has been compromised by the difficulties in culturing *P. carinii* in vitro. A number of in vitro assays that use feeder cell lines have been reported, but reproducibility among laboratories is poor (1, 4, 5, 20). Furthermore, these assays primarily rely on morphologic quantitation of *P. carinii*, a tedious procedure that cannot be used reliably to assess the viability of *P. carinii* organisms.

Recently, studies examining the ability of *P. carinii* to incorporate radiolabeled glucose, amino acids, and nucleotides have suggested that *P. carinii* is able to use these precursors in vitro (3, 19). Utilization of radiolabeled precursors to assess the viability of *P. carinii* in culture clearly has advantages over conventional counting methods: incorporation of the radiolabel unambiguously demonstrates that the organisms are metabolically active, quantitation of metabolic activity is simple, and, since quantitation is rapid and not labor-intensive, large numbers of samples can be processed simultaneously. One problem with this approach is that *P. carinii* is invariably contaminated with host-derived cells, which may also be metabolically active. The use of precursors specific for *P. carinii*, such as *para*-aminobenzoic acid, avoids the problem associated with mammalian cell metabolism (12).

We undertook the development of a short-term in vitro cultivation assay, based on utilization of [³⁵S]methionine during protein synthesis, that would allow for the rapid screening of compounds with potential antipneumocystis activities. Since we had recently purified the major surface glycoprotein of *P. carinii* and developed antibodies to this

protein, the assay could be validated by demonstrating incorporation of the radiolabel into this protein (16). The major surface protein of rat *P. carinii* is a glycoprotein of approximately 116 kDa that can easily be visualized with standard protein stains and specifically identified by immunoblotting techniques (3, 7-9, 13, 15, 16, 22).

In the study described here, we unambiguously demonstrated that de novo protein synthesis by *P. carinii* does occur in vitro during short-term cultures and that such synthesis can be inhibited by clinically effective antipneumocystis agents.

MATERIALS AND METHODS

Organisms. *P. carinii* isolates were obtained from the lungs of immunosuppressed rats with heavy *P. carinii* infections, as assessed by Diff-Quik staining, and were partially purified by Ficoll-Hypaque density gradient centrifugation as described previously (13). An aliquot of 5 to 10 µl was plated onto a sheep blood agar plate, and isolates from rat lungs that revealed more than a few colonies of bacteria or fungi were discarded.

In vitro culture. Partially purified organisms from a single lung were washed in phosphate-buffered saline (PBS), pelleted, and resuspended in 25 ml of methionine-free RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Organisms were transferred to a sterile 24-well culture plate (1 ml per well; Costar) or 25-cm² tissue culture flask (10 ml per flask) and were pulsed overnight with 50 µCi of [³⁵S]methionine/cysteine (specific activity, 1,075 mCi/mmol; ICN) per ml.

Similarly, *P. carinii* organisms cultivated in RPMI 1640 medium without glucose were pulsed with 10 µCi of the following radiolabeled carbohydrates per ml: D-[2,6-³H]mannose (specific activity, 47.1 Ci/mmol; ICN), D-[¹⁴C]mannose (specific activity, 270 mCi/mmol; ICN), and D-[¹⁴C]glucose (specific activity, 288 mCi/mmol; ICN). *P. carinii* cultured in complete RPMI 1640 medium was pulsed as described above

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with 20 μ Ci of [3 H]uracil (specific activity, 457 mCi/mmol; Amersham) per ml and [3 H]thymidine (specific activity, 20 Ci/mmol; Amersham).

For drug and cytokine inhibition studies, organisms were incubated with drugs for 4 h prior to pulsing with [35 S]methionine. Drug and cytokine studies in 24-well plates were performed in triplicate or quadruplicate; six to eight wells with diluent alone or no drug were included on each plate as controls.

Organisms were harvested from 24-well plates after 18 h, washed twice in PBS, and resuspended in 0.5 ml of PBS. One hundred microliters from each well was counted in a liquid scintillation counter.

Immunoprecipitation studies. *P. carinii* that was cultured in 25-cm² flasks was harvested after 18 to 24 h, washed twice in PBS, resuspended in 1.25 ml of PBS, and frozen and thawed three times. Samples were pelleted, and the supernatant was removed. The pellet was resuspended in 1 ml of PBS, treated for 18 h at 37°C with 84 U (per ml) of lyticase (Sigma), an enzyme with 1,3- β -glucanase as well as protease activity, and centrifuged at 14,000 $\times$ g for 15 min.

For immunoprecipitation, 60 μ l of the lyticase-treated supernatant, which contained solubilized major surface glycoprotein, was added to 200 μ l of immunoprecipitation buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 25% gelatin) and 20 μ l of hyperimmune serum from a rabbit immunized with purified major surface glycoprotein, serum from a rat immunized with whole *P. carinii* organisms, or preimmune serum. After mixing at 4°C overnight, 100 μ l of a 25% suspension of protein A-Sepharose CL-4B (Pharmacia) in PBS was added, and the solution was mixed at 4°C for 30 min. Samples were then centrifuged at 14,000 $\times$ g for 15 min and washed three times with 200 μ l of immunoprecipitation buffer, pelleted, and resuspended in 100 μ l of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.125 M Tris-HCl [pH 8.0], 2% SDS, and 0.5 M dithiothreitol). SDS-PAGE was performed as described previously (16) on the basis of the methods of Lammeli by using a 4% stacking gel and a 10% running gel.

Drugs and cytokines. Drugs were dissolved to give 10⁻² M stock solutions, unless otherwise indicated. Pentamidine isethionate (Lyphomed, Melrose Park, Ill.) was dissolved in normal saline. Amphotericin B (GIBCO Bethesda Research Laboratories Life Technologies Laboratories, Gaithersburg, Md.) was dissolved in sterile, deionized water (250 μ g/ml). Trimethoprim (Hoffmann-La Roche, Nutley, N.J.), dapsone (Jacobus Pharmaceutical Co.), and piritrexim (10⁻⁴ M) (Burroughs Wellcome Co., Research Triangle Park, N.C.) were dissolved in 100% methanol. Sulfamethoxazole (7.9 $\times$ 10⁻² M) (Sigma) and atovaquone (a new hydroxynaphthoquinone; Burroughs Wellcome Co.) were dissolved in 100% ethanol (10). RO 11-8958 (Hoffmann-La Roche, Basel, Switzerland) was dissolved in dimethyl sulfoxide. Clarithromycin (Abbott Laboratories, North Chicago, Ill.) was dissolved in methanol at concentrations of 30 and 100 μ g/ml immediately before each experiment. Recombinant mouse tumor necrosis factor (TNF; 5 μ g/ml) and gamma interferon (10⁵ U/ml) were obtained from Boehringer Mannheim, Indianapolis, Ind. Rat interferon (1.5 $\times$ 10⁵ U/ml), which contained both alpha and beta but not gamma interferon, was obtained from Sigma.

Data analysis. Results are expressed as the mean $\pm$ standard error. The significance of differences was calculated by Dunnett's test to compare treated with control (untreated) *P. carinii*. Pentamidine was not included as a

treatment since it served as a positive control. One-tailed *P* values of <0.05 were considered statistically significant.

RESULTS

In the initial phase of the present study, cultures of *P. carinii* were demonstrated to incorporate [35 S]methionine when grown in either 24-well plates or a 25-cm² flask. Incorporation by organisms preincubated with pentamidine (10⁻⁴ M) or atovaquone (10⁻⁴ M), two drugs with clinical activity against *P. carinii*, was inhibited by greater than 90% (Fig. 1). Treatment of radiolabeled organisms with lyticase, an enzyme that solubilizes the major surface glycoprotein, resulted in the solubilization of over 10% of the incorporated label, and incorporation into the solubilized fraction was also inhibited by pentamidine and atovaquone (Fig. 1B).

To document that incorporation of [35 S]methionine was by *P. carinii*, immunoprecipitation of the major surface glycoprotein was performed. Following SDS-PAGE and autoradiography, multiple bands were seen in the crude whole *P. carinii* preparations (data not shown), including a major band of approximately 116,000 Da that corresponds to the major surface glycoprotein. Pulsing of short-term cultures of uninfected rat lung preparations did not show incorporation into a similar protein (data not shown). Lyticase treatment solubilized the major surface glycoprotein, which has previously been shown to have a slightly lower molecular weight following such treatment (16). This protein could be immunoprecipitated with either rabbit or rat hyperimmune serum, providing unambiguous evidence that *P. carinii* in short-term cultures is capable of de novo protein synthesis (Fig. 2). No bands were seen following immunoprecipitation with preimmune sera (Fig. 2). Treatment with atovaquone (10⁻⁴ M) or pentamidine (10⁻⁴ M) prevented incorporation of [35 S]methionine into the major surface glycoprotein as well as other proteins (Fig. 2, lanes 4, 5, 8, and 9).

Because *P. carinii* is metabolically active in short-term cultures and this activity was inhibited by agents that are clinically active against *P. carinii*, we assessed the utility of [35 S]methionine pulsing as an assay for screening drugs for antipneumocystis activity. These studies were conducted in 24-well plates, and incorporation of [35 S]methionine was determined by counting aliquots of washed whole organisms from each well.

We first evaluated the effect of amphotericin B, which causes alteration of membrane permeability because of its binding to a sterol of the plasma membrane (14). This polyene antifungal agent is routinely included in cultures of *P. carinii* to prevent fungal contamination (12, 19). As shown in Table 1, a dose of 1 μ g/ml, which has been used in previous in vitro culture studies, caused a 67% decrease (*P* < 0.05) in [35 S]methionine incorporation compared with that in controls with no amphotericin B. Doses of 0.1 μ g/ml caused no significant inhibition, nor was a synergistic effect seen at different concentrations (10⁻⁷ to 10⁻⁴ M) of pentamidine and atovaquone (data not shown). There was no morphologic evidence of fungal contamination observed by phase-contrast microscopy in controls or amphotericin B-treated cultures. Amphotericin B (0.1 μ g/ml) was included as an antifungal agent in all subsequent experiments.

Similarly, a number of clinically active antipneumocystis drugs and potential new agents were assessed. In a series of experiments (Table 1), treatment with pentamidine (1 $\times$ 10⁻⁴ M) and trimethoprim-sulfamethoxazole (1 $\times$ 10⁻⁴ and 7.9 $\times$ 10⁻⁴ M, respectively) resulted in a greater than 75% inhibition (*P* < 0.05) of [35 S]methionine incorporation by *P.*

FIG. 1. (A) [³⁵S]methionine incorporation by *P. carinii*. *P. carinii* was incubated for 4 h in three 25-cm² flasks containing the following: controls (untreated), pentamidine (10⁻⁴ M), and atovaquone (10⁻⁴ M). The flasks were then pulsed with [³⁵S]methionine (50 μ Ci/ml) and were harvested after 18 h. Aliquots of intact organisms (100 μ l) from each flask were counted by liquid scintillation; data are presented as percent incorporation compared with that of the control. The total count in the control flask was 415,300 cpm. (B) Incorporation of [³⁵S]methionine into lyticase-sensitive proteins of *P. carinii*. After pulsing as described above for panel A, the organisms were freeze-thawed and pelleted; the pellet was treated overnight with lyticase, and an aliquot of the lyticase-solubilized supernatant was counted. Preincubation with pentamidine (10⁻⁴ M) and atovaquone (10⁻⁴ M) resulted in a substantial decrease in incorporation of [³⁵S]methionine. The total count in the lyticase-solubilized control was 53,365 cpm.

FIG. 2. Immunoprecipitation of the major surface glycoprotein of *P. carinii* from cultures pulsed with [³⁵S]methionine. The major surface glycoprotein was solubilized by treatment with lyticase as described in the text. Prior to lyticase treatment, *P. carinii* was incubated with no anti-pneumocystis agent (lanes 3, 6, and 7), pentamidine (10⁻⁴ M) (lanes 1, 4, and 8), or atovaquone (10⁻⁴ M) (lanes 2, 5, and 9) and was then pulsed with [³⁵S]methionine. Samples were immunoprecipitated with serum (see below) and were evaluated by SDS-PAGE and then autoradiography. Lanes 1 to 3, immunoprecipitation with rat serum from an unimmunized (nonimmune) rat; lanes 4 to 6, immunoprecipitation with rat serum from a rat immunized with *P. carinii*. No band is seen with nonimmune serum or in either the pentamidine- or atovaquone-treated specimens (lanes 4 and 5, respectively). In lane 6 (control), the major surface glycoprotein band is easily identified. Lyticase-treated specimens prior to immunoprecipitation are shown in lanes 7 to 9. Multiple bands, including the broad band that corresponds to the major surface glycoprotein, are visualized in the untreated control (lane 7); following treatment with pentamidine (10⁻⁴ M) or atovaquone (10⁻⁴ M), no protein bands were seen.

carinii. Sulfamethoxazole alone (7.9 $\times$ 10⁻⁴ M; 200 μ g/ml) yielded a 64% reduction (P < 0.05), while dapsone (1 $\times$ 10⁻⁵ M; 2.9 μ g/ml) or trimethoprim (1 $\times$ 10⁻⁴ M) was found not to inhibit [³⁵S]methionine incorporation, although the combination was inhibitory. Significant inhibition (P < 0.05) was detected when *P. carinii* was treated with two new dihydrofolate reductase (DHFR) inhibitors, RO 11-8958 and piritrexim. Clarithromycin, a new macrolide, was found to have no significant activity (83% $\pm$ 8% of control) at 100 μ g/ml (1.4 $\times$ 10⁻⁴ M).

Recently, TNF, but not gamma interferon, was reported to be directly cytotoxic to *P. carinii* in an assay examining ¹⁴CO₂ production by organisms (18). We examined the abilities of these cytokines to inhibit [³⁵S]methionine incorporation. Uptake by *P. carinii* treated with mouse TNF at 500 ng/ml and mouse gamma interferon at 1,000 U/ml, concentrations used in a previous study (18), were 110 and 105% of the uptake by the control, respectively, while pentamidine (10⁻⁴ M) resulted in a marked decrease in incorporation of [³⁵S]methionine (Table 1). Furthermore, no decrease in incorporation was seen with TNF or gamma interferon when they were used concurrently or with rat interferon (1,000 U/ml), which contained both alpha and beta but not gamma interferon (data not shown).

Because the major surface glycoprotein contains mannose as well as glucose, we examined the ability of *P. carinii*, incubated in medium with or without glucose, to incorporate [¹⁴C]glucose, [³H]mannose, or [¹⁴C]mannose (6, 16). Minimal incorporation was seen when aliquots of cultures were

TABLE 1. Inhibition of [³⁵S]methionine incorporation by drugs and cytokines^a

Drug or cytokine	Concn	% Incorporation compared with that of control ^a
Amphotericin B^b		
Amphotericin B	1 µg	33 ± 2 ^c
Amphotericin B	0.5 µg	43 ± 14 ^c
Amphotericin B	0.25 µg	58 ± 18 ^c
Amphotericin B	0.10 µg	91 ± 17
Atovaquone	1 × 10 ⁻⁴ M	19 ± 2 ^d
Pentamidine	1 × 10 ⁻⁴ M	18 ± 0.5 ^d
Antipneumocystis agents^e		
Dapsone	1 × 10 ⁻⁵ M	102 ± 4
Sulfamethoxazole	7.9 × 10 ⁻⁴ M	36 ± 5 ^c
Trimethoprim-sulfamethoxazole	1 × 10 ⁻⁴ M/(7.9 × 10 ⁻⁴ M)	16 ± 2 ^c
Trimethoprim-dapsone	1 × 10 ⁻⁴ M/1 × 10 ⁻⁵ M	31 ± 1 ^c
Piritrexim	1 × 10 ⁻⁷ M	17 ± 4
Trimethoprim	1 × 10 ⁻⁴ M	96 ± 6
RO11-8958	1 × 10 ⁻⁴ M	14 ± 2 ^c
Clarithromycin	1.4 × 10 ⁻⁴ M	83 ± 8
Cytokines^f		
TNF	500 ng/ml	110 ± 7
Gamma interferon	1,000 U/ml	105 ± 5
Pentamidine	1 × 10 ⁻⁴ M	10 ± 1 ^d

^a Values are percentage of control and represent the means ± standard errors of the means of four wells per assay per experiment. Four to 16 control wells were included in each experiment.

^b Inhibition of [³⁵S]methionine incorporation by amphotericin B at 0.1 to 1.0 µg/ml. For comparison, inhibition with pentamidine (10⁻⁴ M) and atovaquone (10⁻⁴ M) from the same experiment are shown. Data represent the means ± standard errors of the means of two experiments.

^c *P* < 0.05.

^d Served as a positive control.

^e Inhibition of [³⁵S]methionine incorporation by *P. carinii* by known or potential antipneumocystis agents. Data are from three experiments.

^f Inhibition of [³⁵S]methionine incorporation by cytokines. Cultures were incubated with no cytokine (control), mouse TNF (500 ng/ml), or mouse gamma interferon (1,000 U/ml). Pentamidine (10⁻⁴ M) was included as a control with known antipneumocystis activity. Data represent the means ± standard errors of the means of one experiment. In three additional experiments, no decreased incorporation was seen when TNF (mouse or human) and gamma interferon were used concurrently or with rat interferon.

counted, and no bands were seen by autoradiography when whole organism and lyticase-solubilized fractions were evaluated by SDS-PAGE. Similarly, no incorporation was seen when *P. carinii* cultures were pulsed with [³H]uracil and [³H]thymidine.

DISCUSSION

This report has described a new assay system for rapidly screening agents with potential activity against *P. carinii* and has shown that agents known to be active against *P. carinii* clinically are also active in this system. The assay relies on the ability of *P. carinii* to synthesize proteins and incorporate [³⁵S]methionine during the first 24 h in short-term culture. We have previously shown that *P. carinii* is metabolically active and can synthesize folates de novo during this period (12). Unambiguous evidence that *P. carinii* synthesizes proteins was provided by immunoprecipitation studies demonstrating the incorporation of [³⁵S]methionine into the major surface glycoprotein of *P. carinii*.

The major advantage of the current system over our previously described system, which relies on the incorporation of [³H]*para*-aminobenzoic acid by organisms, is that quantitation requires minimal processing prior to scintillation counting. The major advantage over other reported systems is that quantitation does not depend on visual counting of organisms, a tedious and laborious process; furthermore, this system directly examines metabolic activity, rather than relying on morphology. A disadvantage is that no feeder cells can be used, since they would also incorporate [³⁵S]methionine. However, in previous studies from our group and elsewhere, a feeder layer does not appear to be essential for maintaining the viability of *P. carinii* during short-term cultures (2, 3, 12). Although contaminating mammalian cells may incorporate label, their contribution appears minimal on the basis of the poor incorporation by control lung preparations that were free of *P. carinii*.

Our observations of protein metabolism are similar to those of Cushion and Ebbets (3), who reported that [³⁵S]methionine incorporated into several protein bands presumably originating from *P. carinii*. In contrast to our experience, Cushion and Ebbets (3) also found substantial uptake of [³H]uracil and [³H]thymidine when using an NGP culture medium (1% neopeptone and *N*-acetylglucosamine medium). Differences in the in vitro conditions selected for the present study, such as incubation times, the organism load per well, and different culture media, may account for the differences in the results presented in the two reports. In our system, incorporation of [³⁵S]methionine appeared to be consistently greater than that seen in the earlier study (3) and was likely due to the use of methionine-free medium.

In developing the assay described here, inhibition by compounds with known activity against *P. carinii* was examined at concentrations that are achievable in patients. Significant inhibition was seen with trimethoprim-sulfamethoxazole, atovaquone, pentamidine, dapsone-trimethoprim, and piritrexim. Sulfamethoxazole (200 µg/ml) caused less inhibition when it was used alone; at clinically achievable levels (10⁻⁵ M), dapsone was not found to be inhibitory. The poor inhibition seen in this assay with inhibitors of dihydropteroate synthetase (dapsone and sulfamethoxazole) is likely due to the short duration of the cultures and the high concentration of *p*-aminobenzoic acid (10⁻⁵ M) present in this medium, which probably does not allow for depletion of folate precursors. Amphotericin B at a dose (1 µg/ml) used in a number of other in vitro assays resulted in significant inhibition. Interestingly, Cushion and Ebbets (3) found a 4-fold increase in radiolabeled uptake and 10-fold increase in organism number in *P. carinii* cultured in NPG medium (which did not contain any amphotericin B); however, organisms maintained in Dulbecco's minimal essential medium which contained 0.5 µg of amphotericin B per ml failed to incorporate [³⁵S]methionine, despite a 4-fold increase in *P. carinii*. A new DHFR inhibitor, RO 11-8958, also resulted in significant inhibition. Clarithromycin, a new macrolide, did not cause any significant inhibition when compared with that of diluent-treated control specimens. This macrolide, when used alone, has also been found to be ineffective in preventing pneumocystis pneumonia in immunosuppressed rats (11).

Unlike the previous report of a direct toxic effect of TNF on pneumocystis (18), no suppression of [³⁵S]methionine incorporation was observed when *P. carinii* was incubated with mouse TNF. Although the findings of the present study do not support a direct toxic effect of TNF, a theoretical

constraint of the assay is the inability to identify compounds with significant antipneumocystis properties that do not directly or indirectly affect de novo pneumocystis protein synthesis within a 24-h period. Under the current culture conditions, metabolic activity could not be established with the use of other metabolic labels.

A major goal of our study was to develop a high-processivity assay for identifying agents that are active against *P. carinii*. Rapid screening of large numbers of compounds at multiple concentrations could be performed by adapting the described assay to microtiter plates. A similar adaptation of the [³H]para-aminobenzoic acid incorporation assay has been reported recently (2). Compounds that appear promising on the basis of their ability to inhibit protein synthesis could be further evaluated by autoradiography to demonstrate that inhibition of synthesis of the major surface glycoprotein (a specific *P. carinii* protein) does occur.

The methods used in the study described here can also be used to examine the processing and metabolism of the major surface glycoprotein of *P. carinii*. This protein may play an important role in *P. carinii*-host cell interactions, perhaps through interactions with fibronectin, mannose-binding protein, or surfactant A protein (6, 21, 23).

With the increased number and prolonged survival of severely immunocompromised persons, safe, economic, and clinically effective therapeutic and prophylactic modalities capable of eradicating *P. carinii* are needed. The rapid in vitro assay described here should be a useful adjunct in the development of new antipneumocystis agents.

REFERENCES

1. Bartlett, M. S., P. A. Verbanac, and J. W. Smith. 1979. Cultivation of *Pneumocystis carinii* with WI-38 cells. *J. Clin. Microbiol.* **10**:796-799.
2. Comely, J. C., R. J. Mullin, L. A. Wolfe, M. H. Hanlon, and R. Ferone. 1992. Microculture screening assay for primary in vitro evaluation of drugs against *Pneumocystis carinii*. *Antimicrob. Agents Chemother.* **35**:1965-1974.
3. Cushion, M. T., and D. Ebbets. 1990. Growth and metabolism of *Pneumocystis carinii* in axenic culture. *J. Clin. Microbiol.* **28**:1385-1394.
4. Cushion, M. T., J. J. Ruffolo, M. J. Linke, and P. D. Walzer. 1985. *Pneumocystis carinii*: growth variables and estimates in the A549 and WI-38 VA13 human cell lines. *Exp. Parasitol.* **60**:43-54.
5. Cushion, M. T., and P. D. Walzer. 1984. Growth and serial passage of *Pneumocystis carinii* in the A549 cell line. *Infect. Immun.* **44**:245-251.
6. Ezekowitz, R. A. B., D. J. Williams, H. Koziel, M. Armstrong, A. Warner, F. Richards, and R. M. Rose. 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. *Nature (London)* **351**:155-158.
7. Gigliotti, F. 1992. Host species-specific antigenic variation of a mannosylated surface glycoprotein of *Pneumocystis carinii*. *J. Infect. Dis.* **165**:329-336.
8. Gigliotti, F., D. C. Stokes, A. B. Cheatham, D. S. Davis, and W. T. Hughes. 1986. Development of murine monoclonal antibodies to *Pneumocystis carinii*. *J. Infect. Dis.* **154**:315-322.
9. Graves, D. C., S. J. McNabb, M. A. Worley, T. D. Downs, and M. H. Ivey. 1986. Analyses of rat *Pneumocystis carinii* antigens recognized by human and rat antibodies by using Western immunoblotting. *Infect. Immun.* **54**:96-103.
10. Hudson, A. T. 1993. Atovaquone—a novel broad-spectrum anti-infective agent. *Parasitol. Today* **9**:66-68.
11. Hughes, W. T. 1991. Macrolide-antifol synergism in anti-*Pneumocystis carinii* therapeutics. *J. Protozool.* **38**(6):160S.
12. Kovacs, J. A., C. J. Allegra, J. Beaver, D. Boarman, M. Lewis, J. E. Parrillo, B. Chabner, and H. Masur. 1989. Characterization of de novo folate synthesis in *Pneumocystis carinii* and *Toxoplasma gondii*: potential for screening therapeutic agents. *J. Infect. Dis.* **160**:312-320.
13. Kovacs, J. A., J. L. Halpern, J. C. Swan, J. Moss, J. E. Parrillo, and H. Masur. 1988. Identification of antigens and antibodies specific for *Pneumocystis carinii*. *J. Immunol.* **140**:2023-2031.
14. Kucers, A., and N. M. Bennett. 1987. Amphotericin B, p. 1441-1477. In A. Kucers and N. M. Bennett (ed.), *The use of antibiotics*. J. B. Lippincott, Philadelphia.
15. Linke, M. J., M. T. Cushion, and P. D. Walzer. 1989. Properties of the major antigens of rat and human *Pneumocystis carinii*. *Infect. Immun.* **57**:1547-1555.
16. Lundgren, B., G. Y. Lipschik, and J. A. Kovacs. 1991. Purification and characterization of a major human *Pneumocystis carinii* surface antigen. *J. Clin. Invest.* **87**:163-170.
17. Masur, H., H. C. Lane, J. A. Kovacs, C. J. Allegra, and J. C. Edman. 1989. *Pneumocystis pneumonia*: from bench to clinic. *Ann. Intern. Med.* **111**:813-826.
18. Pesanti, E. L. 1991. Interaction of cytokines and alveolar cells with *Pneumocystis carinii* in vitro. *J. Infect. Dis.* **163**:611-616.
19. Pesanti, E. L., and C. Cox. 1981. Metabolic and synthetic activities of *Pneumocystis carinii* in vitro. *Infect. Immun.* **34**:908-914.
20. Pifer, L. L., D. Woods, and W. T. Hughes. 1978. Propagation of *Pneumocystis carinii* in Vero cell culture. *Infect. Immun.* **20**:66-68.
21. Pottratz, S. T., and W. J. Martin II. 1990. Mechanism of *Pneumocystis carinii* attachment to cultured rat alveolar macrophages. *J. Clin. Invest.* **86**:1678-1683.
22. Walzer, P. D., and M. J. Linke. 1987. A comparison of the antigenic characteristics of rat and human *Pneumocystis carinii* by immunoblotting. *J. Immunol.* **138**:2257-2265.
23. Zimmerman, P. E., D. R. Voelker, F. X. McCormack, J. R. Paulsraud, and W. J. Martin II. 1992. 120-kD surface glycoprotein of *Pneumocystis carinii* is a ligand for surfactant protein A. *J. Clin. Invest.* **89**:143-149.