Effect of Amphotericin B and Rifampin Against *Coccidioides immitis* In Vitro and In Vivo¹

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Amphotericin B, the principal drug used for treating systemic mycoses, possesses undesirable toxic properties. The ability of this antibiotic to potentiate antifungal activity of other compounds suggests that lower doses of amphotericin B could be used in combination with a second drug without loss of therapeutic efficacy. In vitro tests demonstrated that amphotericin B potentiated rifampin against the mycelial growth phase of *Coccidioides immitis* but not against the spherule-endospore phase. Therapy for murine coccidioidomycosis with a combined amphotericin B-rifampin regimen was not better than treatment with amphotericin B alone; in fact, combined drugs may have been even less effective. This would have clinical significance for therapy of concurrent infections.

The hypothesis that drugs that disrupt cell membrane integrity can potentiate the antifungal activity of other antibiotics has received experimental support from both in vitro (2, 3, 8, 9, 17-19, 21-22, 27, 29) and in vivo studies (4, 16, 33). Polyenes, the principal potentiating antibiotics studied, combine with sterols in fungal cell membranes, creating pores that increase permeation by both intracellular and extracellular substances (1, 14, 15). Other antibiotics, which are without effect against intact fungus cells, penetrate the cell membrane damaged by polyenes and inhibit cell synthetic mechanisms. For example, the in vitro combination of amphotericin B with rifampin exhibits synergistic activity against many fungi (2, 3, 17, 18, 22, 27; G. S. Kobayashi et al., Am. Rev. Respir. Dis. 107:1083-1084, 1973).

Since administration of amphotericin B is usually accompanied by toxic effects, combined drug therapy deserves consideration as a means for reducing the dose required. Potentiation by amphotericin B against *Coccidioides immitis* has been reported when this antibiotic was combined in vitro with lysozyme (7, 8), polymyxin B (9), and rifampin (27) and in vivo with tetracycline (16). In our experience, however, the combination of rifampin and amphotericin B had not been more effective than amphotericin B alone against murine coccidioidomycosis. The present report provides the basis for this conclusion as well as comparison of the responses of the mycelial and spherule-endospore

phases of *C. immitis* to combinations of amphotericin B and rifampin in vitro.

MATERIALS AND METHODS

Chemicals. Amphotericin B was obtained from E. R. Squibb as the Fungizone preparation for intravenous infusion. Rifampin was obtained from Ciba Pharmaceutical Co. (Rimactane, 300-mg capsules) and from Dow (lot D-1337, 983 µg/mg).

In vitro tests. Separate growth conditions were required for the mycelial phase and the spherule-endospore phase. The mycelial phase of *C. immitis* (Silveira) was maintained on GYE agar slants (1% glucose, 0.5% yeast extract, 1.5% agar). After several weeks of incubation (34 C), arthrospores were harvested by washing slants with sterile distilled water. The spore suspensions were kept at 4 C. The spherule-endospore growth phase of *C. immitis* (Silveira) was maintained in the chemically defined modified Converse medium (MCM) containing the anionic detergent Tamol N (Robin and Haas) according to the procedure of Levine et al. (20). Tamol N was deleted (MCM-T) when sensitivity to antibiotics was determined. Under these conditions, the fungus remained in the spherule-endospore phase (7).

Susceptibility of the mycelial phase of *C. immitis* to the antibiotics was determined in Sabouraud glucose broth. Amphotericin B was reconstituted at a concentration of 4.0 mg/ml in sterile distilled water. Rifampin was solubilized in Sabouraud glucose broth at a concentration of 80 µg/ml and sterilized by membrane filtration. Tests were performed in duplicate by adding 0.1 ml of arthrospore suspension containing 4.6×10^4 spores/ml to 2.0 ml of Sabouraud glucose broth containing twofold dilutions of amphotericin B in Sabouraud glucose broth in the presence of 0 or 40 µg of rifampin per ml of Sabouraud glucose broth. Tubes were incubated without

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shaking at 35 C. The minimum inhibitory concentration (MIC) was considered to be the lowest concentration of amphotericin B that completely suppressed growth.

The effect of rifampin on the amphotericin B susceptibility of the spherule-endospore phase of *C. immitis* was determined in MCM-T medium. Rifampin was solubilized in 50% ethanol (2 mg of rifampin per ml), and 2 ml was added to duplicate 100-ml volumes of MCM-T (final concentration, 40 μ g of rifampin per ml of MCM-T plus 1% ethanol). Rifampin-free flasks received 2 ml of 50% ethanol. An additional series of MCM-T flasks contained neither ethanol nor rifampin. Dilutions of amphotericin B in MCM-T were added, and flasks were inoculated with approximately 5×10^7 7-day-old endospores, which yielded barely visible turbidity. Flasks were incubated at 34 C on a gyratory shaker operating at 180 rpm. The MIC again was the lowest concentration completely suppressing growth.

In vivo studies. In vivo studies were performed independently in the two laboratories and results were not compared until after experiments were terminated. In one study, male mice (Namru strain, 8 to 10 weeks old) were inoculated intranasally (i.n.) or intraperitoneally (i.p.) with 100 arthrospores of *C. immitis* (Silveira). Forty-eight hours later (to allow time for conversion of arthrospores to the in vivo spherule-endospore phase), treatment was begun with rifampin at 10 or 20 mg/kg of body weight with or without amphotericin B at doses of 0.043, 0.22, or 0.43 mg/kg. The mice received the medications subcutaneously, each drug being injected separately. The medications were administered for 6 days consecutively, with no treatment on day 7 and resumption for 6 days through the 30-day period of the experiment.

In the second study, male mice (dba strain) were purchased from Jackson Laboratories, Bar Harbor, Me. Mice were weighed and distributed at random and held for a minimum of 2 weeks before use in experiments. Weighings were repeated weekly, and food and water were available ad libitum. Any mice exhibiting clinical signs of illness or weight loss during the quarantine period were excluded from experiments. After the quarantine period, the population of animals was made more homogeneous by including only those mice weighing within ± 2 standard deviations of the average for the entire population. The average weights and standard deviations in three separate experiments were 24.2 ± 1.6 , 24.0 ± 3.8 , and 25.4 ± 2.8 g. The infection inoculum was prepared from a second strain of *C. immitis* (C34) grown on GYE at room temperature for 1 to 2 months. Arthrospore suspensions were harvested in minimal amounts of sterile saline by the spinning bar technique described earlier (16). Suspensions were adjusted to contain 50×10^8 spores/ml (visual count), and this was verified for viability by duplicate quantitative dilutions, plating, and averaging the number of colonies on all countable plates. The average viable counts for three separate experiments were 45.0×10^8 , 53.6×10^8 , and 50.0×10^8 .

Each experiment consisted of 140 mice distributed, infected, and treated as indicated in Table 1. The mice were anesthetized lightly by injection (i.p.) with sodium pentobarbital (54 mg/kg) and infected (i.n.) by inhalation of 0.02 ml of the arthrospore suspension (approximately 10^8 viable particles). The noninfected mice (Table 1, groups 1-4) were sham infected with 0.02 ml of sterile saline. The 100 infected mice in each experiment were distributed at random (five mice/cage), and the cages were assigned to treatment groups (two cages/group) according to a table of random numbers. Therapy was initiated on day 5 postinfection, at which time mice were sick (i.e., weight loss, lethargy, ruffled fur). Treatment was continued for a total of 5 days. Amphotericin B, diluted with sterile 5% glucose solution, was administered by single daily injection (0.5 ml, i.p.) and rifampin (8 mg/kg in 0.2 ml of sterile water per os) was given on the same schedule. The same volumes, routes, and schedule were used for diluents replacing antibiotics. Deaths were recorded daily and weights were recorded at weekly intervals for 40 days, when survivors were sacrificed. At necropsy lungs were removed and tissue sections were prepared for histopathological study. Confirmation of infection was obtained by demonstration of endosporeulating spherules in lung tissues.

The significance of differences among treatments was evaluated by nonparametric tests in terms of survival times, total survivors, and 50% end points. Gehan's generalized Wilcoxon test (12) was used for survival time, and a logarithmic-probit graph plot was used for 50% end points (23). When the total

TABLE 1. Distribution, infection, and treatment for mice infected with *C. immitis* (C34)

Group ^a	Infection	Treatment ^b	
		Amphotericin B (mg/kg)	Other
1	No	None	Glucose solution and rifampin
2	No	None	Glucose solution and water
3	No	0.4	Rifampin
4	No	0.4	Water
5	Yes	None	Glucose solution and rifampin
6	Yes	None	Glucose solution and water
7	Yes	0.400	Rifampin
8	Yes	0.100	Rifampin
9	Yes	0.025	Rifampin
10	Yes	0.006	Rifampin
11	Yes	0.400	Water
12	Yes	0.100	Water
13	Yes	0.025	Water
14	Yes	0.006	Water

^a Each group consisted of 10 mice.

^b See text for doses, routes, and schedules.

number of mice was 40 or less, the Fisher exact probability test (31) was used for evaluation of total survivors. When the number exceeded 40, the chi-square test was used (Monroe program no. 3123NS).

RESULTS

In vitro studies. Potentiation by combined antibiotics was apparent with the mycelial phase of *C. immitis* (Silveira) but not with the spherule-endospore phase (Fig. 1). After 6 days of incubation, the MIC of amphotericin B for the mycelial phase was fourfold greater in the absence of rifampin than when rifampin was present, and this difference was maintained through 15 days of incubation. In contrast, the MIC of amphotericin B for the spherule-endospore phase was not influenced by rifampin. At

FIG. 1. Effect in vitro of rifampin plus amphotericin B on mycelial and spherule phases of *C. immitis* (Silveira). Symbols: ○, mycelial phase, no rifampin; ●, mycelial phase plus 40 µg of rifampin per ml; □, spherule-endospore phase, no rifampin; ■, spherule-endospore phase plus 40 µg of rifampin per ml and 1% ethanol; △, spherule-endospore phase, no rifampin, 1% ethanol.

both 0 and 40 µg of rifampin per ml, the MIC of amphotericin B in the presence of 1% ethanol was identical at each time interval throughout 2 weeks of incubation. Flasks containing 1% ethanol showed less growth than the ethanol-free control (spherule-endospore phase with amphotericin B but without rifampin or ethanol). In the presence of 1% ethanol susceptibility to amphotericin B was increased approximately twofold.

In vivo studies with Namru mice and *C. immitis* (Silveira). No potentiation was found with combined drug therapy of mice infected by i.n. or i.p. routes with 100 arthrospores (Table 2). Combinations of amphotericin B with either 10 or 20 mg of rifampin per kg provided no advantage over amphotericin B alone. Indeed, there was some evidence indicating inhibition of the protective effect of amphotericin B. Survival for 30 days was obtained among mice infected i.n. and treated with 0.22 or 0.43 mg of amphotericin B per kg alone compared with 10% (4/39) mortality among mice when rifampin was added to the treatment. Similarly, for mice infected i.p. protection was obtained with 0.043 mg (and greater doses) of amphotericin B per kg alone, whereas adding rifampin to the therapy resulted in 8% (5/60) mortality.

In vivo studies with dba mice and *C. immitis* (C34). All of the sham-infected animals (Table 1, groups 1-4) gained weight and remained apparently healthy throughout the duration of experiments. There was no gross evidence of toxicity caused by any of the antibiotics in the concentrations and combinations used.

When results of three separate experiments were compared, there were no statistically significant differences among the following replications: (i) infected mice not treated with amphotericin B regardless of whether supplemental therapy was rifampin or water (Table 1, groups 5-6); (ii) infected mice treated with amphotericin B and water for each of the four doses of antibiotic (Table 1, groups 11-14); (iii) infected mice treated with amphotericin B and rifampin for each dose of amphotericin B (Table

TABLE 2. Effect of rifampin plus amphotericin B on Namru mice infected with *C. immitis* (Silveira)

i.n. inoculation (100 spores)					i.p. inoculation (100 spores)				
Rifampin (mg/kg)	Amphotericin B (mg/kg) ^a				Rifampin (mg/kg)	Amphotericin B (mg/kg) ^a			
	0	0.043	0.22	0.43		0	0.043	0.22	0.43
0	8/10 ^b	4/5	0/5	0/5	0	9/10	0/5	0/5	0/10
10	4/5	6/10	2/9	0/10	10	4/5	4/10	1/10	0/10
20	4/5	8/10	0/10	2/10	20	5/5	0/10	0/10	0/10

^a Number dead/total mice in group.

^b Mortality by day 30 after infection.

1, groups 7-10). These replicated groups have been combined, and the results in the accompanying tables and figures are based on 60 infection control mice (Table 1, groups 5-6), 30 mice in each group at each dose of amphotericin B supplemented with water, and 30 mice in each group treated with amphotericin B and rifampin.

The effect of these several treatments of infected mice is illustrated in Fig. 2 and 3 in terms of cumulative percentage of mortality over the 40-day postinfection period. It was apparent that the only treatment providing consistently effective chemotherapy was with 0.4 mg of amphotericin B per kg. The addition of rifampin showed no increased therapeutic efficacy at lower doses of amphotericin B. Statistical evaluations demonstrated significant differ-

FIG. 2. Cumulative mortality for dba mice infected with *C. immitis* (C34) and treated daily for 5 days with amphotericin B (i.p.) and water (per os). Symbols: —, controls, 5% aqueous glucose (i.p.) and water (per os); - - -, amphotericin B, 0.006 mg/kg; ····, amphotericin B, 0.025 mg/kg; - · - ·, amphotericin B, 0.100 mg/kg; - - - - -, amphotericin B, 0.400 mg/kg.

FIG. 3. Cumulative mortality for dba mice infected with *C. immitis* (C34) and treated daily for 5 days with amphotericin B (i.p.) and 8 mg of rifampin per kg (per os). Symbols: —, controls, 5% aqueous glucose (i.p.) and water (per os); - - -, amphotericin B, 0.006 mg/kg; ····, amphotericin B, 0.025 mg/kg; - · - ·, amphotericin B, 0.100 mg/kg; - - - - -, amphotericin B, 0.400 mg/kg.

ences only for comparisons shown in Tables 3 and 5 for survival times and in Tables 4 and 6 for total survivors. All other comparisons were not significant ($P > 0.05$), including the amphotericin B-water combination versus amphotericin B-rifampin for both survival times and total survivors (shown in tables). It was apparent that addition of rifampin did not improve the

TABLE 3. Comparison of survival times among dba mice infected (i.n.) with *C. immitis* (C34): treatment groups versus controls

Amphotericin B (mg/kg/day)	Treatment per os ^a	Significance of survival times		
		Day 20	Day 30	Day 40
0.006	Water	NS ^b	NS	NS
0.025	Water	NS	NS	NS
0.100	Water	0.04	0.04	NS
0.400	Water	<0.001	<0.001	<0.001
0.006	Rifampin	NS	NS	NS
0.025	Rifampin	NS	NS	NS
0.100	Rifampin	NS	NS	NS
0.400	Rifampin	0.002	0.001	0.002

^a Water or rifampin (8 mg/kg per day), single treatment daily for 5 days.

^b NS, Not significant; $P > 0.05$ in all comparisons.

TABLE 4. Comparison of total number of survivors among dba mice infected (i.n.) with *C. immitis* (C34): treatment groups versus controls

Amphotericin B (mg/kg/day)	Treatment per os ^a	Significance of total survivors		
		Day 20	Day 30	Day 40
0.006	Water	NS ^b	NS	NS
0.025	Water	NS	NS	NS
0.100	Water	0.02	NS	NS
0.400	Water	<0.001	<0.001	<0.001
0.006	Rifampin	NS	NS	NS
0.025	Rifampin	NS	NS	NS
0.100	Rifampin	<0.05	NS	NS
0.400	Rifampin	<0.001	<0.001	<0.001

^a Water or rifampin (8 mg/kg per day), single treatment daily for 5 days.

^b NS, Not significant; $P > 0.05$ in all comparisons.

TABLE 5. Significance of survival times among dba mice infected (i.n.) with *C. immitis* (C34): amphotericin B and water versus amphotericin B and rifampin

Amphotericin B + water ^a (mg/kg/day)	Amphotericin B + rifampin ^a		
	Day 20	Day 30	Day 40
0.006	NS ^b	NS	NS
0.025	NS	NS	NS
0.100	NS	NS	NS
0.400	NS	NS	NS

^a Water or rifampin (8 mg/kg per day), single treatment daily for 5 days.

^b NS, Not significant; $P > 0.05$ in all comparisons.

therapeutic effect obtained with amphotericin B and water. This conclusion was supported by the more precise determination of 50% end points for survival time (ST₅₀) and for survival dose (SD₅₀) of amphotericin B (Tables 7 and 8). The ST₅₀ results were almost identical for each dose of amphotericin B, and the SD₅₀ results at each time interval did not differ markedly; i.e., the ratios for combinations at each time interval were approximately one.

There was some indication that the combined drug therapy was less effective than treatment with amphotericin B plus water. A significant increase in survival time was found with the 0.1-mg/kg amphotericin B-water combination

TABLE 6. Significance of total number of survivors among dba mice infected (i.n.) with *C. immitis* (C34): amphotericin B and water versus amphotericin B and rifampin

Amphotericin B + water ^a (mg/kg/day)	Amphotericin + rifampin ^a		
	Day 20	Day 30	Day 40
0.006	NS ^b	NS	NS
0.025	NS	NS	NS
0.100	NS	NS	NS
0.400	NS	NS	NS

^a Water or rifampin (8 mg/kg per day), single treatment daily for 5 days.

^b NS, Not significant; $P > 0.05$ in all comparisons.

TABLE 7. Comparison of time to 50% survival for dba mice infected with *C. immitis* (C34)

Amphotericin B (mg/kg/day)	ST ₅₀ ^a (days) with amphotericin B plus:	
	Water	Rifampin
0	11.7	10.8
0.006	11.2	11.3
0.025	11.5	11.0
0.100	15.9	15.6
0.400	39.9	35.3

^a Standard errors for all values were ± 0.5 to ± 2.8 at the greatest dose of amphotericin B.

TABLE 8. Dose of amphotericin B required for survival of 50% of dba mice infected with *C. immitis* (C34)

At day	SD ₅₀ ^a (mg/kg/day) for amphotericin B plus:	
	Water	Rifampin
20	0.104	0.088
30	0.136	0.112
40	0.224	0.196

^a Standard errors of all values were ± 0.004 to 0.012.

through day 30, but a similar effect was not apparent with the 0.1-mg/kg amphotericin B-rifampin combination (Table 3). In terms of total survivors, however, this difference was not obvious (Table 4). A reasonable interpretation of these results would be that addition of rifampin to therapy with amphotericin B in this model of coccidioidomycosis produced an indifferent effect at best.

Pathological findings. No difference was noted in the gross or microscopic lesions of mice whether amphotericin B was used alone or in combination with rifampin. The lungs from mice treated with the lowest dose of amphotericin B contained lesions of varying size and age. The oldest lesions contained mature granulomata encapsulated by collagen and with central necrosis and abscesses. The histiocytic response was moderate, but giant cells were scant. Lesions of intermediate age were composed primarily of epithelioid cells with early peripheral collagen deposition. The youngest lesions consisted of polymorphonuclear cells usually clustered around a spherule and situated at the margins of older lesions. Frequently, a break was noted in the collagen barriers of many of the older lesions near the younger lesions, suggesting that the latter were satellite lesions resulting from escape of organisms from older lesions.

The complete spherule-endospore cycle was readily apparent in the lesions of mice receiving the lowest dose of amphotericin B. These could be found not only in areas immediately adjacent to older lesions but also within aveoli and bronchi, suggesting a continuing and active dissemination of the infection. Old, intermediate, and young lesions were found also in lungs of mice treated with higher doses of amphotericin B, but there were fewer satellite lesions. In addition, there was a marked increase in the number of immature (non-endospore-forming) spherules relative to mature spherules although the total number of identifiable fungus cells was apparently not reduced. Thus, treatment with amphotericin B appeared to restrict maturation of spherules.

DISCUSSION

When chemotherapy has been required for systemic mycoses, amphotericin B usually has been the drug of choice because of necessity for treatment rather than satisfaction with this antibiotic. The drug induces both immediate and long-term toxic effects, must be administered by slow infusion, and is primarily fungistatic. Successive courses of therapy are common and the cumulative dose can result in

permanent tissue damage. Nevertheless, successful and presumably life-saving therapy has been achieved with amphotericin B.

Since no other drug has proven more consistently effective, amphotericin B has retained its status as the principal drug for treating systemic mycoses and one must seek improved methods for using this antibiotic. The *in vitro* demonstration by Medoff, Kobayashi, and their colleagues (17-19, 21, 22, 29) that amphotericin B potentiated the antifungal activity of other drugs, which were ineffective when used alone, suggested the possibility that combined drug therapy might achieve successful results with lower doses of amphotericin B. This has been reported for murine models of mycoses with combinations of amphotericin B-tetracycline (16) and amphotericin B-5-fluorocytosine (4, 33), but combined drug therapy with rifampin as the supplemental antibiotic has yielded indifferent results at best in the experiments reported here. The evidence supporting this conclusion is too strong to permit any other interpretation. The combined antibiotics regimen did not significantly increase survival times or total number of survivors and did not reduce the amount of amphotericin B required to protect 50% of the infected mice in comparison with that needed with amphotericin B alone. In addition, linear regression analysis of the ST_{50} (Table 7) for each type of combined therapy produced a correlation coefficient of 0.99 (slope = 0.85) for the amphotericin B-water combination compared with amphotericin B-rifampin. The same results (correlation coefficient = 0.99, slope = 0.91) were obtained with linear regression analysis for the SD_{50} (Table 8). The almost perfect correlations indicate that these therapeutic modalities show parallel increases in ST_{50} with respect to increasing dose of amphotericin B and corresponding increases in SD_{50} with respect to time. These correlations, combined with the evidence that the ratios of ST_{50} at each dose of amphotericin B and those of the SD_{50} for each time interval approximate unity, indicate that these treatments do not differ.

Further evidence supporting this conclusion derives from the pathological studies of lesions in survivors. In our opinion, treatment with amphotericin B appeared to inhibit the maturation from endospore to spherule and thereby limited the spread of the infection. This was seen also in another study, not reported here but using the same experimental animal model, in which whole spleens were homogenized and cultured. Thus all the spleens of infected placebo-treated mice yielded *C. immitis* by day 10. In contrast, spleens from infected animals treated with 0.4 mg of amphotericin B

per kg did not yield positive cultures until day 20, i.e., 10 days after cessation of therapy. The important point with respect to the present study was that inclusion of rifampin with amphotericin B did not influence the histopathological appearance or extent of lesions.

An explanation for the failure of rifampin to enhance the therapeutic effectiveness of amphotericin B against coccidioid infection in mice can be only speculative at the present time. On a theoretical basis with supporting experimental evidence (2, 3, 17, 18, 22, 27), the combination of amphotericin B and rifampin should be synergistic against eukaryotic fungi. Although rifampin inhibits ribonucleic acid synthesis by binding to the deoxyribonucleic acid-dependent ribonucleic acid polymerase in prokaryotic organisms and has no detectable effect in eukaryotic 80S ribosomes, it does inhibit ribonucleic acid synthesis by the mitochondrial 70S ribosomes of eukaryotic cells that are similar in size and antibiotic susceptibility to prokaryotic ribosomes (5, 6, 11). Therefore, one would anticipate that rifampin could exert an antifungal effect after penetrating the fungal cytoplasmic membrane through pores created by amphotericin B (1, 14, 15).

The relation of the regimen of rifampin used here in the murine model of coccidioidomycosis to that in therapy of humans should be considered. Rifampin was administered subcutaneously in one of our *in vivo* studies and orally in the other. Although rifampin is used only orally for treating humans because of convenience, studies in experimental bacterial infections in mice have demonstrated that the same effective dose for 50% of the animals is obtained by either route (10, 30). The commonly used doses of rifampin have been 5 to 10 mg/kg per day for animal studies (10, 30) and 450 to 600 mg/kg per day for humans, or approximately 6 to 9 mg/kg per day (24, 26). These are comparable to the doses used in the experiments presented here and have been effective for treating experimental murine tuberculosis (10, 30, 34).

It is evident, however, that the mycelial phase of *C. immitis* responds differently than the spherule-endospore phase to the combination of rifampin and amphotericin B. Thus, in agreement with the findings of Rifkind et al. (27), the mycelial phase is inhibited by lower concentrations of amphotericin B in the presence of rifampin than in its absence. In contrast, the response of the spherule-endospore phase was not affected by the presence of rifampin for one strain by *in vitro* test and for this and a second strain by the *in vivo* experiments. In addition, these two growth forms differ in susceptibility to amphotericin B; the MIC of

this antibiotic was higher for the mycelial phase than for the spherule-endospore phase. The difference in response of mycelial and spherule-endospore growth forms has been recognized in other ways, e.g., susceptibility to lysozyme (7, 8) and efficacy as protective vaccines (20). Since it has been demonstrated here for only two isolates with respect to the effect of combined amphotericin B and rifampin, it will be necessary to test additional cultures of *C. immitis* to determine whether the lack of potentiation by combined drugs on the spherule-endospore stage is common for this species of fungus. The molecular bases for the failure of amphotericin B to potentiate antifungal activity of rifampin against the spherule-endospore growth phase remains unexplained. Since this growth form is composed of cells within cells, it is possible that higher concentrations of antibiotics might have demonstrated synergism. Such higher concentrations of antibiotics, however, would be unrealistic on a practical basis.

This difference in antibiotic susceptibility of the two growth phases should be considered when assessing the susceptibility of a given patient's strain of *C. immitis* or the putative effectiveness of the serum level of amphotericin B. In this connection, it is of interest that the in vitro susceptibility of the spherule-endospore phase (MIC, approximately 0.16 $\mu\text{g}/\text{ml}$) falls within the protective doses of 0.1 to 0.4 mg/kg or 0.1 to 0.4 $\mu\text{g}/\text{g}$, assuming uniform distribution of antibiotic in tissues of the host.

A second possible explanation for the indifferent effect of combined amphotericin B-rifampin therapy might lie both in the nature of the experimental disease and in drug effects secondary to antifungal activity. The in vivo model of coccidioidomycosis is far more complex and probably less sensitive in its capacity for detecting synergism than in vitro tests. The ultimate outcome will depend not only on the virulence of the organism, but also on the efficacy of antimicrobial therapy and the competence of the host's immunological response. In the present model, the immunosuppressive activity of rifampin (13, 28) may have counterbalanced any specific antifungal activity. This was anticipated by Rifkind et al., who commented that cell-mediated immunity was "central to the defense against fungal infections" and that "its suppression would adversely affect recovery" (27). There is some evidence that amphotericin B also may have immunosuppressive activity (32). A combined anti-immune effect of these two antibiotics would have worked against an antifungal activity and might explain the limited evidence in this report that the combination appeared less efficacious than

amphotericin B alone (Tables 2 and 3). If this should prove to be true, then it would be clinically significant since it would be a potential problem for patients receiving rifampin therapy for other infections, e.g., concomitant tuberculosis and coccidioidomycosis. The depressing effect of rifampin on the cell-mediated immune response could contribute to potentiation of coccidioidal disease, although no definitive evidence for this was provided by our studies.

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