

Treatment of Experimental Murine Cryptococcosis: a Comparison of Miconazole and Amphotericin B

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Miconazole was compared with amphotericin B in the treatment of murine cryptococcosis. Both subcutaneous and intraperitoneal administration of miconazole produced serum levels higher than the minimum inhibitory concentration for the challenge strain. However, maximal tolerable doses of miconazole gave no increase in survival. When combined with amphotericin B, miconazole demonstrated neither additive nor antagonistic effects on survival. Spleen and brain counts of cryptococci were not lowered by miconazole; also, miconazole did not alter the effect of amphotericin B on reducing tissue counts. In vitro studies confirmed that the strain of *Cryptococcus neoformans* was quite susceptible to both miconazole and amphotericin B. However, miconazole had a delayed onset of antifungal activity. This was apparent even at miconazole levels 20 times greater than the minimum inhibitory concentration. Also, the antifungal activity of miconazole was markedly inhibited by serum. Delayed antifungal activity and serum inhibition may limit the in vivo effectiveness of miconazole in murine cryptococcosis.

Before the introduction of amphotericin B, cryptococcal meningitis was thought to be 100% fatal, although the course often ran a protracted period (2). At present, amphotericin B remains the mainstay of antifungal chemotherapy. Although many patients have dramatically responded, the treatment is long, arduous, and complicated by both outright failures and late relapses after apparent responses (1, 2). More recently the antimetabolite 5-fluorocytosine has been added in an effort to reduce the total dose (and toxicity) of amphotericin B (10). Although this has been largely achieved, 5-fluorocytosine has brought its own forms of gastrointestinal and hematological toxicity and therefore is not totally benign. Furthermore, there are still patients who resist combined therapy with amphotericin B and 5-fluorocytosine.

A few of this last group of patients have been treated with miconazole, a newly introduced member of the imidazole family of drugs. Miconazole has been used with great success in treating murine coccidioidomycosis. On the basis of these studies, miconazole was given to patients with a variety of fungal infections, including a few with cryptococcosis (7, 8; S. C. Deresinski, R. B. Lelly, and H. B. Levine, Program Annu. Meet. Am. Thoracic Soc., Abstr., p. 71, 1976; R. J. Duma, J. F. Fisher, and S. Markowitz, Clin. Res. 25:56A, 1977; J. R. Graybill and H. B. Levine,

Arch. Intern. Med., in press). Results have been inconclusive in a small number of patients. The present study was designed to evaluate miconazole therapy in experimental murine cryptococcosis. In vivo and in vitro studies were conducted to determine the effect of miconazole on a susceptible strain of *Cryptococcus neoformans* and also to explore its interaction with amphotericin B.

MATERIALS AND METHODS

Challenge and survival studies. Five-week-old male BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Amphotericin B was obtained from E. R. Squibb & Sons, Princeton, N.J., as the commercial powder and reconstituted in sterile water before use. Miconazole was a gift of Janssen Research and Development Corp., New Brunswick, N.J. It was used undiluted. Miconazole-free carrier was used as a placebo control for miconazole.

All studies were conducted with *C. neoformans* strain B, obtained from the cerebrospinal fluid of a patient with cryptococcal meningitis (J. R. Graybill and R. L. Taylor, Int. Arch. Allergy Appl. Immunol., in press). This strain was highly virulent for BALB/c mice, with a 50% lethal dose of approximately 600 cryptococci when challenged by the intraperitoneal (i.p.) route. However, pilot studies indicated that the strain was far less virulent by the nasal challenge route; only 70% of nasally challenged mice were dead by 120 days postchallenge with cryptococci. All i.p.

challenged mice were dead by day 36. Therefore, the i.p. route was used. For challenges, cryptococci were grown for 2 days in Sabouraud broth at 30°C, washed twice with sterile phosphate-buffered saline, and re-suspended in phosphate-buffered saline. Inocula were given in doses of 0.5 ml/mouse. The inoculum size was determined with a hemocytometer and confirmed with colony counts of serial dilutions as colony-forming units (CFU). These usually approximated hemocytometer counts.

After challenge, some groups of mice were observed for survival up to 60 days postchallenge. Others were sacrificed 10 days after challenge, and cryptococcal tissue counts on brain and spleen were determined from colony counts of tissue homogenates. Survival was compared with life table analysis, and tissue levels were compared with the rank sum test. $P < 0.05$ determined significance.

In vitro studies. Amphotericin B susceptibility of cryptococci was determined in the Littman modification of Sabouraud broth, using serial tube dilutions. The sensitivity was 0.098 µg/ml. Miconazole susceptibility was determined by a previously described method and was 0.65 µg/ml (5). Serum specimens for miconazole levels were obtained from cardiac blood at various intervals after subcutaneous or i.p. miconazole injection. Each specimen represented pooled sera of two mice, and each serum miconazole level was the mean of two identically collected specimens. Serum levels were performed by a cup well diffusion assay (5).

Finally, in vitro growth curves for *C. neoformans* were performed in fresh mouse serum or unmodified Sabouraud broth with inocula of either 10^3 , 10^4 , or 10^5 cryptococci per ml. Various concentrations of amphotericin B or miconazole or both were added to the broth, and incubations were conducted in a shaking water bath at 37°C. For growth curves in serum, pooled normal mouse serum was sterilized by filtration through a 0.45-µm Nalgene filter (Nalge Sybron Corp., Rochester, N.Y.). Several studies compared growth in serum with growth in Sabouraud broth. In any given study, both media were adjusted to the same pH with 0.1 N NaOH or HCl. Studies were conducted at pH 5.6 (usual pH for Sabouraud broth) or 7.4 (physiological pH). Additionally, in two studies conducted at pH 7.4, growth in serum was evaluated with and without supplementation of ferrous ammonium sulfate (300 µg of iron per 100 ml). Amphotericin B, miconazole, or both were then added to the serum or Sabouraud broth, and growth curves were carried out over a 48-h period.

Treatment. Either 1 day before or 1 or 2 days after challenge miconazole or amphotericin B or combined therapy was begun. Treatment was terminated on postchallenge day 9 in some studies and on day 12 in others. Amphotericin B was given in 0.1 ml of sterile distilled water, injected i.p. once per day. Controls for some studies included mice treated with 0.1 ml of distilled water i.p. daily. Miconazole or miconazole placebo was given twice daily. It was given subcutaneously into the thigh in some studies and i.p. in others. At 1 day after completion of drug or placebo therapy, some groups of mice were sacrificed for tissue counts. The remainder were followed for survival.

RESULTS

Preliminary studies with subcutaneous miconazole at 225 mg/kg twice daily produced a 60% mortality within 3 days. The same dose given i.p. produced a 10% mortality. This was due to miconazole itself and not to the polyethoxylated castor oil vehicle. At 125 mg/kg subcutaneously twice daily, mortality was approximately 10% from miconazole alone, and at 100 mg/kg twice daily, no mice died from miconazole. Serum levels of miconazole after various doses are shown in Table 1. These indicate that miconazole achieved the minimum fungicidal levels of our strain of *C. neoformans* 1 h after a 100-mg/kg dose, but fell below that level within 4 h. Because of these relatively low levels, other groups of mice were treated with either 100 or 200 mg of miconazole per kg i.p. twice daily. This was given to provide maximal drug levels at the challenge site. The other antifungal agent, amphotericin B, was not lethal to mice in i.p. doses of up to 6 mg/kg per day for a 9-day course of treatment and subsequent 50-day observation period. Blood levels of amphotericin B were not measured.

The initial survival studies are shown in Fig. 1. Ten mice per group received 125 mg of miconazole per kg subcutaneously or no drug. The first study (schedule 1) was conducted with an extremely high challenge of 10^8 CFU, and others (schedules 2 and 3) were conducted with a more modest challenge of 10^6 CFU. The latter dose was chosen because deaths from cryptococcosis did not begin until the week after treatment was discontinued. This permitted some distinction of toxicity deaths from cryptococcosis deaths. In all three studies, even when miconazole was begun a day before challenge, miconazole-treated mice did not survive longer than did controls. When spleen and brain counts were done on miconazole-treated and untreated controls, no differences were found (Table 2). Therefore, miconazole itself appeared to have no benefit in the dose of 125 mg/kg twice a day.

TABLE 1. Mouse serum miconazole levels after a single dose either subcutaneously (s.c.) or i.p. into the right thigh

Time after dose (h)	Serum miconazole level (µg/kg) after following dose (mg/kg):				
	100 (s.c.)	100 (i.p.)	125 (s.c.)	150 (s.c.)	200 (i.p.)
1	0.56				
2	0.50	0.55	0.64	0.97	5.7
4	0.27				
6	0.19				

A second series of studies examined possible synergism or antagonism of miconazole and amphotericin B. At a very high dose of 6.25 mg/kg per day, amphotericin B caused no mortality,

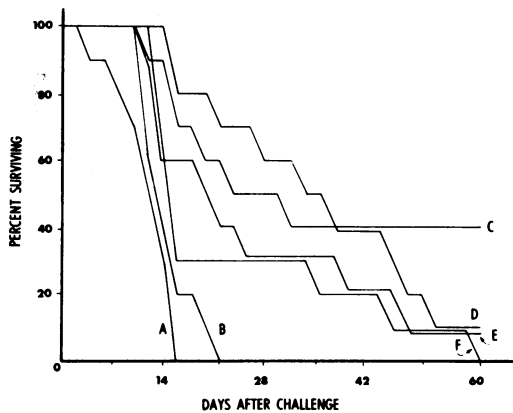


FIG. 1. Failure of protection from subcutaneous miconazole therapy (125 mg/kg twice daily) given in three different programs. There were 10 mice per treatment group. Schedule 1 included a challenge of 10^6 CFU and treatment on days 3 through 12 postchallenge. Group A was the control, and group B received miconazole. Schedule 2 included a challenge of 10^6 CFU and treatment on days 3 through 12 postchallenge. Group C was the control, and group D received miconazole. Schedule 3 included a challenge of 10^6 CFU and treatment from 1 day prechallenge to 10 days postchallenge. Group E was the control, and group F received miconazole.

TABLE 2. Tissue levels of *C. neoformans* at 10 days after challenge^a

Tissue	Tissue level (CFU/g)	
	Control	Miconazole ^b
Brain	1.7×10^4	1.1×10^4
Spleen	3.4×10^6	5.5×10^6

^a Mean counts of four mice per group. Miconazole treatment was begun at 1 day before challenge with 1×10^6 CFU and terminated at 9 days after challenge.

^b Rank sum test: $P > 0.05$ compared with controls (nonsignificant).

caused most challenged mice to survive the full 60-day postchallenge observation period (Fig. 2), and dramatically reduced the brain and spleen counts of cryptococci, usually to undetectable levels (Table 3). Mice treated with 0.1 ml of distilled water i.p. daily (data not shown) did not survive longer than did unmanipulated controls. In this study, when miconazole was given alone, even at a modest dose, survival was shortened compared with controls. The cause of shortened survival might have been the combination of miconazole toxicity and mortality from cryptococcosis. Mean tissue counts in brain and spleen were lower in miconazole-treated mice than they were in controls, but differences were not statistically significant. Of interest, there was no in vivo evidence of antagonism of miconazole on mice receiving these large doses of amphotericin B. Animals survived for the full period of observation and were as free of cryptococci as were those receiving only amphotericin B.

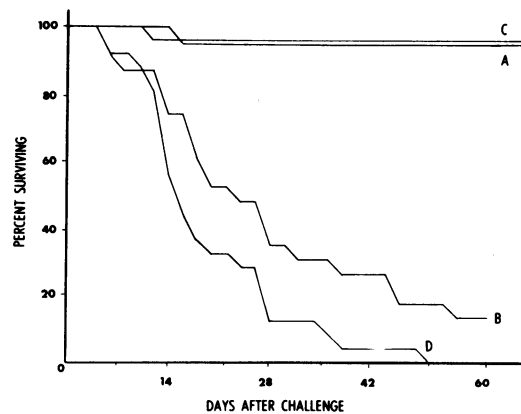


FIG. 2. Effect of combination therapy on survival after a challenge of CFU of *C. neoformans* i.p. Amphotericin B (6.25 mg/kg per day i.p.) and/or miconazole (125 mg/kg twice daily subcutaneously) were given from days 2 through 10 postchallenge. A total of 24 mice were given amphotericin B (A); 23 were control mice (B); 21 mice received both amphotericin B and miconazole (C); and 25 mice received miconazole (D).

TABLE 3. Tissue levels of *C. neoformans* at 10 days after challenge^a

Tissue	Tissue level (CFU/g)			
	Amphotericin B ^b (6.25 mg/kg per day)	Miconazole (125 mg/kg twice daily)	Amphotericin B ^b + miconazole	Control
Brain	$<1.7 \times 10^1$	5.6×10^2	$<1.7 \times 10^1$	1.4×10^4
Spleen	$<3.0 \times 10^2$	2.8×10^3	$<3.0 \times 10^2$	3.2×10^5

^a Mean of six mice per group.

^b $P < 0.05$ compared with control by rank sum test. Amphotericin B (i.p.) and miconazole (subcutaneous) were begun at 1 day after challenge and continued for 8 days.

The above results clearly showed no benefit of miconazole given at a site remote from the cryptococcal challenge and also no antagonism of amphotericin B activity. Therefore, other groups of mice were treated with either 125 or 200 mg of miconazole per kg i.p. twice daily, beginning on the day after cryptococcal challenge. As shown in Fig. 3, even with the miconazole injected i.p. (directly into the challenge site), there was no benefit. Failure was particularly impressive because the 200-mg/kg i.p. dose achieved a peak blood level of 5.6 $\mu\text{g}/\text{ml}$. This was nine times the minimum inhibitory level of miconazole. A final effort was made to demonstrate miconazole effect on survival. In this study, groups of 10 mice were challenged with 3×10^6 cryptococci, an inoculum that produced 80% mortality in controls by 60 days. Mice were treated i.p. with amphotericin B or miconazole or both from postchallenge days 2 through 10. Mice receiving amphotericin B at 0.6 mg/kg per day, a modest dose, all survived 60 days postchallenge. The addition of 125 mg of miconazole per kg twice daily did not increase mortality (no antagonism). In another experiment involving groups of 10 mice, mice treated with 0.1 mg of amphotericin B per kg per day had 40% mortality by 60 days postchallenge. When miconazole was added to this low dose of amphotericin B, the cumulative mortality was 80%, or the same as untreated controls. Therefore, even when miconazole was placed directly in apposition to amphotericin B, with both drugs injected into

the site of cryptococcal challenge, there was no demonstrable benefit from adding miconazole.

Further studies were then conducted on the in vitro effects of amphotericin B and miconazole on *C. neoformans*. As shown in Fig. 4, amphotericin B at 0.1 $\mu\text{g}/\text{ml}$ was rapidly fungicidal, with 95% killed in less than 4 h. However, at 0.01 $\mu\text{g}/\text{ml}$, amphotericin B had no effect, and cryptococci grew at the same rate as did the control. Miconazole at 5 and 0.5 $\mu\text{g}/\text{ml}$ was also fungicidal, but the effect was delayed. There was no apparent decrease in cryptococcal colony counts for at least 8 h after exposure to the drug. Thereafter, CFU declined gradually but steadily. Miconazole at 0.1 $\mu\text{g}/\text{ml}$ did not inhibit the growth of *C. neoformans* (data not shown). The same patterns occurred at 10^3 , 10^4 , and 10^5 CFU per ml of inoculum, so the delayed and gradual "kill" from miconazole did not appear to be inoculum dependent.

Figure 4 also shows that combinations of miconazole and amphotericin B showed no synergism or antagonism at the doses studied. When miconazole was added to large concentrations of amphotericin B, there was no delayed fungal killing by amphotericin B, and when miconazole was added to small concentrations of amphotericin B, there was no acceleration in the speed of miconazole killing.

The effects of both amphotericin B and miconazole were rather serum dependent (Fig. 5). This was possibly due to protein binding, which occurs with both drugs. At pH 5.6, cryptococci grew as well or better in mouse serum than in Sabouraud broth. However, although both amphotericin B and miconazole were able to kill cryptococci in Sabouraud broth, much higher levels were required in serum. The inhibitory effect of miconazole was as gradual in serum as it was in Sabouraud broth, indicating that this effect was not dependent on the culture medium.

At pH 7.4, cryptococci grew as rapidly in Sabouraud broth as they did at pH 5.6. However, at pH 7.4, serum inhibited the growth of cryptococci. By 30 h of incubation, cryptococcal counts had fallen to between 50 and 10% of the initial inoculum size. The pH dependence of the serum inhibitory activity suggested to us that transferrin might be involved. Free iron is important for growth of *Candida albicans* in serum and has also been suggested for *C. neoformans*. At pH 7.4, transferrin removes free iron, making it unavailable, whereas at a pH of less than 6 transferrin is unable to bind iron and it is available (3, 9). We tested the possible importance of transferrin by comparing growth in serum with and without added iron. In serum to which iron was added, growth was as rapid

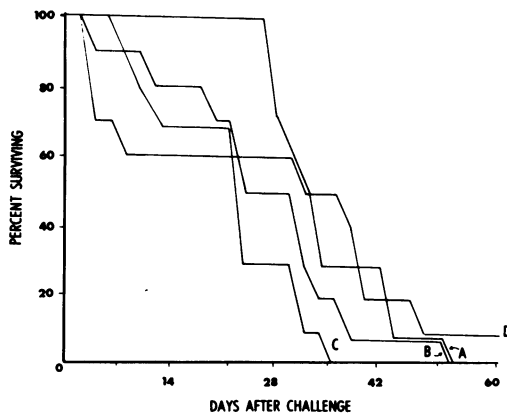


FIG. 3. Effect of i.p. miconazole on survival after a challenge of 10^6 CFU of *C. neoformans*. Miconazole was given twice daily from days 2 through 10 postchallenge. There were 10 mice per group. Group A received 125 mg of miconazole per kg; group B received 0.25 ml of miconazole placebo; group C received 200 mg of miconazole per kg; and group D was nonmanipulated.

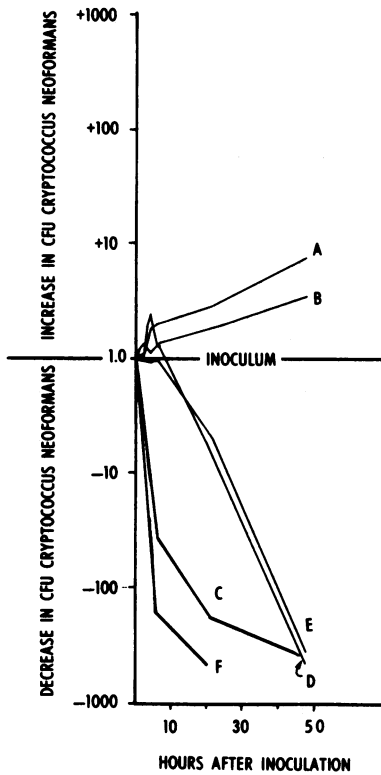


FIG. 4. Effect of amphotericin B and miconazole on growth of *C. neoformans* in Sabouraud broth after an inoculum of 10^4 CFU/ml and incubation at 37°C . The vertical axis indicates growth or killing of cryptococci on a logarithmic scale. (A) Control; (B) amphotericin B, $0.01\ \mu\text{g/ml}$; (C) amphotericin B, $0.1\ \mu\text{g/ml}$; (D) miconazole, $0.5\ \mu\text{g/ml}$; (E) miconazole, $0.5\ \mu\text{g/ml}$ plus amphotericin B, $0.01\ \mu\text{g/ml}$; and (F) miconazole, $0.5\ \mu\text{g/ml}$ plus amphotericin B, $0.1\ \mu\text{g/ml}$.

at pH 7.4 as it was at pH 5.6. Miconazole inhibition by serum was not affected by the addition of iron; results with added iron were quite similar to those shown in Fig. 5. In addition to mouse serum, several studies were conducted using pooled human serum from healthy AB+ donors and from a healthy subject working in this laboratory. Kinetics of growth in human serum and miconazole inhibition by human serum were similar to those in mouse serum.

In a final study, miconazole or amphotericin B was given i.p. to groups of five mice. At 2 h after drug administration, the mice were exsanguinated and growth curves of *C. neoformans* were performed in 100% mouse serum at pH 5.6 (Fig. 6). Amphotericin B at $6.25\ \text{mg/kg}$ was rapidly fungicidal. On the other hand, only the 200-mg/kg miconazole dose produced inhibitory activity in the serum, and this was less than 1

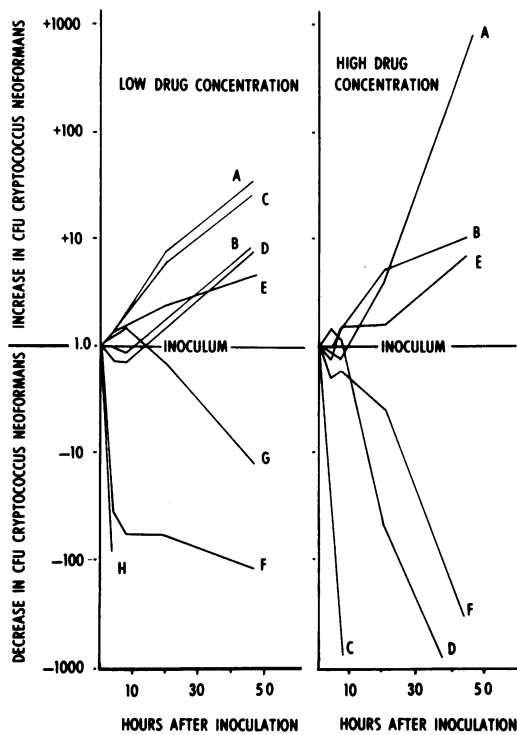


FIG. 5. Growth of *C. neoformans* in either Sabouraud broth or 100% mouse serum, pH 5.6, after an inoculum of 10^4 CFU/ml and incubation at 37°C . The vertical axis indicates growth or killing of cryptococci on a logarithmic scale. For low-drug-concentration study, A = control serum; B = amphotericin B, $0.1\ \mu\text{g/ml}$, serum; C = miconazole, $0.5\ \mu\text{g/ml}$, serum; D = amphotericin B, $0.1\ \mu\text{g/ml}$, plus miconazole, $0.5\ \mu\text{g/ml}$, serum; E = control, Sabouraud broth; F = amphotericin B, $0.1\ \mu\text{g/ml}$, Sabouraud broth; G = miconazole, $0.5\ \mu\text{g/ml}$, Sabouraud broth; and H = amphotericin B, $0.1\ \mu\text{g/ml}$, plus miconazole, $0.5\ \mu\text{g/ml}$, Sabouraud broth. For high-drug-concentration study, A = control serum; B = amphotericin B, $0.1\ \mu\text{g/ml}$, serum; C = amphotericin B, $2.0\ \mu\text{g/ml}$, serum; D = miconazole, $10\ \mu\text{g/ml}$, serum; E = control, Sabouraud broth; and F = miconazole, $10\ \mu\text{g/ml}$, Sabouraud broth.

log decrease. A portion of the same serum had $5.6\ \mu\text{g}$ of miconazole per ml, an extremely high level (Table 3). This poor serum fungicidal activity correlated with the failure of even this large dose to prolong survival, as previously shown in Fig. 3.

DISCUSSION

Miconazole has been dramatically effective in vitro against a number of fungi. Studies were extended in vivo to murine coccidioidomycosis, where the drug proved to be an excellent thera-

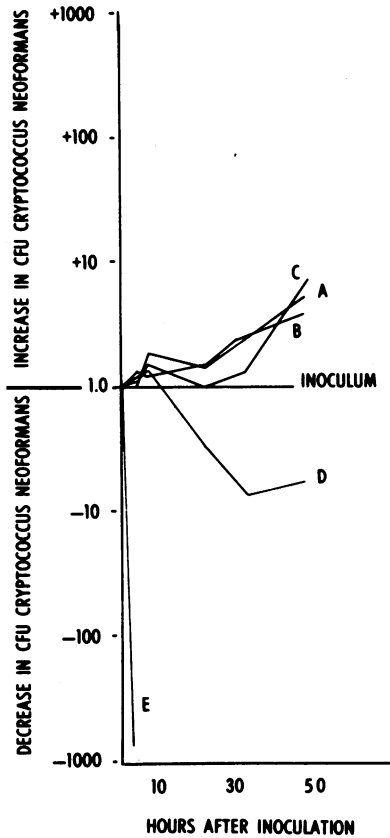


FIG. 6. Growth of cryptococci in serum obtained 2 h after i.p. miconazole or amphotericin B administration to mice. The vertical axis indicates growth or killing of cryptococci on a logarithmic scale. The inoculum size was 10^5 CFU/ml. (A) Miconazole, 125 mg/kg i.p.; (B) miconazole placebo, 0.25 ml i.p.; (C) control; (D) miconazole, 200 mg/kg i.p.; and (E) amphotericin B, 6.25 mg/kg i.p.

peutic agent. The mouse studies predicted the efficacy of miconazole in humans, where it is now undergoing extensive evaluation and appears to be a significant therapeutic agent (7, 8; Deresinski et al., Program Annu. Meet. Am. Thoracic Soc., Abstr. no. 71).

Another likely candidate for miconazole therapy would be *C. neoformans*. This is because (i) patients may be refractory to conventional therapy, and (ii) several strains of *C. neoformans* including our own appear to be susceptible in vitro to miconazole. However, we could not confirm that miconazole is of protective value in mice challenged with this strain of *C. neoformans*. Furthermore, although amphotericin B is clearly protective against cryptococcosis, we could not demonstrate any additive (or antago-

nistic) effect of miconazole on amphotericin B therapy in murine cryptococcosis.

There could be several reasons for this discrepancy between in vitro susceptibility and in vivo resistance. First, because miconazole is known to penetrate poorly into human cerebrospinal fluid, it was possible that miconazole might sterilize the peritoneal site of inoculation but not a remote protected site such as the central nervous system. We found no support for this because the spleen counts of cryptococci were not significantly different between miconazole-treated mice and nontreated controls. Also, amphotericin B (which penetrates the cerebrospinal fluid in minimal amounts) dramatically lowered cryptococcal counts in both spleen and brain in treated mice. Very low doses of i.p. amphotericin B protected mice, whereas very high doses of i.p. miconazole failed.

As a second possibility, the miconazole might have held the cryptococci in check as a static agent only to have the fungi grow unchecked after the drug was stopped. If this were the case, one would still expect at least a modest delay in mortality from miconazole and a reduction in spleen counts compared with controls (mice were sacrificed at 1 day after the treatment was stopped). Neither occurred.

A third possible explanation of the failure of miconazole is that we selected an unsuitable animal model for the demonstration of miconazole activity. There are three arguments against this. The first is that BALB/c mice have been used successfully in studies of protective immunization against cryptococcosis in this laboratory (Graybill and Taylor, in press). The second is that another chemotherapeutic agent, amphotericin B, was quite satisfactory in the present studies. The third is the success of miconazole in murine coccidioidomycosis.

A fourth possibility would be that the strain of cryptococcus selected was "inappropriate" and did not reflect a "majority" of cryptococci that are susceptible to miconazole in vitro and in vivo. Although this has not been excluded, it still does not explain the discrepancy between in vitro susceptibility and in vivo resistance seen with this strain. It is also not likely that the challenge dose was the critical factor, because the effect was apparent at doses that were quite satisfactory in showing protection in concurrent mice treated with amphotericin B.

Fifth, one may argue that not enough miconazole was given, because the maximal serum levels after a subcutaneous dose of 125 mg of miconazole per kg only exceeded the minimum inhibitory concentration for a relatively short time. However, injection of 200 mg of miconazole

per kg i.p. provided a 5.7- μ g/ml peak blood level, and by inference, even higher i.p. levels. Survival was still not prolonged in mice so treated. Furthermore, we usually did not detect any cryptococcal brain infection until 7 days postchallenge (unpublished data in our laboratory). Treatment was thus begun when there were few intracerebral cryptococci. Finally, the daily doses used were equivalent to those found protective against murine coccidioidomycosis. Thus, although we cannot absolutely exclude "inadequate" dose, we used maximal miconazole within the limits of toxicity, with no benefit.

Furthermore, even if miconazole had limited value by itself, it might be anticipated to have some effect on mice concurrently treated with amphotericin B. Over a broad dose range of amphotericin B, we could find no benefit of added miconazole therapy. It should be added that we also did not find antagonism either in vivo or in vitro. This may also be important, in view of recent observations that patients on miconazole plus amphotericin B therapy appear to have lower serum antifungal activity than do those on amphotericin B alone (6). Our observations do not directly refute these studies as the experimental methods were not closely comparable.

Finally, it is possible that the kinetics of miconazole activity might be biologically important. The effects of amphotericin B on membrane permeability of fungi are apparent within moments of exposure to the drug, well before any killing occurs (4). When the relatively crude test of colony counts was used, miconazole exerted no apparent effect for up to 6+ h of exposure to the drug. It is possible that prolonged contact at pharmacological levels must be achieved for any killing to occur. In addition to this delayed and "slow" antifungal activity of miconazole, the drug is much less effective in the presence of serum. This was demonstrated with in vitro studies in human serum as well as mouse serum. If prolonged in vivo contact of

miconazole and cryptococci is required at levels exceeding the minimum inhibitory concentration and if serum inhibition is important in vivo, it is reasonable that these two effects could negate even larger doses of miconazole than those we used.

In any case, the dramatic effects of miconazole in murine coccidioidomycosis could not be reproduced with a mouse model of cryptococcosis. There is very little experience with miconazole in human cryptococcosis. Although both responses and failures have been reported, it is too early to determine whether these studies will have predictive value on the ultimate status of miconazole in human cryptococcosis.

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