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Effect of Severity of Meningitis on Fungicidal Activity of Flucytosine Combined with Fluconazole in a Murine Model of Cryptococcal Meningitis

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We studied the effect of the severity of meningitis on the response to therapy with fluconazole and flucytosine in a murine model of cryptococcal meningitis. Meningitis was established by intracerebral injection of *Cryptococcus neoformans*. The severity of meningitis was varied by delaying the onset of treatment from 3 to 7 days. Animals were sacrificed after 14 days of treatment, and the numbers of *C. neoformans* per gram of brain tissue were quantified. The range of effective dose combinations of fluconazole and flucytosine became progressively reduced as the severity of meningitis increased. The magnitude of treatment effect, as measured by the numbers of CFU/gram of brain tissue, was also reduced with increasing severity of meningitis. In this model, as the severity of meningitis increases, higher doses of fluconazole are required to achieve equivalent levels of activity. The combination of fluconazole and flucytosine appears to have the most-potent antifungal effects. This is most readily observed in animals with more-severe meningitis.

A variety of approaches to the treatment of cryptococcal meningitis have been tried, most with limited success. Among patients with AIDS-related cryptococcal meningitis who are treated with amphotericin B or fluconazole for 10 weeks, approximately one-third will resolve their meningitis, one-third will have active meningitis, and nearly one-third will die (3, 4, 16, 18). Combining flucytosine with fluconazole or amphotericin B results in improved rates of success, usually in the range of 55 to 65% in patients with and without human immunodeficiency virus (HIV) coinfection (2, 12–14, 16). Both animal and in vitro studies have demonstrated that the combinations of flucytosine with amphotericin B and with fluconazole have significantly improved antifungal activity compared to that for each drug used alone (1, 11, 17, 19). However, patients with more-severe meningitis tend to fare far worse (18). The present study was designed to evaluate the effect that the severity of meningitis has on response to therapy with fluconazole and flucytosine in a murine model of cryptococcal meningitis.

MATERIALS AND METHODS

Animal protocol. Pathogen-free BALB/c male mice, approximately 6 weeks of age and weighing 20 to 26 g, were used in all experiments. The animals were weighed individually, housed in isolation cages (four or five per cage), and given free access to food and water. The mice were briefly anesthetized with CO_2 narcosis and challenged intracerebrally with 5 to 15 CFU of *Cryptococcus neoformans* in 0.06 ml (isolate no. 1597, a clinical isolate responsive to fluconazole and flucytosine). The inoculum was delivered in a volume of 0.06 ml through a 27-gauge needle by direct puncture through the cranial vault approximately 6 mm posterior to the orbit. The animal protocol was approved by the University Institutional Animal Care and Use Committee.

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Chemotherapy. Mice were randomly assigned to treatment groups 3 days after intracerebral challenge, and treatment was initiated on days 3, 5, and 7 for the low-, medium-, and high-fungal-burden groups, respectively, with the assigned concentrations of fluconazole and flucytosine dissolved in the sole source of drinking water. Water intake was recorded daily for each cage. Treatment water was replaced every 3 to 4 days; drug concentrations were recalculated on the basis of the weights of the animals in each treatment cage, measured water intake during the preceding days, and assigned drug doses. Treatment was continued for 14 days. The doses tested were as follows: flucytosine, 0 to 140 mg/kg of body weight/day; fluconazole, 0 to 40 mg/kg/day (0 to 50 mg/kg for the day 7 group). One cage of four to five animals was treated at each dose combination tested in each of the onset of treatment groups.

Mycologic procedures. The *C. neoformans* isolate was obtained from a patient with AIDS-associated cryptococcal meningitis that responded to treatment with fluconazole and flucytosine (isolate no. 1597). Two days prior to use, the isolate was plated on Sabouraud dextrose agar. Twenty-four hours prior to infection of the mice, 1 CFU was placed in brain heart infusion broth, and the infusion was incubated at 35°C overnight. The organisms were washed twice with normal pyrogen-free saline before suspension in saline. The concentration of organisms injected into the mice was confirmed by serial 10-fold dilutions of the initial suspension and by counting the numbers of CFU on plates prepared from 0.06 ml of the suspension ejected from the inoculation syringe just before and after inoculation of the mice from each cage.

For measurement of fungal burden in the brain, the animals were sacrificed, and their brains were removed, weighed, and homogenized in 1.0 ml of normal saline. Serial dilutions of the whole brain homogenate were prepared for quantitative counts of CFU. A 0.01-ml aliquot from each dilution was plated on Sabouraud dextrose agar, the agar was incubated at 35°C for 72 to 96 h, and the numbers of CFU were recorded. The remaining homogenate was plated on a large agar plate, the plate was incubated at 35°C for 72 to 96 h, and the numbers of CFU were recorded.

Measurements of efficacy. Treatment activity was measured by the following endpoints: survival, weight change relative to initial weight, and mycologic evaluation of brain tissue. Survival was considered in two forms: firstly, as duration of survival time and, secondly, as the proportion of animals treated at each dose combination which were alive at the end of treatment. Weight change for each animal was based on weight at the time of sacrifice relative to the initial weight recorded at the time of infection.

Groups of four to five control mice were sacrificed on days 3, 5, and 7 and at additional time points following infection to define the levels of fungal burden present at the onset of treatment. Animals exhibiting signs of distress were sacrificed, and the day following the date of sacrifice was considered the date of death. Survival time for animals alive on the scheduled day of sacrifice was censored at that day. Cases in which animals died or were found moribund prior

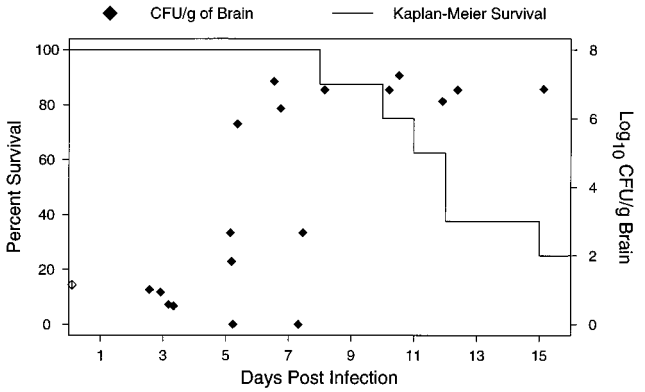

FIG. 1. Kaplan-Meier survival and numbers of CFU of *C. neoformans* per gram of brain tissue by day postinfection for controls.

to the scheduled day of sacrifice were considered deaths, with survival time equal to the day of death or the day following sacrifice, respectively.

Statistical analysis. The primary objectives of these experiments were to identify the dose combinations with the highest fungicidal activity and to determine the effect of delaying treatment on the degree of fungicidal activity. Loess regression was used to estimate the dose-response surface for each measure of response (2, 6, 7). Unlike classical least-squares regression methods, Loess regression uses locally weighted linear or quadratic regression to fit the dose-response surface at each dose combination so that the patterns of association are not forced to be the same over the entire range of doses. Loess regression is nonparametric in that it does not require specifying an equation for the association between response and dose levels. The Loess method does not require the assumption of normally distributed errors. Robust resistant iterative methods are used to minimize distortion of the estimated surface by unusually large or small observations (5, 9).

The relative association of potentially explanatory variables with response was assessed by robust analysis of variance (7, 9). Pointwise 99% confidence intervals (CIs) for the estimated response were used to identify the most effective region of dose combinations for each measure of efficacy (2). The duration of survival for untreated controls was estimated by the Kaplan-Meier method (10). CIs for the proportion surviving up to a time point were estimated by Greenwood's formula by using the method described by Link (8, 15). Analysis of survival for treated animals was based on the proportion of animals alive at the end of the 14-day treatment period for each dose combination separately by onset of treatment. Analysis of fungicidal activity for treated animals was based on the numbers of CFU/gram of brain tissue recovered at the end of 14 days of treatment. Descriptive statistics were based on medians and robust 99% CIs (9). All statistical analyses were performed with S-plus (20, 21). Because of the exploratory nature of these analyses, only P values of less than 0.001 were considered significant.

RESULTS

Survival for controls. Figure 1 shows the Kaplan-Meier survival curves for control animals. Median survival was 11 to 12 days; the Kaplan-Meier estimate of the proportion alive at day 10 was 70% (99% CI, 46 to 100%).

Growth curve of *C. neoformans*. The median inoculum level was 14 CFU/g of brain (99% CI, 11 to 18). On day 3 (corresponding to onset of treatment for the low-fungal-burden group), 2 to 9 CFU/g of brain were recovered from untreated controls; on day 5 (medium-fungal-burden group), the range was 0 to $>10^5$; and on day 7 (high fungal burden group), the range was 0 to $>10^6$. By day 8, the fungal burden had reached 10^7 CFU/g of brain tissue, regardless of the initial level. Mice tolerated as much as 7 to 8 days of untreated infection, resulting in 10^7 CFU/g of brain tissue, before exhibiting signs of distress or dying.

Survival for treated mice. When used alone, flucytosine did not provide complete protection for survival, regardless of onset of treatment (Fig. 2A). For animals in the day 3 and day 5 groups receiving flucytosine in combination with >5-mg/kg/day fluconazole, 100% were alive at the end of treatment,

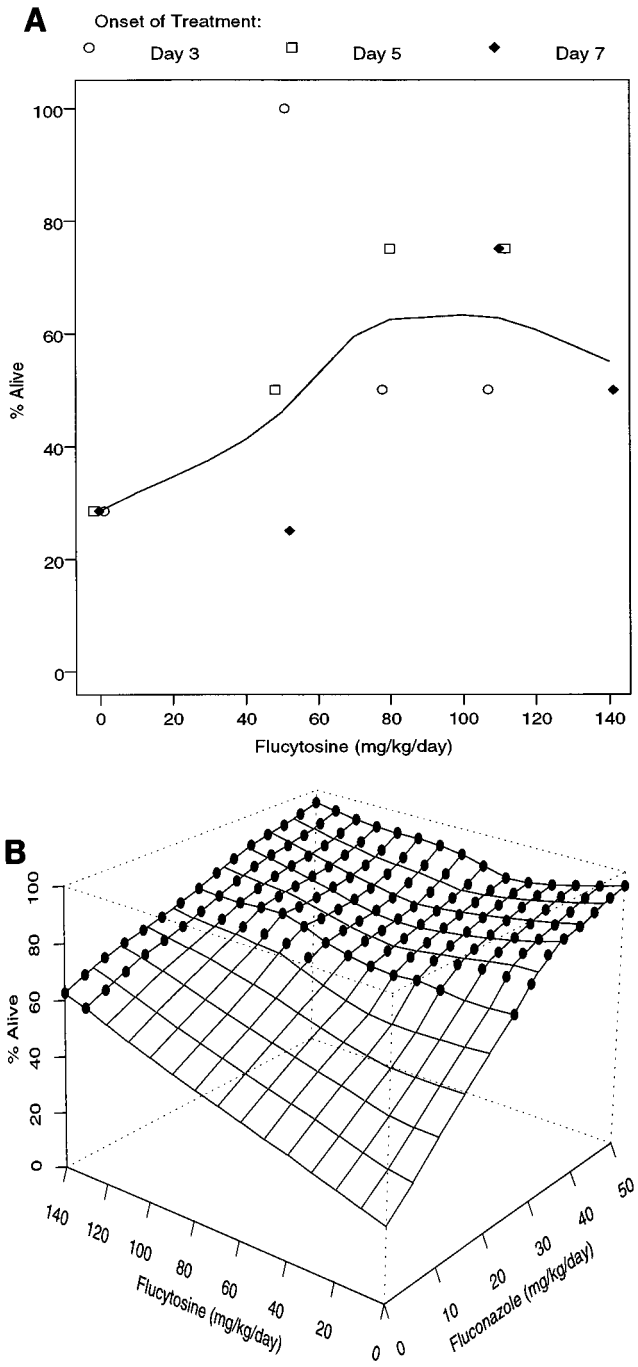

FIG. 2. (A) Survival for flucytosine alone. Loess fit of the association between the proportion of animals alive at the end of treatment and dose level of flucytosine for animals receiving flucytosine alone. (B) Survival for onset of treatment at day 7. Response surface showing the Loess fit of the association between the proportion of animals alive at the end of treatment and dose levels of fluconazole and flucytosine for the day 7 group. The Loess fit used a neighborhood of 75% with local regression linear in both fluconazole (P = 0.01) and flucytosine (P = 0.23). Dots, upper limits of 99% CIs of 95 to 100%.

regardless of the dose level of flucytosine (data not shown). For animals in the day 7 group, 75 to 100% survival was seen at the higher doses of fluconazole (>25 mg/kg/day), regardless of the dose level of flucytosine (Fig. 2B).

Chemotherapy. Analysis of the daily water intake for each cage indicated that water intake was very uniform across as-

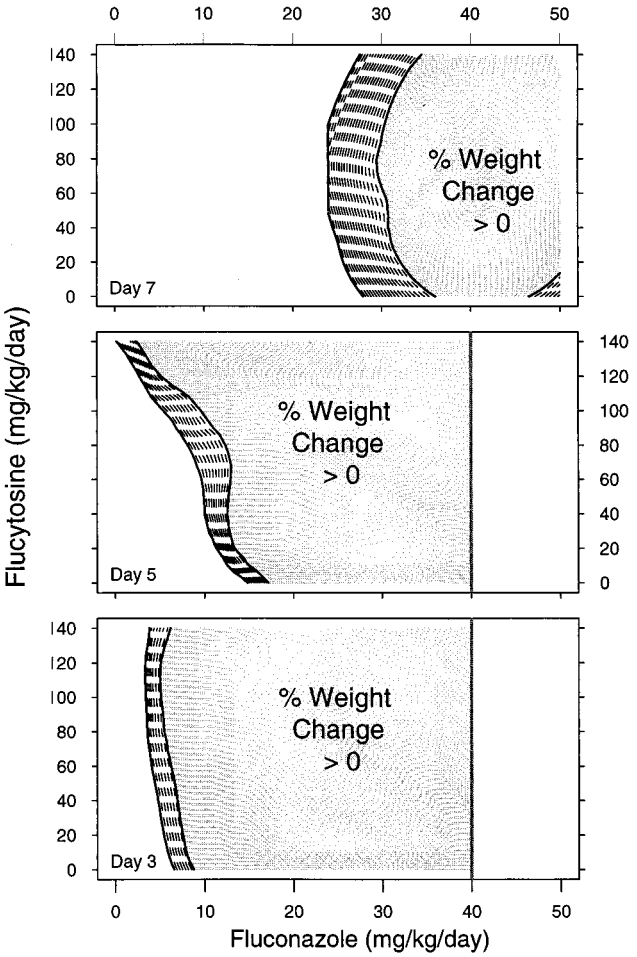

FIG. 3. Regions of dose combinations which prevented weight loss by treatment onset. Light shaded areas, dose combinations for which the lower limits of the 99% CI for weight change were >0%; areas with heavy broken lines, dose combinations for which the lower limits of the 99% CI for weight change were between −5 and 0%. The lower limits were <−5% for all dose combinations to the left of the shaded regions for each group. The maximum dose tested for the day 3 and 5 groups was 40-mg/kg/day fluconazole.

signed dose combinations, days postinfection, and severity of meningitis (data not shown).

Weight change. Untreated control animals lost as much as 30% of their initial weights; median weight change for treated animals ranged from −30 to +25%. When treatment was started on day 3 or 5, the dose levels of both fluconazole and flucytosine were strongly associated with weight change (fluconazole, $P < 0.00001$; flucytosine, $P < 0.0001$). For the day 7 group, the dose levels of fluconazole and flucytosine were weakly associated with weight change ($P < 0.01$ and 0.12, respectively). The regions of dose combinations sufficient to maintain weight (lower limits of the 99% CI for weight change were >0%) are shown in Fig. 3. For the day 3 and 5 groups, these regions covered a wide range, including fluconazole alone in the range 10 to 40 mg/kg/day (Fig. 3, lower and middle panels, respectively). For the day 7 group, the dose combinations required to maintain weight were restricted to >35-mg/kg/day fluconazole alone or >30-mg/kg/day fluconazole in combination with 50 to 100-mg/kg/day flucytosine (Fig. 3, top panel).

Fungicidal activity. The dose level of fluconazole was strongly associated with the numbers of CFU/g of brain tissue recovered after treatment regardless of onset of treatment ($P < 0.00001$). The dose level of flucytosine had a strong association with fungicidal effect for the day 3 and 5 groups ($P < 0.001$) but was weakly associated with fungicidal effect for the day 7 group ($P = 0.045$). The observed numbers of CFU/g of brain ranged from 10^8 for untreated control animals to less than 10 for animals treated with fluconazole, which is a 10^7-fold reduction.

When treatment was started on day 3, the upper limits of the 99% CI for the numbers of CFU/g of brain were below 10 for fluconazole alone in the range 25 to 35 mg/kg/day (Fig. 4, lower panel). For the day 5 group, the upper limits of the 99% CI for the numbers of CFU/g of brain were below 10 for fluconazole in the range 30 to 35 mg/kg/day and for flucytosine doses of 10 to 80 mg/kg/day (Fig. 4, middle panel). For both the day 3 and 5 groups, numbers of CFU/g of brain were <100 for fluconazole in the range of 20 to 40 mg/kg/day, regardless of the dose of flucytosine. However, a decrease in fungicidal activity was

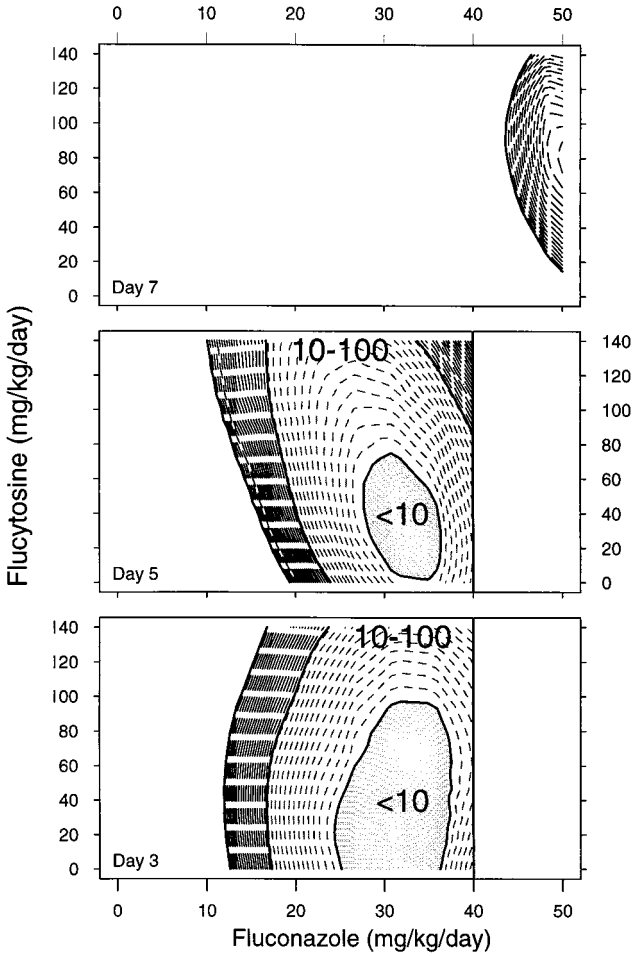

FIG. 4. Regions of dose combinations with greatest fungicidal activity by treatment onset. Lightly shaded areas, dose combinations for which the upper limits of the 99% CI for fungicidal activity were >10 CFU/g of brain; areas with light broken lines, dose combinations for which the upper limits of the 99% CI were between 10 and 100 CFU/g; areas with heavy broken lines, dose combinations for which the upper limits were between 100 and 1,000 CFU/g. The upper limits were >1,000 CFU/g for all dose combinations to the left of the shaded regions for each group. The maximum dose of fluconazole tested for the day 3 and 5 groups was 40 mg/kg/day.

seen with 40-mg/kg/day fluconazole in combination with >80-mg/kg/day flucytosine in the day 5 group. When the onset of treatment was delayed to day 7, the upper limits of the 99% CI for the numbers of CFU/g of brain were >100 at all dose combinations tested; the maximum fungicidal effect that could be achieved was in the range 100 to 1,000 CFU/g (Fig. 4, top panel). This effect was seen for fluconazole at 45 mg/kg/day in combination with 60 to 90-mg/kg/day flucytosine and for fluconazole at >45 mg/kg/day in combination with 30- to 120-mg/kg/day flucytosine.

DISCUSSION

Treatment with combinations of antifungal agents offers the promise of significant improvement in the outcome for patients with AIDS-related cryptococcal meningitis. However, patients with more-severe meningitis tend to fare far worse (18). In our murine model of cryptococcal meningitis, we used delay in the onset of treatment to study the effect of severity of meningitis on the fungicidal activity of fluconazole in combination with flucytosine. When used alone, flucytosine did not prevent weight loss or death in animals infected with cryptococcal meningitis, regardless of the severity of meningitis. However, in animals with mild to moderate meningitis, even when fluconazole was used alone at relatively low doses, it prevented weight loss and death. Fluconazole used alone at high doses had potent fungicidal activity in animals with mild cryptococcal meningitis. For animals with more-severe meningitis, high doses of fluconazole combined with moderate doses of flucytosine were required in order to achieve maximal antifungal effects. Furthermore, the level of maximum response which could be achieved decreased as the severity of meningitis increased. Overall, a 10^7-fold reduction in the numbers of CFU/g of brain tissue was achieved with combination therapy.

Previous murine models of cryptococcal meningitis have demonstrated a dramatic effect of fluconazole on the fungicidal activity of flucytosine (11). Flucytosine at dose levels of as much as 200 mg/kg/day alone or in combination with low doses of fluconazole had minimal fungicidal activity, whereas in combination with fluconazole at 24 to 40 mg/kg/day, flucytosine sterilized the brains in 45 to 65% of the animals treated at doses of 40 to 100 mg/kg/day. This striking effect of fluconazole on the fungicidal activity of flucytosine against cryptococcus has also been observed in in vitro studies (17). The impact of fluconazole on the fungicidal activity of flucytosine is also consistent with the results of clinical trials which evaluated flucytosine alone and in combination with fluconazole (13, 14, 16).

One of the important factors affecting clinical outcome for patients with AIDS-related cryptococcal meningitis is the severity of central nervous system infections (18). In order to improve the clinical relevance of the results in animal models, we used delay in the onset of treatment in the murine model to incorporate the effects of severity of meningitis. In this report, we have evaluated easily determined endpoints at a wide range of dose combinations. We estimated the association between response and the dose combination of fluconazole and flucytosine using a regression method specifically developed for data such that the nature of this association might vary depending on the dose region, such as synergism at low dose levels of each drug and antagonism at high doses (6, 7). We used the CIs from the estimated dose-response surface to identify regions of dose combinations with the most promising activity (3). We then used these regions to determine the effect of the severity of meningitis on the fungicidal activity of flucytosine when it is combined with fluconazole.

Conclusions. In this model, as the severity of meningitis increases, higher doses of fluconazole are required to achieve equivalent levels of fungicidal activity. The combination of fluconazole and flucytosine appears to have the most-potent antifungal effects. This is most readily observed in animals with more-severe meningitis. The ability to achieve equivalent or improved fungicidal activity with lower doses of flucytosine when it is combined with higher doses of fluconazole is seen consistently across a wide range of meningitis severity. The effect of fluconazole seen in the present study's murine model of cryptococcal meningitis is consistent with the effect of fluconazole on the fungicidal activity of flucytosine observed in previous murine models (11) and on the in vitro inhibitory action of flucytosine reported by Nguyen and colleagues (17). These results suggest that in the clinic, combining higher doses of fluconazole with lower doses of flucytosine could improve the treatment of cryptococcal meningitis with less toxicity. These results also demonstrate that animal models which incorporate clinically important factors can be designed to identify regions of dose combinations with promising activity. These regions can provide more useful information for the design of subsequent clinical evaluations than statistical tests comparing activities at a specific dose combination with those of controls.

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