The Combined Effect of 5-Fluorocytosine and Amphotericin B in the Therapy of Murine Cryptococcosis (37049)

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There is a clear need for more effective and less toxic therapy for cryptococcal disease in man. To date, amphotericin B (AmB) is the single most effective drug for the treatment of this infection. For the most part, treatment regimens of AmB are empirically derived, carry with them a significant incidence of toxicity, and in 30% of the cases the infection recurs once therapy is discontinued (1). 5-Fluorocytosine (5-FC) is a new oral antifungal agent which has been used with limited success in the treatment of cryptococcal disease (2–5). It has been associated with few adverse effects, and these are usually transient or reversible upon cessation of therapy (4). However, the effectiveness of 5-FC is limited by the development of profound drug resistance among the infecting cryptococcal organisms during therapy (5). This is in contrast to AmB therapy in which relapse or failure has not been associated with drug resistance (6).

There are several reasons to consider the use of 5-FC and AmB together in cryptococcosis. If the drugs were either additive or synergistic, the incidence of treatment failure with combined therapy might be less than that with either drug alone and the reduced dose of AmB could provide a substantial reduction in toxicity due to that agent. However, even at a reduced dose, AmB would need to inhibit the appearance of 5-FC-resistant mutants. Preferably, the dose of AmB required in the combination would be low enough so that AmB-induced nephrotoxicity would not elevate 5-FC blood concentrations, a problem encountered with conventional doses of AmB (7). Another potential problem with such a combined drug regimen is the possibility that AmB might increase intracellular penetration of 5-FC and enhance 5-FC toxicity as well as chemotherapeutic effect (8).

In the sole study on this subject, *in vitro* synergism between 5-FC and AmB was reported for three isolates of *Cryptococcus neoformans* (9). Although this was encouraging, an *in vivo* model promised to clarify more of the problems posed in the preceding paragraph. There are no widely accepted definitions of synergism in *in vitro* studies, and this lack of consensus is even more pronounced for *in vivo* systems. The model suggested by Gaddum (10) seemed acceptable to us, and the results of such a study are reported here.

Materials and Methods. Murine cryptococcosis model. Saline suspensions of *C. neoformans* for mouse inoculation were made from 48-hr cultures grown on Sabouraud agar at 30°. Mice were infected by injection into a lateral tail vein of 0.1 ml containing from 5.3 to 5.9 $\times$ 10^6 viable yeast cells. Female, general purpose (NIH) white mice weighing 17–20 g were housed in groups of 10 and given food and water *ad libitum*. Ten mice were infected for each test group and for each control group. Mice were observed daily for survival. Therapy was begun 72 hr after infection. Both 5-FC and AmB were diluted in 5% dextrose in water (D5W). The 5-FC was injected intraperitoneally (ip) in 1.0 ml once daily for 4 wk while AmB was given intravenously (iv) in 0.1 ml three times per week for 4 wk. Controls received 1.0 ml of D5W ip daily and/or 0.1 ml D5W iv three times per week. On Day 34 postinfection, the surviving mice were killed and brain cultures were made on Sabouraud agar containing chloramphenicol

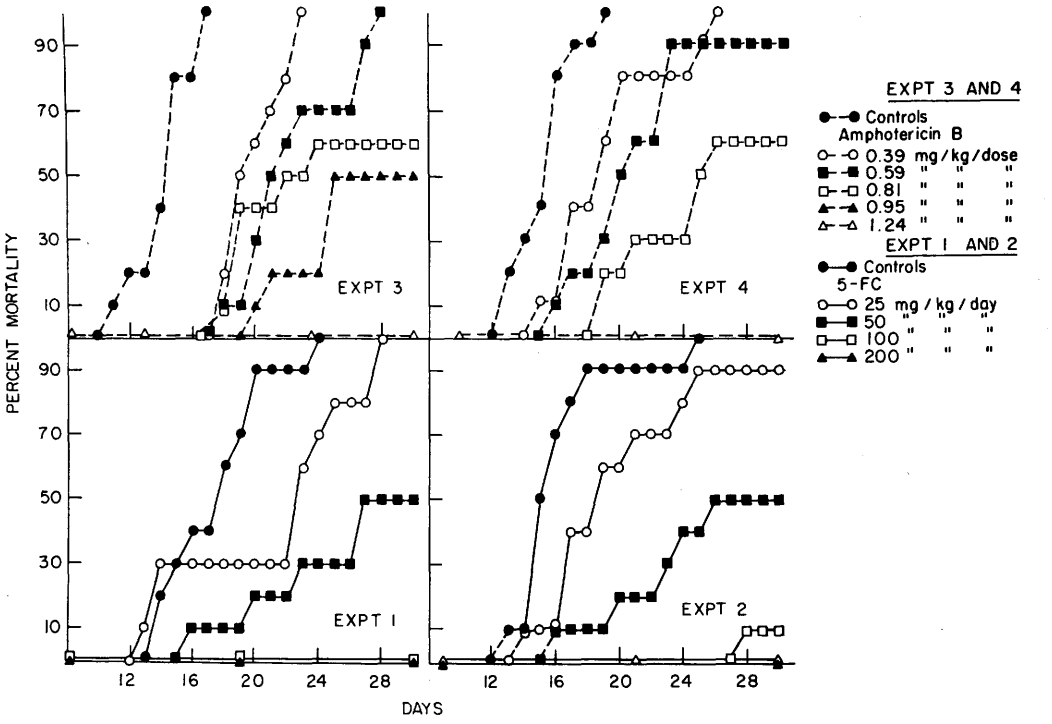

FIG. 1. Dose–response curves for 5-FC (Expts 1 and 2) and amphotericin B (Expts 3 and 4) in the therapy of murine cryptococcosis.

400 μg/ml.

Single and double drug dose–response curves for 5-FC and AmB. Four logarithmically spaced dosage levels of 5-FC and five logarithmically spaced doses of AmB were tested separately in duplicate trials (Expts 1–4). From the dose–response curves for 5-FC and AmB alone, the dosage required to protect approximately 50% of the infected mice from death (PD_{50}) was estimated for each drug using the Spearman-Karber method (11). With these data, five combined-drug and 2 single-drug dosage regimens were chosen and tested in duplicate (Expts 5 and 6):

Group A

1. 1/3 PD_{50} 5FC and 2/3 PD_{50} AmB;
2. 2/3 PD_{50} 5FC and 1/3 PD_{50} AmB;
3. 1/4 PD_{50} 5FC and 3/4 PD_{50} AmB;
4. 3/4 PD_{50} 5FC and 1/4 PD_{50} AmB;
5. 1/2 PD_{50} 5FC and 1/2 PD_{50} AmB;

Group B

1. PD_{50} 5FC;
2. PD_{50} AmB.

The survival data from Groups A and B were then compared to determine whether there was any potentiation or antagonism betewen 5-FC and AmB.

The 5-FC minimum inhibitory concentration (MIC) was determined by tube dilution testing on 44 isolates from the survivors of Expt 6. In this test, a final concentration of approximately 5×10^4 cryptococci/ml was incubated at 34° on a rotating drum for 48 hr with twofold serial dilutions of 5-FC extending from 2.5 to 320 μg/ml in yeast nitrogen base (Difco) broth with the addition of 2% dextrose. The MIC was defined as the lowest concentration of drug in which no visible growth is observed. Skipped tubes were noted on two occasions, and in both instances the organisms in these tubes were found to be resistant to 1000 μg/ml 5-FC.

Results. In vivo studies. Cryptococcal infection of the mouse induced by iv injection afforded a reproducible experimental model in which all controls could be made to die from Days 11 to 26 postinfection. Dose–re-

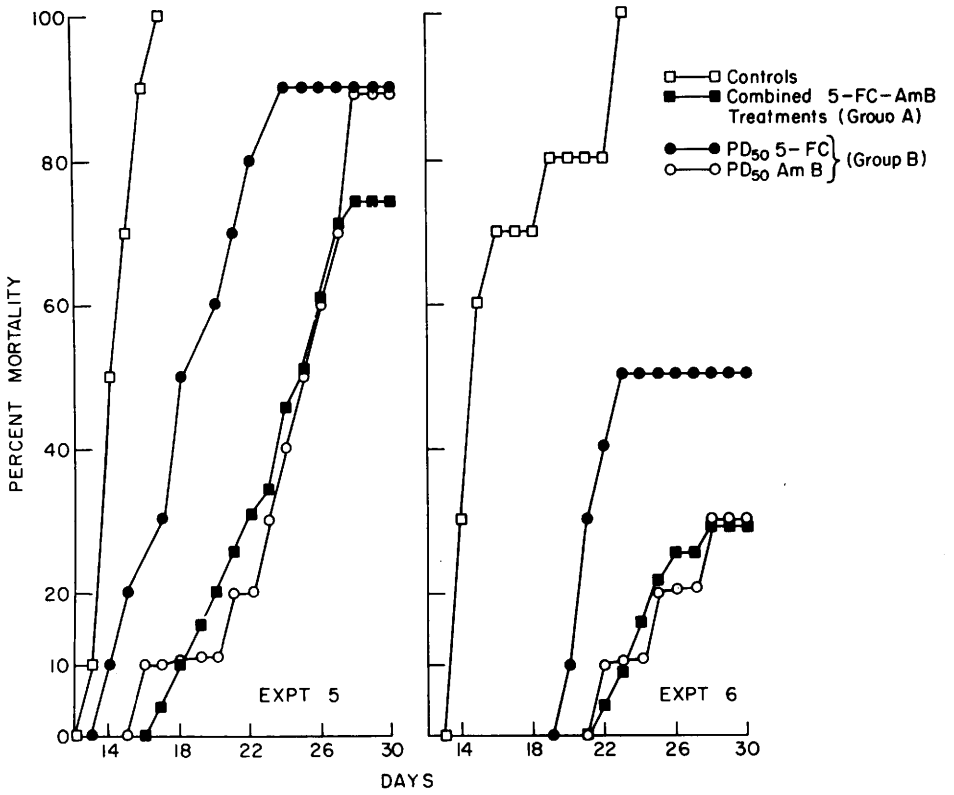

FIG. 2. Cumulative mortality curves from two separate experiments comparing the therapeutic results of untreated controls and three separate treatment regimens in murine cryptococcosis. Group A results in each experiment represent the summation of 5 separate dosage combinations (see Materials and Methods).

sponse curves for single drug therapy are presented in Fig. 1. Mortality was prevented completely by triweekly iv AmB 1.24 mg/kg/dose or daily 5-FC 200 mg/kg. The PD_{50} for AmB was calculated to be 0.86 mg/kg/dose iv and that for 5-FC was 50 mg/kg/dose ip.

In Fig. 2, the cumulative mortality curves for the combined-drug (Group A) and the single-drug regimens from Expts 5 and 6 are graphically depicted. The deviation from the expected 50% mortality in Expt 5 is not easily explained. However, this high mortality (90% in both single-drug regimens) does not alter our subsequent analysis or conclusions which are based on the shifts in the mortality curves rather than percentage survival on Day 30 (10, 12). In Expt 6 with the same infecting organism, inoculum size, host, and treatment regimens, the expected 50% mortality value for the 2 single-drug groups was closely approximated.

Although survival data differed between Expts 5 and 6, within each experiment the survival data from the 5 combined-drug treatment groups were quite similar to one another (Table I). This permitted pooling of these results within each experiment. The cumulative mortality curve based on the pooled data from Group A in each experiment was then compared to the cumulative mortality for the 2 single-drug groups in the same experiment (Fig. 2). The survival with PD_{50} AmB alone was significantly prolonged when compared to that seen with PD_{50} 5-FC in both experiments [$p < 0.05$, Wilcoxon test for censored data (12)]. In such a situation, if 5-FC and AmB were simply additive, the cumulative mortality curve for Group A would be expected to lie intermediate to the 2 single-drug mortality

TABLE I. Number of Deaths on Each Day in the 5 Separate Treatment Regimens Within Group A.

Combined drug treatment groups	Postinfection (days)[a]											
	17	18	19	20	21	22	23	24	25	26	27	28
	Expt 5											
3/4 PD_{50} 5-FC + 1/4 PD_{50} Am B	0	2	1	0	1	0	0	2	0	0	2	0
2/3 PD_{50} 5-FC + 1/3 PD_{50} Am B	1	0	1	1	2	2	0	1	0	0	0	1
1/2 PD_{50} 5-FC + 1/2 PD_{50} Am B	0	1	0	0	0	0	0	0	0	3	1	0
1/3 PD_{50} 5-FC + 2/3 PD_{50} Am B	1	0	0	1	0	1	1	1	1	0	1	1
1/4 PD_{50} 5-FC + 3/4 PD_{50} Am B	0	0	1	0	0	0	0	2	2	1	1	0
	Expt 6											
3/4 PD_{50} 5-FC + 1/4 PD_{50} Am B	0	0	0	0	0	0	0	3	0	2	1	0
2/3 PD_{50} 5-FC + 1/3 PD_{50} Am B	0	0	0	0	0	1	1	0	0	0	1	0
1/2 PD_{50} 5-FC + 1/2 PD_{50} Am B	0	0	0	0	0	0	0	1	0	0	1	0
1/3 PD_{50} 5-FC + 1/3 PD_{50} Am B	0	0	0	0	0	0	0	0	1	0	0	1
1/4 PD_{50} 5-FC + 3/4 PD_{50} Am B	0	0	0	0	0	0	0	0	2	0	0	0

[a] All deaths occurred between the 17th and 28th days postinfection.

curves (13). Antibiotic synergism would be present if the Group A mortality curve were shifted to the right from this expected position. In our model, without further assumptions, there is no way to determine whether a small amount of rightward shift in the Group A mortality curve constitutes synergism or simply additivity. However, if the Group A curve were to lie significantly to the right of the PD_{50} AmB curve, the 2 drugs would necessarily be synergistic (10). In both experiments the Group A curve was indistinguishable from that seen with PD_{50} AmB alone, despite 25–75% less AmB in the combined drug regimens. These results, when taken together, indicate at least an additive effect between 5-FC and AmB in our *in vivo* model. Slight synergism was suggested but could not be proven with certainty.

Results of MIC testing. All brain cultures from surviving mice in Expts 1 through 6 were positive for *C. neoformans*. The 5-FC MIC was determined for the isolates from all survivors in Expt 6. Markedly resistant cryptococci (MIC > 320 μg/ml) were found in 3 of 5 survivors treated with the PD_{50} 5-FC alone. In the remaining 39 survivors, including those from the PD_{50} AmB group, 37 had 5-FC sensitivities similar to the infecting microorganism (5 μg/ml). Isolates from the other 2 survivors exhibited skipped tube phenomena and were therefore arbitrarily designated as resistant to > 320 μg/ml 5-FC.

Discussion. The use of an *in vivo* model to study the combined therapeutic effect of 5-FC and AmB has not been described in the literature. Our results indicate at least additive and perhaps slight synergistic action between these two drugs in murine cryptococcosis. This is in keeping with previously reported studies (8, 9) in which slight to definite *in vitro* synergism was demonstrated for several isolates of *C. neoformans*. While our experience was encouraging, it was limited to observations on a single isolate of *C. neoformans*. Further *in vivo* studies are needed to determine whether this drug combination is equally effective for additional isolates of *C. neoformans*.

The mechanism by which these two drugs might potentiate one another is not known. Medoff *et al.* (8) have demonstrated increased penetration of 5-FC through the cytoplasmic membrane of a single isolate of *Candida albicans* in the presence of AmB. No comparable study with *C. neoformans* has been reported. AmB seemed to inhibit the *in vivo* development of 5-FC resistance in murine cryptococcosis. Sixty percent of survivors treated with the PD_{50} 5-FC in Expt 6 had markedly resistant isolates (MIC > 320 μg/ml) while only 6% of survivors from the 5 combined-drug treatment groups had isolates with a similar degree of resistance. The comparative toxicity of single versus double drug regimens was not studied. We observed no deaths in our studies which appeared related to drug toxicity. All dying

animals, irrespective of their treatment group, exhibited the signs of far-advanced cryptococcal disease. No search was made for histologic or biochemical evidence of drug toxicity in any of our mice. Future controlled clinical investigation is needed to determine whether the benefits from the combined therapy with 5-FC and AmB outweigh the potential synergistic toxicity.

Summary. 5-Fluorocytosine and amphotericin B were demonstrated to be at least additive in the therapy of murine cryptococcosis. The mechanism for this effect is not known. However, AmB seemed to inhibit the *in vivo* development of 5-FC resistance among *C. neoformans* in our model. These results raise the possibility that combined-drug therapy might reduce AmB toxicity and/or the appearance of 5-FC resistant cryptococci without compromising therapeutic efficacy in cryptococcal disease.

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