Treatment of Murine Candidiasis and Cryptococcosis with Amphotericin B Incorporated into Egg Lecithin-Bile Salt Mixed Micelles

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Amphotericin B (AmB) with deoxycholate (Fungizone) and AmB incorporated into mixed micelles (AmB-mixMs) composed of egg lecithin with glycocholate, deoxycholate, or taurocholate were compared as treatments for murine infections. For mice infected with *Candida albicans*, treatment consisted of a single intravenous injection; for mice infected with *Cryptococcus neoformans*, treatment consisted of two intravenous injections. The maximal tolerated doses of AmB as Fungizone were 1.25 mg/kg of body weight in mice with candidiasis and 2.5 mg/kg of body weight in mice with cryptococcosis. The AmB-mixMs were nontoxic to mice at doses of 80 and 100 mg/kg of body weight and were therapeutically more active than the maximal tolerated dose of Fungizone in both models of infection. However, when Fungizone or AmB-mixMs were administered at equivalent doses of AmB, AmB-mixMs were more active in treating murine candidiasis, whereas Fungizone was more active in treating murine cryptococcosis.

Amphotericin B (AmB) formulated with deoxycholate and sodium phosphate is used clinically as Fungizone. It is highly effective in the treatment of systemic fungal infections, but its usefulness is limited by its toxicity to patients (10). The problem is further aggravated in immunocompromised patients, in whom opportunistic fungal infections caused by *Cryptococcus neoformans* and various species of *Candida* may be life-threatening (30).

In experimental studies in animals, the therapeutic index of AmB has been improved by incorporating it into several types of lipid-based formulations such as multilamellar lipid vesicles composed of saturated phospholipids (22) or small unilamellar vesicles composed of egg lecithin and cholesterol (1, 14) or by complexing AmB to open lipid structures composed of saturated phosphatidylcholine (9) or cholesteryl ester (18). The preparation and standardization of some of these formulations are associated with a range of technical and commercial problems (32) which may make long-term use difficult. While some of these formulations have undergone successful clinical trials in humans (2), treatment failures have been reported (24). Moreover, the safety of the long-term use of synthetic saturated phospholipids in the clinical setting has not been determined. While progress in the development of rational approaches to the development of delivery systems is impressive (19, 21, 22, 28), these efforts have been hampered because of the deficiency in our understanding of the molecular basis of the biological activities of formulations containing AmB. Therefore, a vehicle which retains optimal therapeutic properties and that can be used safely is still being sought.

In previous in vitro studies, we observed that mixed micelles prepared from unsaturated phosphatidylcholine (egg yolk lecithin) and bile salt (glycocholate) significantly decreased the toxicity of Fungizone for human erythrocytes and cultured L cell fibroblasts, while it retained potent activity against *Candida albicans* and *C. neoformans* (6). In the present study, we prepared formulations of AmB with egg lecithin and glyco-

cholate (Egam), deoxycholate (Edam), and taurocholate (Etam) that were nontoxic to mice up to doses as high as 100 mg/kg of body weight. Here we report a comparison of the therapeutic efficacies of these formulations and Fungizone in the treatment of murine models of candidiasis and cryptococcosis. In the accompanying report (5) we consider the molecular and cellular aspects of the therapeutic activities of these formulations.

(The results described here were presented in part at the 92nd General Meeting of the American Society for Microbiology, New Orleans, La., 26 to 30 May 1992 [7].)

MATERIALS AND METHODS

Antibiotics and chemicals. Fungizone (E. R. Squibb & Sons, Princeton, N.J.), a sterile lyophilized powder containing 50 mg of AmB, 41 mg of sodium deoxycholate, and 20.2 mg of sodium phosphate per vial, was obtained from the Barnes Hospital Pharmacy (St. Louis, Mo.). Egg yolk lecithin (100 mg/ml dissolved in chloroform), AmB without deoxycholate (80% pure, according to the manufacturer), and sodium salts of glycocholic, deoxycholic, and taurocholic acids were purchased from Sigma Chemical Co. (St. Louis, Mo.). All reagents and chemicals were used without further purification. All doses of AmB refer to those of crude AmB; the molecular weight ascribed to AmB is 924.

Preparation of AmB-mixMs. The formulations of AmB as AmB and mixed micelles (AmB-mixMs) were prepared by a modification of the procedure of Son and Allan (31) by incorporating the suggestions of New (27). Briefly, 38.7 mg of egg lecithin in 387 μ l of chloroform was added to a sterile screw-cap scintillation vial; this was followed by the addition of a bile salt. The amount of glycocholate added to the egg lecithin in chloroform was the same as that in the previous study (31) (20.5 mg). Deoxycholate was added in the amount of 7.75 mg. Deoxycholate is more toxic to mammalian cells (26) and more damaging to mammalian cell membranes (33) than glycocholate, and probably for this reason, in order to obtain nontoxic formulations of AmB, it had to be used in lower amounts. Taurocholate was added in the amount of 4.0 mg. At

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TABLE 1. Molar ratios of AmB to egg lecithin and proportion of AmB, lecithin, glycocholate, deoxycholate, or taurocholate in Egam, Edam, and Etam^a

Index	Molar ratio of AmB to egg lecithin	% (wt/wt)								
		Egam			Edam			Etam		
		AmB	Lecithin	Glycocholate	AmB	Lecithin	Deoxycholate	AmB	Lecithin	Taurocholate
2.5	0.009	0.7	65.0	34.0	0.8	82.7	16.5			
5.0	0.017	1.3	64.6	34.0	1.6	82.0	16.4			
10.0	0.034	2.6	63.8	33.7	3.2	80.6	16.1			
20.0	0.068	5.0	62.0	32.8	6.3	78.0	15.6	6.8	84.5	8.7
40.0	0.135	9.5	59.0	31.3	11.8	73.5	14.7	12.7	79.1	8.2
80.0	0.270	17.0	54.0	28.6	21.0	65.7	13.0	22.6	70.2	7.2
100.0	0.333	20.0	52.0	27.6	24.0	63.0	12.6	26.0	67.1	6.9

^a Molar ratios were lecithin:glycocholate, 1.2 (Egam); lecithin:deoxycholate, 2.5 (Edam); and lecithin:taurocholate, 7.1 (Etam).

lower ratios, taurocholate did not disperse in the egg lecithin-chloroform mixture. To the dispersion of egg lecithin-bile salts in chloroform was added AmB in doses ranging between 0 and 15 mg per vial, and the mixtures were shaken by hand. The chloroform was evaporated in a stream of nitrogen gas at room temperature and was then desiccated for 24 to 48 h over anhydrous calcium chloride under reduced pressure.

To hydrate the samples, 0.2-ml portions of sterile distilled water were added to the desiccated film and the mixtures were carefully vortexed. This was followed by the addition of 1.3 ml of 5% glucose and vortexing for an additional 2 min. Since the final product was not sterilized, aseptic conditions were used at all steps. Formulations containing glycocholate were termed Egam, those with deoxycholate were termed Edam, and those with taurocholate were termed Etam. The composition and drug/lipid ratios of the various AmB-mixMs are given in Table 1. The doses of all formulations were expressed in terms of AmB doses. The mice received the following doses of AmB (in milligrams per kilogram) in constant doses of egg lecithin and the bile salt that was assayed: 1.2, 2.5, 5.0, 10, 20, 40, 80, and 100. The effects of modulations of the AmB to egg lecithin ratio on the stability and biological activities of Egam and Edam are under investigation. All samples containing AmB were protected from light exposure during preparation, storage, and application.

Source and preparation of organisms for in vivo studies. *Candida albicans* B311 (ATCC 32354) and *Cryptococcus neoformans* 145A (ATCC 62070), both part of our stock culture collection, were maintained on Sabouraud dextrose agar. To prepare inocula for studies in mice, an overnight culture of yeast grown at 37°C in Sabouraud dextrose broth was harvested, washed three times in saline, and resuspended to a final density in saline of 2.5×10^6 cells per ml for *C. albicans* or 1×10^6 cells per ml for *C. neoformans*. Portions of the inocula were plated to determine the number of CFU. Cell viability for *C. albicans* was in the range 80 to 90%, and that for *C. neoformans* was between 75 and 85%.

Animals. Female CF1 mice (age, 7 to 8 weeks; weight, 20 to 25 g) were purchased from Sasco Farms (O'Fallon, Mo.) and were held in our animal facilities for at least 1 week prior to use. They were fed mouse chow (Purina Farms, St. Louis, Mo.) and were provided water ad libitum. Acute systemic fungal infections were established by intravenous injection of 0.2 ml of inoculum into the dorsal tail vein.

Therapeutic protocols. The therapeutic efficacies of the various treatments were measured as prolongation of survival over that of sham-treated controls or as a decrease in residual infections in surviving mice. For mice infected with *C. albicans*, treatment was begun 24 h after infection and consisted of a

single intravenous injection; for mice infected with *C. neoformans*, treatment consisted of two intravenous injections: the first was given 48 h after infection and the second was given 24 h later. The formulations of AmB as Fungizone, Egam, Edam, or Etam were prepared in 5% glucose solutions, and 0.2-ml portions were injected into the dorsal tail veins of infected mice. Controls were treated with glucose, were given empty micelles of the type and dose corresponding to those administered with AmB, or were given deoxycholate in the dose corresponding to the dose given with Fungizone. No differences in the effects of treatment on the number of survivors and the load of infections of the three groups of controls were observed.

Evaluation of therapeutic regimens. A census of surviving animals in the various treatment groups was taken every day for 31 to 35 days postinfection.

In separate experiments on the day when all control mice were dead but all AmB-mixM-treated mice were still alive, the experiments were terminated and all mice were necropsied. Organs were aseptically removed, and groups of three kidneys or three brains were homogenized in 3 ml of sterile saline by using a hand-held tissue homogenizer. Serial 10-fold dilutions were plated onto Sabouraud dextrose agar. After incubation at 37°C for 48 h, the number of CFU in the kidneys of mice infected with *C. albicans* and the brains of those infected with *C. neoformans* were determined and compared with the number of yeasts in comparable organs of Fungizone-treated survivors.

Statistical analysis. Survival curves were calculated by the Kaplan-Meier method, and tests for differences in survival distributions were based on a generalized Wilcoxon test; data for load of infection in organs of infected mice were compared by analysis of variance and nonparametric tests (12). Differences were considered significant if *P* was <0.05.

RESULTS

Evaluation of AmB-mixMs in treatment of murine candidiasis. Figure 1A illustrates the dose-response of AmB as Fungizone when it was used to treat mice infected with *C. albicans*. The rate of death of infected mice treated with 0.6 mg of AmB per kg was comparable to that of glucose-treated controls. Mice that received 1.2 mg of Fungizone per kg survived longer than controls. Administration of 2.5 mg of Fungizone per kg had variable effects; in some experiments there were immediate deaths and in others there was an increase in survival. All mice given 5.0 mg of Fungizone per kg died during the first hour after injection. On the basis of these results, we concluded that the maximal tolerated dose of AmB

FIG. 1. Survival of mice infected with *C. albicans* and treated with a single dose of glucose (■), Fungizone, or Egam (doses of Fungizone or Egam in milligrams of AmB per kilogram). (A) Fungizone at 0.6 (●), 1.2 (▲), 2.5 (◆), and 5.0 (□) mg/kg. (B) Fungizone at 1.2 (●), Egam at 1.2 (▲), Egam at 2.5 (◆), Egam at 5.0 (□), and Egam at 10 (○) mg/kg. Panel A shows composite results; data in panel B are from one representative experiment.

as Fungizone in our model for the treatment of murine candidiasis is 1.2 mg/kg.

The survival rates of infected mice treated with Egam at a dose of 1.25 mg/kg, which was equivalent to the maximal tolerated dose of Fungizone, and in twofold increasing increments to 10 mg/kg are shown in Fig. 1B. At the 1.25-mg/kg dose, Egam was more effective than Fungizone in prolonging the lives of infected mice. At a dose of 2.5 mg/kg, Egam increased the survival rate, and at 5 and 10 mg of Egam per kg, all infected mice survived to the end of the period of observation (35 days postinfection). During the entire study (six to seven independent experiments), Fungizone was consistently superior to glucose in prolonging the survival of infected mice ($P < 0.0001$). Egam at doses of 1.2, 2.5, 5, and 10 mg/kg was superior to Fungizone and glucose ($P < 0.0001$). We observed, however, three deaths in mice treated with Egam at 5 mg/kg and two deaths in mice treated with Egam at 10 mg/kg. In all experiments, all infected mice treated with 20 to 100 mg of Egam, Edam, or Etam per kg survived to the end of the period of observation (data not shown).

TABLE 2. Effects of Fungizone, Egam, or Edam on load of infection in kidneys of mice infected with *C. albicans*^{a,b,c}

Treatment	Dose (mg/kg)	Total no. of mice assayed	log ₁₀ colonies/kidney (mean ± SE)
Fungizone	1.2	17	6.32 ± 1.39
Egam	20.0	18	3.92 ± 0.98
Egam	80.0	15	3.66 ± 0.49
Fungizone	1.2	17	7.28 ± 1.45
Edam	20.0	29	3.96 ± 1.11
Edam	80.0	29	4.22 ± 0.71

^a Experiments were done on days 24 to 26 postinfection.

^b $P < 0.0001$ for Fungizone versus Egam at 20 mg/kg or Egam at 80 mg/kg. For Egam at 20 mg/kg versus Egam at 80 mg/kg, the difference was not significant.

^c $P < 0.0001$ for Fungizone versus Edam at 20 mg/kg or Edam at 80 mg/kg. For Edam at 20 mg/kg versus Edam at 80 mg/kg, the difference was not significant.

The therapeutic effects of Fungizone, Egam, and Edam were also compared by determining the number of CFU of yeasts per kidney of treated mice. At the time of necropsy, the load of infection in the kidneys of mice infected with *C. albicans* and treated with Egam or Edam at doses of 5 and 10 mg/kg did not differ significantly from that found in mice treated with Fungizone at the maximal tolerated dose (data not shown). Table 2 summarizes the results obtained for mice treated with Fungizone at the maximal tolerated dose with Egam or Edam at doses of 20 and 80 mg/kg. A significant decrease was seen in the load of infection in the kidneys of mice treated with either Egam or Edam at doses of 20 and 80 mg/kg in comparison with that found in mice treated with Fungizone. The higher doses (80 mg/kg) were no more effective than the lower doses (20 mg/kg). It is of interest that others have found that above a certain dosage, the efficacy of liposomal AmB (AmBisome) for treating murine candidiasis does not improve significantly (1). In summary, our data suggest that in our model of murine candidiasis, the optimal therapeutic dose of Egam is 20 mg/kg. At lower doses, sporadic deaths occurred, whereas there was no further improvement in the lowering of the load of infection at higher doses.

Evaluation of AmB-mixMs in treatment of murine cryptococcosis. Figure 2A illustrates the dose-response of AmB as Fungizone on the survival of mice infected with *C. neoformans*. Mice treated with 0.6 mg of Fungizone per kg had a death rate similar to that of control mice. Mice treated with 1.2 and 2.5 mg of Fungizone per kg showed an increasing survival rate that was dose dependent. An increase in the dose of AmB to 5.0 mg/kg resulted in immediate deaths of 20% of the treated animals, and the surviving mice died at a rate similar to those that received 2.5 mg of AmB per kg. Immediate deaths were observed in 60% of mice that received 7.5 mg of Fungizone per kg, with the remaining 40% surviving up to 22 days postinfection. On the basis of these results, a dose of 2.5 mg/kg was selected as the maximal tolerated dose of AmB as Fungizone.

Figure 2B and C illustrates the survival of infected mice treated with different doses of Egam and the maximally tolerated dose of Fungizone. Mice treated with 2.5 mg of Fungizone per kg did better than those treated with Egam or Edam at doses of 2.5, 5.0, 10, and 20 mg/kg. However, the survival of mice treated with Egam at doses of 40, 80, and 100 mg/kg was better than the survival of those treated with Fungizone ($P < 0.0001$).

Mice that were treated with Edam at doses of 5, 10, and 20 mg/kg did as well as those treated with comparable doses of Egam (data not shown). In contrast to Egam, a dose of 40 mg of Edam per kg was no better than treatment with Fungizone.

FIG. 2. Survival of mice infected with *C. neoformans* and treated with two doses of glucose (■), Fungizone, or Egam (doses of Fungizone or Egam in milligrams of AmB per kilogram). (A) Fungizone at 0.6 (●), 1.2 (▲), 2.5 (◆), 5.0 (□), and 7.5 (○) mg/kg. (B) Fungizone at 2.5 (●), Egam at 2.5 (▲), Egam at 5.0 (◆), and Egam at 10.0 (□) mg/kg. (C) Fungizone at 2.5 (●), Egam at 20.0 (▲), Egam at 40.0 (◆), Egam at 80.0 (○), and Egam at 100.0 (□) mg/kg. Each point is an average of three independent experiments.

However, treatment with Edam at 80 and 100 mg/kg was superior to all treatment regimens ($P < 0.0001$).

In two experiments, treatment with Etam at 80 and 100 mg/kg was superior to that with Fungizone in prolonging the lives of infected mice.

TABLE 3. Effects of Fungizone, Egam, or Edam on load of infection in brains of mice infected with *C. neoformans*^{a,b,c}

Treatment	Dose (mg/kg)	log ₁₀ colonies/brain (mean ± SE)
Fungizone	1.2	11.42 ± 0.59
Egam	20.0	12.03 ± 0.90
Edam	80.0	6.27 ± 2.39
Fungizone	1.2	10.50 ± 2.0
Edam	20.0	11.23 ± 1.55
Edam	80.0	3.17 ± 0.26

^a Experiments were done on day 15 postinfection; the total number of mice assayed in each group was nine.

^b $P < 0.0001$ for Egam at 80 mg/kg versus Fungizone or versus Egam at 20 mg/kg. For Fungizone versus Egam at 20 mg/kg, the difference was not significant.

^c $P < 0.0001$ for Edam at 80 mg/kg versus Fungizone and for Edam at 80 mg/kg versus Edam at 20 mg/kg. For Fungizone versus Edam at 20 mg/kg, the difference was not significant.

Table 3 summarizes the results obtained at the time of necropsy for mice treated with Fungizone compared with the results for those treated with two doses of Egam or Edam. The numbers of viable *C. neoformans* cells cultured from mice treated with Fungizone at a dose of 2.5 mg/kg and Egam or Edam at a dose of 20 mg/kg were not statistically different. However, there was a 3-log-unit or greater decrease in the number of viable yeasts in the brains of mice treated with Egam and Edam at doses of 80 mg/kg compared with those in the brains of mice treated with Fungizone at a dose of 2.5 mg/kg and Egam or Edam at doses of 20 mg/kg. In summary, in our model of murine cryptococcosis, the optimal therapeutic doses were the highest assayed doses of Egam and Edam.

DISCUSSION

We previously demonstrated that preformed egg lecithin-glycocholate mixed micelles potentially decrease the toxicity of AmB (Fungizone) to mammalian cells, while activity against fungal cells is retained (6). These observations prompted us to evaluate the effects of mixed micelles on the toxicity of Fungizone to mice. In our experiments we did not see any differences in the toxicity of Fungizone administered with or without mixed micelles (4a). Other investigators (3) were also unsuccessful in attempts to decrease the toxicity of AmB in uninfected mice by these micelles. We postulated that a possible reason for the discrepancies between the in vitro and in vivo effects was that AmB formed complexes with mixed micelles in vitro that resulted in decreased toxicity to mammalian cells. However, when these complexes were injected into blood, AmB dissociated readily from the other components of the complexes. Thus, the toxicities of AmB-mixMs in vivo were comparable to that of Fungizone, which dissociates rapidly and completely to AmB and deoxycholate when injected into blood (13).

By adding powdered AmB to egg lecithin and bile salt in chloroform, we obtained formulations of AmB with egg lecithin and glycocholate (Egam), deoxycholate (Edam), and taurocholate (Etam) that were nontoxic to mice up to doses of 100 mg/kg, the highest dose assayed. Egam, Edam, and Etam at doses of 80 and 100 mg/kg were more effective than Fungizone given to *C. albicans*- and *C. neoformans*-infected at maximal tolerated doses of 1.25 and 2.5 mg/kg, respectively, in prolonging life and reducing the number of yeasts in the kidneys of mice infected with *C. albicans* or the brains of mice infected with *C. neoformans*.

In cellular studies, we found that AmB dissociated from Egam and Edam only as monomeric species and that Egam and Edam were nontoxic to mammalian cells but retained antifungal activity (5). These findings are consistent with those of Bolard et al. (4) and Legrand et al. (23), who demonstrated that only self-associated species are toxic to mammalian cells, whereas both self-associated and monomeric species exhibit antifungal activities. Our cellular findings, in turn, could be associated with the attenuation of the toxicity of AmB-mixM to mice and the manifestation of therapeutic activity in fungal infections.

Improvement of antifungal activity by using lipid-based AmB formulations whose toxicities allow administration of higher drug doses than those achievable with Fungizone is a common feature of various delivery systems (9, 18, 20, 25). Thus, the wide range of therapeutic doses of AmB-mixMs contrasts favorably with the narrow range of therapeutic doses of Fungizone.

When Egam, Edam, and Fungizone were used at equivalent doses, Egam was more effective in treating murine candidiasis but had less effect in treating cryptococcosis. In models of candidiasis, comparable results for other delivery systems and Fungizone administered in equivalent doses have been reported (1, 20, 25), while others have found lipid-based formulations to be more effective (9, 12, 33).

Several reports (1, 15, 18) show the comparable therapeutic activities of lipid-based formulations and Fungizone when used at equivalent doses in murine models of cryptococcosis; however, the AmB-lipid complex was less effective than Fungizone (9). Thus, our results resemble those obtained with the AmB-lipid complex (9) in showing that at equivalent doses the same formulation may be therapeutically more potent than Fungizone in *C. albicans*-infected mice and less potent in *C. neoformans*-infected mice. It is noteworthy that when used at equivalent doses, AmB complexed to cholesteryl sulfate and Fungizone were similarly effective in treating murine cryptococcosis (18) but Fungizone was more effective in treating coccidioidomycosis (11).

We considered two possible reasons for the differences in the relative therapeutic activities of Fungizone and Egam in our two models of infections. It has been reported that incorporation of AmB into multilamellar liposomes (17, 29) or complexation to the monolauryl ester of sucrose (8) or to cholesteryl sulfate (16) affects the antifungal activity of AmB in vitro. Thus, it could be hypothesized that, in comparison with Fungizone, incorporation of AmB into mixed micelles increases the in vivo activity of AmB against *C. albicans* and decreases its activity against *C. neoformans*.

Although this hypothesis is not compatible with our findings in vitro, that is, that the decreases in damage to fungal cells by AmB-mixMs were comparable when *C. albicans* cells or *C. neoformans* cells were assayed (5), it cannot be completely rejected. It is possible that our assay in vitro was not predictive of the in vivo situation or, alternatively, that lower antifungal activity was not important in one model but was decisive in another.

The second possible reason for the differences in the relative therapeutic efficacies of Fungizone and Egam is linked to our finding that Egam is more stable than Fungizone in the presence of serum lipoprotein (5). It can be assumed that Egam stays in blood longer and is scavenged more effectively by cells of the reticuloendothelial system than the AmB which has dissociated from Fungizone. These differences may affect the pharmacokinetics and the distribution of AmB in the bloodstream in a way that is advantageous for the treatment of candidiasis but is disadvantageous for the treatment of cryp-

tococcosis. Whatever the reason, the finding that the relative therapeutic potencies of Fungizone and AmB-mixMs depend on the model of infection being treated deserves further attention.

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