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Amphotericin B Incorporated into Egg Lecithin-Bile Salt Mixed Micelles: Molecular and Cellular Aspects Relevant to Therapeutic Efficacy in Experimental Mycoses

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The cellular activities of amphotericin B (AmB) used as Fungizone were compared with those of AmB complexed to either egg lecithin and glycocholate (Egam) or egg lecithin and deoxycholate (Edam). Under conditions in which leakage of K^+ from erythrocytes and cultured L cells treated with Fungizone was almost complete, Egam and Edam containing concentrations of AmB severalfold greater than the concentration of AmB in Fungizone had no effect but retained the ability to decrease the level of retention of K^+ in fungal cells. Analysis by absorption and circular dichroism spectroscopy demonstrated that when these formulations containing AmB at concentrations of less than 10^{-5} M were added to buffer, the AmB dissociated slowly as monomers from Egam or Edam and dissociated rapidly as a mixture of monomers and self-associated species from Fungizone. We propose that in Egam and Edam, the absence of free AmB in the self-associated form reduces the toxicity of AmB to mammalian cells, whereas the presence of monomeric AmB results in the retention of the antifungal activities of these complexes.

Amphotericin B (AmB) formulated with deoxycholate and sodium phosphate as Fungizone is highly effective in the treatment of systemic fungal infections, but its usefulness is limited by toxicity to patients (13, 31). The toxicity of AmB can be reduced by incorporating it into liposomes (29, 30) or by complexing it to various lipids (1, 14, 32), and in these less toxic formulations, AmB can be given in higher, more effective doses. These formulations have been successful in treating systemic fungal infections in clinical trials (2), but despite current efforts to rationalize the research on AmB delivery systems (17, 25, 26), the molecular and cellular bases of the differences in the therapeutic efficacies of these lipid-based formulations and that of Fungizone are not completely elucidated.

Recently, the therapeutic efficacies of novel formulations of AmB complexed to either egg lecithin and glycocholate (Egam) or to egg lecithin and deoxycholate (Edam) were compared with that of AmB as Fungizone in murine models of candidiasis and cryptococcosis (11). These formulations were nontoxic at doses 80-fold higher than the maximal tolerated doses in mice infected with *Candida albicans* and 40-fold higher than the maximal tolerated doses in mice infected with *Cryptococcus neoformans*. At these doses, Egam and Edam were therapeutically more effective in both models of infection than Fungizone given at maximal tolerated doses.

The present study was designed to gain insights into the cellular and molecular bases of the different effects of AmB as Fungizone or complexed with egg lecithin and bile salt. To this end, we characterized the physical states of AmB dispersed in buffer as Fungizone, Egam, or Edam. AmB has characteristic absorption and circular dichroism (CD) spectra (5, 12, 15), and from spectral analyses of the various formulations, we were able to obtain information about the time and concentration dependencies of AmB dissociation from the complexes and about the types of AmB species that evolve in buffer.

We also compared Fungizone, Egam, and Edam for (i) stability of complexes in the presence of lipoproteins, (ii) stability of AmB antifungal activity during illumination, (iii) selectivity in damaging action to mammalian and fungal cells, and (iv) stimulatory and toxic action on the growth of cultured mouse fibroblast L cells.

We found that the difference in the physical state of AmB in aqueous solutions of Egam or Edam in comparison with that of AmB as Fungizone may explain the differences in cellular activity seen in vitro. The decreased toxicity to mice and the improved therapeutic indices of Egam and Edam compared with that of Fungizone described in the companion report (11) may in turn be associated with these in vitro findings.

(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

Antibiotic 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.). Stock solutions of Fungizone were prepared daily in water. 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.). Stock solutions of AmB were prepared daily in dimethyl sulfoxide (Me_2SO). 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. Protein was measured by using the bicinchoninic acid protein reagent from Pierce (Rockford, Ill.) according to the protocol of the manufacturer.

Lipoprotein fractions were separated from human serum by

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ultracentrifugation at 15°C and then dialysis at 4°C against phosphate-buffered saline (PBS; pH 7.0).

Source and preparation of organisms for in vitro studies. *C. albicans* B311 (ATCC 32354) and *C. neoformans* 145A (ATCC 62070), both part of our stock culture collection, were maintained on Sabouraud dextrose agar and were transferred monthly to fresh medium.

Preparation of Egam and Edam. Egam and Edam were prepared as described in the companion report (11). Briefly, 387 μl of chloroform containing 38.7 mg (0.05 mmol) of egg lecithin and then either glycocholate or deoxycholate were added to sterile scintillation vials. AmB was added to the egg lecithin-bile salt dispersions in amounts of 0, 3.1, 12.5, or 15.0 mg, and the mixtures were shaken by hand. The chloroform in the egg lecithin-bile salt-AmB mixture was removed by placing the vial in a stream of nitrogen gas at room temperature. Each sample was further dried by placing the vials in a vacuum desiccator for 24 to 48 h. The dried films were hydrated by the addition of 0.2 ml of water and were subjected to careful vortexing. This was followed by the addition of 0.4 ml of water and vortexing for another 2 min.

The following preparations were used in the studies: AmB-egg lecithin-glycocholate at a molar ratio of 1:14.8:12.5 (Egam 20) or 1:3.7:3.1 (Egam 80) and AmB-egg lecithin-deoxycholate at a molar ratio of 1:14.8:5.4 (Edam 20) or 1:3.7:1.3 (Edam 80). As measured with a nanosizer, we obtained Egam and Edam as polydispersed particles whose sizes evolve with time. These observations resemble the findings of other investigators (23, 24) who measured the levels of dispersity of aggregates formed by AmB and deoxycholate.

The concentrations of all formulations used are expressed in terms of the concentrations of AmB. To facilitate comparison with previous studies, concentrations of AmB in the cellular experiments are designated in micrograms of AmB per milliliter, whereas in the spectrophotometric experiments, concentrations are given as molar AmB, with the initial concentrations calculated from spectral analysis.

Spectroscopic measurements. Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer, and CD spectra were recorded with a Jobin-Yvon Mark V dichrograph. Both apparatuses were thermostated at 37°C. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2 mol^{-1}).

Stability of complexes in the presence of serum lipoproteins. Egam or Edam were dissolved in PBS, added to lipoproteins dispersed in the same buffer (final concentrations in lipoproteins, 1.2 mg/ml), and incubated for 1 h at 37°C. The CD spectra were then compared with those of Egam and Edam incubated in buffer for the same amount of time.

Stability of antifungal action during illumination. Aliquots containing the assayed formulations of AmB were dissolved in Sabouraud liquid medium to a final AmB concentration of 0.25 μg/ml and were incubated in the dark or were illuminated with a fluorescent lamp (F15T8-CW 15-W cool white; General Electric Co., Schenectady, N.Y.) at a distance of 10 cm. After 3 h, *C. albicans* cells dispersed in Sabouraud medium were added to a final concentration of 2×10^7 cells per ml. After 2 h of incubation at 37°C, the cells were separated by sedimentation and the amount of cell-associated K^+ was determined in a flame photometer as described previously (8).

Effect of AmB on retention of K^+ by fungal cells and erythrocytes. Preparation of fungal cells and assays were done as described previously (8). Cultures of *C. albicans* B311 and *C. neoformans* grown on Sabouraud dextrose agar slants were taken from our stock collection and were inoculated into flasks of freshly prepared Sabouraud dextrose broth; the flasks were incubated at 37°C for 18 to 24 h with constant shaking. The cells were harvested by sedimentation, counted in a hemacytometer, resuspended in Sabouraud medium, and adjusted to a density of 2×10^7 cells per ml.

To prepare erythrocytes, blood was taken from healthy volunteers and placed in tubes containing 1 mg of EDTA or 250 U of heparin per ml, mixed, and centrifuged at $800 \times g$ for 10 min, and the plasma and buffy coat were removed. The erythrocytes were washed with PBS. The final suspension in PBS was counted in a hemacytometer and was adjusted with PBS to a density of 10^8 cells per ml.

The AmB in the assayed formulations was added to cell dispersions, and samples were incubated in 37°C for 1 h for fungal cells and 0.5 h for erythrocytes. Cells were harvested by sedimentation and were lysed with lithium diluent. Concentrations of cell-associated K^+ were measured in a flame photometer.

Effects of AmB on retention of K^+ and growth of mouse L-929 fibroblast cells. L-929 cells were maintained in monolayers in growth medium (Eagle's minimal essential α-medium with 10% fetal calf serum) in a humidified atmosphere of 5% CO_2–95% air at 37°C. To measure the effects of AmB on the retention of K^+, cells were grown to 60 to 70% confluency and then overlayed with minimal essential α-medium without serum; Fungizone, Egam, or Edam was added and samples were incubated for 1 h. At the end of the incubation period, the medium was removed by aspiration and the cells were rinsed with 0.1 M $MgCl_2$. Cell-associated K^+ was measured in a flame photometer as described previously (9).

To measure the effects of AmB on cell growth, L-929 cells were seeded at concentrations of 2×10^4 cells per ml of medium with serum. After 3 h of growth, Fungizone or Edam was added. On each of the following 4 days, medium was aspirated from the cell monolayers and fresh medium containing the various formulations of AmB was added to the cells. On the fourth day, the medium was removed, cells were rinsed with 0.1 M $MgCl_2$, and the protein content per dish was measured (9).

RESULTS

Dissociation of AmB from Fungizone, Egam, and Edam. AmB was dissolved in a small amount of organic solvent (*N,N*-dimethyl formamide [Me_2F] or Me_2SO) and was added to buffer. The CD and absorption spectra of these species differed greatly. A strong CD at about 350 nm (with dichroic doublet) and weak absorption at about 420 nm are characteristic for self-associated AmB, whereas a weak CD at about 320 nm and strong absorption at 409 nm are characteristic for monomeric AmB (4, 27). Therefore, by analyzing the absorption and CD spectra, we could follow the evolution of both species from AmB complexes.

Absorption spectra. When AmB was added to buffer as an aqueous solution of Fungizone at a final concentration greater than 10^{-6} M, the spectra measured after 5 min were slightly different from those obtained by the addition of AmB, which was dissolved in organic solvent, to buffer. This finding indicated an interaction of AmB with deoxycholate. However, when Fungizone was added at AmB concentrations of less than 10^{-6} M, no spectral differences were observed. Thus, under these conditions, the AmB-deoxycholate interaction did not occur.

To observe the effects of concentration and time on the dissociation of AmB from AmB-mixed micelles Egam 20, Egam 80, Edam 20, and Edam 80 were dispersed in PBS at 37°C at concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M. The

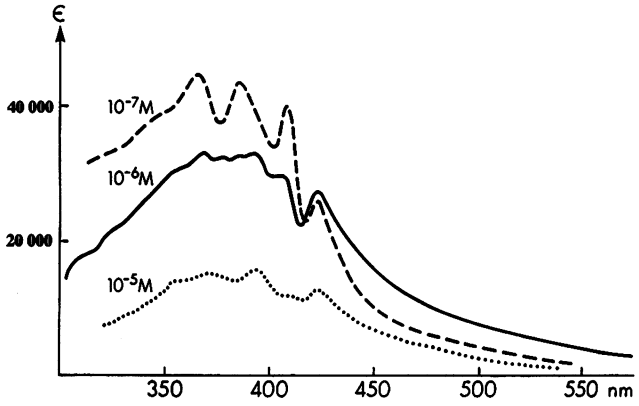

FIG. 1. Absorption spectra of Egam 20 dispersed in PBS at different dilutions. Spectra were recorded at 37°C immediately after dispersion (time zero). AmB concentrations are given above each curve.

spectra were recorded at time zero and at 10, 30, and 60 min after dispersion.

At the highest concentration, 10^{-5} M, the absorption spectrum was the superposition of a scattering background and a wide band presenting maxima at 420, 392, 370, and 358 nm (Fig. 1). The extinction coefficient was about 10,000. For decreasing concentrations (10^{-6} and 10^{-7} M), the scattering background decreased (although it increased in comparison with those of the bands at about 400 nm) and new bands appeared at 408, 385, 365, and 347 nm. After 10 min of centrifugation at 6,000 × *g*, only these bands remained, while the wide band with smaller maxima at 420, 392, 370, and 358 nm disappeared. When time dependence was considered, it appeared that the first set of bands was progressively superseded by the second set (Fig. 2).

The absorption bands observed at high concentrations and short incubation times can be assigned to AmB incorporated in the lipidic medium. The bands that appeared at lower concentrations and/or longer incubation times corresponded to free monomeric AmB in aqueous medium (5). Their extinction coefficient, ε, was high (about 120,000 at 406 nm). Consequently, low amounts of monomeric free AmB could easily be detected in the presence of bound AmB (in the lipidic medium), the ε of which was 10 times smaller.

From the absorption spectra of Egam or Edam dispersed in buffer, we calculated the concentrations of free monomeric AmB evolving after 1 h at 37°C, either directly from the mixture or from the supernatant obtained after 10 min of centrifugation at 6,000 × *g*. At an AmB concentration of 1 × 10^{-7} M these concentrations were 5 × 10^{-8} M (Egam 80), 4 × 10^{-8} M (Egam 20), 2.5 × 10^{-8} M (Edam 80), and 1.5 × 10^{-8} M (Edam 20). For higher total concentrations, the concentrations of soluble AmB increased and reached, for instance, 7 × 10^{-7} and 1.75 × 10^{-5} M for Egam 20 at total AmB concentrations of 0.1 × 10^{-3} and 1 × 10^{-3} M, respectively. The stabilities of the last three preparations are therefore comparable to that of AmB bound to small unilamellar vesicles from dipalmitoylphosphatidylcholine (19, 20), which is strong.

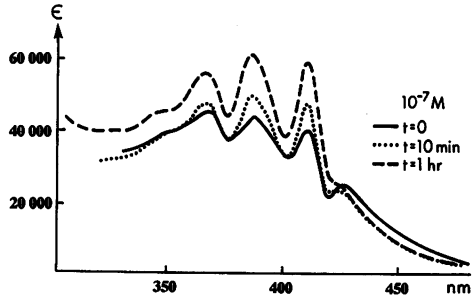

FIG. 2. Time (*t*) dependence of the absorption spectra of Egam 20 dispersed in PBS at 37°C (AmB concentration, 10^{-7} M).

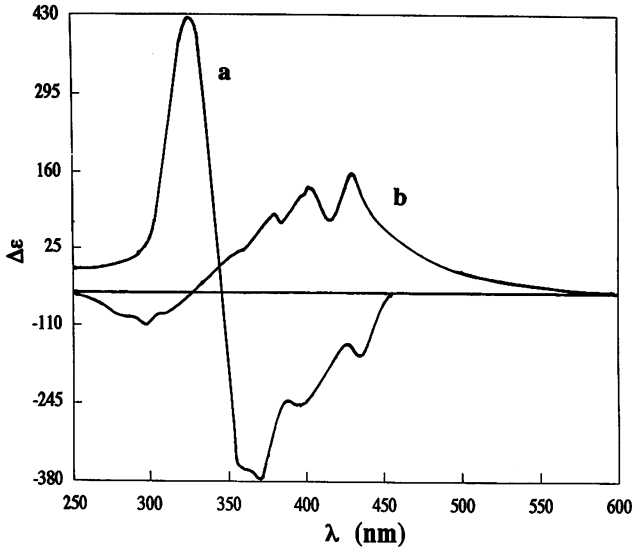

FIG. 3. CD spectra of 10^{-5} M AmB in PBS (curve a) or as Edam 20 (curve b).

CD spectra. The shapes of the CD spectra of AmB added to buffer as solution in Me_2F or Me_2SO or as an aqueous solution of Fungizone to a final concentration 10^{-6} M were similar, with an intensive doublet centered at about 340 and 330 nm, respectively. We (5) and others (12) attributed this doublet to self-associated AmB. It is noteworthy that no trace of free self-associated AmB in water, which would be detected by a dichroic doublet at 340 nm, was observed when Egam or Edam was added to buffer, even at concentrations of soluble fractions higher than the threshold of self-association of AmB (that is, approximately 2 × 10^{-7} M).

In an aqueous dispersion of free monomeric AmB of less than 10^{-7} M, the CD spectrum was weak (Δε, about 10 at 409 nm) and negligible in comparison with that of self-associated AmB (Δε, above 600).

The CD spectra of Egam and Edam added to buffer consisted of three intensely positive bands at 430, 390, and 370 nm (Δε, about 150) (Fig. 3). Their intensities were weakly dependent on both the concentration, which decreased with dilution, and the incubation time. After 1 h of incubation, they were approximately 10% weaker.

From these data we attributed the increase in intensity of the absorption spectra at 408, 385, 365, and 347 nm from Egam and Edam incubated at a low concentration for 1 h in buffer to an increased presence of free monomeric AmB.

Therefore, the finding that the CD spectra of Egam and Edam progressively decreased after dispersion in PBS, considered together with the concomitant increase in the absorption band at 409 nm, indicated that upon dilution and/or incubation, AmB is progressively released from these formulations as a free monomeric species.

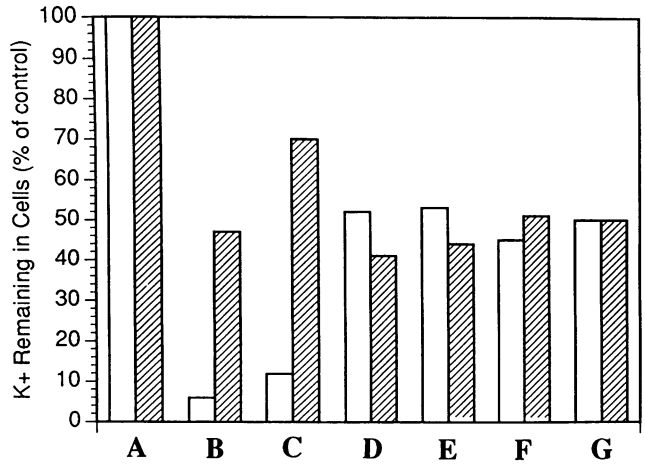

FIG. 4. Effect of light on retention of antifungal activity by different formulations of AmB used at an initial concentration of 0.25 μg/ml. Each set of bar graphs is as follows: A, control; B, AmB; C, Fungizone; D, Egam 80; E, Edam 80; F, Egam 20; G, Edam 20. Open bars, formulation kept in the dark; shaded bars, formulation exposed to light. Data are from one representative experiment done in duplicate. Similar results were obtained in two other experiments.

Stability of the complexes in the presence of lipoproteins. Previously, we studied the binding of AmB to serum lipoproteins by measuring the effects of lipoproteins on the CD spectra of AmB added as a solution in organic solvent to buffer alone or supplemented with liposomes (6, 21). In both situations, the presence of lipoproteins induced drastic changes in the CD spectra of AmB, in particular, the disappearance of the dichroic doublet of the antibiotic, which is characteristic of its self-associated species. This disappearance was interpreted as an indication of AmB binding to lipoproteins. In the present study, after incubation of Egam 80 or Edam 80 in buffer with serum lipoproteins, almost no changes in the CD spectra could be detected. We interpreted this observation to be an indication that there is no transfer of AmB from complexes to lipoproteins. Thus, the stabilities of Egam and Edam in the presence of lipoproteins are greater than the stabilities of liposomal formulations assayed before.

Although AmB can bind to various proteins, it is known that it binds preferentially to lipoproteins in serum (6, 34). Therefore, the stabilities of Egam and Edam in the presence of serum lipoproteins suggest that they would also be stable in blood.

Stability of antifungal activity during illumination. AmB decomposes during storage, with a resultant decrease in its anticellular activity. In earlier studies, we and others have shown that the decomposition of AmB is due to auto-oxidation and can be accelerated by exposing AmB to fluorescent light (10). We tested how incorporation of AmB into egg lecithin-bile salt micelles affects the stability of AmB's antifungal activity. We compared the decrease in retention of K^+ by *C. albicans* yeast cells treated with aliquots of AmB that were either exposed to illumination over a period of 3 h or protected from light for the same period of time.

Figure 4 shows that *C. albicans* cells treated with samples of AmB dissolved in Me_2SO or with Fungizone and maintained in the dark retained no more than 10% of their intracellular K^+, in comparison with about 40% cell-associated K^+ in cells that had been treated with preparations that were illuminated. In contrast, the effects of AmB used either as Egam or Edam were not decreased by illumination. Thus, AmB in Egam and Edam retained antifungal activity under conditions detrimental for AmB added in organic solvent or as Fungizone.

Effect of AmB on retention of K^+ by mammalian and fungal cells. One of the first damaging effects of AmB on cells is a decrease in the cell's ability to retain K^+ against a concentration gradient (18). We compared the decrease in the retention of K^+ by mammalian and fungal cells induced by AmB preparations added to samples as a dispersion in Me_2SO or aqueous solutions of Egam 80, Egam 20, Edam 80, or Edam 20. Figure 5 summarizes the effects on the retention of K^+ by *C. albicans*, *C. neoformans*, erythrocytes, and L cells. The concentrations required to reduce K^+ retention by 50% of control values were comparable in fungal and mammalian cells for AmB added in Me_2SO or as Fungizone. About 30-fold higher concentrations of Egam 80, Egam 20, and Edam 80 than of Fungizone were required to induce comparable decreases in K^+ retention in fungal cells. Edam 20 was slightly more potent than Egam 80, Egam 20, and Edam 80 in inducing K^+ leakage from fungal cells. The difference was greater at higher concentrations than at lower concentrations. Egam 80, Egam 20, and Edam 80 did not have any effect on mammalian cells, but Edam 20 retained some activity. The concentration of Edam 20 required to induce a 50% decrease in the level of K^+ in cells was about 100-fold greater than the concentrations of Fungizone required to induce comparable toxicity. Thus, the differences in damage to fungal and mammalian cells were greater for Egam and Edam than for Fungizone. No effect on K^+ retention by any cell type was seen after exposure of cells to deoxycholate or egg lecithin-bile salt mixed micelles without AmB at concentrations corresponding to those introduced with Fungizone or Egam and Edam, respectively.

None of the formulations assayed had any hemolytic effects on erythrocytes. The absence of hemolysis in erythrocytes exposed to AmB-lipid complexes was observed previously for AmB complexed to cholesteryl ester (14) or open lipid structures (32).

Effects of Egam and Edam on growth of L-929 cells. Fungizone increases the uptake of metabolic precursors by L-929 cells, but its stimulatory action is limited by its toxicity to cells (9). To test the relationship between the toxic and stimulatory effects of Egam and Edam, we compared their effects on the growth (protein content per dish) of L-929 cells. Figure 6 shows that under conditions when Fungizone at concentrations of about 4 μg/ml induced a decrease in protein content to 50% of the value found in control cells, Egam 80 was nontoxic up to the highest concentration assayed (25.0 μg/ml), and in the range of concentrations assayed (1.5 to 25 μg/ml), Egam 80 caused an increase in the protein content per dish. Similar results were obtained in cells treated with Edam 80 (data not shown). Thus, nontoxic AmB-mixed micelles stimulated the growth of L-929 cells at AmB concentrations toxic for cells treated with Fungizone.

DISCUSSION

In our murine models of candidiasis and cryptococcosis, the two novel formulations of AmB, Egam and Edam, were less toxic to mice and could be used at higher, more therapeutically effective doses than Fungizone (11). In the present study, we measured the effects of Egam and Edam on cells and characterized the dissociation of AmB from these complexes. We consider the molecular basis of the cellular findings reported here to be pertinent to our in vivo results.

The first question that we asked was why are Egam and

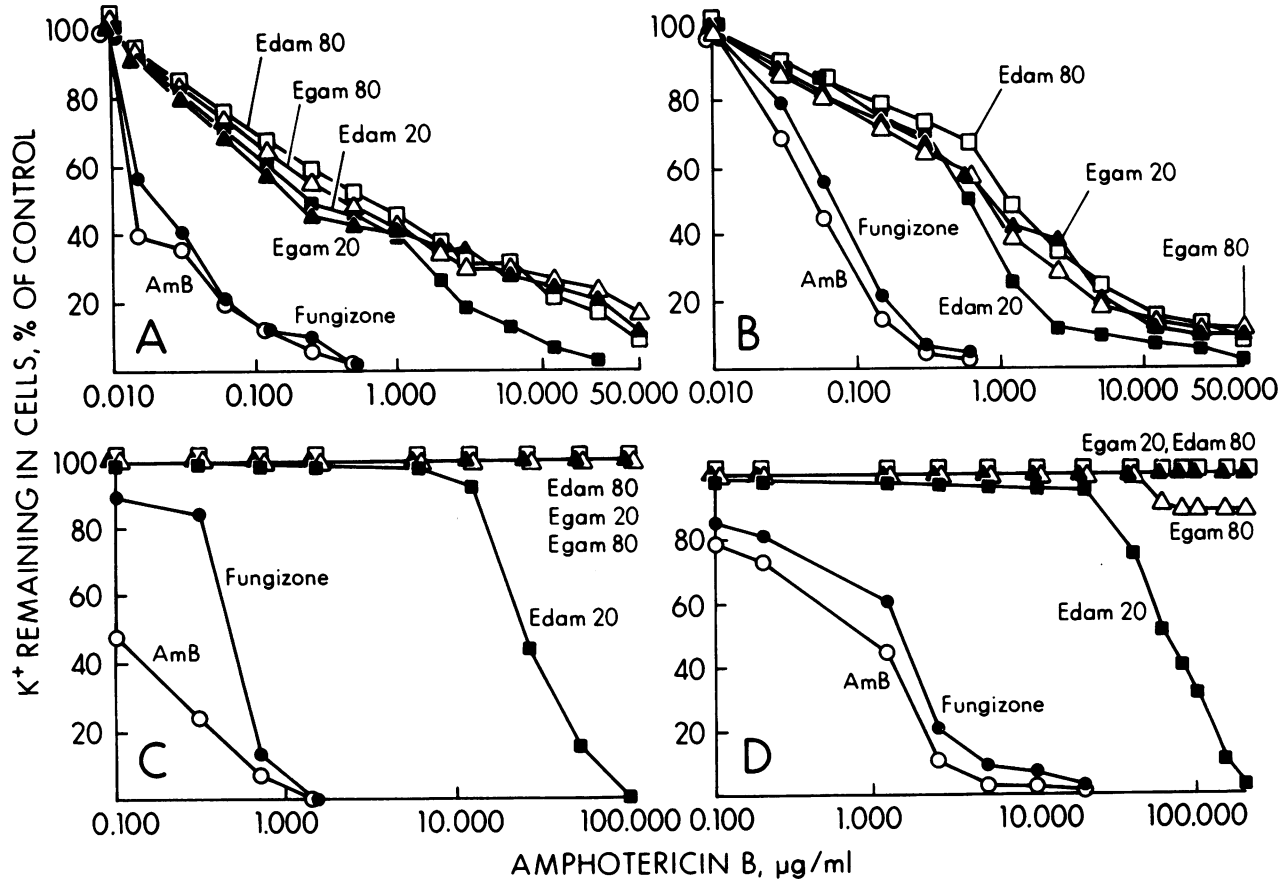

FIG. 5. Retention of K^+ by cells exposed to AmB added as a dispersion in Me_2SO or as a dispersion of Fungizone (AmB plus deoxycholate), Egam, or Edam in water. *C. albicans* B311, *C. neoformans* 145A, erythrocytes, and L-929 cells were assayed. Data are from one representative experiment done in duplicate. Similar results were obtained in two other experiments.

Edam nontoxic to mice? Our cellular experiments showed the absence, or at least a tremendous reduction, of toxicity of Egam or Edam compared with that of Fungizone to mammalian cells. Our spectrophotometric studies demonstrated the absence of self-associated species of AmB in dispersions of Egam and Edam in buffer at all concentrations assayed.

AmB damages mammalian cells by interacting with their cholesterol-containing membranes (17, 18). This interaction depends on (i) the presence of free AmB unbound to carrier, because bound AmB is poorly toxic to the cells, and (ii) the aggregation state of this unbound AmB. AmB in aqueous dispersion forms various species, and it has been proposed that only self-associated, water-soluble species are able to induce leakage of K^+ through cholesterol-containing membranes, which is one of the first manifestations of cell damage by AmB (4, 27).

Thus, we attributed the absence of toxicity to mice to the absence of toxic species of AmB in the dissociation products of Egam and Edam. This conclusion is in agreement with those of others (3), who observed in uninfected mice decreased toxicity of AmB deaggregated by detergents.

Another mechanism for the reduction of AmB toxicity for mammalian cells can be considered. It has been shown that injection of AmB bound to lipoproteins is much more toxic than Fungizone for rabbits (22). Furthermore, cellular studies have shown that AmB does not act solely at the membrane level but also acts by endocytosis (27a); the receptor to lipoproteins appears to be more important in this mechanism. Moreover, it has been demonstrated that AmB binds to lipoproteins (6, 34) and probably enters human fibroblast cells through a low-density lipoprotein receptor pathway (28). Hence, it may be assumed that the very weak binding of Egam or Edam to lipoproteins in comparison with that of Fungizone may contribute to their lower levels of toxicity.

The second question was why are Egam and Edam therapeutically active? Previous studies indicated that nonbound (free) AmB, both as the monomeric and as the self-associated species, damages ergosterol-containing membranes and has antifungal activity (4). Direct antifungal activity is generally considered to be the main mechanism of its therapeutic action (13). Therefore, it can be concluded that the presence of free, monomeric AmB in dispersions of Egam and Edam in buffer explains the levels of retention of antifungal activity by these formulations. The activities of Egam and Edam were lower than that of Fungizone, probably because the concentration of free AmB was lower in the dispersions of Egam and Edam than in the dispersion of Fungizone.

In addition to its anticellular action, AmB is known to stimulate various cells (33), and its stimulatory effects are restricted by its toxicity (9). Although the mechanism of cell stimulation by AmB is not clear, the observed level of stimulation of thymocytes by AmB derivatives which self-associate less than AmB (16) suggests that monomeric AmB may also have stimulatory action. In agreement with this assumption, we

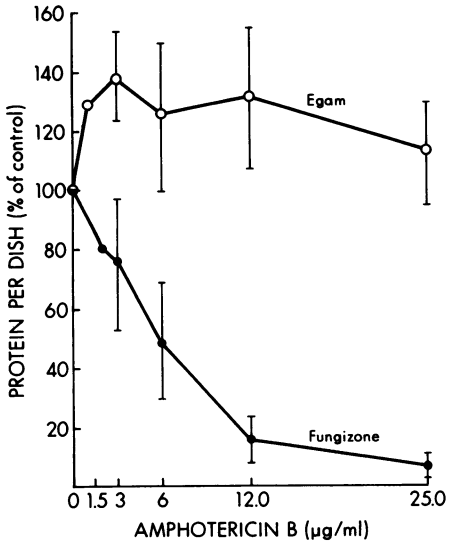

FIG. 6. Growth of L cells (protein content per well) of L-929 cells treated with AmB added as Fungizone (●) or Egam 80 (○). Data are means ± standard errors from four independent experiments.

observed that Egam and Edam stimulated the growth of L cells without being toxic.

ACKNOWLEDGMENTS

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REFERENCES

1. **Andriole, V. T., and T. F. Paterson.** 1989. The role of liposomal amphotericin B in the treatment of systemic fungal infections. Eur. J. Clin. Oncol. **25**(Suppl. 2):S63–S68.
2. **Bangham, H. C.** 1992. Liposomes: realizing their promise. Hosp. Pract. **27**:51–62.
3. **Barwicz, J. S., S. Christian, and I. Gruda.** 1992. Effects of the aggregation state of amphotericin B on/its toxicity to mice. Antimicrob. Agents Chemother. **36**:2310–2315.
4. **Bolard, J., P. Legrand, F. Heitz, and B. Cybulska.** 1991. One sided action of amphotericin B on cholesterol containing membranes is determined by its self association in the medium. Biochemistry **30**:5707–5715.
5. **Bolard, J., M. Seigneuret, and G. Boudet.** 1980. Interaction between phospholipid bilayer membranes and the polyene antibiotic amphotericin B. Biochim. Biophys. Acta **599**:280–293.
6. **Brajtburg, J., S. Elberg, J. Bolard, G. S. Kobayashi, R. A. Levy, R. E. Ostlund, Jr., D. Schlessinger, and G. Medoff.** 1984. Interaction of plasma proteins and lipoproteins with amphotericin B. J. Infect. Dis. **149**:986–997.
7. **Brajtburg, J., S. Elberg, G. S. Kobayashi, and J. Bolard.** 1992. Characterization and cellular effects of amphotericin B (AmB) formulated with egg lecithin-bile salt mixed micelles, abstr. A-42, p. 8. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
8. **Brajtburg, J., S. Elberg, G. S. Kobayashi, and J. Medoff.** 1990. Inhibition of amphotericin B (Fungizone) toxicity to cells by egg lecithin-glycocholic acid mixed micelles. Antimicrob. Agents Chemother. **34**:2415–2416.
9. **Brajtburg, J., S. Elberg, J. Medoff, G. S. Kobayashi, D. Schlessinger, and J. Medoff.** 1984. Stimulatory, permeabilizing, and toxic effects of amphotericin B on L cells. Antimicrob. Agents Chemother. **26**:892–897.
10. **Brajtburg, J., S. Elberg, D. R. Schwartz, A. Vertut-Croquin, D. Schlessinger, G. S. Kobayashi, and G. Medoff.** 1985. Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. Antimicrob. Agents Chemother. **27**:172–176.
11. **Brajtburg, J., S. Elberg, S. J. Travis, and G. S. Kobayashi.** 1993. Treatment of murine candidiasis and cryptococcosis with amphotericin B incorporated into egg lecithin-bile salt mixed micelles. **38**:294–299.
12. **Ernst, C., J. Grange, H. Rinhert, G. Dupont, and J. Lematre.** 1981. Structures of amphotericin B aggregates as revealed by UV and CD spectroscopies. Biopolymers **20**:1575–1578.
13. **Gallis, H. A., R. H. Drew, and W. W. Pickard.** 1990. Amphotericin B: 30 years of clinical experience. Rev. Infect. Dis. **12**:308–329.
14. **Guo, L. S. S., R. Fielding, D. D. Lasic, R. H. Hamilton, and D. Mufson.** 1991. Novel antifungal drug delivery: stable amphotericin B-cholesteryl sulfate discs. Int. J. Pharm. **75**:45–54.
15. **Henger, R. P., T. Kaplan, and J. Gray.** 1983. Structure of amphotericin B aggregates based on calculations of optical spectra. Biopolymers **22**:911–918.
16. **Henry-Toulme, N., M. Seman, and J. Bolard.** 1989. Interaction of amphotericin B and its *N*-fructosyl derivative with murine thymocytes: a comparative study using fluorescent membrane probe. Biochim. Biophys. Acta **982**:245–252.
17. **Janoff, A.** 1992. Lipids, liposomes, and rational drug design. Lab. Invest. **66**:655–658.
18. **Joly, V., J. Bolard, and P. Yeni.** 1992. In vitro models for studying toxicity of antifungal agent. Antimicrob. Agents Chemother. **36**: 1799–1804.
19. **Jullien, S., J. Brajtburg, and J. Bolard.** 1990. Affinity of amphotericin B for phosphatidylcholine vesicles as a determinant of the in vitro cellular activity of liposomal preparations. Biochim. Biophys. Acta **1201**:39–45.
20. **Jullien, S., A. Contrepois, J. E. Sligh, Y. Domart, P. Yeni, J. Brajtburg, G. Medoff, and J. Bolard.** 1989. Study of the effect of liposomal amphotericin B on *Candida albicans*, *Cryptococcus neoformans*, and erythrocytes by using small unilamellar vesicles prepared from saturated phospholipids. Antimicrob. Agents Chemother. **33**:345–349.
21. **Jullien, S., A. Vertut-Croquin, J. Brajtburg, and J. Bolard.** 1988. Circular dichroism for the determination of amphotericin B binding to liposomes. Anal. Biochem. **172**:197–202.
22. **Koldin, M. H., G. S. Kobayashi, J. Brajtburg, and G. Medoff.** 1985. Effects of elevation of serum cholesterol and administration of amphotericin B complexed to lipoproteins on amphotericin B-induced toxicity in rabbits. Antimicrob. Agents Chemother. **28**: 144–145.
23. **Lamy-Freund, M. T., V. F. N. Ferreira, and S. Schreier.** 1989. Polydispersity of aggregates formed by the polyene antibiotic amphotericin B and deoxycholate. A spin label study. Biochim. Biophys. Acta **981**:207–212.
24. **Lamy-Freund, M. T., S. Schreier, R. M. Peitzch, and W. F. Reed.** 1991. Characterization and time dependence of amphotericin B: deoxycholate aggregation by quasielastic light scattering. J. Pharm. Sci. **80**:262–266.
25. **Lasic, P. D.** 1992. Mixed micelles in drug delivery. Nature (London) **355**:279–280.
26. **Lasic, P. D.** 1992. Liposomes. Am. Sci. **80**:20–30.
27. **Legrand, P., E. A. Romero, B. E. Cohen, and J. Bolard.** 1992. Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes. Antimicrob. Agents Chemother. **36**: 2518–2522.

27a. **Legrand, P., A. Vertut-Doi, and J. Bolard.** Submitted for publication.

28. **Levy, R. A., R. E. Ostlund, Jr., and J. Brajtburg.** 1985. The effects of amphotericin B on lipid metabolism in cultures of human skin fibroblasts. In Vitro Cell Dev. Biol. **21**:26–31.
29. **Lopez-Berestein, G.** 1988. Liposomes as carriers of antifungal drugs. Ann. N.Y. Acad. Sci. **544**:590–597.
30. **Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh, and R. Juliano.** 1983. Treatment and prophylaxis of disseminated *Candida albicans* infection in mice with liposome encapsulated amphotericin B. J. Infect. Dis. **147**:939–945.

31. **Lyman, C. L., and T. Y. Walsh.** 1992. Systemically administered antifungal agents: a review of their clinical pharmacology and therapeutic applications. Drugs **44:**9–35.
32. **Perkins, W. R., S. R. Minchey, L. T. Boni, C. E. Swenson, M. C. Popescu, R. F. Pasternack, and A. S. Janoff.** 1992. Amphotericin B-phospholipid interactions responsible for reduced mammalian cell toxicity. Biochim. Biophys. Acta **1107:**271–282.
33. **Sullivan, G. W., H. T. Carper, and G. L. Mandell.** 1992. Pentoxifylline modulates activation of human neutrophils by amphotericin B in vitro. Antimicrob. Agents Chemother. **36:**408–418.
34. **Wasan, K. M., G. A. Brazeau, A. Keyhani, A. C. Hayman, and G. Lopez-Berstein.** 1993. Roles of liposome composition and temperature in distribution of amphotericin B in serum lipoproteins. Antimicrob. Agents Chemother. **37:**246–250.