0026-895X/87/010001-11$02.00/0

MOLECULAR PHARMACOLOGY, 31:1-11

Mechanism of the Selective Toxicity of Amphotericin B Incorporated Into Liposomes

R. L. JULIANO, CHRISTOPHER W. M. GRANT, KATHRYN R. BARBER, and MARY ANN KALP

Department of Pharmacology, University of Texas Medical School, Houston, Texas 77025 (R.L.J., M.A.K.) and Department of Biochemistry, University of Western Ontario, London, Ontario, Canada (C.W.M.G., K.R.B.)

Received July 8, 1986; Accepted October 15, 1986

SUMMARY

Previously, it has been shown that incorporation of the membrane channel-forming polyene antibiotic, amphotericin B (AMB), into liposomes composed of dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylglycerol (7:3 ratio) results in reduced drug toxicity to animals with full retention of therapeutic activity against systemic fungal infections. In this report we explore the cellular and biochemical bases of the enhanced therapeutic index of liposomal amphotericin B (L-AMB). AMB and L-AMB are equally potent and both promptly induce rapid cation efflux from *Candida albicans* cells. By contrast, AMB, but not L-AMB, induces cation efflux and cell lysis in mammalian erythrocytes, demonstrating the selectivity of L-AMB at the cellular level. The characteristics of the lipid of the erythrocyte membrane seem to be the most important determinant of cellular sensitivity, since AMB, but not L-AMB, induces cation release from large unilamellar liposomes composed of red cell membrane lipids, thus paralleling the observations on intact cells. The ability of L-AMB to induce cation release and cause toxicity to erythrocytes, however, can be modulated by changing the lipid composition of the liposome carrier. Thus, AMB-containing liposomes composed of phospholipids with saturated acyl chains are nontoxic, whereas AMB liposomes composed of phospholipids containing unsaturated acyl chains are almost as toxic as AMB itself. The acyl chain composition rather than the head group composition seems most important, although substitution of anionic phosphatidylglycerols for phosphatidylcholines contributes somewhat to the protective effect. Analysis of several types of liposomes containing AMB at concentrations up to 5 mol %, using electron paramagnetic resonance and freeze fracture electron microscopy, shows that the drug is incorporated in the lipid bilayer but produces only modest disruptive effects on bilayer structure. Current results are interpreted in terms of a selective transfer of AMB from "donor" liposomes to "target" cell membranes. The transfer process probably occurs by diffusion of AMB through the solvent but is regulated by the physical properties of both donor and target membranes.

AMB, a polyene antibiotic, is the drug of choice for therapy of systemic fungal infections (1). Diseases of this type occur primarily in individuals with defective immune responses and thus are becoming more prevalent as the population of immunosuppressed AIDS, cancer, and transplant patients increases (2). The mechanism underlying the toxicity of AMB to fungal cells has been intensively investigated. The drug is an amphiphilic compound which binds to membranes and, in the presence of sterols, forms transmembrane channels or pores which allow the egress of ions and small metabolites from the cells (1, 3, 4). Although some investigators have found that AMB-induced ion fluxes are not closely correlated with cellular toxicity (5, 6), much of the literature suggests that the ionic and osmotic alterations produced by the formation of AMB channels contribute to the cytotoxic effects of the drug (7–9). In any case, AMB-induced ion fluxes provide the investigator with a convenient means for monitoring the interaction of AMB with cellular membranes and its possible toxicity to cells.

The ability to employ AMB as a therapeutic agent probably rests on the fact that the drug binds somewhat more strongly to ergosterol, the primary fungal sterol, than to cholesterol, its mammalian cell counterpart (1, 3, 10–12). However, the clinically utilized formulation of AMB (Fungizone), in which the drug is administered as a deoxycholate micelle, has a number of serious adverse side effects, most especially a severe nephrotoxicity (1, 13). The adverse actions of the drug in humans are likely to be due, at least in part, to the ability of AMB to interact with cholesterol and form pores in the cellular membranes of critical organ systems such as the kidney and cardiovascular system.

This work was supported by grants from the National Institutes of Health (Grant CA 36840) and the Elsa Pardee Foundation to R. L. J. and by a grant from the University of Western Ontario Development Fund to C. W. M. G.

ABBREVIATIONS: AMB, amphotericin B; DMPC, dimyristoyl phosphatidylcholine; DMPG; dimyristoyl phosphatidylglycerol; L-AMB, liposomal amphotericin B; f-AMB, free amphotericin B; DLPC, dilauroyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; DPOPC, dipalmitoleoyl phosphatidylcholine; PC, phosphatidylcholine; PBS, phosphate-buffered saline; DMFA, dimethylformamide; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; PE, phosphatidyl ethanolamine; LUVs, large unilamellar vesicles; PG, phosphatidylglycerol.

Recently, it has been shown that the incorporation of AMB into certain types of phospholipid vesicles (liposomes) can markedly reduce the toxicity of the drug, including its nephrotoxicity, without loss of antifungal potency (14). Studies in infected animals (15) and in patients (16) support the idea that AMB in multilamellar liposomes composed of DMPC and DMPG remain therapeutically effective while being much less toxic than Fungizone. The reduced toxicity of L-AMB *in vivo* might be due to a variety of causes including slow or sustained release of the drug, altered tissue kinetics and distribution, or effects on the immune system (17, 18). However, we have observed that incorporation of AMB in liposomes has a very direct effect in terms of reducing the toxicity of the drug to mammalian cells *in vitro*. Thus, erythrocytes (19) and macrophages (20) are protected against the lytic and cytotoxic effects of AMB by incorporation of the drug into DMPC/DMPG multilamellar liposomes. By contrast, fungal cells remain equally susceptible to AMB or L-AMB (21). Thus, the incorporation of AMB into certain liposomes seems to increase the selective cellular toxicity of the drug; that is, its ability to discriminate between host cells and pathogens. We suspect that the selective toxicity of L-AMB at the cellular level may be an important determinant of the reduced toxicity of this agent *in vivo*. In this communication, we explore the mechanistic basis for the selective toxicity of L-AMB *in vitro*. It is hoped that this will provide insights which may eventually be used to further improve the therapeutic actions of AMB and of other membrane-active antibiotics.

Experimental Procedures

Materials. Amphotericin B (Squibb Pharmaceuticals) and its deoxycholate micellar form (Fungizone) were obtained courtesy of Dr. G. Lopez-Berestein, M. D. Anderson Hospital (Houston, TX). The drugs were assayed for purity by reverse phase high pressure liquid chromatography in a methanol/water solvent and were 95–97% pure. Synthetic and natural phospholipids were obtained from Avanti Biochemicals (Birmingham, AL) and were tested for purity by thin layer chromatography. The following components were used: DLPC, DMPC, DMPG, dimyristoyl phosphatidyl ethanolamine, DPPC, DSPC, DOPC, DPOPC, dioleoyl phosphatidylglycerol, dioleoyl phosphatidyl ethanolamine, and egg lecithin (egg PC). $^{86}Rb^+$ as RbCl was obtained from Amersham (specific activity = 1–8 mCi/mg). Yeast agar and yeast inoculate (*Candida albicans* strain 336) were obtained from Dr. R. Hopfer, M. D. Anderson Hospital.

Liposome preparation. Multilamellar lipid vesicles containing AMB were prepared essentially as previously described (15). The initial drug/lipid mixture was usually 3.5 mg of AMB and 50 mg of total phospholipid (10–50 mg/ml in chloroform); this was suspended in 7 ml of methanol and dried as a film in a round bottom flask by rotary evaporation. Buffer (8 ml of isotonic PBS, pH 7.2) was added to the flask and liposomes formed by swirling and vortexing during 30 min incubation. Unincorporated AMB was removed by washing the vesicles several times with 8 ml of PBS and sedimenting the vesicles at 20,000 rpm for 15 min in a JA20 rotor (Beckman J21 centrifuge). In some cases, vesicle populations were made more homogenous in size by high pressure filtration through straight channel polycarbonate membrane filters (0.6 μm) (Unipore), as described elsewhere (22). Liposome formation and filtration were always performed above the phase transition temperature of the relevant lipids. Based on previous experience with amphiphilic drugs, it was anticipated that AMB would intercalate into the bilayer membranes of the liposomes (23).

Assay of AMB and stability studies. The AMB content of liposomes was determined by dissolving an aliquot of the lipid/drug complex in methanol and measuring the AMB absorbance at 410 nm versus AMB standards in methanol; the absorbance blanks consisted of equivalent amounts of lipid dissolved in methanol. AMB content in some samples was confirmed by HPLC; the two methods gave close agreement. The stability of liposomes in terms of AMB loss was evaluated as follows. Samples of L-AMB were divided in two. The first sample was immediately assayed for AMB content. The second sample was incubated for various periods in a 10-fold excess of PBS. This sample was then centrifuged at 20,000 rpm for 15 min and the pellet of AMB liposomes was resuspended in PBS at the original sample volume; the AMB content of the incubated, washed sample was determined as above and compared to the original AMB content. In most cases a 3-hr incubation at 25° was used; in some cases L-AMB samples were reassayed after several weeks of storage at refrigerator temperature.

Ion fluxes in yeast. Procedures for measuring cation fluxes in yeast cells are adapted from those described by Gale (8), by McDonald-Armstrong and Rothstein (24), and others (25). *C. albicans* 336 cells were loaded with $^{86}Rb^+$ (RbCl) by incubating 3.5×10^6 colony-forming units/ml of cells in sterile saline with 1 μCi/ml of isotope for 3 hr at 37°. The cells were pelleted by centrifugation at $1400 \times g$ and washed three times in 10 volumes of PBS to remove unincorporated isotope. For efflux experiments, dilutions of f-AMB or L-AMB were made in 3.5 ml of PBS in a series of Microfuge tubes. The f-AMB was maintained as a stock solution in DMFA and then diluted in DMFA, and equal aliquots were added to each assay tube. The DMFA concentration was kept below 5% in all assays. Fungizone and L-AMB were diluted directly into the assay tubes. To the 3.5 ml of AMB solution was added 0.5 ml of $^{86}Rb^+$-loaded *Candida* cells and the samples were then incubated at 37° for 90 min. The samples were then centrifuged at 1500 rpm, the supernatant was recovered by careful aspiration, and the pellet was lysed with 1% sodium dodecylsulfate; thereafter both pellet and supernatant were transferred to counting vials and the radioactivity was determined. The percentage efflux was determined according to the formula

$$\% \text{ efflux} = \frac{S - B}{S + P - 2B} \times 100$$

where S = supernatant cpm, P = the pellet (cell) cpm, and B = the blank cpm. All determinations of $^{86}Rb^+$ efflux were done in triplicate.

Ion fluxes in erythrocytes. The procedures generally follow those described by Kotyk and Janacek (26). Blood from normal volunteer donors was collected into Alsever's solution and centrifuged at $1100 \times g$ for 10 min, and the red cells were washed twice in Alsever's and once in PBS. The packed cells were resuspended in a 4-fold volume of PBS plus 1% glucose with 100 μCi of $^{86}Rb^+$ (RbCl) and incubated overnight at 37°. The isotope-loaded cells were washed four times with 20 ml of PBS. Dilutions of L-AMB, f-AMB, or Fungizone were made in PBS; 2.0 ml of each dilution were placed in a centrifuge tube; at the outset of the experiment 100 μl of $^{86}Rb^+$-labeled red cells were added to each tube and the incubation was allowed to proceed for various periods (usually 60 min) at 25°. Samples were then diluted to 10 ml with PBS and centrifuged at $1100 \times g$ for 5 min, and the pellet (cells) and supernatant were collected and assayed for radioactivity. The percentage efflux was calculated using the formula given above. Generally, the isotope-loading procedures described here for yeast cells and red cells provided 10^4–10^5 cpm/sample, thus permitting very accurate determination of ion efflux, with replicate samples usually agreeing to within 5%. In all ion flux experiments involving L-AMB, increased doses of AMB also imply increased amount of lipid. In general, all lipid compositions tested were nontoxic for yeast cells. However, very large amounts of certain liposome types caused a modest degree of ion leakage and hemolysis in red cells; this was controlled for by keeping the amount of lipid below the toxic level.

Preparation of large unilamellar liposomes composed of erythrocyte lipids. Total red cell membrane lipids were extracted with chloroform/isopropanol (7:11) according to the procedure of Rose and Oklander (27). The thin layer chromatographic pattern of this

extract was comparable to those reported previously (28), with the predominant components being PC, sphingomyelin, phosphatidyl serine, phosphatidyl ethanolanine, and cholesterol; the lipid extract was pale yellow, indicating only minor contamination with heme pigments. These lipids were used to form large unilamellar liposomes employing the reverse phase procedure of Szoka and Papahadjopoulos (29) with $CHCl_3$ as a solvent; $^{86}Rb^+$ was encapsulated by inclusion of the isotope in the aqueous phase.

Ion fluxes in liposomes. The incorporation of $^{86}Rb^+$ into liposomes composed of RBC lipids was accomplished using standard techniques for LUV preparation and loading as described above (23, 29, 30). The release (efflux) of $^{86}Rb^+$ from lipid vehicles was monitored by separating released and retained isotope via passage of the sample through Dowex cation exchange columns as described (31). In preliminary experiments, this procedure was shown to result in the retention of 99.9% of the free $^{86}Rb^+$ by the column while greater than 95% of the liposome material passed through.

Phase transition studies. EPR spectroscopy was used to measure the effect of incorporated AMB upon its lipid host matrix. TEMPO was synthesized as described by Rozantsev (32). Liposome suspensions in saline were mixed with (TEMPO) spin label at a lipid-to-spin label molar ratio of 125:1 (spin label added from a 5×10^{-3} M aqueous stock solution). These were sealed in Corning 50-μl microsampling pipettes and held in the Dewar insert of a Varian E12 EPR spectrometer equipped with Tm_{110} cavity using an insert described by Gaffney and McNamee (33). Sample temperature was monitored with a copper/constantan thermocouple in the Dewar insert. Data treatment was as recommended by Shimshick and McConnell (34).

Electron Microscopy. Samples of liposomes to be studied by freeze-fracture electron microscopy were handled in normal saline. They were rapidly quenched from 20° by plunging suspension droplets on gold discs into liquid freon cooled in liquid nitrogen. The frozen specimens were fractured at −105 to −110° in a Balzers BAF 300 high vacuum coating unit equipped with electron beam guns. Replicas were cleaned initially in bleach, rinsed with distilled water, and picked up from 1:1 acetone/ethanol. Replicas were examined using a Phillips 300 electron microscope.

Results

Dose-effect relationships for f-AMB and L-AMB. Fig. 1A depicts the release of $^{86}Rb^+$ from *Candida* cells caused by various concentrations of AMB, during a fixed time interval. Thus, AMB incorporated in DMPC/DMPG liposomes and AMB-deoxycholate micelles (Fungizone) were equally potent, with approximately 0.03 μg/ml of either form of drug causing 50% release of the radioactive marker. Interestingly, "free" AMB, that is, a DMFA stock of AMB diluted into buffer, gave a concentration-effect curve somewhat right shifted from the curves for Fungizone and L-AMB. This suggests that not all of the "free" AMB is available for interaction with the cells; this may be due to the low solubility of AMB in aqueous buffers and its tendency to form multimeric complexes under these conditions (35, 36). By contrast, the results with L-AMB and Fungizone suggest that the AMB incorporated in liposomes is as readily and as completely available to interact with *Candida* cells as the AMB in Fungizone.

The AMB-induced release of $^{86}Rb^+$ from erythrocytes is depicted in Fig. 1B. In contrast to *Candida* cells, there are marked differences in the potency of f-AMB and L-AMB in this system. Thus, the concentration of "free" AMB producing 50% release of the radioactive marker was approximately 0.5 μg/ml, whereas concentrations of L-AMB of 10 μg/ml or more produced no discernable release of $^{86}Rb^+$ (note that Fungizone is even more potent than "free" AMB in inducing ion release in red cells; data not shown). Fig. 1B also shows data for hemolysis of red cells induced by "free" AMB. As reported by others, (5, 37) the curve for lysis is right shifted with respect to the curve for cation release, indicating that effects other than ion channel formation may be of importance in the cytolytic mechanism of AMB.

The results of Fig. 1, using $^{86}Rb^+$ release as a measure of cellular cytotoxicity, clearly illustrate the characteristic selective toxicity of the liposomal form of AMB. Thus, L-AMB is at least as toxic as "free" AMB or Fungizone to fungal cells, but is much less toxic to mammalian cells. This suggests that the AMB in liposomes can completely and effectively interact with fungal cell membranes but is not available to interact with mammalian cell membranes.

Kinetics of interaction of f-AMB or L-AMB with fungal cells. A possible explanation for the reduced toxicity and useful therapeutic effects of L-AMB *in vivo* is that the inclusion of the AMB in liposomes results in a slow or sustained release of drug. We wished to explore this possibility with experiments *in vitro* which were designed to indicate how quickly either "free" AMB or L-AMB could act to form ion channels in fungal cell membranes. Fig. 2A depicts the spontaneous rate of $^{86}Rb^+$ release from *Candida* cells and the drug-induced rate of $^{86}Rb^+$ release for the case of f-AMB. Fig. 2B depicts identical studies for L-AMB. The basal rate of cation efflux from yeast cells is very low (24); thus, one can establish a basal rate and then treat cells with drug to determine how quickly the efflux increases to a higher rate. The time required for the conversion from the slow basal rate to the faster drug-induced efflux rate will presumably reflect both the time needed for the AMB to transfer from solution (or from the liposomes) to the cell membrane and the time needed for AMB channel formation within the membrane. The latter aspect, the time for channel formation within the membrane, should be identical in Fig. 2, A and B, and, thus, the change in efflux rate should reflect the transfer of AMB to the membrane.

As seen in Fig. 2, both f-AMB and L-AMB rapidly increased the $^{86}Rb^+$ efflux rate from *Candida* cells. In both cases, approximately 5 min were required to convert the cells from the slow basal rate to the faster drug-induced rate. The concentrations of "free" AMB (0.3 μg/ml) and of L-AMB (0.03 μg/ml) used in these experiments were selected since they both resulted in approximately 50% release of $^{86}Rb^+$ during a long (90-min) incubation (see Fig. 1). These results strongly suggest that L-AMB does not constitute a slow release form of the drug. Rather, AMB seems to transfer with equal rapidity from liposomes or from solution to the fungal cell membrane.

Membrane lipids as a determinant of the selective toxicity of L-AMB. The results of Fig. 1, as well as our previous findings (19, 20), suggested that the membranes of erythrocytes and of other mammalian cells are not susceptible to the actions of AMB when the drug is presented in liposomal form. We wished to determine whether the relative resistance of the erythrocyte membrane to L-AMB as compared to f-AMB could be accounted for by the properties of the membrane lipids, or whether membrane protein components played a role in conferring resistance. To this end, we prepared extracts of total red cell plasma membrane lipids (RBC lipid), as described in Experimental Procedures, and used this material to form LUVs containing entrapped $^{86}Rb^+$. We then tested the ability of f-AMB and L-AMB to induce $^{86}Rb^+$ efflux from the RBC

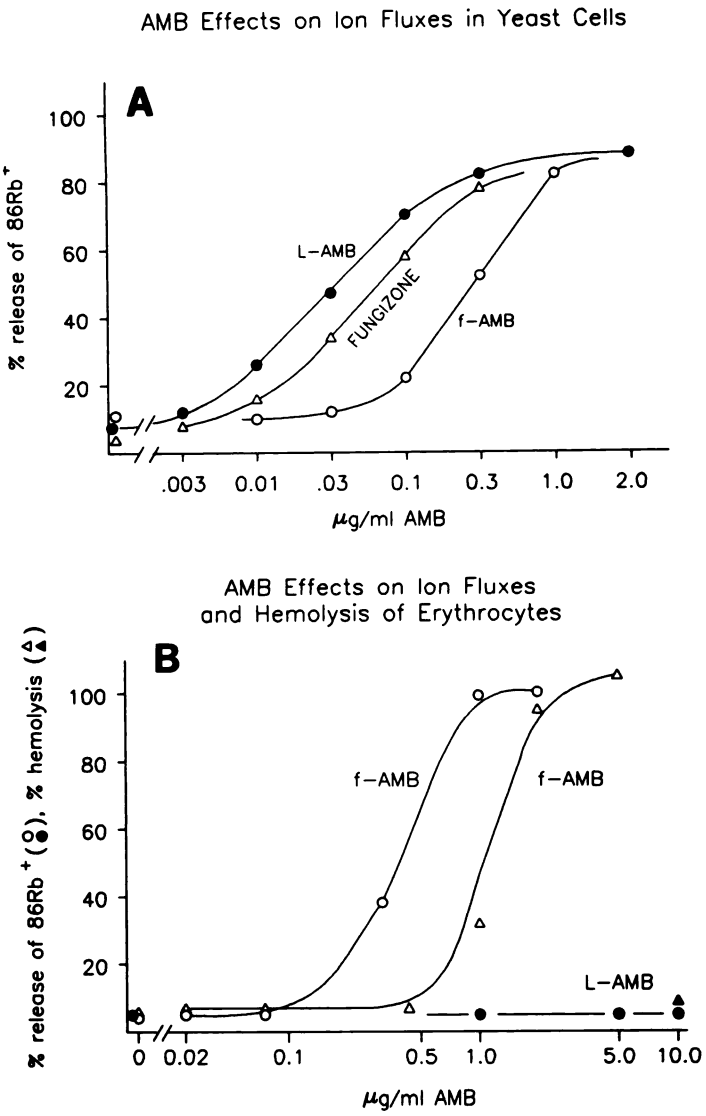

Fig. 1. AMB effects on ion fluxes in yeast cells and erythrocytes. A. For *Candida* (yeast) cells, the release of $^{86}Rb^+$ during a 90-min interval at 37° caused by various concentrations of f-AMB or L-AMB was measured as described in Experimental Procedures. *Points* are means of triplicate determinations. ●, L-AMB; △, Fungizone; ○, f-AMB. B. For erythrocytes, the release of $^{86}Rb^+$ and the hemolysis induced by various concentrations of f-AMB or L-AMB during 60 min incubation at 25° were determined as described in Experimental Procedures. *Points* are means of triplicate determinations. ○, f-AMB ^{86}Rb release; △, f-AMB hemolysis; ●, L-AMB ^{86}Rb release; ▲, L-AMB hemolysis.

lipid LUVs. Fig. 3A illustrates that the electron microscopic characteristics of the RBC lipid liposome preparations are consistent with a population mainly comprised of LUVs. As seen in Fig. 3B, f-AMB induced 50% release of $^{86}Rb^+$ at a concentration of 42 μg/ml, whereas L-AMB failed to induce appreciable cation release at concentrations of 160 μg/ml. Thus, LUVs composed solely of red cell lipids and devoid of any cell protein are more affected by f-AMB than by L-AMB, just as is the case with intact cells. This suggests that the physical characteristics of the red cell lipid bilayer are the prime determinants of the lack of susceptibility of the red cell membrane to L-AMB. The observation that far more f-AMB is required to produce $^{86}Rb^+$ leakage from red cell lipid LUVs than from red cells themselves is probably accounted for by the much higher concentration of total cell lipid present in the experiments using LUVs as drug "targets" than in those using red cells as targets. Although it would be of considerable interest, we have not attempted the reciprocal experiments of testing f-AMB and L-AMB on lipid vesicles prepared from *Candida* lipids. The reason for this lies in the fact that, unlike the case of the red cells, it is quite difficult to prepare pure yeast plasma membranes. Thus, lipid extracts of whole yeast cells or of partially fractionated membranes would be severely contaminated with components derived from intracellular membranes and from lipid storage droplets and, therefore, might not be relevant to our investigations of the actions of AMB on plasma membranes.

The role of donor liposome composition in determining the effects of L-AMB. Most of the experiments thus far with liposome-incorporated AMB (15, 16, 19, 20) have utilized a single liposome form and composition, namely, multilamellar vesicles composed of a 7:3 molar ratio of DMPC/DMPG. We wished to investigate the question of whether changes in the composition of the donor liposome would alter the effects of L-AMB on cells. Thus, we incorporated AMB into liposomes composed of PCs of differing acyl chain types; as well, we examined mixtures of PCs with PEs and PGs. We tested the ability of AMB containing multilamellar liposomes of differing compositions to induce $^{86}Rb^+$ efflux from red cells. As seen in Fig. 4, there are wide variations in the potency of different preparations of L-AMB in terms of the induction of cation fluxes in the red cell. Thus, AMB incorporated into vesicles

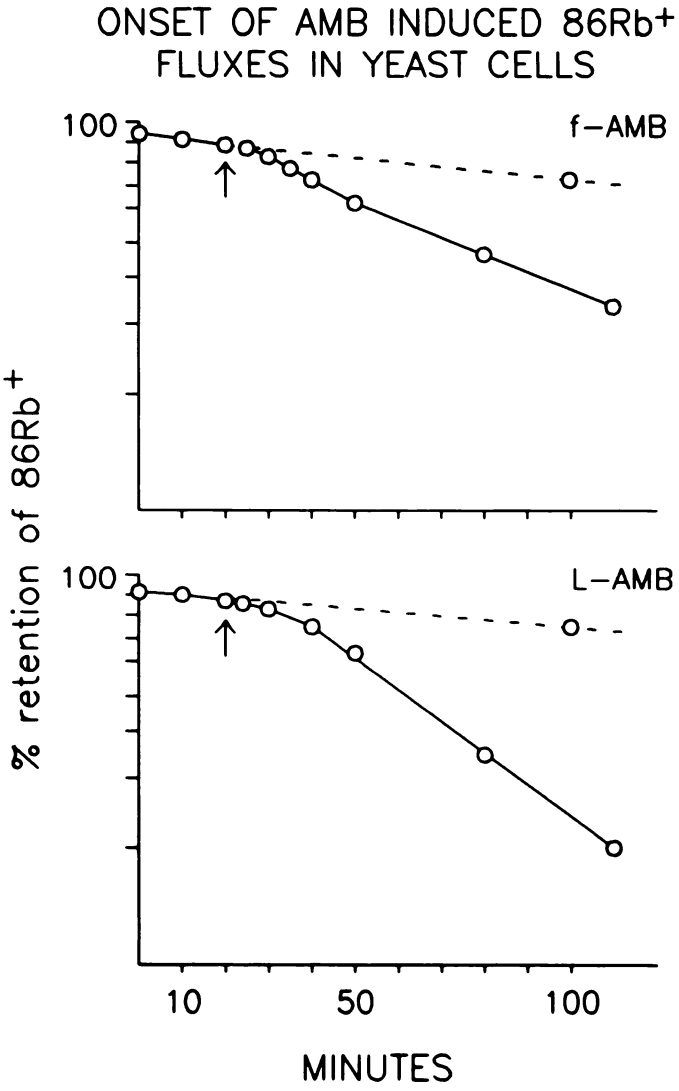

Fig. 2. Onset of $^{86}Rb^+$ flux induced by AMB in *Candida*. The basal flux of $^{86}Rb^+$ and the AMB-induced flux were determined as described in Experimental Procedures. *The ordinate* is a log scale. ---, basal fluxes;—, the flux subsequent to AMB addition ↑, the point at which AMB was added. The doses of f-AMB and L-AMB selected were approximately equally effective in inducing $^{86}Rb^+$ loss from yeast cells during a 90-min incubation (see Fig. 1). *Upper panel*, effect of 0.3 μg/ml f-AMB; *lower panel*, effect of 0.03 μg/ml L-AMB.

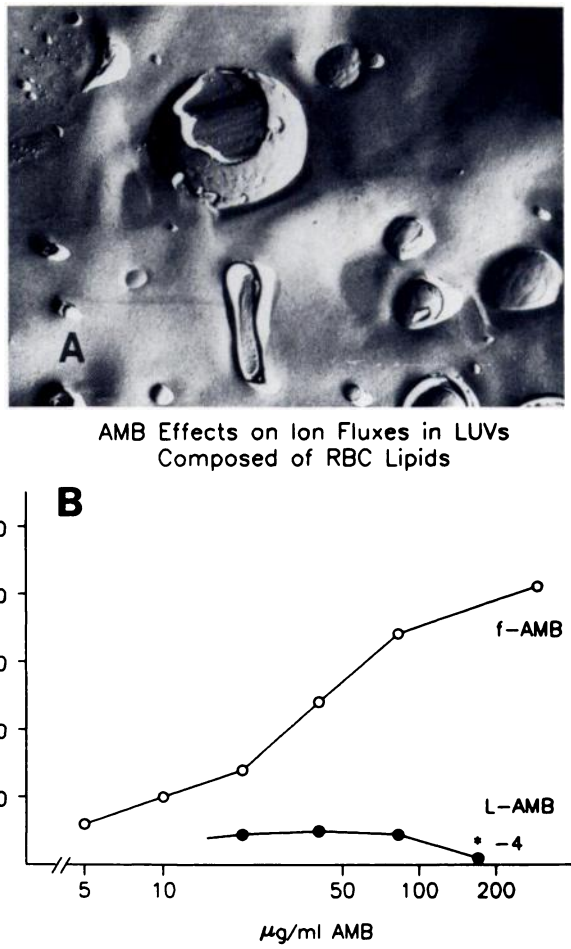

Fig. 3. AMB effects on $^{86}Rb^+$ efflux in LUVs prepared from red cell lipids. A. Freeze-fracture electron micrograph of LUVs composed of red cell lipids. B. Release of $^{86}Rb^+$ from LUVs composed of red cell lipid was determined as described in Experimental Procedures. Results are means of triplicate determinations. O, f-AMB; ●, L-AMB. *, the value for this point was less than that of the control.

composed of saturated PCs (DMPC, DLPC) caused little ion efflux from red cells, whereas AMB incorporated into unsaturated DOPC vesicles was almost as potent as f-AMB in inducing ion fluxes.

The role of donor liposome composition in determining AMB effects on erythrocytes is explored in more detail in Table 1, where the AMB concentration required for 50% leakage of $^{86}Rb^+$ from the cells is shown for various types of AMB liposomes. In general, vesicles composed of saturated PCs protect red cells against AMB actions while vesicles composed of unsaturated PCs do not protect as well. The acyl chain composition rather than the head group seems to be the more important determinant. Thus, partial substitution of PE for PC of similar acyl chain type seems to have little effect. However, the partial substitution of anionic PG derivatives for PC seems to increase somewhat the ability of unsaturated liposomes to protect red cells against AMB toxicity. Equal mixtures of saturated and unsaturated PCs behave like the saturated compound in terms of red cell protection. It should be noted that all of the compositions of L-AMB tested in Table 1 effectively inhibited growth (21) of *C. albicans* 336 with minimum inhibitory concentrations ranging from 0.4 μg/ml to 0.8 μg/ml as compared to 1.0 μg/ml for "free" AMB. Thus, the various L-AMB preparations of Table 1 show dramatic differences in toxicity for mammalian erythrocytes while displaying similar toxicities to fungal cells.

Stability of AMB liposomes. The toxicity of various preparations of AMB liposomes to red cells does not correlate in any way with the stability of the AMB-liposome complexes in aqueous solution. Thus, in Table 2, one sees that all of the liposomes used display only minimal release of AMB during incubation in buffer solutions. Moreover, the liposome preparations showing the greatest loss of AMB during incubation (DLPC, DSPC) are both very efficient in protecting red cells. Finally, we have maintained preparations of L-AMB of various compositions at refrigerator temperature for many weeks without appreciable loss of AMB, indicating that the drug is associated with the liposomes in a very stable form (data not shown). These data suggest that the effects of various preparations of L-AMB on red cells and on yeast cells are not simply

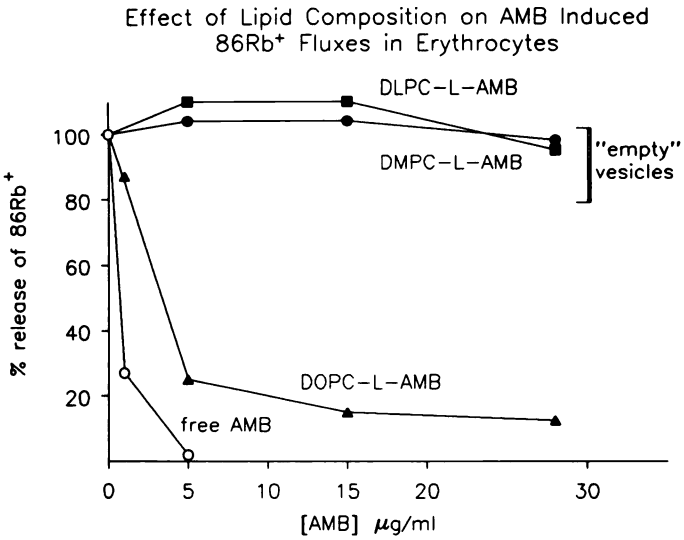

Fig. 4. Effect of donor lipid composition on AMB-induced $^{86}Rb^+$ release from red cells. The release of $^{86}Rb^+$ from red cells during 60 min incubation at 25° was determined as described in Experimental Procedures. *Points* represent the means of triplicate determinations. The brackets ([]) indicate the range of $^{86}Rb^+$ release caused by various compositions of "empty" liposomes. ○, f-AMB; ■, AMB in DPLC liposomes; ●, AMB in DMPC liposomes; ▲, AMB in DOPC liposomes.

TABLE 1

Concentration of L-AMB required for 50% leakage of $^{86}Rb^+$ from erythrocytes

Vesicles were passed through 0.6-μm filters to promote size uniformity in the different preparations. The values given are estimated from concentration response curves similar to those of Fig. 4 for the various lipid compositions and represent means of duplicate experiments differing by less than 5%. We have not explored toxicities of all preparations of L-AMB at concentrations greater than 30 μg/ml since, with some vesicle preparations, higher AMB doses require correspondingly large amounts of lipids which are themselves toxic to red cells.

Liposome composition (molar ratio)	[AMB]
	μg/ml
(Fungizone)	(0.5)
DLPC	>30
DMPC	>30
DPPC	>30
DSPC	>30
DMPC/DMPE (4/1)	>30
DMPC/DMPE (1/1)	>30
DMPC/DMPG (7/3)	>30
Egg PC	>30
DPOPC	10
DOPC	7
DOPC/DOPE[a] (4/1)	5
DOPC/DOPE (1/1)	3
DOPC/DOPG (4/1)	26
DOPC/DOPG (1/1)	14
DPOPC/DPPC (1/4)	>30
DPOPC/DPPC (1/1)	>30

[a] Abbreviations not defined elsewhere are: DOPE, dioleoyl phosphatidyl ethanolamine; DOPG, dioleoyl phosphatidylglycerol.

due to instability of the liposome and consequent release of the drug.

Diffusional versus collisional transfer. An important question in this study was whether the AMB was transferred from donor liposomes to cells by means of vesicle-cell collisions or whether the transfer process involved diffusion of AMB monomers or oligomers through the solvent phase. If a collisional process were operating, then, for a fixed amount of AMB,

TABLE 2

Stability of L-AMB preparations

L-AMB samples were incubated for 3 hr at 25°, diluted 10-fold, and centrifuged at 20,000 rpm. The AMB content in the pellets was compared to the original AMB content. Values represent the means of duplicates differing by less than 5%. See Table 1, Footnote *a*, and text abbreviations footnote for lipid abbreviations.

Vesicle composition (molar ratio)	Percentage of AMB retained
DLPC	87
DMPC	108
DSPC	89
DMPC/DMPE (1/1)	104
DMPC/DMPG (4/1)	98
DOPC	92
DPOPC	98
DOPC/DOPE (1/1)	101
DOPC/DOPG	90
DOPC/DPPC (1/1)	102

TABLE 3

Retention of $^{86}Rb^+$ in erythrocytes treated with L-AMB: effect of varying the number of liposomes

Liposomes (multilamellar vesicles) were prepared using three different ratios of phospholipid (DOPC) to AMB and passed through 0.6-μm Unipore filters. The incorporated AMB in each sample was determined as described in Experimental Procedures. The liposomes were diluted in Tris-NaCl buffer so as to provide concentrations of AMB in the final assay mixture of either 10 μg/ml or 20 μg/ml. The lipid content of the diluted samples was measured by organic phosphorus determination and the AMB/lipid ratio was calculated. Erythrocytes loaded with $^{86}Rb^+$ were then exposed to the AMB liposomes for 60 min at 25° and the percentage of release of isotope was determined. Varying the AMB/lipid ratio at fixed AMB concentration and fixed liposome size entails a variation of the total number of liposomes in the preparation. Data represent means and standard errors for triplicate determinations.

AMB/DOPC ratio	% of $^{86}Rb^+$ retained by cells with [AMB]	
	10 μg/ml	20 μg/ml
μg/mg		
46	24.0 ± 1.6	7.0 ± 0.6
21	19.0 ± 2.4	6.0 ± 0.6
4.4	15.0 ± 0.9	11.0 ± 2.0
Control (no treatment) = 100%		

increasing the number of carrier vesicles should increase the rate of AMB transfer, since the collision probability should be proportional to particle number. If transfer occurred primarily by diffusion, then the transfer rate would depend on the concentration of AMB but would not directly depend on particle number. To test these alternatives, we prepared liposomes having three different AMB/lipid ratios. Erythrocytes, preloaded with $^{86}Rb^+$, were then treated with these liposomes at fixed AMB concentrations of 10 μg/ml or 20 μg/ml and the percentage of isotope release was determined. Thus, the cells were exposed to two fixed concentrations of AMB, but the drug was contained in different amounts of lipid; assuming that size profiles of the liposome populations are similar in all cases, the treatment at high AMB/lipid ratios implies fewer carrier vesicles, whereas low AMB/lipid ratios imply more numerous carrier vesicles. As seen in Table 3, varying the AMB/lipid ratio (and, thus, the number of carrier liposomes), over a 10-fold range had only modest effects on AMB-induced cation leakage from red cells. This experiment has been repeated several times at different AMB concentrations with qualitatively similar results. Thus, the data suggest that AMB transfer from liposomes to cells does not involve a collisional mechanism. There are, however, inherent limitations in this type of study since it

is only feasible to vary the donor particle number over a limited range and it is technically quite difficult to vary the target particle (cells) over a wide range. Both of these parameters should be carefully evaluated prior to reaching firm conclusions on the transfer mechanism. Thus, the preliminary data here tentatively support a noncollisional mechanism as the rate-limiting step in AMB transfer.

Phase behavior of AMB-containing liposomes. The lipid bilayer membrane is crystalline in nature (or so-called "liquid crystal" in the case of fluid membranes). The mode of assembly of AMB into such ordered lattices is relevant to this work since it might be expected to influence the rate of drug transfer from liposome membranes. In particular, we were interested in the possible disruptive effect of the drug upon its various host matrices, and whether there might be some related peculiarity of the DMPC/DMPG matrix. To this end we monitored the melting behavior of these matrices with and without AMB at the concentration employed for liposomal drug delivery—the reasoning being that AMB-induced changes in lattice energies would show up in bilayer phase behavior. Our approach was to monitor, as a function of temperature, the EPR spectrum of a small amphiphilic spin label (TEMPO) that partitions reversibly between lipid and aqueous phases. This is a widely recognized technique for studying membrane thermodynamic properties since the partition coefficient has been shown to be a sensitive function of lipid order, with the EPR spectrum permitting quantitative assessment of the amount in each environment (34). The parameter generally plotted is the height ratio of a peak assigned to spin label in lipid, to total spin label present. For DOPC, whose phase transition temperature is below 0°, we have plotted the ratio of highfield to midfield line width. This ratio would normally decrease with increasing temperature, hence its abrupt increase at the phase transition, due to growth of the incompletely resolved peak arising from label in lipid, is quite recognizable. The phase transition temperatures determined for 7:3 DMPC/DMPG, DMPC, DSPC, and DOPC were 23.9°, 24.6°, 53.2°, and −19.4°, respectively. Corresponding values found for the liposomes with AMB are slightly shifted to lower temperatures, giving 23.9°, 23.5°, 52.5°, and −21.2°, respectively. In the presence of 2 mM Ca^{2+}, membranes containing the acidic phospholipid, DMPG, are known to become measurably more rigid (38, 39); and, indeed, the measured membrane melting curves were smoothly shifted to higher temperatures by 2.7° and 2.1° for the samples without and with AMB, respectively. Typical experimental data are plotted in Fig. 5 for the above phospholipid bilayers together with data derived at the same time for the mixtures with AMB for comparison. The phase behavior plots for all of the lipid mixtures tested were affected very similarly by the presence of AMB in the concentration range of our experiments.

Liposome examination by electron microscopy. Freeze-fracture electron microscopy employs a sample preparation technique in which a droplet of sample suspension is rapidly frozen to liquid nitrogen temperatures and fractured prior to platinum shadowing. The fracture plane exposes membrane hydrophobic interior. In this work, a short period of etching has also preceded the shadowing step in order to expose a thin rim of liposome outer surface. The technique resolves features down to 2.5 nm. This is the technique of choice for visualizing lipid bilayer membranes—providing 100 times higher resolution than light microscopy and avoiding the need for organic solvent extraction, drying, and staining characteristic of other electron microscopy procedures. Results are presented in Figs. 3A, 6, and 7. Fig. 3A is a low power view of the liposomes made from extracted red blood cell lipids and used as a target for drug-induced leakage. Figs. 6 and 7 are detailed views of liposomes used in the biological experiments. Bilayers of rigid DMPC (Fig. 7C) are well known to have a rippled appearance characteristic of the $P_{\beta'}$ phase (40). This same pattern is present in most of the liposomes of 7:3 DMPC/DMPG (Fig. 6A). Van Dijck *et al.* (39) observed that liposomes of 1:1 DMPC/DMPG are typically smooth in freeze-fracture preparations. Indeed, the ripple pattern in our 7:3 DMPC/DMPG samples is absent in some liposomes or portions of liposomes. Presumably, this reflects the fact that the 7:3 ratio is close to the concentration of DMPG that converts the DMPC $P_{\beta'}$ phase to L_α phase (40), and that there is some minor heterogeneity of liposome composition. Incorporation of 5 mol % AMB largely converted the ripple structure to a smooth bilayer appearance characteristic of the L_α phase (e.g., Fig. 6B). The rest of the liposome types studied were seen to be (smooth) L_α phase. Note that the liposome preparation technique produced multilamellar structures as anticipated (Figs. 6 and 7).

There is the question as to whether AMB in these preparations incorporates into the bilayers, or whether it may simply coexist with them as free drug and/or non-bilayer mixtures with phosphatidylcholine. However, extensive scrutiny of numerous preparations showed no evidence of non-bilayer or amorphous structures. All liposomes have the classic fracture face and etch face appearance unique to the lipid bilayer. Nevertheless, there is evidence that AMB is a significantly disruptive force within the bilayer. For instance, fracture faces are less extensive than those seen for lipid without drug, the fracture plane having jumped back and forth between concentric lamellae rather than remaining within the same bilayer from one side of a liposome to the other. Also, the bilamellar sheets are less smoothly continuous than in the absence of drug. Similar features are observed in all the preparations (Figs. 6 and 7).

Discussion

We have demonstrated that the inclusion of AMB in certain types of liposomes can markedly protect mammalian erythrocytes against AMB-induced ion efflux and cytolysis (Fig. 1B, Table 1). This protective effect may underlie the reduced *in vivo* toxicity of L-AMB observed previously (15, 16). Liposome compositions such as DMPC/DMPG, which are quite nontoxic to red cells, remain effective in promoting ion fluxes and cytotoxicity in *Candida* (Fig. 1A); thus, they display a high degree of selective toxicity to fungal cells as compared to mammalian cells. The interaction of the liposomal form of AMB with fungal cell membranes is rapid and complete. Thus, both free and liposomal drug induced ion fluxes in *Candida* with equal rapidity (Fig. 2); furthermore, AMB in liposomes is as potent as or more potent than "free" AMB or the deoxycholate micelle of AMB (Fungizone) in its actions on *Candida* cells (Fig. 1A). Thus, the liposomal form of AMB does not seem to owe its reduced toxicity to a slow or limited release of drug from the liposomal carrier; rather, the drug transfers selectively from the liposomes to fungal cell membranes but not to mammalian cell membranes.

The relative resistance of mammalian erythrocyte mem-

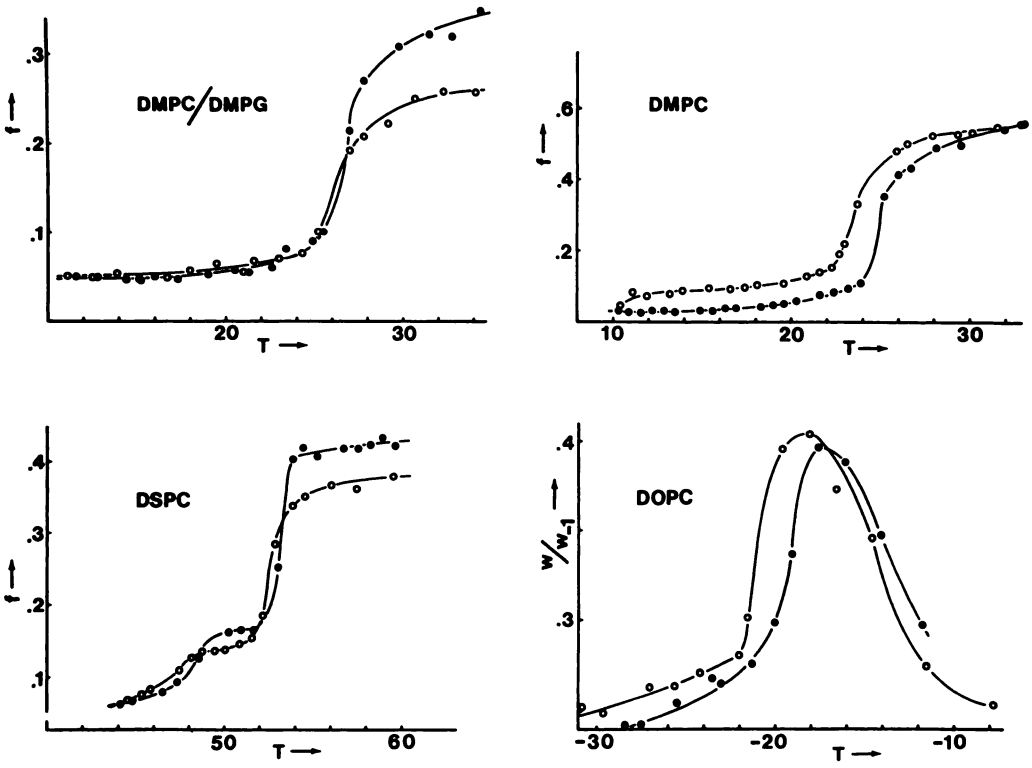

Fig. 5. Phase behavior profiles for liposomes with and without AMB. Curves derived from EPR spectral data displaying the melting characteristics of liposomes used are shown. ●, lipid alone, ○, in the presence of AMB. The TEMPO spectral parameter, *f*, of Shimshick and McConnel (34) has been plotted for 7:3 DMPC/DMPG (5.0 mol % AMB), DMPC (3.1 mol % AMB), and DSPC (5.0 mol % AMB) as a function of temperature, *T*, in °C. For DOPC (and its mixture containing 4.9 mol % AMB), the ratio, w/w_{-1}, of midfield to highfield spectral line width has been plotted. All samples were in normal saline; the data shown for DMPC/DMPG were derived in the presence of 2 mM Ca^{2+} at pH 7/4. Without Ca^{2+} the curves for DMPC/DMPG and DMPC/DMPG/AMB were virtually identical to those shown, but shifted to lower temperatures by 2.7° and 2.1°, respectively (see the text).

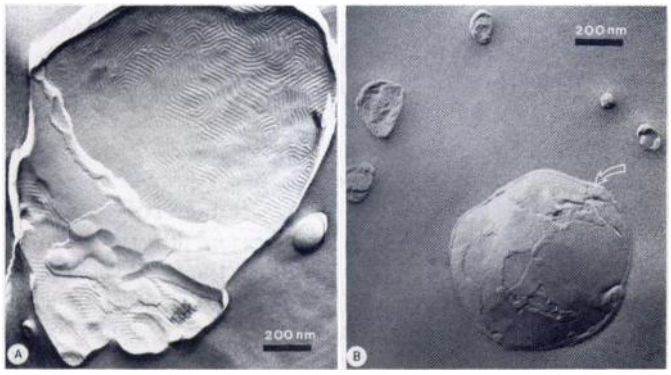

Fig. 6. Freeze-fracture electron micrographs of liposomes formed by hydration in normal saline of a 7:3 (molar ratio) mixture of DMPC/DMPG *without* (A) and with (B) 5 mol % AMB. Samples were quenched from 20°. The large liposome in A shows the ripple pattern characteristic of some synthetic PCs below their phase transition temperatures. The *arrow* in B points to a rim of each face (liposome outer surface), which is of normal morphology as is the etch face/fracture face junction. There is some evidence of bilayer disruption by the presence of drug (compare A and B). Shadow direction is from bottom to top. Magnification × 100,000.

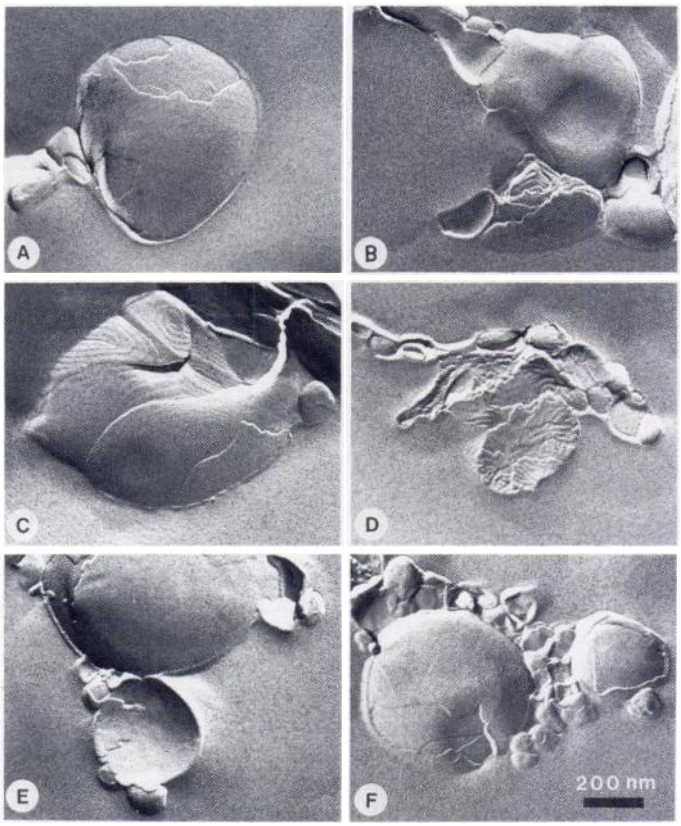

Fig. 7. Freeze-fracture electron micrographs of liposomes of DOPC (A), DMPC (C), and DSPC (E) formed by hydration of dry films with normal saline. The accompanying micrographs show DOPC with 4.9 mol % AMB (B), DMPC with 3.05 mol % AMB (D), and DSPC with 2.7 mol % AMB (F). Samples were quenched from 20°. Shadow direction is from bottom to top. Magnification × 71,333.

branes to the effects of L-AMB seems to be determined by the chemical and physical characteristics of the cell membrane lipids themselves. Thus, as shown in Fig. 3, f-AMB, but not L-AMB, can induce cation fluxes in LUVs prepared from erythrocyte lipids. We have not fractionated the mixed lipids of the red cells to see if any of the components were particularly important in controlling the interaction with the liposomal form of AMB. A more precise definition of the chemical and physical characteristics of the "target" membrane that confer sensitivity or resistance to the effects of L-AMB will clearly be of interest for future investigations.

In this study we have partially delineated the characteristics

of the "donor" liposome membranes which determine ability to transfer AMB to erythrocytes. Thus, Fig. 4 and Table 1 show that AMB incorporated into unsaturated PC liposomes is much more potent in inducing ion fluxes in red cells than AMB incorporated into saturated PC liposomes. These experiments suggest that saturation rather than fluidity seems to be important, since liposomes of either DLPC, which is fluid at 25°, or DSPC, which is in solid phase at 25° (29), can confer protection against AMB-induced cation efflux. However, one should keep in mind that the fluidity of a DMPC liposome above its phase transition will be quantitatively different from that of a liposome composed of unsaturated PC. Thus, additional, more quantitative studies will be required to evaluate the role of fluidity. The fatty acyl chain composition rather than the polar head group seems to be the most important determinant of the protective effect; thus, AMB liposomes prepared from mixtures of PCs and PEs were similar to AMB liposomes prepared solely from PC, so long as the acyl chain compositions were similar. However, the inclusion of anionic PG into vesicles seemed to confer some additional protection, perhaps via interaction with the polar mycosamine moiety of AMB. In the case of PC/PE vesicles, there is a possibility that the initial PC/PE liposome structure may have undergone a phase separation because of the marked differences in phase transition temperatures for PCs and homologous PEs (29), thus allowing the AMB to segregate into a PC-rich phase and escape effects of the PE. The relative toxicities of AMB liposome preparations did not correlate with any measure of overall instability; most preparations of L-AMB retain the drug for long periods of time during storage in buffer solutions at 25° or 0°. Thus, the ability of liposome formulations containing unsaturated PCs to cause ion fluxes in red cells seems due to their ability to transfer or "donate" AMB to the red cells rather than to any overall instability.

We compared the effects of AMB, at the concentrations used in flux studies, upon liposomes composed of different phospholipids. Dufourc *et al.* (41) have recently reported a study, by NMR spectroscopy, of DMPC bilayers bearing AMB at high concentrations. They concluded that, above the phase transition temperature, an aggregate phase of 1:1 AMB/DMPC coexisted with a phase of DMPC. The aggregate phase was relatively more ordered, and the phase transition temperature for the bulk matrix was lowered 5–10° by the inclusion of 30 mol % AMB. Our approach involved monitoring the EPR spectra of samples in the presence of small amounts of the spin label, TEMPO, whose partition coefficient between membrane and surrounding water is a sensitive function of membrane order. The temperature profiles obtained in each case showed a decrease in host matrix transition temperature by less than 2° in the presence of AMB. Such a lowering of lipid bilayer phase transition temperature by incorporation of small amounts of some other lipid (AMB in this case) is generally attributed to minor crystal lattice disruption due to imperfect "fit" of the new species. A comparison of the temperature profiles of the various lipid types showed no obvious differences that might form a basis for the demonstrated differential toxicities. This latter observation argues against the possibility that AMB simply has a more disruptive fit into bilayers such as DOPC that leads to greater transfer to other membranes. The DMPC/DMPG mixture is an interesting one in terms of phase behavior in that the transition temperature of each pure species is some 23–24° and, when combined, a mixture is formed that also melts sharply in this range (38, 39). It also is known to be measurably rigidified by Ca^{2+}, as already mentioned (39). Fig. 5 demonstrates that neither of these phenomena altered the effect of AMB significantly, relative to other phospholipids studied.

The freeze-etch approach lends itself particularly well to the problem of liposome structure determination, affording views of features related to molecular interactions. We were able to demonstrate that the bilayer nature of liposomes used is fully preserved in all cases. Both fracture and etch faces had normal morphology in the presence of 5 mol % AMB. Signs of drug-related alterations in host matrix crystal packing were evident in some liposomes. However, no amorphous masses or particles were identified that would indicate f-AMB or local drug concentrations so high as to totally disrupt the host bilayer matrix.

The behavior of L-AMB illustrates that the selectivity, toxicity, and therapeutic efficacy of polyene antibiotics can be modulated through use of appropriate lipid vesicle carriers. The high degree of selective cellular toxicity displayed by certain forms of L-AMB can probably be best described in terms of a rapid and extensive transfer of AMB from "donor" liposomes to fungal cell membranes and consequent formation of transmembrane channels or pores; by contrast, transfer from donor liposomes to mammalian cells is less rapid and/or less extensive. The selective transfer of AMB from liposomal membranes to cell membranes can probably be understood in the context of previous investigations of the characteristics of AMB-induced membrane channel formation (42–44), the binding of AMB to sterols and to phospholipid membranes (11, 35, 45, 46) and the characteristics of membrane to membrane transfer of AMB (36) and other amphiphilic molecules (47–49).

The transmembrane channels or pores formed by AMB-sterol complexes are not static entities. Rather, pore formation is a dynamic process involving assembly and disassembly steps and the transient opening and closing of the pore; these processes have been clearly discerned via single-channel conductance measurements of AMB acting on planar lipid membranes (43, 44). These kinetic processes are also reflected in a complicated series of forms of the AMB-sterol complex, as indicated by studies using NMR or CD (35, 41). Since the rate of ion translocation mediated by a conducting AMB channel is extremely high (44, 50), the kinetics of ion loss through a membrane exposed to AMB is not limited by the conductance of the AMB pore per se, but rather by the relative rates of pore assembly/disassembly. This process in turn depends, in a highly cooperative manner, on the content of AMB and of sterols within the membrane, and on the physical characteristics of the membrane environment (42, 45). Since AMB pore formation is highly cooperative, the formation of such pores will not occur unless a "threshold" level of AMB is reached in the membrane (37, 43). In the present experimental situation, the characteristics of the "target" membranes are fixed; they are the ergosterol-rich membranes of fungal cells or the cholesterol-rich membranes of erythrocytes. Thus, the extent of pore formation in either of these two membranes, under differing experimental circumstances, will depend largely on the amount of AMB available in the membrane. This in turn will depend on the net transfer to AMB, either from solution or from donor liposomes, to the target membrane. Thus, the nature of the

AMB transfer processes for f-AMB and for L-AMB are critical to an understanding of the selective toxicity of L-AMB.

It is well known that AMB binds strongly to phospholipid membranes as well as to membranes containing both phospholipid and sterol (10). As an amphiphilic compound, AMB is almost totally insoluble in aqueous solution and, thus, in the presence of lipid membranes, most of the drug probably rapidly becomes membrane associated, with a small residual soluble pool in the form of AMB monomers and multimers (35). It is also clear that the rate of transfer of AMB from solution to sterol-containing membranes depends on both the stereochemistry of the sterol and on the characteristics of the surrounding phospholipid (42, 45); in other words, the transfer rate of "free" AMB from solution will depend on the physical characteristics of the "target" membrane. In the case of membrane-to-membrane transfer of AMB, the situation is even more complex. The studies of Bolard *et al.* (36) strongly suggest that AMB can transfer from one liposome population to another. Under some circumstances, this transfer can be extremely rapid ($t_{1/2}$ = 30 sec). However, rapid transfer does not take place in all cases; thus, at 22°, AMB could readily transfer between two populations of fluid egg PC vesicles, between two populations of gel phase DPPC vesicles, or from egg PC to DPPC vesicles; transfer from DPPC to egg PC vesicles was not detected. This suggests that the physical characteristics of both donor and target (acceptor) liposome populations are important in determining AMB transfer.

The nature of membrane-to-membrane transfer processes for amphiphiles has been addressed most clearly in the case of phospholipid molecules. Some of the concepts derived in those studies can probably be applied to an understanding of transfer processes for AMB. Thus, Nichols and Pagano (47) and Roseman and Thompson (48) have both demonstrated that phospholipids transfer between two vesicle populations, not by collisional processes, but, rather, as monomers (or multimers) diffusing through aqueous solution. However, although the process occurs via diffusion, the physical characteristics of both the donor and the acceptor vesicles determine the net rate of transfer. This is due to the fact that the vesicle physical properties determine both the rate of association and the rate of dissociation of the monomer from the bilayer membrane. Thus, the equation describing net transfer contains terms which reflect the physical characteristics of both donor and acceptor vesicle populations (47).

These concepts can readily be applied to the present case of AMB transfer from lipid vesicle membranes to cell membranes. The data of Table 3 suggest that the rate-limiting step of the AMB transfer process seems to proceed not by a collisional process, but probably by diffusion of monomers, in agreement with previous observations on transfer of lipids (47, 48). Furthermore, the physical characteristics of both the donor membrane (liposome) and the acceptor membrane (cell) determine the extent of the transfer process. Thus, manipulation of the composition of the donor liposome can alter the degree of AMB transfer, as shown in Table 1. It seems likely that the striking differences in the toxicity of L-AMB to fungal or mammalian cells may also depend on the attainment of a "threshold" level of AMB transfer (42, 50) before AMB pore function can occur. So long as the transfer does not exceed the threshold, the ion fluxes and cytolysis will be minimal; once the threshold is exceeded, then AMB pore formation and toxicity will ensue. Thus, the cellular selective toxicity of L-AMB can probably be understood in terms of a selective transfer process, which likely occurs by diffusion, but which is regulated by the physical characteristics of donor and target membranes.

Acknowledgments

The authors thank Paul Stoufflet and Reeta Mehta for their assistance and Sandra Hobbs for secretarial assistance.

References

1. Medoff, G., J. Brajtburg, G. S. Kobayashi, and J. Bolard. Antifungal agents useful in therapy of systemic fungal infections. *Annu. Rev. Pharmacol. Toxicol.* **23**:303–304 (1983).
2. Singer, C., M. H. Kaplan, and D. Armstrong. Bacteremia and fungemia complicating neoplastic disease. A study of 364 cases. *Am. J. Med.* **62**:731–742 (1971).
3. Hamilton-Miller, J. M. Chemistry and biology of the polyene macrolide antibiotics. *Bacteriol. Rev.* **37**:166–169 (1973).
4. Norman, A. W., A. M. Spielvogel, and R. G. Wong. Polyene antibiotic-sterol interaction. *Adv. Lipid Res.* **14**:127–170 (1976).
5. Brajtburg, J., S. Elberg, D. R. Schwartz, A. Vertut-Croquin, D. Schlessinger, G. S. Kobayashi, and G. Medoff. Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. *Antimicrob. Agents Chemother.* **27**:172–176 (1985).
6. Chen, W. C., D. L. Chou, and D. S. Feingold. Dissociation between ion permeability and the lethal action of polyene antibiotics on *Candida albicans*. *Antimicrob. Agents Chemother.* **13**:914–917 (1978).
7. Holz, R. W. Mechanism of action of antieukaryotic and antiviral compounds, in *Antibiotics* (D. Gottlieb and P. D. Shaw, eds.), Vol. 2. Springer-Verlag, New York, 313–340 (1979).
8. Gale, E. F. The release of potassium ions from *Candida albicans* in the presence of polyene antibiotics. *J. Gen. Microbiol.* **80**:451–465 (1974).
9. Kerridge, D. The polyene macrolide antibiotics. *Postgrad. Med. J.* **55**:653–656 (1979).
10. Chen, W. C. and R. Bittman. Kinetics of association of amphotericin B with vesicles. *Biochemistry* **16**:4145–4149 (1977).
11. Readio, J. D., and R. Bittman. Equilibrium binding of amphotericin B and its methyl ester and borate complex to sterols. *Biochim. Biophys. Acta* **685**:219–224 (1982).
12. Edwards, D. I. *Antimicrobial Drug Action.* University Park Press, Baltimore (1980).
13. Pratt, W. B. *Chemotherapy of Infection.* Oxford University Press, New York (1977).
14. Juliano, R. L., G. Lopez-Berestein, R. L. Hopfer, R. Mehta, K. Mehta, and K. Mills. Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. *Ann. N. Y. Acad. Sci.* **446**:390–402 (1985).
15. Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, U. Fainstein, M. Luna, E. M. Hersh, and R. L. Juliano. Treatment and prophylaxis of disseminated *Candida albicans* infections in mice with liposome encapsulated amphotericin B. *J. Infect. Dis.* **147**:939–945 (1983).
16. Lopez-Berestein, G., V. Fainstein, R. Hopfer, M. Sullivan, M. Rosenblum, R. Mehta, M. Luna, E. Hersh, J. Reuben, K. Mehta, R. L. Juliano, and G. Bodey. A preliminary communication: treatment of systemic fungal infections in cancer patients with liposome encapsulated Amphotericin B. *J. Infect. Dis.* **151**:704–710 (1985).
17. Poznansky, M. and R. L. Juliano. Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol. Rev.* **36**:277–336 (1984).
18. Juliano, R. L. Pharmacokinetics of liposome encapsulated drugs, in *Liposomes: From Physical Structure to Therapeutic Application* (G. Knight, ed). Elsevier, Cambridge, England, 21–32 (1981).
19. Mehta, R., G. Lopez-Berestein, R. Hopfer, K. Mills, and R. L. Juliano. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. *Biochim. Biophys. Acta* **770**:230–234 (1984).
20. Mehta, R., K. Mehta, G. Lopez-Berestein, and R. L. Juliano. Effect of liposomal amphotericin B on murine macrophages and lymphocytes. Infect. Immun. **47**:429–433 (1985).
21. Hopfer, R. L., K. Mills, R. Mehta, G. Lopez-Berestein, V. Fainstein, and R. L. Juliano. *In vitro* anti-fungal activities of amphotericin B and liposome encapsulated amphotericin B. *Antimicrob. Agents Chemother.* **25**:387–389 (1984).
22. Hope, M. J., M. B. Bally, C. Webb, and P. R. Cullis. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* **812**:55–65 (1985).
23. Juliano, R. L., and D. Stamp. Interaction of drugs with lipid membranes: characteristics of liposomes containing polar or non-polar anti-tumor drugs. *Biochim. Biophys. Acta* **586**:137–145 (1979).
24. McDonald-Armstrong, W., and A. Rothstein. Discrimination between alkali metal cations by yeast. II. Cation interactions in transport. *J. Gen. Physiol.* **50**:967–988 (1967).
25. Borst-Pauwels, G. W., P. Schnetkamp, and P. van Well. Activation of Rb +

and Na + uptake into yeast by monovalent cations. *Biochim. Biophys. Acta* **291**:274–279 (1973).
26. Kotyk, A., and K. Janacek. *Cell Membrane Transport: Principles and Techniques*. Plenum Press, New York (1975).
27. Rose, H. G., and M. Oklander. Improved procedure for the extraction of lipids from human erythrocytes. *J. Lipid Res.* **6**:428–431 (1965).
28. Ways, P., and D. J. Hanahan. Characterization and quantification of red cell lipids in normal man. *J. Lipid Res.* **5**:318–327 (1964).
29. Szoka, F., Jr., and D. Papahadjopoulos. Comparative properties and methods in preparation of lipid vesicles (liposomes). *Annu. Rev. Biophys. Bioeng.* **9**:467–508 (1980).
30. Juliano, R. L., and D. Stamp. Pharmacokinetics of liposome encapsulated anti-tumor drugs. *Biochem. Pharmacol.* **27**:21–27 (1978).
31. Garty, H., B. Rudy, and S. D. Karlish. A simple and sensitive procedure for measuring isotope fluxes through ion specific channels in heterogenous populations of membrane vesicles. *J. Biol. Chem.* **258**:13094–13099 (1983).
32. Rozantsev, E. G. *Free Nitroxyl Radicals*. Plenum Press, New York (1970).
33. Gaffney, B. J., and C. M. McNamee. Spin label measurements in membranes. *Methods Enzymol.* **32**:161–198 (1974).
34. Shimshick, B. J., and H. M. McConnell. Lateral phase separations in phospholipid membranes. *Biochemistry* **12**:2351–2360 (1973).
35. Bolard, J., M. Seigneuret, and G. Boudet. Interaction between phospholipid bilayer membranes and the polyene antibiotic amphotericin B: lipid state and cholesterol content dependence. *Biochim. Biophys. Acta* **599**:280–293 (1980).
36. Bolard, J., A. Vertut-Croquin, B. E. Cybulska, and C. M. Gary-Bobo. Transfer of the polyene antibiotic amphotericin B between single-walled vesicles of dipalmitoylphosphatidylcholine and egg-yolk phosphatidylcholine. *Biochim. Biophys. Acta* **647**:241–248 (1981).
37. Ahnert-Hilger, G., G. S. Chhatwal, H. J. Hessler, and E. Habermann. Changes in erythrocyte permeability due to palytoxin as compared to amphotericin B. *Biochim Biophys. Acta* **688**:486–494 (1982).
38. Findlay, E. J., and P. G. Barton. Phase behavior of synthetic phosphatidylglycerols and binary mixtures with phosphatidylcholines in the presence and absence of calcium ions. *Biochemistry* **17**:2400–2405 (1978).
39. Van Dijck, E., L. R. Van Damme, P. W. van Dijck, B. de Kruijff, A. J. Verkleij, L. L. van Deenen, and J. de Gier. Comparative studies on the effects of pH and Ca2+ on bilayers of various negatively charged phospholipids and their mixtures with phosphatidylcholine. *Biochim. Biophys. Acta* **512**:84–96 (1978).
40. Tardieu, A., V. Luzzati, and F. C. Roman. Structure and polymorphism of the hydrocarbon chains of lipids: a study of lecithin-water phases. *J. Mol. Biol.* **75**:711–733 (1973).
41. Dufourc, E. J., I. C. Smith, and H. C. Jerrell. Interaction of amphotericin B with membrane lipids as viewed by 2H-NMR. *Biochim. Biophys. Acta* **778**:435–442 (1984).
42. van Hoogevest, P., and B. de Kruijff. Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didocosenoyl phosphatidylcholine. A refinement of the model for the formation of pores by amphotericin B in membranes. *Biochim. Biophys. Acta* **511**:397–407 (1978).
43. Sasumov, K. M., M. P. Borisova, L. N. Ermishkin, V. M. Potseluyev, A. Y. Silberstein, and V. A. Vainshtein. How do ion channel properties depend on the structure of polyene antibiotic molecules. *Biochim. Biophys. Acta* **551**:229–237 (1979).
44. Finkelstein, A., and R. Holz. Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B in *Membranes*. Vol 2: *Lipid Bilayers and Antibiotics* (G. Eisenman, ed.). Marcel Dekker, Inc., New York, 377–408 (1973).
45. Clejan, S., and R. Bittman. Rates of amphotericin B and filipin association with sterols. A study of changes in sterol structure and phospholipid composition of vesicles. *J. Biol. Chem.* **260**:2884–2889 (1985).
46. Dufourc, E. J., I. C. Smith, and H. C. Jarrell. Amphotericin and model membranes. The effect of amphotericin B on cholesterol-containing systems as viewed by 2H-NMR. *Biochim. Biophys. Acta* **776**:317–329 (1984).
47. Nichols, J. W., and R. E. Pagano. Kinetics of soluble lipid monomer diffusion between vesicles. *Biochemistry* **20**:2783–2789 (1981).
48. Roseman, M. A., and T. E. Thompson. Mechanisms of the spontaneous transfer of phospholipids between bilayers. *Biochemistry* **19**:439–444 (1980).
49. Wharton, S. A., and C. Green. Effect of sterol structure on the transfer of sterols and phospholipids from liposomes to erythrocytes *in vitro*. *Biochim. Biophys. Acta* **711**:398–402 (1982).
50. Herve, M., B. Cybulska, and C. M. Gary Bobo. Cation permeability induced by valinomycin, gramicidin D and amphotericin B in large unilamellar vesicles studied by ^{31}P-NMR. *Eur. Biophys. J.* **12**:121–128 (1985).

Send reprint requests to: Dr. R. L. Juliano, Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514.