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Susceptibilities of Phospholipid Vesicles Containing Different Sterols to Amphotericin B–Loaded Lysophosphatidylcholine Micelles

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To investigate the susceptibilities of fungal and mammalian cells to amphotericin B (AmB), AmB-loaded lysophosphatidylcholine (LPC)micelles as drug delivery vehicles were incubated at 37°C with phosphatidylcholine vesicles containing different sterols as model systems for fungal and mammalian cells. The binding and kinetics of AmB to sterols in the membranes were judged by UV-visible spectroscopy. In the 91% monomeric form, AmB interacted rapidly with ergosterol and slowly with 7-dehydrocholesterol (7-DHC), while it did not interact with cholesterol. In the 50% monomeric form, AmB formed complexes more rapidly with ergosterol or 7-DHC than in the monomeric form, whereas it did not still interact with cholesterol. The interaction was also characterized by resonance energy transfer between the fluorescent probe trimethylammonium diphenylhexatriene (TMA-DPH) and AmB. In the 91% monomeric form, AmB caused initial fluorescence quenching in bilayer membranes containing any sterol as well as sterol-free bilayer membranes due to the release of AmB and its incorporation within the membranes. However, a second phase of increasing fluorescence was found in the case of ergosterol alone. On the other hand, in the 47% monomeric form, AmB gave a biphasic intensity profile in membranes containing any sterol as well as sterol-free membranes. However, the extent of the second phase of increasing fluorescence intensity was markedly dependent upon sterol composition. Studies using sterol-containing vesicles provide important insights into the role of the aggregation state of AmB in its effects on cells.

Key words: amphotericin B, drug delivery system, lysophosphatidylcholine micelle, resonance energy transfer, sterol.

Abbreviations: AmB, amphotericin B; DMPC, 1,2-dimyristoyl-*sn*-glycero- 3-phosphocholine; DMSO, dimethyl sulfoxide; 7-DHC, 7-dehydrocholesterol; EPC; egg-yolk phosphatidylcholine, LPC; egg-yolk lysophosphatidylcholine, TMA-DPH; 1-[4-(trimethylammonia)phenyl]-6-phenylhexa-1,3,5-triene.

Amphotericin B is the most potent and effective antibiotic for the treatment of systemic fungal infections, despite its unpleasant toxicity (*1*). Such systemic fungal infections, notably caused by species *Candida* and *Aspergillus*, are a major cause of death in patients suffering from leukemia, lymphoma and a number of immunodeficiency diseases including AIDS (*2–4*). Recently, AmB has been shown to delay the accumulation of the pathological form of PrP (*5*, *6*). An interesting recent work also involved expanding the therapeutic role of AmB to diseases other than fungal infections (*7*). Prevailing models of the mechanism of action of AmB postulate the formation of ion-permeable membrane channels arising from the complexing of AmB with ergosterol present in fungal cell membranes as the direct cause of its activity and toxicity (*8*). While complexes can be formed with cholesterol in mammalian cell membranes, the selective toxicity of AmB for fungi is believed to result from its greater affinity for ergosterol (*9–12*). However, the molecular bases for the interactions of AmB with ergosterol and cholesterol are not known in detail, and neither is the relationship between the aggregation states of AmB and its better selectivity toward ergosterol. On the other hand, with respect to the interaction between AmB and sterols, it has been recently proposed that 7-dehydrocholesterol (7-DHC), the last metabolic intermediate in the cholesterol biosynthesis pathway, has a higher affinity for AmB than cholesterol (*13*). The absence of feedback regulation in cholesterol biosynthesis in hepatomas suggests the substitution of cholesterol with its biosynthetic precursors such as 7-DHC (*14*). Therefore, Feigin (*15*) has proposed to a way to replace cholesterol in hepatomas with 7-DHC as a new application for AmB in chemotherapy.

It has been proposed that the toxicity of AmB in the monomeric state toward mammalian cells is significantly decreased without impairment in its antifungal activity, whereas AmB in the aggregated state is nonselective and toxic to both kinds of cells (*16*, *17*). It has been confirmed pharmacologically that the formulation of monomeric AmB greatly reduces the acute and chronic side effects of the parent drug (*18*). This recent understanding has lead to renewed interest in the formulation of AmB, in particular the delivery of monomer or deaggregated AmB (*19*). The main strategy involves the development of drug delivery systems such as surfactant micelles (*20–22*), liposomes (*17*, *23*, *24*), and lipid emulsions (*25*, *26*).

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Unfortunately, a definitive anti-mycotic drug delivery system without side effects has not yet been developed.

Recently, we developed a novel drug delivery system in which AmB-loaded lysophosphatidylcholine (LPC) micelles adopt a more favorable monomeric form than that in any drug formulation (*27*). With LPC micelles containing little aggregated AmB, the formation of AmB-ergosterol complexes occurs progressively with increasing ergosterol but the formation of AmB-cholesterol complexes is slight. In contrast, with LPC micelles containing large amounts of aggregated AmB, the formation of AmB-cholesterol as well as AmB-ergosterol complexes occurs rapidly. These results indicate that the selectivity of AmB appears when used in its monomeric or, preferably, slightly aggregated form. Thus, the aggregation state of AmB can be regulated by using AmB in LPC micelle systems.

Conventional methods of sterol-AmB binding analysis have not been applied to the sterol-AmB system. However, spectrometry is applicable because the absorption spectrum of AmB is very sensitive to conformational changes induced by the aggregation of the polyene or by its complexation with other compounds. Absorption spectral changes in AmB with a decrease in the band at the longest wavelength occur when its forms complexes with sterols (*27–29*). On the other hand, in accordance with the general observations of heptaenes, AmB does not fluoresce (*1*), so that quantitative measurements of AmB binding to sterols incorporated into phosphatidylcholine vesicles is technically difficult. However, the molecular actions of AmB on the cell membranes of fungal and mammalian cells have been investigated using the fluorescent membrane probe 1-[4-(trimethylammonia)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) to screen fungal cells for susceptibility to AmB (*30–33*). Hence, we utilized both the absorbance of AmB and the fluorescence properties of TMA-DPH to monitor the time course of binding of AmB-loaded LPC micelles as a drug delivery system to sterol-containing phospholipid vesicles as membrane models of fungal and mammalian cells. To further investigate the mechanism of action of AmB-loaded delivery systems, we have investigated the ratio of free monomeric AmB with its action against fungal and mammalian cells.

It is the purpose of the present work to explore the susceptibilities of phospholipid vesicles containing different sterols as models for fungal and mammalian cells to AmB-loaded LPC micelles as a drug delivery system. We first describe the formation of AmB complexes with sterols by measuring the absorption spectra. We then utilized the fluorescence properties of TMA-DPH to monitor the formation of AmB complexes with sterols. Finally, we discuss the results in terms of the structural differences in the sterols.

MATERIALS AND METHODS

Materials—Amphotericin B (AmB) was purchased from Sigma (St. Louis, MO). Cholesterol, ergosterol, and 7-dehydrocholesterol (7-DHC) were from Sigma, and had purities of 99+%, approx. 90%, and approx. 85%. Ergosterol and 7-DHC were recrystallized twice from ethanol. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Sigma with a purity of approximately 99%. Egg-yolk lysophosphatidylcholine (LPC) was from Wako Pure Chemical. LPC contained 66% palmitic, 24% stearic, 6% oleic, and 1% linolic acids at position 1, with an average molecular weight of 504. Egg-yolk phosphatidylcholine (EPC) was from Nacalai Tesque. The fluorescent probe TMA-DPH was purchased from Sigma with a purity of approximately 95%. Stock solutions of TMA-DPH were prepared in methanol and kept at 4°C. The concentration of TMA-DPH was determined using an extinction coefficient at 355 nm of 7.4×10^4 $M^{-1}\cdot cm^{-1}$ (*33*). All solvents and other reagents were of the highest purity available and used without further purification.

Preparation of AmB-Loaded LPC Micelles—A stock solution in which AmB was dissolved in 1-propanol/DMSO (1:1 v/v) at a concentration of 1 mM was prepared. LPC was added to the water (0.1 mg/ml), and the mixture was sonicated in a Bath-type sonifier (Branson Model Sonicator, Yamato) at 50°C. Subsequently, the stock solution of AmB was added to various amounts of a LPC dispersion so that the concentration of AmB was 20 μM. Finally, the mixtures were sonicated at 50°C for 30 min. Three concentrations of AmB-loaded LPC micelles, in LPC to AmB molar ratios of 100, 50, and 25, were used for UV-visible absorption spectroscopy. Also two concentrations of AmB-loaded LPC micelles (LPC to AmB ratios of 100 and 25) were used for fluorescence spectroscopy.

Preparation of Sterol-Containing Phospholipid Vesicles—DMPC (or EPC) and variable amounts of sterols (cholesterol, ergosterol, or 7-DHC) were codissolved in chloroform, and aliquots of the solutions were transferred to sample tubes, and the solvent was completely evaporated at 70°C *in vacuo* for 1 h to give thin, homogeneous films. Subsequently, each sample was hydrated in water, then sonicated at 50°C for 30 min. In this procedure, the dispersions were shown to consist of small unilamellar vesicles, as described in our previous paper (*27*). The concentrations of sterol and DMPC used for UV-visible absorption spectroscopy were 160 and 147 μM, respectively. The EPC vesicles labelled with TMA-DPH as a fluorescent probe were used for fluorescence measurements. TMA-DPH dissolved in methanol was dispersed into the sample at a concentration of approx. 10 μM. The concentration of methanol in the sample was 0.025%, which was previously shown to have no significant effect on cell viability (*33*). The quantum yield of this probe in aqueous solution is virtually zero, yet, as the probe is incorporated into the hydrophobic environment of the membrane, the fluorescence intensity increases dramatically. The concentrations of sterol and EPC used for fluorescence measurement were 40 μM and 0.011%, respectively.

AmB Binding as Measured by Absorption Spectroscopy—By forming complexes of AmB with sterol, the absorbance at 413 nm decreases enormously (*28*), because free AmB in the monomeric form exhibits a very high molar extinction coefficient ($\varepsilon = 1.7 \times 10^5$ $M^{-1}\cdot cm^{-1}$), while complexes exhibit a low value ($\varepsilon = 2 \times 10^4$ $M^{-1}\cdot cm^{-1}$) (*28*, *30*) with an acceptable level of error (±5%). Hence, the formation of AmB complexes with sterols can be monitored by the decrease in absorbance at 413 nm. For monitoring the kinetics of the formation of AmB complexes with sterols, LPC micelles containing AmB and phos-

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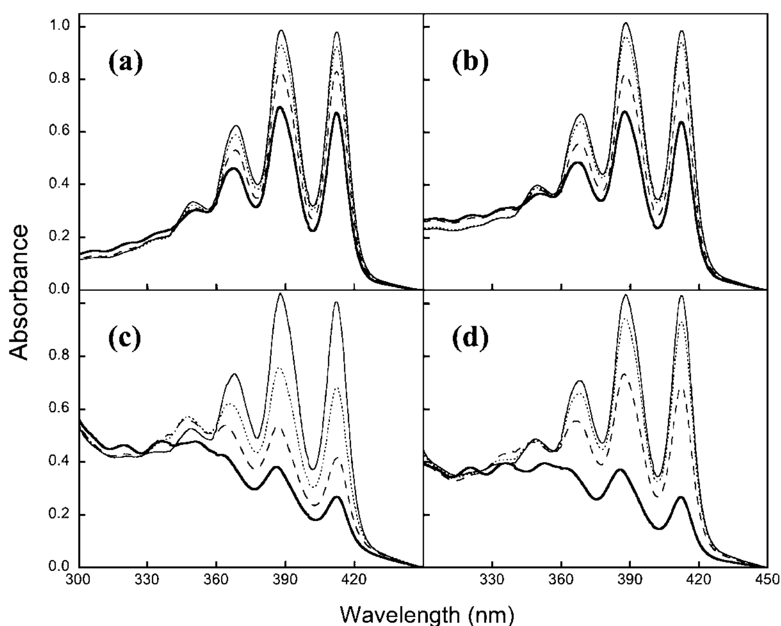

Fig. 1. **Changes in the UV-visible absorption spectrum of AmB when 91% monomeric AmB-loaded LPC micelles are incubated with cholesterol-, ergosterol-, or 7-DHC-containing DMPC vesicles, or with sterol-free DMPC vesicles, at 37°C.** Curves (a)–(d) correspond to sterol-free, cholesterol, ergosterol, and 7-DHC, respectively. The ratio of LPC to AmB was 100. Samples were prepared as described in "MATERIALS AND METHODS." All representative spectra are shown at constant concentrations of AmB (10 μM), sterol (80 μM), and DMPC (74 μM). From top to bottom, the depicted spectra correspond to incubation times of 0, 1, 6, and 24 h.

phatidylcholine vesicles containing the different types of sterols were separately preincubated at 37°C for 5 min. Then, equal volumes of the dispersions were mixed, and the mixtures were incubated at 37°C for the desired periods. Subsequently, aliquots were taken from the reaction system and used immediately for spectrometry. The final concentrations of AmB, sterol, and DMPC in all samples were 10, 80, and 74 μM, respectively. Absorption spectra (between 300 and 450 nm) of AmB were measured at room temperature against a DMPC vesicle as a blank. Absorption spectra were recorded with a double-beam spectrophotometer (type Ubest V-530, JASCO). All measurements were taken in 1.0 cm path length quartz cuvettes.

AmB Binding as Measured by Fluorescence Spectroscopy—TMA-DPH has been used as a specific marker in the plasma membranes of various types of cells (*30*). The fluorescence energy transfer from TMA-DPH to AmB was used to judge the binding of AmB to sterols in the phosphatidylcholine vesicles, according to the procedure described by Jullien *et al*. (*31*). Since the emission spectrum of TMA-DPH overlaps the absorption spectrum of AmB, energy transfer can occur between the two molecules when AmB is near the probe. As AmB does not fluoresce, the energy transfer results in a quantifiable decrease in the fluorescence of TMA-DPH proportional to the quantity of AmB bound to the membranes. Resonance energy transfer efficiency is a function of the surface density of the energy acceptor. For fluorescence spectroscopy, EPC was used in the place of DMPC, because no difference between EPC and DMPC was found in a preliminary experiment. After aliquots of TMA-DPH solution were added to sterol-containing EPC vesicles, the mixtures were sonicated in a Bath-type sonifier at 60°C for 1 h, then quickly cooled to room temperature. Under these experimental conditions, it has been shown that substantially all TMA-DPH is incorporated into the vesicles according to a partition equilibrium (*34*). For measuring the energy transfer from TMA-DPH to AmB, the AmB-loaded LPC micelles and the probe-labelled EPC vesicles containing sterols were preincubated separately at 37°C for 5 min. After equal volumes of the dispersions were mixed, the mixture was incubated at 37°C for the desired periods. Subsequently, aliquots were taken from the reaction system and used immediately for fluorescence measurement. The final concentrations of AmB, TMA-DPH, sterol, and EPC were 10 μM, 5 μM, 20 μM, and 0.0055%, respectively. Fluorescence measurements were performed on a fluorescence spectrophotometer (type FP-175, JASCO). The excitation and emission wavelengths were 365 and 427 nm, respectively.

RESULTS

Susceptibilities of Sterols in Vesicles to AmB as Judged by Changes in the Absorption Spectra—It has been proposed that monomeric AmB is nontoxic, but causes fungal cells to leak, presumably due to its selective interaction with ergosterol, while aggregated AmB is nonselective, *i.e.*, forms pores in both mammalian and fungal cell membranes (*35*, *36*). The degree of aggregation of AmB can be monitored by the absorption spectrum characteristics of AmB (*1*, *36–39*). We have shown previously that in LPC micelles, 90% or more of the AmB is in a monomeric form when the LPC to AmB ratio approaches 200, but in all drug formulations, has less than 60% of the AmB is in a monomeric form. Even AmBisome, regarded as one of the best drug systems, less than 80% of the AmB is in a monomeric form (*27*). Thus, AmB in LPC micelles adopts a more favorable monomeric form than that of any drug formulation. Figure 1 shows changes in the absorption spectra of AmB when AmB-loaded LPC micelles (LPC to AmB ratio 100) are incubated with cholesterol-, ergosterol-, or 7-DHC-containing DMPC vesicles, or with sterol-free DMPC vesicles, at 37°C. The spectrum of AmB-loaded LPC micelles alone has been found to exhibit four bands with absorbance maxima at 350 (band 1), 368 (band 2), 388 (band 3), and

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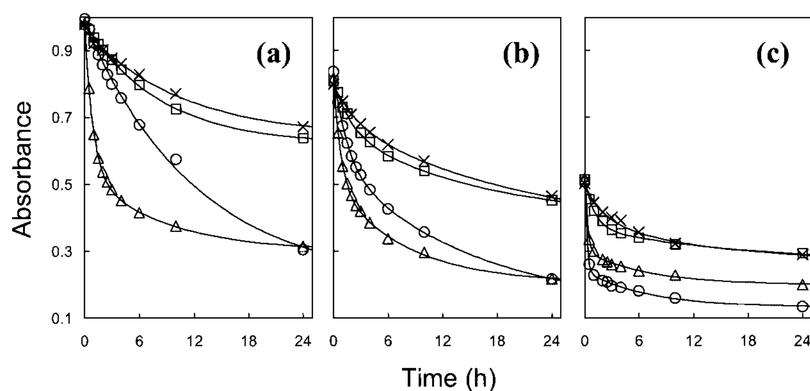

Fig. 2. **Time course of the absorbance of band 4 of AmB when 91% (a), 74% (b), and 47% (c) monomeric AmB-loaded AmB are incubated with cholesterol-, ergosterol-, or 7-DHC-containing DMPC vesicles, or with sterol-free DMPC vesicles, at 37°C.** Data derived from Fig. 1. Curves (×), (squares), (circles), and (triangles) correspond to sterol-free, cholesterol, 7-DHC, and ergosterol, respectively. The values are the averages of three experiments.

413 nm (band 4) (*27*). Band 1 is regarded as characteristic of AmB in the aggregated state, while band 4 is regarded as characteristic of AmB in its monomeric form, because AmB in an aggregated form has no band 4 (*27*, *38–40*). As shown in Fig. 1, no significant band 1 was detected in the AmB-loaded LPC micelles prior to incubation, showing that AmB is essentially completely dissociated into the monomeric form. The proportion of monomeric AmB can be obtained from the absorbance ratio of band 4 in micelles to that in 1-propanol/dimethylsulfoxide (*27*). In Fig. 1, the proportion of monomeric AmB was found to be 91% in LPC micelles. On the other hand, a decrease in band 4 is attributable to the formation of complexes between AmB and sterols, as described under "MATERIALS AND METHODS." When virtually monomeric (91%) AmB-loaded micelles were incubated with sterol-free DMPC vesicles, a small decrease in band 4 was observed over the course of incubation, reflecting the incorporation of AmB into the phospholipid bilayers and the subsequent formation of aggregates. When the virtually monomeric AmB-loaded LPC micelles were incubated with ergosterol-containing vesicles, three bands (bands 2, 3, and 4) decreased significantly with time, but band 1 remained unaltered, due to the incorporation of AmB into the phospholipid bilayers and the subsequent formation of the complexes with ergosterol. On the other hand, with cholesterol-containing vesicles, the decrease in band 4 was almost the same as in the case of the sterol-free vesicles over the course of incubation.

Figure 2a shows changes in the absorbance of band 4 when the 91% monomeric AmB-loaded LPC micelles were incubated with cholesterol-, ergosterol-, or 7-DHC-containing DMPC vesicles, or with sterol-free DMPC vesicles, at 37°C, the data derived from Fig. 1. With the AmB-loaded LPC micelles alone, band 4 remained almost unaltered over the course of incubation (data not shown). However, when the AmB-loaded LPC micelles were incubated with sterol-free DMPC vesicles, band 4 decreased slightly over the time course. This suggests that AmB is incorporated from the interior of micelles into the vesicle membranes, and undergoes subsequent self-aggregation, because the absorbance of band 4 obviously decreases when monomeric AmB forms aggregates in which the polyene chromophores are stacked so as to interact electronically (*24*, *41*). When the AmB-loaded LPC micelles were incubated with ergosterol-containing vesicles, band 4 decreased rapidly with time as compared with sterol-free vesicles. The result shows that AmB interacts rapidly with ergosterol in the membranes of vesicles. On the other hand, when the AmB-loaded LPC micelles were incubated with 7-DHC-containing vesicles, band 4 decreased slowly with time and the decrease in absorbance approached the value of the ergosterol-containing vesicles. This implies that AmB interacts slowly with 7-DHC-containing vesicles. However, when the AmB-loaded LPC micelles were incubated with cholesterol-containing vesicles, the decrease in the absorbance of band 4 over time was almost same as in sterol-free vesicles. This result suggests that virtually monomeric AmB hardly interacts with cholesterol in membranes.

Figure 2, b and c, shows the changes in the absorbance of band 4 when AmB-loaded LPC micelles (LPC to AmB ratios of 50 and 25) were incubated with choleserol-, ergosterol-, or 7-DHC-containing DMPC vesicles, or with sterol-free DMPC vesicles, at 37°C. The proportions of monomeric AmB in the micelles at LPC to AmB ratios of 50 and 25 were found to be 74% and 47%, respectively. With the slightly aggregated AmB (74% monomeric form), a rapid decrease in band 4 occurred that is attributable to the formation of complexes between AmB and both ergosterol and 7-DHC (Fig. 2b). Especially, the decrease in band 4 with the slightly aggregated AmB/7-DHC-containing vesicles was more pronounced that with the virtually monomeric AmB/7-DHC-containing vesicles. However, when the slightly aggregated AmB-loaded LPC micelles were incubated with cholesterol-containing vesicles the change in band 4 over time was almost the same as for sterol-free vesicles, showing that no significant formation of complexes between AmB and cholesterol occurred. With highly aggregated AmB (47% monomeric form), the formation of complexes between AmB and 7-DHC was slightly higher than with ergosterol (Fig. 2c). However, no significant complex formation between AmB and cholesterol occurred even when the highly aggregated AmB was used.

Susceptibilities of Vesicles Containing Sterols to AmB as Measured by TMA-DPH Fluorescence—TMA-DPH solubilized in methanol was found to incorporate readily into the vesicle membranes as evidenced by a significant increase in fluorescence intensity upon the addition of the probe. This increase in fluorescence intensity is characteristic of TMA-DPH embedded in a lipid environment since the quantum yield of TMA-DPH in the membrane is much higher than that in water (*33*). Figure 3 depicts the typical fluorescence emission spectrum of TMA-DPH when incorporated into EPC vesicles, together with the

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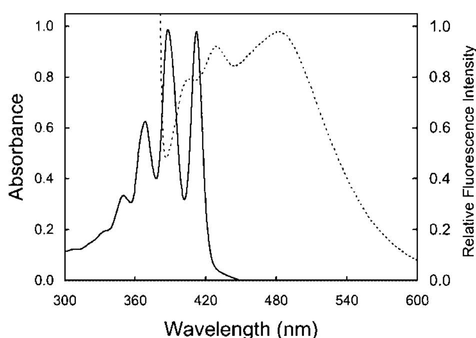

Fig. 3. **Absorption spectrum of AmB-loaded LPC micelles (solid curve) and the fluorescence spectrum of EPC vesicles containing TMA-DPH (broken curve).** The concentrations of AmB and TMA-DPH were 10 μM and 5 μM, respectively. Excitation wavelength was 365 nm.

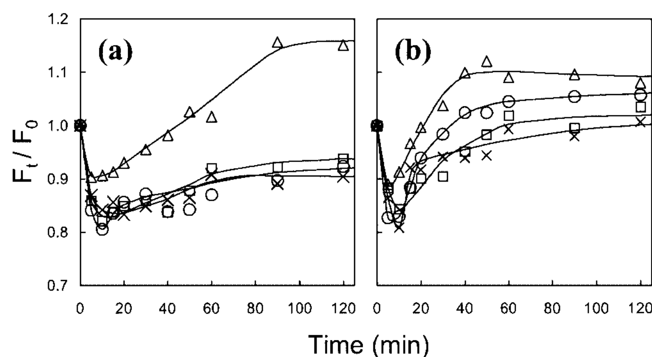

Fig. 4. **Changes in the relative fluorescence intensity (*Ft*/*F*0) of TMA-DPH when 91% (a) and 47% (b) monomeric AmB-loaded LPC micelles were incubated with cholesterol-, ergosterol-, or 7-DHC-containing EPC vesicles, or with sterol-free EPC vesicles, at 37°C.** Curves (×), (squares), (circles), and (triangles) correspond to sterol-free, cholesterol, ergosterol, and 7-DHC, respectively. For 91% and 41% monomeric AmB, the ratios of LPC to AmB were 100 and 25, respectively. Samples were prepared as described in "MATERIALS AND METHODS." All spectra are shown at constant concentrations of TMA-DPH (5 μM), AmB (10 μM), sterol (40 μM), and EPC (0.011%). The values are the averages of three experiments.

absorption spectrum of AmB-loaded LPC micelles. The emission spectrum of TMA-DPH overlaps the absorption spectrum of AmB over the range of 400–420 nm, showing that fluorescence energy transfer from TMA-DPH to AmB should occur when the two molecules are in close contact. The positively charged TMA group is at the lipid/water interface of the phospholipid bilayer where the lipids are in direct contact with membrane sterols. Moreover, TMA-DPH has an emission spectrum that partially overlays the absorption spectrum of AmB. When TMA-DPH and AmB are in close proximity, resonance energy transfer between TMA-DPH and AmB occurs, which leads to a decrease in TMA-DPH fluorescence.

Figure 4a shows changes in the relative fluorescence intensity (*F*t/*F*0) of TMA-DPH when the 91% monomeric AmB-loaded LPC micelles are incubated with cholesterol-, ergosterol-, or 7-DHC-containing EPC vesicles, or with sterol-free EPC vesicles, at 37°C. The addition of the AmB-loaded LPC micelles caused the fluorescence intensity of TMA-DPH in the bilayer membranes containing any sterol as well as in the sterol-free bilayer membranes to undergo an initial decrease for several minutes. For the ergosterol-containing membranes, this was followed by an increase in fluorescence intensity that eventually reached a saturation point. However, with the bilayer membranes containing 7-DHC or cholesterol, or with sterol-free membranes, the fluorescence intensity leveled off over the range measured.

Figure 4b shows the *F*t/*F*0 of TMA-DPH when the 47% monomeric AmB-loaded LPC micelles were incubated with cholesterol-, ergosterol-, or 7-DHC-containing EPC vesicles, or with sterol-free EPC vesicles, at 37°C. The addition of the AmB-loaded LPC micelles resulted in a sharp decrease in the fluorescence intensity of TMA-DPH in all sterol-containing membranes. For all the sterol-containing vesicles, as well as the sterol-free vesicles, this was followed by an increase in fluorescence intensity that eventually reached a saturation point. Surprisingly, the biphasic intensity profile was observed with sterol-free membranes. Moreover, the biphasic profile of the cholesterol-containing vesicles was very similar to that of the sterol-free vesicles.

DISCUSSION

Experimental evidence gathered in the past decade has shown that a close correlation exists between the aggregation state of AmB in the delivery system and its toxicity (*22*, *35*, *42*). Aramwit *et al*. (*19*) have shown that the dose-limiting toxicity of AmB depends on its self-aggregation state, the critical aggregation concentration of AmB coinciding with its concentration at the onset of toxicity. Thus, the equilibrium between monomers and aggregates seems to play a key role in drug activity (*43*). Therefore, various formulations of monomeric or, preferably, slightly aggegated AmB have been developed. We have previously demonstrated that a new formulation, LPC micelles, monomerizes more favorably than formulations such as lauryl sucrose or AmBisome (*27*). Unfortunately, an attempt to examine the pharmacological efficacy in fungal and mammalian cells with AmB-loaded LPC micelles has still not been made. However, it is deducible from the facts described herein that LPC micelles substantially decrease the toxicity of AmB to mammalian cells, while retaining the antifungal activities.

Surfactants such as lauryl sucrose and palmitoyl mannose as well as liposomes such as AmBisome, which induce a high degree of monomerization of AmB, have been shown to enhance the cellular selectivity of AmB and to decrease its toxicity to animals. Lauryl sucrose decreases the binding of AmB to cholesterol without interfering with its binding to ergosterol. For example, the toxicity of AmB toward mouse erythrocytes and cultured mouse fibroblasts is significantly decreased by low concentrations of lauryl sucrose (*22*). In another instance, the concentrations of lauryl sucrose required to inhibit the antifungal effect of AmB were about 1,000-fold higher than the concentrations that inhibited the toxicity to mammalian cells (*42*). In a further instance, lauryl sucrose micelles, in which AmB was in an almost entirely

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monomeric form, were found to be half as toxic after 24 h and about one-sixth as toxic after 1 week than the corresponding micelles of Fungizone (*44*). With a micellar formulation of amphotericin B complexed with palmitoyl mannose, similar to lauryl sucrose, the cytotoxicity of AmB was found to be decreased towards fungal and mammalian cells, while its fungistatic potential increased, its inflammatory properties were conserved, and its acute toxicity was significantly diminished (*45*). On the other hand, a liposomal formulation, AmBisome, greatly reduces the acute and chronic side effects of the parent drug as expressed by potassium efflux (*18*). AmBisome is also less toxic than Fungizone, while it maintains the antifungal activity of Fungizone. Furthermore, AmBisome is more than 1,000-fold less hemolytic to erythrocytes *in vitro* than Fungizone (*46*).

The activity of AmB depends on the presence of sterols in the membranes and the interaction between membrane sterols and AmB is responsible for the selectivity of the drug (*47*). Gruda and Dussault (*28*) have proposed that ergosterol does not react with monomeric AmB but that dimeric AmB is responsible for the formation of complexes with ergosterol in aqueous systems. When traces of dimeric AmB are present in the medium, the complexes formed most likely have the structure AmB-ergosterol-AmB. Other investigators have proposed that AmB in the monomeric or dimeric state does not react with cholesterol, but in the aggregated state does react with cholesterol (*16*, *17*). On the other hand, AmB also interacts with sterol-free bilayers when present in an aggregated form (*11*, *48–50*). Moreover, Huang *et al*. (*51*) have proposed that when AmB is present in a relatively highly aggregated state it forms channels in bilayer membranes. Thus, it is still uncertain whether AmB forms complexes with cholesterol in bilayer membranes or not. Therefore, the present work was undertaken to investigate the formation of complexes upon AmB uptake from LPC micelles into phospholipid vesicles containing sterols.

The selectivity of AmB complex formation among sterols as judged by spectral analysis differed markedly when virtually monomeric AmB was used, that is, rapid complex formation of AmB with ergosterol occurred. However, with both virtually monomeric AmB and highly aggregated AmB, no significant complex formation with cholesterol was observed over the time course studied (Fig. 2). Therefore, our results imply that AmB does not form complexes with cholesterol, even in its aggregated form. This is also the interpretation of the fluorescence measurements, as described below.

The detailes of the interaction of AmB with sterols in cell membranes, as well as the formation of transmembrane pore structures, are still imperfectly understood. Hervé *et al*. (*48*) have postulated that AmB binds to ergosterol in the fungal cell membrane and to cholesterol in mammalian cells via hydrogen bonds and van der Waals forces. It is mainly claimed that hydrophobic interactions occur between the steroid skeleton and the alkyl tail of sterols and the rigid polyenic part of the macrocyclic ring of AmB. The presence of the double bond at the C-22 position in the terminal hydrocarbon chain of ergosterol makes its structure more rigid than that of cholesterol. Such rigidity may be the reason why the contraction is greater in the former system than in the latter. Moreover, Baginski *et al*., based on molecular dynamics simulations (*52*) recently proposed that the interaction between AmB and ergosterol through intermolecular hydrogen bonds is more specific than between the drug and cholesterol. These results suggest that the binding affinity of AmB for cholesterol is weak even for aggregated AmB. The present work demonstrates the markedly higher affinity of AmB for ergosterol than cholesterol in any AmB formulation. Therefore, the selective toxicity of AmB for fungi is assumed to be the result of its capacity to bind more strongly to ergosterol, the principal fungal sterol, than to cholesterol, the principal sterol in mammalian cells.

7-DHC is the metabolic intermediate immediately preceding cholesterol in the biosynthetic pathway. Smith-Lemli-Opitz syndrome, an autosomal recessive inherited birth defect, is caused by deficient enzyme activity that leads to a reduction in cholesterol and an accumulation of the cholesterol precursor, 7-DHC, in plasma and tissues (*53*). 7-DHC is structurally similar in all respects to cholesterol, except for the presence of a double bond, as in ergosterol, at position 7 of the nucleus. However, 7-DHC shows a markedly different feature in terms of its affinity for AmB from cholesterol (Fig. 2). The formation of AmB complexes with 7-DHC occurred slowly when virtually monomeric AmB was used but more rapidly when the highly aggregated AmB was used. Charbonneau *et al*. (*13*) have proposed that the affinity of AmB for 7-DHC is even greater than its affinity for ergosterol in an aqueous organic solvent. Feigin (*15*) observed a strong correlation between the sensitivity of bilayers to AmB and the presence of a 5,7-dien system in the sterol molecule. Membranes containing any sterol with a 5,7-dien system, such as ergosterol and 7-DHC, are at least ten times more sensitive to AmB than membranes containing a sterol without this feature. The presence or absence of a double bond or a methyl group in the side-chain of the sterol molecule has no effect on its sensitivity to AmB. Therefore, it can be concluded that the higher binding affinity of AmB for 7-DHC than cholesterol will be found in any drug formulation. Our findings on 7-DHC support the idea that AmB can be used in a therapeutic strategy against some forms of cancer provided that 7-DHC, through diet and adequate medication, can indeed accumulate in the membranes of malignant cells.

The addition of virtually monomeric AmB-loaded LPC micelles caused a decrease in the fluorescence intensity of TMA-DPH in phospholipid membranes containing not only any sterol but also sterol-free membranes in several minutes (Fig. 4a). This decrease in TMA-DPH fluorescence results from the resonance energy transfer between TMA-DPH as donor and AmB as acceptor. Such a profile can be interpreted as a resonance energy transfer between AmB incorporated into membranes and membrane-bound TMA-DPH (*30*, *33*). Therefore, it can be assumed that the binding of AmB to vesicle membranes is a spontaneous and relatively fast process. On the other hand, only for ergosterol-containing membranes, the fluorescence decrease was followed by an increase in intensity. The second phase of the biphasic intensity profile may be related to AmB-induced membrane alterations (*33*). Once inside the membrane, AmB not only interacts with TMA-DPH, but also forms complexes with ergos-

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terol via van der Waals interactions. The formation of AmB-ergosterol complexes is then followed by a lateral diffusion, leading to a segregation of the probe from the complex in the membranes. In general, the efficiency of resonance energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor. Therefore, this segregation probably decreases the energy transfer between AmB and the probe, thereby increasing fluorescence intensity. However, with 7-DHC- and cholesterol-containing membranes, as well as sterol-free membranes, no biphasic intensity profile was observed. This shows that the quenching of TMA-DPH fluorescence by AmB was not reversed over the time course measured (within 2 h). Therefore, it is probable that AmB in the virtually monomeric form does not interact with cholesterol. Also, no significant complex formation of the virtually monomeric AmB with 7-DHC seems to occur in several minutes. This is also deduced from the finding that AmB in the virtually monomeric form interacts slowly with 7-DHC-containing membranes as shown in Fig. 2.

The addition of the highly aggregated AmB produced a biphasic behavior not only for all sterol-containing vesicles but also with sterol-free vesicles (Fig. 4b). This implies that when aggregated AmB is incorporated into bilayer membranes lacking sterol, it binds to TMA-DPH and then forms channels in bilayer membranes, as proposed by Huang *et al*. (*51*). On the other hand, the extent of the second phase of increasing fluorescence intensity was dependent upon the sterol composition of the bilayer membranes. Moreover, the level of fluorescence intensity in the second phase was greater than the initial value. Hayenes *et al*. (*33*) have interpreted this as reflecting probe repartitioning through AmB-induced membrane pores. Once formed, these pores would induce TMA-DPH molecules remaining in the aqueous phase to partition into membranes. They have also proposed that the extent of energy transfer is due to the magnitude of pore formation via the aggregation of AmB-sterol complexes (*33*). Therefore, highly aggregated AmB is likely to interact more easily with ergosterol than 7-DHC. However, the extent of energy transfer for the cholesterol-containing vesicles was very similar as that for sterol-free vesicles. This suggests that no significant formation of AmB complexes with cholesterol occurs, as judged from the absorption spectra.

In conclusion, the mechanism underlying the formation of complexes between AmB and sterols is revealed by AmB uptake from AmB-loaded LPC micelles into sterol-containing phospholipid vesicles. From the absorption and fluorescence characteristics, it is obvious that there is a considerably higher affinity of AmB for ergosterol and 7-DHC than for cholesterol.

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