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Complexation of Amphotericin B With Egg Phosphatidylcholine Liposomes

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The complexation and physical characteristics of egg phosphatidylcholine (PC) liposome containing amphotericin B (AmB) were investigated through circular dichroism (CD) spectra, the size distribution, the turbidity change, and the calcein release. CD spectra of AmB-containing egg PC mixture exhibited a positive peak around 330 nm indicative of complexation of AmB and four negative peaks. The positive peak increased up to 2.2 millidegree/μg AmB as AmB contents increased up to 12 % (w/w), suggesting that AmB-phospholipid complexation was promoted by the antibiotics. The effective diameter of liposomes by dynamic light scattering decreased from 450 nm to 220 nm as the amount of AmB in liposomes increased from 0 to 30 % (w/w). The complexation may be responsible for the reduction in size. On the other hand, at around 1 mM deoxycholate (DOC), the relative turbidities of 5 and 10 % (w/w) AmB-containing liposome suspension were less than 1 probably due to the solubilization of the complex, while those of pure PC liposome suspension were larger than 1 at the same concentration. Deoxycholate-induced release of calcein entrapped in AmB-containing liposomes was lower than those of pure egg PC liposomes, indicating the intercalation of the drug into the bilayers. Therefore, it is concluded that in AmB/egg PC/water system, AmB-phospholipid complex coexists with AmB-containing liposomes.

Key words: Amphotericin B, Egg phosphatidylcholine, Circular dichroism, Complexation, Hemolytic ability

INTRODUCTION

For most systemic fungal infections, AmB, a polyene macrolide antibiotic, is widely used (Edwards *et al.*, 1978; Horn *et al.*, 1985). This compound has a greater avidity for ergosterol which is preponderantly present in fungi membranes than for cholesterol, the major sterol in mammalian membranes (Metha *et al.*, 1984; Archer, 1976; Chen and Bittman, 1977; Vertut-Croquin *et al.*, 1984). It is known that the antifungal activity and mammalian cell toxicity arise from the formation of a conducting pore, composed of AmB and sterol by interacting with sterol-containing membranes (Khutorsky, 1992; Hoogevest and Kruijff, 1978; De Kruijff and Demel, 1974; Bittman *et al.*, 1974). Despite a certain degree of the selectivity for fungal cells, the intravenous administration of AmB induces adverse effects (Joly *et al.*, 1992; Lopez-Berestein and Juliano, 1987). The incorporation of AmB in phospholipid vesicles resulted in a marked reduction in the toxicity of the drug in vivo and in vitro maintaining its antifungal activity (Metha *et al.*, 1984; Lopez-Berestein *et al.*, 1983; Tremblay *et al.*, 1984; Lopez-Berestein *et al.*, 1985). The reduced toxicity of AmB in vitro is ascribed to phospholipid-AmB complex concurrent with AmB-containing liposomes (Perkins, *et al.*, 1992; Janoff *et al.*, 1986). The transitions of PC liposome by nonionic surfactants and bile salts were also reported with the structure-dependence of the added surfactant and bile salts (Kim and Kim, 1991; Lee *et al.*, 1992). In this report, the evidence of AmB-phospholipid complex was presented through CD spectra and the physical properties of liposomal AmB were investigated in terms of size distribution, hemolysis, X-ray diffraction and DOC-induced solubilization.

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MATERIALS AND METHODS

Materials-PC from egg yolk, AmB, DOC and calcein were purchased from Sigma Chemical Co. RBC was obtained form the Red Cross of Chung Nam. All other reagents were analytical grade.

Preparations of AmB-containing PC liposomes-The method for preparing multilamellar liposomes containing AmB, had been described previously (Lopez-Berestein *et al.*, 1983). Amphotericin B dissolved in methanol was added to a chloroform solution of egg PC. Organic solvent was removed by a rotary evaporator fitted with an aspirator at 40°C. The lipid film was dispersed in phosphate-buffered saline (PBS, pH 8.0) and then sonicated in a tip sonicator for ten 30-sec cycles with a 30-sec rest period at room temperature. For a calcein release experiment, 50 mM calcein was used as a fluorescence marker, and the untrapped calcein was removed by a Bio-Gel A-0.5 M column (1 cm×45 cm) chromatography.

Circular dichroism-Concentration of AmB was adjusted to 20 MM and scanned using a JASCO J-720 circular dichroism spectrophotometer. Spectra of 0, 3, 6, 9 and 12 % (w/w)-AmB containing liposomes were recorded at 25°C with 1-cm path length cell.

Size distribution measurements of liposomes by dynamic light scattering (DLS)-The effective size and the size distribution of 0, 10, 20, and 30 % (w/w) AmB-containing liposomes in PBS (pH 8.0) were measured on the light scattering spectrophotometer (Brook Haven) at room temperature under dust-free conditions.

Hemolytic ability-Fresh concentrated RBC was added, at a final concentration of 2.7%, to a tube containing variable amounts of liposomal AmB, each suspended in PBS (pH 7.4). The samples were incubated for 10 hr at 37°, centrifuged at 3000 rpm for 10 min. The supernants were filtrated through membrane with a pore diameter of 220 nm to remove cells and cell debris. Hemoglobin in the supernant was determined by its absorbance at 550 nm. The absorbance of hypotonic lysis of the same amount of cells in H_2O was measured to determine complete hemolysis. The percent hemolysis was determined as follows.

$$\text{Hemolysis } \% = H_t / H_o \times 100$$

Here, H_o is the absorbance of the completely hemolysed solution and Ht is the absorbance of the solution from the sample containing 2.7% RBC and variable amount of liposomal AmB at a given time.

X-ray diffraction-Liposomal suspension was centrifuged to remove excess water and the precipitate of liposome was contained in a sample holder. The X-ray source was CuKα radiation (wavelength=1.5418 Å) and the scanning rate was 2°/min.

Solubilization of liposomes by DOC-The solubilization of liposomes by DOC was observed through its turbidity change. Various amounts of DOC were added to the suspensions of 0, 5, and 10 % (w/w) AmB-containing liposomes. The turbidity change was measured 30 min after mixing the suspension by a Hewlett Packard 8452A Diode array spectrophotometer at 582 nm where the absorbance of AmB did not occur. Lipid concentrations in all samples were adjusted to 0.02 % (w/w).

DOC-induced release of calcein from liposome-The bile salt-induced release of calcein entrapped in pure PC and 5 % (w/w) AmB-containing liposome was determined, immediately after DOC was added to each 0.02 % (w/w) liposome suspension so that the final DOC concentrations are 0, 0.6, 0.8, and 1 mM. Fluorescence was measured at 5 sec interval for 30 min with a Perkin-Elmer LS 50B spectrophotometer at 20°. The percent release of calcein was determined as follows.

$$\% \text{ release}=[(F-F_o)/(F_t-F_o)]\times 100$$

Where F_o and F are the calcein fluorescences of the liposome suspension at the initial and specific conditions, respectively. Ft is the total calcein fluorescence measured after the addition of DOC to a final concentration of 0.12 % (w/w).

RESULTS AND DISCUSSION

CD spectra-In Fig. 1, all the spectra exhibit one broad positive peak around 330 nm and four negative peaks around 356, 370, 393 and 422 nm. The positive peak increased up to 2.2 millidegree/μg AmB as AmB content was increased up to 12 % (w/w). The broad positive peak around 330 nm is an indication of the level of aggregation of AmB and can be used to measure the presence of complexed AmB (Borlard *et al.*, 1980). With AmB in dimyristoylphosphatidylcholine (DMPC)/dimyristoylphosphatidylglycerol (DMPG) (7:3, mol:mol), the population exhibiting intense positive CD peak around 330 nm comes from the drug-phospholipid complex (Perkins, *et al.*, 1992). Thus, it is believed that AmB was complexed with phospholipid and the complexation was promoted by increasing the contents of AmB.

Size distributions-The effective diameters of liposomes decreased linearly from 450 nm to 220 nm and both lower and higher bands were shifted to the left as the contents of AmB increased from 0 to 30 % (w/w) (Fig. 2). Certainly, the marked reduction in size comes from the drug addition but the effect of the drug on the size is questionable. However, AmB is hydrophobic and membrane-active and, thus, it may intercalate into the bilayer to give an AmB-phospholipid complex. Above the local concentration of ap-

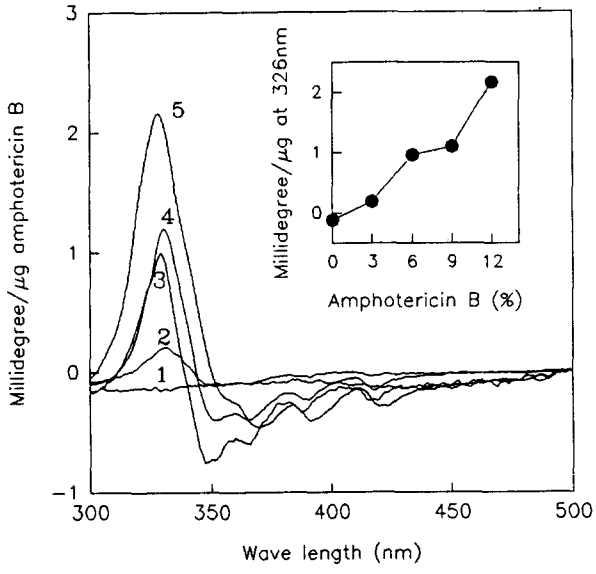

Fig. 1. Circular dichroism spectra of 0, 3, 6, 9 and 12%(w/w) AmB-containing liposomes. Concentrations of the antibiotics were adjusted to 20 μM. Plot no. of 1, 2, 3, 4 and 5 correspond to AmB contents of 0, 3, 6, 9 and 12%(w/w), respectively. Inset represents millidegree/μg AmB at 326 nm with AmB contents.

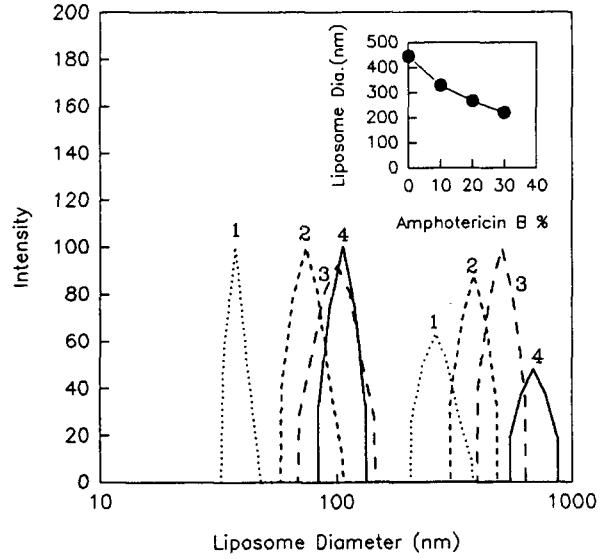

Fig. 2. Effects of AmB contents in egg PC liposomes on the size distribution of liposomes. Inset represents mean diameter of liposomes with AmB contents. 1, 0 %(w/w) 2, 10 %(w/w) 3, 20 %(w/w) 4, 30 %(w/w)

proximately 3 % (w/w) AmB, the lipid bilayer would be sufficiently disrupted into an AmB-phospholipid complex (Perkins, *et al.*, 1992). Therefore, the complexation would be responsible for the reduction in size of liposomes.

To investigate the composition of the lower and the higher bands, the liposomal suspension was filtrated

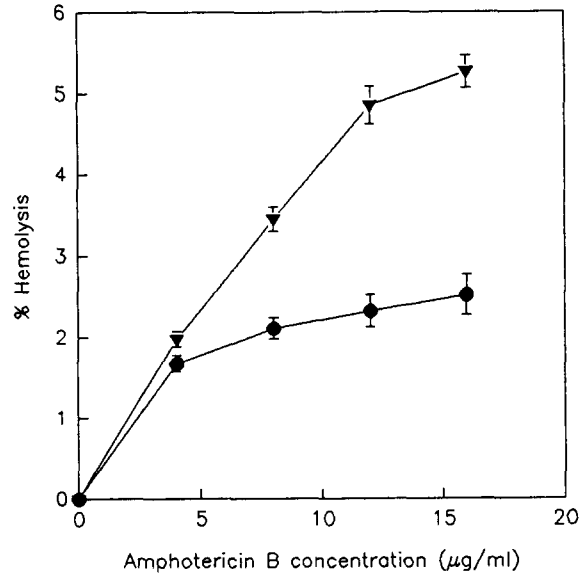

Fig. 3. Hemolysis by cake(●) and filtrate(▼) obtained from filtration of 12%(w/w) AmB-containing liposomes with membrane with pore diameter of 220 nm.

through the membrane with a pore diameter of 220 nm. The filtrate and the cake were assayed for AmB and phospholipid. The contents of AmB in the filtrate was 2.2 % (w/w) and 17.8 % (w/w) in the cake. That is, lower band is AmB-poor and the higher band is AmB-rich. Following the composition of each band, it is likely that the higher band is an AmB-phospholipid complex and the lower band is a liposome with AmB. It was reported that in the mixture of AmB and phospholipid, AmB-rich complexes coexist with AmB-poor liposomes (Perkins, *et al.*, 1992; Janoff *et al.*, 1986).

Hemolytic abilities-The in vitro toxicities of the filtrate and the cake were investigated through hemolysis. In Fig. 3, the degree of hemolysis by filtrate increases with AmB concentration and was higher than that of hemolysis by cake. Thus, it is certain that transfer of AmB from filtrate to RBC was easier than that from cake.

As mentioned previously, the cake was AmB-rich and, thus, may be an AmB-phospholipid complex. The complex takes a configuration of a drug-lipid alteration. In this configuration, AmB may be immobilized by an adjacent phospholipid and its transfer to RBC is unfavorable.

On the other hand, the filtrate was AmB-poor and may be liposomes containing AmB. In liposomes, AmB would be adsorbed onto the surface of liposmes. Since AmB molecules would be required to minimize the exposure of its hydrophobic polyene in water, It will be adsorbed more or less parallel to the plane of the liposome membrane. The hydrophobic site of the

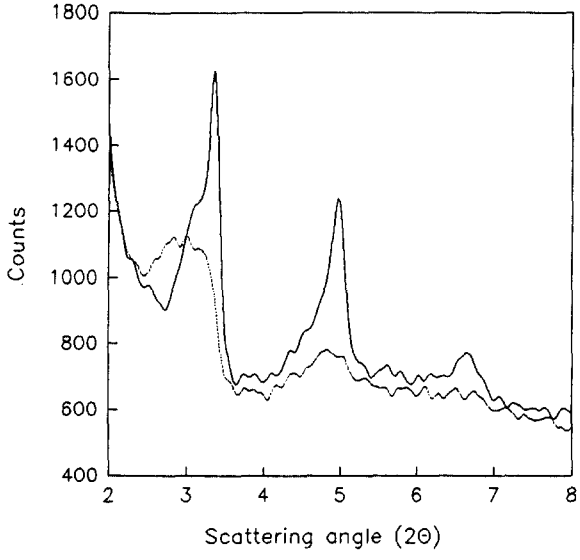

Fig. 4. X-ray diffraction pattern of pure (—) and 6 %(w/w) AmB-containing (---) liposomes.

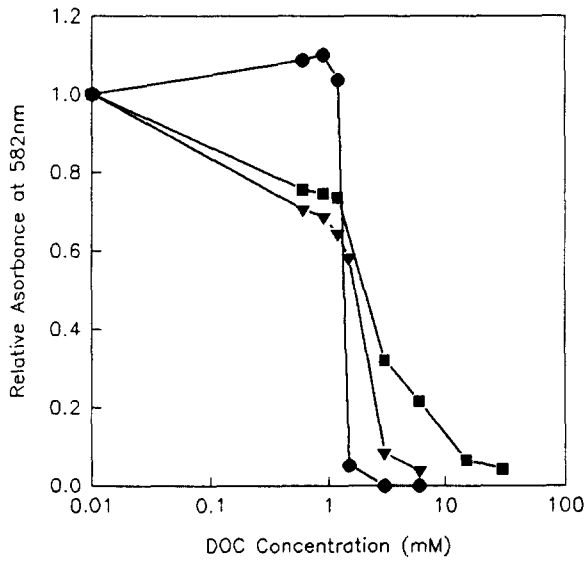

Fig. 5. Relative absorbance change of 0, 5 and 10 %(w/w) AmB-containing liposomes by DOC addition at 582 nm, 20 °C, pH 8.0. Each point is the mean of three measurements and all the SDs fall within 0.008, ● : Pure egg PC liposome, ▼: 5 %(w/w) amp B-containing liposome, ■ : 10 %(w/w) amp B-containig liposome

drug is at the membrane/water interface, while the hydrophobic site, the conjugated double bond system, anchors in the lipid bilayer. In this configuration, the transfer of the drug to RBC may be relatively easy. It was already reported that AmB in complex is more toxic than AmB in liposomes

X-ray diffraction-Fig. 4 shows the x-ray diffraction patterns of pure and 6 % (w/w) AmB-containing liposomes. With pure PC liposomes, the peak of h=2 order occured at a scattering angle 2 of 3.356°, corresponding to the a lamellar repeat distance of 52.7 Å and relatively sharp peaks were obtained. In case of 6 % (w/w) AmB-containing liposomes, the peak for n=2 order occurred at scattering angle 2 of 2.99°, corresponding to the Bragg d-spacing of 59.1 Å and peaks were relatively broad. Since a broad distribution of Bragg d-spacing exhibits Bragg reflection in a somewhat wide range of scattering angles, the broad peak indicates broad distribution of d-spacing. Thus, it is reasonable that the intercalation of AmB into the bilayer produced a broad lamellar repeat distance. The characteristics of the peak reflect only AmB-containing liposomes but not the AmB-phospholipid complex which doesn't have a lamellar structure.

Solubilization of liposome by DOC-Interestingly, at 1 mM DOC, the relative turbidity of pure PC liposome suspensions was larger than 1 but those of 5 and 10% (w/w) AmB-containing phospholipid mixture were less than 1 (Fig. 5). With pure liposomes, some DOC are inserted into the lipid bilayer and disrupt the local structure of the bilayer without loss of liposomal structure, while the others were bound to the surface of liposomes. The adsorbed DOC molecules on the surface of the liposome would produce a relative turbidity larger than 1. With the mixture of AmB and phospholipid, liposomes containing AmB coexist with the AmB-phospholipid complex. Owing to the packing effect of AmB, AmB-containing liposomal membrane is more dense than pure PC liposomal membrane. However, the complex with high curvature have packing defects so that DOC molecules readily solubilize the complex. Thus, the solubilization of the complexes may be responsible for the relative turbidity less than 1.

The relative turbidity of pure PC liposomes completely disappeared at 1.5 mM DOC, whereas in the 5 and 10% (w/w) AmB-containing phospholipid mixtures, the suspensions were more or less turbid even at 1.5 mM DOC. In AmB-containing phospholipid mixture, the remaining tubidity at this concentration of DOC may come from AmB-containing liposomes which weren't subject to the solubilization due to the packing effect of AmB.

DOC-induced calcein release-A poorly packed lipid bilayer is easily transformed into a mixed micelle by DOC. In Fig. 6 and Fig. 7, the release of calcein from liposomes by DOC was investigated as a measure of the transition. The question is that AmB really intercalates into the bilayers and packs them. As the DOC concentration increased from 0 to 1.0 mM, the calcein release increased approximately from 0% to 90% for 30 min in Fig. 6 and 7. Certainly the binding of bile salt to liposome induces the structural change of the liposome to the mixed micelle

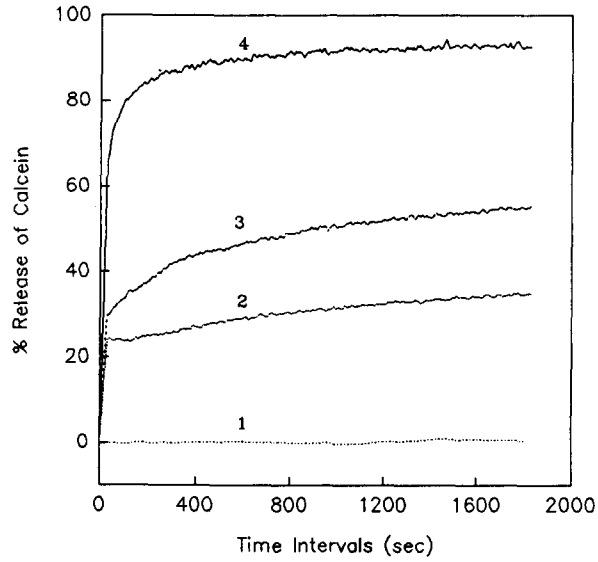

Fig. 6. Release of calcein from pure egg PC liposome by DOC addition at 15°, pH 8.0. 1, 0 mM DOC 2, 0.6 mM DOC 3, 0.8 mM DOC 4, 1.0 mM DOC

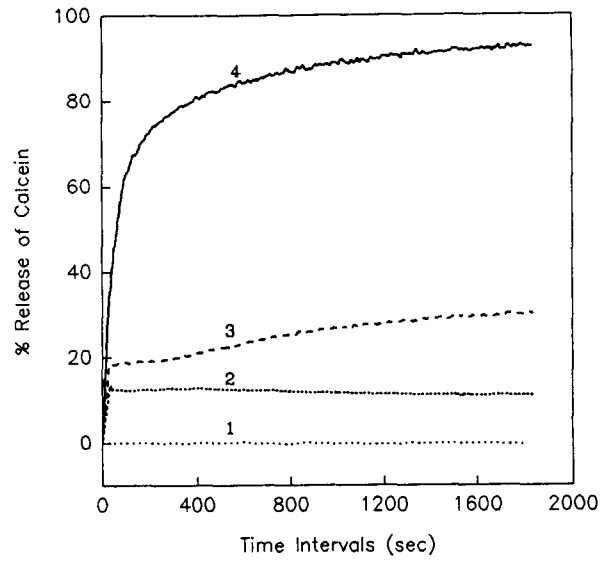

Fig. 7. Release of calcein from 5 %(w/w) AmB-containing egg PC liposome by DOC addition at 15°, pH 8.0. 1, 0 mM DOC 2, 0.6 mM DOC 3, 0.8 mM DOC 4, 1.0 mM DOC

(Schubert *et al.*, 1986; Schubert and Schmidt, 1988). So the approximately 90% leakage may be attributed to the destruction of the liposomes by the formation of mixed micelles. However, the initial rate of calcein release at 1 mM DOC from the liposomes containing 5% drug is lower than from pure PC liposomes (see two initial slopes in Fig. 6 and 7). The dense lipid matrix can be constructed by the bilayer-partitioned drug and, thus, the penetration of DOC molecules into the bilayers becomes difficult. Therefore, the lag of calcein release from AmB-containing liposomes occurs at an early stage of release. At the relatively low DOC concentrations of 0.6 and 0.8 mM, the release of calcein for 30 min was significantly suppressed. The lower level of release indicates that more liposome particles remained intact due to the close packing of the lipid matrix. The closer the lipid packing is, the less the insertion of DOC molecules into lipid bilayer is likely. Thus, it is certain that AmB molecules incorporate into the lipid bilayers and they inhibit the penetration of DOC molecules into the bilayers due to the packing and blocking effect.

In summary, the presence of an AmB-phospholipid complex was confirmed by a positive CD peak at around 330 nm. This indicates that in egg PC/AmB/water systems, AmB-phospholipid complex and AmB-containing liposomes would coexist. According to the result of DLS, a higher complexation gives the particles of smaller size. With assay for AmB and phospholipid, the complex was AmB-rich and the liposome was AmB-poor. The hemolytic ability of the complex was lower than that of AmB-containing liposomes. In the complex, immobilization of the antibiotics by adjacent phospholipid may contribute to the lower hemolytic ability of the complex. In the liposome, the antibiotics adsorbed onto the liposomal membrane give a higher hemolytic abilty due to its easy escape from liposomes. In solubilization experiments, more favorable solubilization of the mixture of AmB and phospholipid than that of pure PC liposomes at around 1 mM DOC also indicates the existence of the complex with high curvature.

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