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Toxicity of Solubilized and Colloidal Amphotericin B Formulations to Human Erythrocytes

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Abstract—The toxicity of a number of solubilized or colloidal amphotericin B formulations to human erythrocytes has been studied in-vitro. All the solubilized formulations studied, using poloxamer F127 or L92, or sodium deoxycholate as solubilizing agents, showed similar toxicity, erythrocyte lysis being greater than 90% for amphotericin B concentrations between 4 to 8 $\mu g\ mL^{-1}$. Emulsion formulations stabilized by poloxamers showed reduced toxicity, while those stabilized by egg lecithin showed less than 5% erythrocyte lysis up to an amphotericin B concentration of 200 $\mu g\ mL^{-1}$. The mechanisms of the differential toxicity is considered to be due to the differences in the equilibrium concentration of free amphotericin B in the aqueous phase.

Amphotericin B is the drug of choice for the treatment of deep-seated disseminated mycoses; it is commonly used in immunosuppressed patients such as AIDS victims and transplant patients (Medoff et al 1983). It is a metabolite of *Streptomyces nodosus* which shows selective cytotoxicity to fungal cells, and is believed to act by forming a pore in the cell membrane (Norman et al 1976). The most refined model to date (De Kruijff & Demel 1974) suggests that the pore consists of a ring of eight sterol molecules alternating with eight amphotericin B molecules in both sides of the cell bilayer. The mode of action of amphotericin B and other polyene antibiotics has recently been reviewed by Bolard (1986).

The amphotericin B complex with ergosterol in fungal cells is more strongly bound than that with cholesterol in mammalian cells, hence the selective action on fungal cells (Gale 1974; Archer 1976; Chen & Bittmann 1977; Readio & Bittmann 1982). However, toxicity to human cells is still present, and amphotericin B therapy is frequently associated with side-effects such as CNS damage, nephrotoxicity, and general malaise. Amphotericin B is clinically administered as Fungizone (Squibb), which is a buffered solubilizate in sodium deoxycholate. This is necessary since amphotericin B is insoluble in most solvents.

Amphotericin B administered in liposomes has been found to have a much reduced toxicity, while retaining its antifungal action in-vitro, in murine models, and more recently in human trials (New et al 1981; Graybill et al 1982; Lopez-Berestein et al 1983, 1985; Mehta et al 1984; Hopfer et al 1984; Juliano et al 1985). More recent studies (Payne et al 1985, 1987; Mehta et al 1986; Juliano et al 1987) have demonstrated that the reduced toxicity is dependent on liposome size, composition, and the presence of added sterols in the formulation.

Recent results from our group (Davis et al 1987) have demonstrated that an emulsion formulation of amphotericin B has a toxicity similar to that of a liposomal preparation, in that it displays reduced toxicity to human erythrocytes, while maintaining activity against *Candida albicans* in a murine model. We have now extended this work to study the erythrocyte toxicity of amphotericin B emulsions at higher concentrations, and that of emulsions stabilized with poloxamers (block copolymers based on polyoxyethylene and polyoxypropylene subunits) instead of lecithin, and additionally of amphotericin B solubilized by poloxamers.

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Materials and Methods

Materials

Amphotericin B was obtained from Sigma Chemical Co. Pluronic F127 and L92 were from BASF Wyandotte. Fungizone was obtained from the Hospital Pharmacy, Queen's Medical Centre. Lecithin was intravenous grade Lipoid E80 kindly donated by Lipoid AG, and consists of 80% phosphatidylcholine, 10% phosphatidylethanolamine and 85% acyl chain unsaturation. Soya oil was purchased from J. Sainsbury PLC. All solvents were of Analar grade and were purchased from BDH.

Three solubilizates and three emulsion formulations were studied, with the following compositions:

(a) Amphotericin B solubilized in sodium deoxycholate as the commercial formulation (Fungizone, Squibb).

(b) Amphotericin B (0·5 mg mL^{-1}) solubilized in Pluronic F127 (10%) or Pluronic L92 (10%). These formulations were produced by dissolving amphotericin B (25 mg) in methanol (100 mL), adding the Pluronic (5 g) and removing the methanol by rotary evaporation. Water (50 mL) was added and the resulting solubilizate was centrifuged to remove any insoluble material.

(c) Amphotericin B (0·5 mg mL^{-1}) in a 10% soya oil emulsion stabilized by egg lecithin (1·2%), Pluronic F127 (1%) or Pluronic L92 (1%). This was produced as described by Davis et al (1987). Typically, amphotericin B (25 mg) was dissolved in methanol (100 mL), and the emulsifier (Pluronic or lecithin) dissolved in water (100 mL) was added. The mixture was evaporated down to 30–40 mL to remove the methanol. In the case of the lecithin-stabilized formulation, it was necessary briefly to sonicate and then to re-evaporate this mixture to release any methanol entrapped in liposomal structures

present. The solubilized amphotericin was made up to 45 mL with water, 5 mL of soya oil added, and the whole emulsified by sonication (50W, 20 min in an ice bath).

Assay of amphotericin B

Amphotericin B was assayed in all formulations by dissolution in dimethyl sulphoxide, aided by sonication, followed by absorption measurement at 412 nm ($E_{412}=124000$) (Kontron Uvikon 860).

Erythrocyte toxicity assay

The erythroctye toxicity assay was conducted essentially as described by Mehta et al (1984). Red blood cells were obtained from a healthy 29 year old group O male with normal blood chemistry, and were washed three times with isotonic saline before being diluted to an absorbance of 1·0 at 540 nm. They were washed freshly each day from a sample of whole blood stored at 4°C.

The amphotericin B formulation was added to washed erythrocytes to produce final amphotericin B concentrations in the range 0–16, or 0–200 $\mu g\ mL^{-1}$ for the lecithin-stabilized emulsion. These were then incubated for 20 min at 38°C in a water bath. Cell lysis was stopped by immersion in ice water, the unlysed cells were removed by centrifugation, (10 min at 2000 rev min^{-1}) and haemoglobin assayed in the supernatant by absorbance at 541 nm. Samples lysed by emulsion formulations were too turbid for spectroscopic assay, and the haemoglobin release was estimated by reference to a set of turbid standards made from dilutions of totally lysed blood and Intralipid 10%. Using this technique, lysis could be estimated to within 5%. Control samples of 0% lysis (no amphotericin B) and 100% lysis (1 mg Fungizone added to cells) were employed in all experiments.

Physical properties of emulsions

The z-average droplet diameters were measured by photon correlation spectroscopy (Malvern K7025 correlator, CBM 3032 computer, Seimens 40 mW He-Ne laser), and the zeta potentials by quasielastic laser light scattering (Malvern Zetasizer II).

Results

Physical properties of emulsions

Table 1 lists the z-average droplet diameters of the emulsion formulations and their zeta potentials at pH7 and an ionic strength of 0·01M. Data for "Intralipid 10%" is included for comparison. Ongoing stability studies have indicated that the emulsion is chemically and physically stable for several months at 4°C.

Table 1. Physical properties of emulsion systems.

Emulsifier	Droplet diam. (nm)	Zeta potential (mV)
Lipoid E80	308+3	39+2
Pluronic L92	280+2	21+2
Pluronic F127	240+2	27+2
Intralipid 10%	267+2	48+2

Solubilizate toxicity

Fig. 1 shows the erythrocyte lysis as a function of amphotericin B concentration for the three formulations solubilized in Pluronics F127 and L92, and sodium deoxycholate (Fungizone). All formulations were highly toxic, total cell lysis occurring at 4–6 $\mu g\ mL^{-1}$ for Fungizone and Pluronic F127 solubilizate. Amphotericin B solubilized in Pluronic L92 showed slightly lower toxicity, cell lysis not being total until an amphotericin B concentration of 12 $\mu g\ mL^{-1}$ was reached.

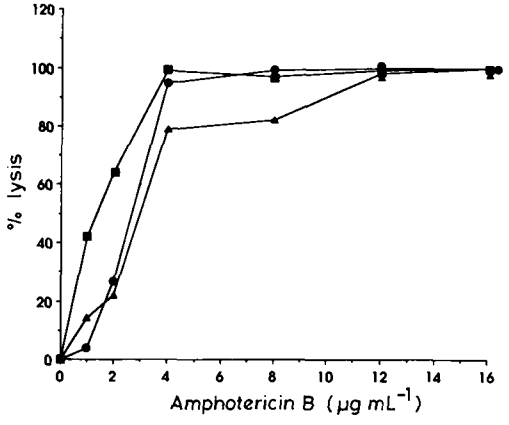

FIG. 1. Erythrocyte lysis caused by solubilized formulations. All data normalized to 100% lysis. All formulations caused 100% lysis above 12 $\mu g\ mL^{-1}$. Solubilizing agent: ■ Fungizone, ▲ Pluronic L92, ● Pluronic F127.

Emulsion toxicity

The erythrocyte lysis of the emulsions studied is shown in Fig. 2, with Fungizone for comparison. The emulsions produced with egg lecithin as stabilizer showed no detectable erythrocyte lysis up to an amphotericin B concentration of 20 $\mu g\ mL^{-1}$ and only 2–5% greater lysis than the control emulsion up to an amphotericin concentration of 200 $\mu g\ mL^{-1}$.

Erythrocytes were incubated for up to 6 h at 38°C with concentrations of lecithin-stabilized amphotericin emulsion corresponding to amphotericin concentrations of 0, (control), 10 and 60 $\mu g\ mL^{-1}$. No significant erythrocyte lysis was

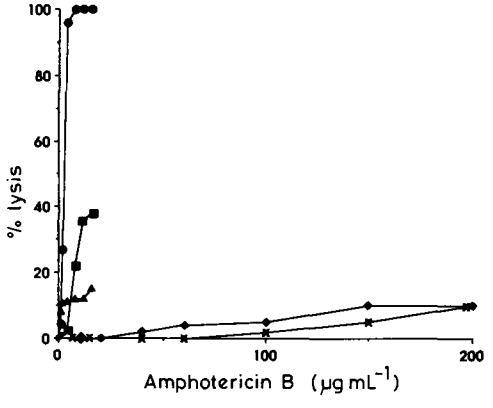

FIG. 2. Erythrocyte lysis caused by emulsion formulations. All data normalized to 100% lysis. Key: × control, ♦ amphotericin B emulsion/lecithin, ● Fungizone, ▲ amphotericin B emulsion/Pluronic L92, ■ amphotericin B emulsion/Pluronic F127.

observed under these conditions over the duration of the experiment.

The emulsion stabilized with Pluronic L92 showed gradually increasing toxicity, to a maximum of 38% lysis at 16 μg mL^{-1} amphotericin B. When Pluronic F127 was used as stabilizer, the cell lysis increased only to 16% over the concentration range studied.

Discussion

The three solubilized formulations of amphotericin B all displayed a high degree of toxicity to erythrocytes. This is in agreement with the results of Mehta et al (1984), who found total lysis at 10 μg mL^{-1} (45 min at 38°C) for 'free' amphotericin B, added as a solution in dimethylformamide. The formulation solubilized in Pluronic L92 showed slightly less toxicity than the other two, perhaps because this more hydrophobic polymer interacts less with the outer hydrophilic layer of the red cell membrane.

All the emulsion formulations examined showed less toxicity than the solubilized formulations. The Pluronic-stabilized formulations showed some toxicity, whereas the lecithin-stabilized emulsion showed very low toxicity, even at high (200 μg mL^{-1}) amphotericin concentrations. The lecithin-stabilized emulsion containing amphotericin B showed only slightly higher toxicity than the control emulsion containing no amphotericin. The highest amphotericin concentration studied (200 μg mL^{-1}) corresponds to diluting the erythrocyte sample with an equal volume of non-isotonic emulsion. This may be the cause of the slight lysis observed at amphotericin concentrations of 50 μg mL^{-1} and above.

It is possible that the low toxicity of colloidal formulations is due to an extended lag phase, so that lysis does not occur within the 20 min duration of the experiment. This was proved not to be the case, since no significant lysis was observed when erythrocytes were incubated for up to 6 h with the lecithin-stabilized amphotericin emulsion corresponding to amphotericin concentrations of 10 and 60 μg mL^{-1}.

The precise mechanism by which liposomal amphotericin B interacts with cells is poorly understood, although Juliano et al (1987) have suggested that transfer from carrier to cell is mediated by the diffusion of free amphotericin B, in a similar manner to the transfer of lipids between liposomes. They also suggest that the toxicity is dependent on the attainment of a 'threshold level' of amphotericin B in the aqueous phase before pore formation in the target cell can occur. We would propose the following refinements to this model:

(i) that the concentration of amphotericin B in the aqueous phase is determined by simple partitioning from the emulsion droplet or liposome in which it is administered. Consequently, the higher the affinity of the carrier, the lower the equilibrium concentration of amphotericin B in the aqueous phase. We would suggest that the process is not kinetically limited, since the studies of Chen & Bittman (1977) show that the transfer of amphotericin B from donor to acceptor is rapid (of the order of seconds). Thus an equilibrium concentration of amphotericin B/sterol pores in the target cell would be rapidly established

(ii) that the threshold concentration for pore formation is dependent only on the acceptor properties of the target cell. Consequently a fungal cell, with strongly binding acceptor sites, due to the presence of ergosterol, has a lower threshold concentration for pore formation than a mammalian cell containing cholesterol.

A final detail of the model is that the concept of a threshold for pore formation is consistent with the pore being an octameric species (De Kruijff & Demel 1974), since simple kinetic considerations suggest that this would lead to pore concentration being proportional to the eighth power of the monomer concentration, i.e. strongly non-linear.

The results can now be rationalized on a comparative basis by considering the affinity of the drug carrier (micelle, emulsion, or liposome) for the amphotericin B. Pluronics probably solubilize amphotericin B in the palisade layer of polyoxyethylene groups. Consequently diffusion out of the micelle is rapid, and a high amphotericin B monomer concentration is established in the continuous phase. This is also the case with the commercial Fungizone formulation. The reason for the low toxicity of the liposomal and lecithin-stabilized emulsions is probably due to the strong amphotericin B-phospholipid interaction as demonstrated by binding studies (Chen & Bittmann 1977; Bolard et al 1980; Witzke & Bittmann 1984; Dufourc et al 1984). These cited studies suggest that amphotericin B binds strongly to a small number of sites in the phospholipid vesicles (possibly to a charged lipid impurity). Addition of cholesterol further increases the affinity of the vesicle for amphotericin by providing a large number of weakly binding sites. Payne et al (1987) have demonstrated that ergosterol decreases the toxicity of liposomal amphotericin B, as would be expected, since ergosterol-amphotericin B complex is more strongly bound than the cholesterol complex. We are currently investigating the exact role of cholesterol in determining the toxicity of emulsion formulations.

The emulsions used in this study were stabilized using egg lecithin, the acyl chains of which are largely unsaturated. Juliano et al (1987) found that liposomes composed of unsaturated phospholipids showed little difference in toxicity to free amphotericin B; consequently the low toxicity observed for the emulsion formulation here is surprising. It is possible that the commercial lecithin used in the present work (a relatively impure mixture of lipids) contains species that can bind amphotericin B strongly.

Our emulsion formulations of amphotericin B should have considerable promise as therapeutic systems. We have already demonstrated the effectiveness of the emulsion formulation against *Candida* in mice (Davis et al 1987); emulsions have an additional advantage over liposomes in that they are straightforward to prepare by established technology.

Acknowledgements

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