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Toxicity of amphotericin B emulsion to cultured canine kidney cell monolayers

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Abstract—The effect of amphotericin B in an emulsion formulation on the integrity of monolayers of kidney cells has been studied. Whereas a conventional solubilized amphotericin formulation (Fungizone, Squibb) caused a loss in monolayer integrity at concentrations above 1 $\mu g\,mL^{-1}$, the emulsion formulation had no measurable effect on confluence at amphotericin concentrations up to 100 $\mu g\,mL^{-1}$. The emulsion retained a comparable antifungal activity to that of Fungizone against *Saccharomyces cerevisiae* in suspension culture. These results parallel the observed erythrocyte lysis data obtained previously using amphotericin B emulsions, and suggest that the emulsion formulation may have a lower toxicity and improved therapeutic potential over existing formulations.

Amphotericin B is an antifungal agent obtained from *Streptomyces nodusus*, which is used in the treatment of a number of systemic mycoses. Its mode of action is believed to involve the formation of cell membrane pores, causing loss of cell viability. The pore is possibly a cyclic complex consisting of eight amphotericin molecules alternating with eight membrane sterol molecules; the high affinity of amphotericin for the fungal membrane sterol ergosterol leads to the antifungal activity. Unfortunately amphotericin also binds, although weakly, to mammalian sterols (most notably cholesterol), and this is the origin of the severe side-effects which can arise when amphotericin is used clinically. These side-effects include general malaise and raised temperature, but most importantly nephrotoxicity, which can lead to severe kidney damage when amphotericin is administered for extended periods.

Amphotericin B administered in liposomes has demonstrated considerably reduced toxicity in-vitro, and in animal and clinical 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). The reasons for this improvement in toxicity profile are still unclear, but may be related to the relative binding affinities of amphotericin for fungal cells, mammalian cells, and for the liposomal carrier, which will serve to control the transfer of the drug between the various phospholipid membranes. Although the liposomal formulation displays considerable promise, it still has drawbacks; these include the relative novelty of liposomal technology, which leads to high development costs, and the need to use highly purified synthetic phospholipids, which appear to be necessary in order to minimize the toxicity of the formulation (Payne et al 1985, 1987; Juliano et al 1987).

Recently, our group (Davis et al 1987; Forster et al 1988; Washington et al 1988) have described the production and properties of parenteral amphotericin formulations based on a conventional intravenous fat emulsion. This appears to address the problems encountered in formulating liposomal amphotericin, in that its preparation is straightforward using well-known technology, and a low toxicity is obtained when the unsaturated egg lecithins normally used for parenteral products are used as emulsifiers. The formulation does not lyse erythrocytes and has good efficacy and low toxicity in a *Candida*-infected mouse model.

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The current communication describes a study of the effects of the amphotericin emulsion formulation on the viability and integrity of canine kidney cells grown in monolayer culture. Toxicity screens on cultured cell monolayers are of widespread use (see e.g. Stammati et al 1981; Wenzel & Cosma 1983). It is expected that the results will reflect the toxicity of the formulation to the kidney and thus be of greater clinical relevance than simpler erythrocyte lysis experiments. In order to confirm that the antifungal efficacy of amphotericin was not compromised by the emulsion formulation, the activity of the emulsion against the amphotericin B bioassay organism *Saccharomyces cerevisiae* NCYC 87 grown in suspension was also followed.

Materials and methods

Amphotericin B was kindly donated by Dumex AG (Copenhagen, Denmark). The preparation and characterization of the amphotericin B emulsion has been described previously (Davis et al 1987). Briefly, the formulation was a conventional lecithin-stabilized triglyceride emulsion with a z-average diameter of 250 nm and a zeta potential of −45 mV, in which the amphotericin was localized on the droplet surface (Washington et al 1988). The emulsion contained 20% of unsaturated soya triglycerides and was stabilized by a parenteral unsaturated egg lecithin. The final concentration of amphotericin in the emulsion before dilution was 1·0 mg mL^{-1}. Fungizone (Squibb, UK) and Intralipid (Kabi-Vitrum, Sweden) were obtained from the hospital pharmacy of the Queen's Medical Centre, Nottingham. The MDCK NBL-2 canine kidney cell line was obtained from Flow Laboratories Ltd, (High Wycombe, Bucks, UK) and maintained in MEM medium (Flow Laboratories Ltd) containing 10% foetal bovine serum, 2 mM L-glutamine, 1% non-essential amino-acids, 5 mg mL^{-1} streptomycin and 5000 int. units mL^{-1} penicillin. *Saccharomyces cerevisiae* NCYC 87 was maintained in suspension culture using medium F (Appendix XIV BP 1988). The constituents of medium F were obtained from Oxoid Ltd (Basingstoke, Hants, UK) and Difco (East Molesley, Surrey, UK).

In-vitro efficacy and kinetics of amphotericin formulations against Saccharomyces cerevisiae. Amphotercin B emulsion or Fungizone was diluted to a stock concentration of 44 $\mu g\,mL^{-1}$ into Hanks balanced salt solution (HBSS) under aseptic conditions. An inoculum of *S. cerevisiae* strain NCYC 87 was prepared at 10^8 cfu mL^{-1} in quarter strength Ringers solution and the amphotericin B formulations added to 1 mL portions of this culture at a concentration of 40 $\mu g\,mL^{-1}$. Samples (50 μL) were removed at 5, 10, 30 and 120 min after inoculation and added to 10 mL of 2% Tween 80 in peptone water. The amphotericin had previously been shown to be neutralized by the Tween 80 under these conditions.

The neutralized solution, and a 10-fold dilution, were plated out using a spiral plater onto medium F (Appendix XIV BP 1988). Colony counts were determined after 48 h incubation at 37°C using a laser counter (Don Whitley Scientific, Shipley, Yorks, UK). Initial viable counts were determined by plating successive dilutions of the original *S. cerevisiae* inoculum.

Toxicity of amphotericin formulations to canine kidney cells. The cell line (MDCK NBL-2) was grown to a confluent monolayer on Millicell HA filters from a seeding inoculum of 10^6 cells mL^{-1}. The integrity of the monolayer was measured via its resistance using an epithelial volt-ohm meter (World Precision Instruments, New Haven, CT, USA). An incubation time of 7 days was required for full confluence to be achieved.

Since the emulsion would flocculate in the presence of divalent cations, the cells on their filter supports were transferred to calcium- and magnesium-free HBSS at the commencement of the amphotericin treatment. This resulted in a gradual loss in monolayer integrity over 48 h which did not, however, obscure the experimental results. Amphotericin B formulations (emulsion and Fungizone) were immediately added to the monolayer bathing media in concentrations up to 100 $\mu g\ mL^{-1}$ from a stock formulation of 1 $mg\ mL^{-1}$. Control experiments were performed with an amphotericin-free emulsion (Intralipid 20%, diluted in modified HBSS to the same disperse phase concentrations as the amphotericin emulsion). The resistance of the monolayer was then followed over 2 days.

Results and discussion

The survival curve (Fig. 1) demonstrated that both amphotericin formulations rapidly kill *S. cerevisiae* in suspension. The kill rate of the amphotericin emulsion was slower than that of the solubilized formulation; this effect may have been due to the time taken for the drug to be transferred from the emulsion droplets to the target cells. The overall efficacy of the amphotericin did not appear to have been compromised by its formulation in an emulsion; the LD50 for both formulations is evidently considerably lower than the concentrations studied here.

Fig. 2 shows the confluence of the cell monolayers, as measured via their resistance, over 48 h after transfer to the

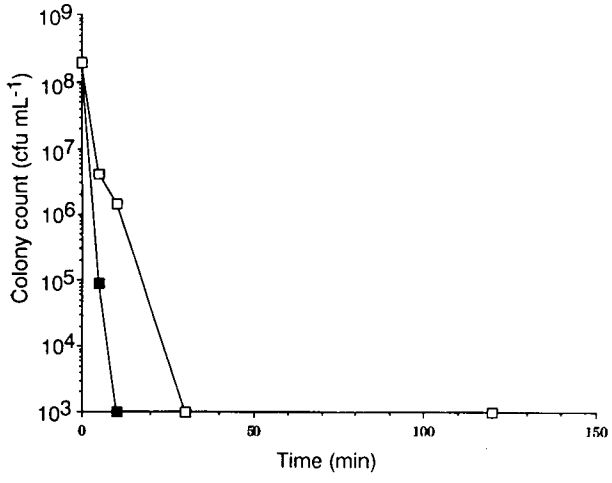

FIG. 1. Fungicidal action of the formulations Fungizone and amphotericin emulsion against *Saccharomyces cerevisiae* in suspension over 3 h. ■ Fungizone, □ amphotericin B emulsion.

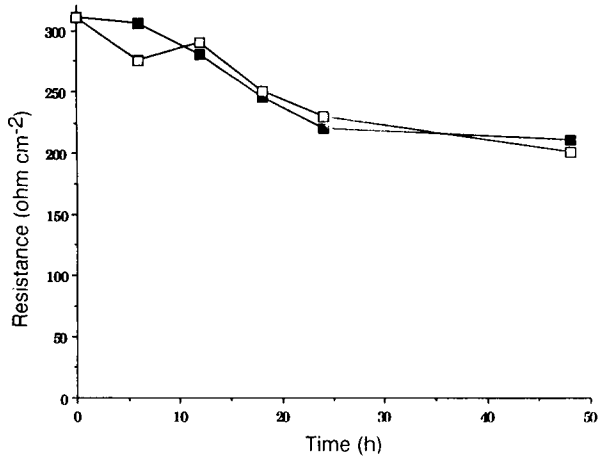

FIG. 2. Control resistances of MDCK NBL-2 monolayers in the presence of modified HBSS and control emulsion (no amphotericin) over 48 h. ■ Emulsion control, □ HBSS.

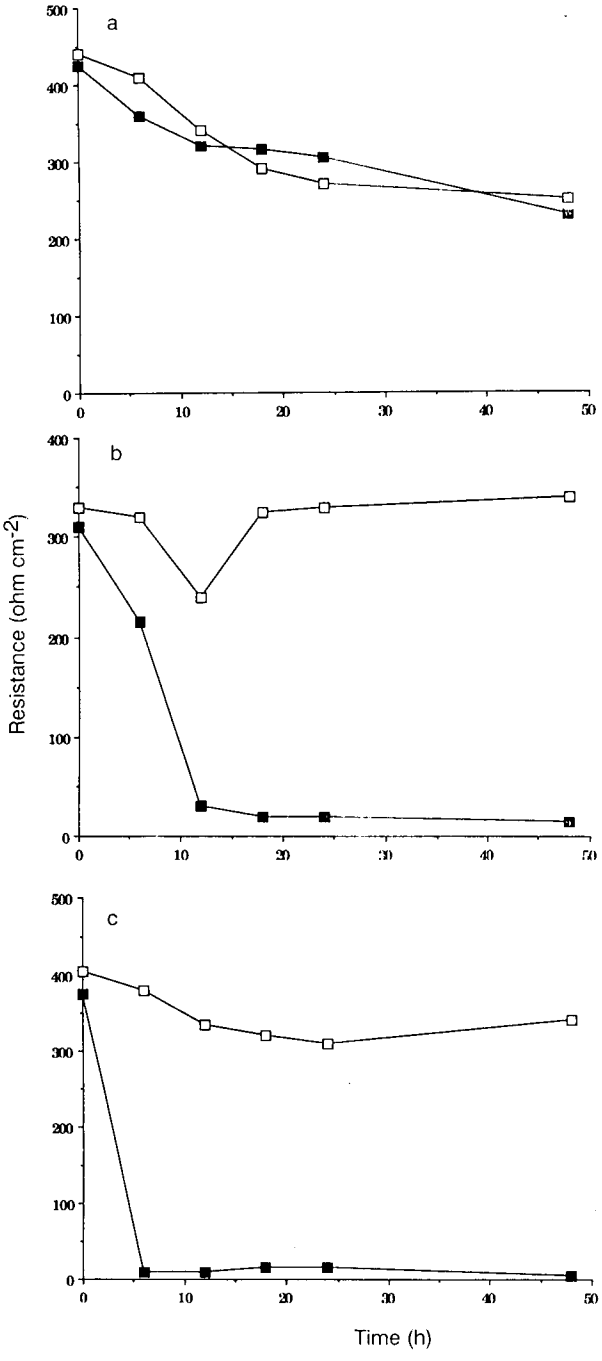

FIG. 3. Resistances over 48 h of MDCK NBL-2 monolayers in the presence of amphotericin B as emulsion or Fungizone, over 48 h. (a) 1 $\mu g\ mL^{-1}$ (b) 10 $\mu g\ mL^{-1}$; (c) 100 $\mu g\ mL^{-1}$. ■ Fungizone, □ amphotericin B emulsion.

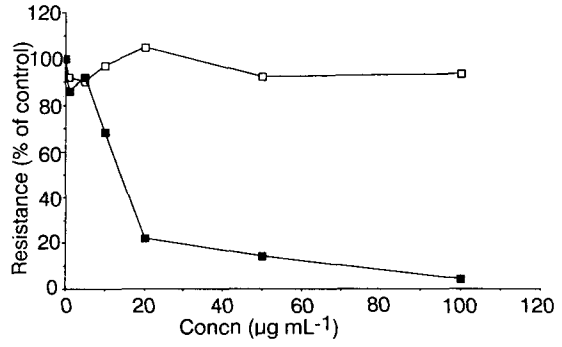

FIG. 4. Dose-response curve of kidney cell monolayers to amphotericin formulations after 6 h exposure. Response is cell monolayer resistance as a percentage of control (untreated) resistance at that time. ■ Fungizone, □ amphotericin B emulsion.

divalent ion-free HBSS medium. The decrease in resistance observed using either the Intralipid control or HBSS control indicated a gradual loss of viability of the cell monolayers. This is presumably due to the cells being transferred to a medium which lacked essential electrolytes (calcium and magnesium). Unfortunately such a medium was necessary since these ions would have caused flocculation of the emulsion over the extended period of the experiment. It is difficult to predict the effect that flocculation would have on the toxicity of the emulsion, although it would not be expected that flocculation would significantly alter the binding of amphotericin to the emulsion. In a clinical situation, it is unlikely that the emulsion would flocculate when injected into the bloodstream, due to dilution and the protective effect of opsonic factors.

Typical plots of resistance per unit area of monolayer against time over 48 h after addition of amphotericin formulations to the MDCK NBL-2 cultures are shown in Fig. 3 (amphotericin concentrations 1, 10 and 100 $\mu g\ mL^{-1}$). The loss of confluence on addition of Fungizone was evident within 6 h at all concentrations above 5 $\mu g\ mL^{-1}$, and was demonstrated by a rapid drop in monolayer resistance to near zero. In contrast, all concentrations of the emulsion formulation displayed only a slow loss in viability similar to that observed with monolayers maintained in the control (Intralipid or HBSS) media.

Dose-response curves were calculated as a percentage of the control resistance after 6 h, and are shown in Fig. 4. No significant differences were detected between the responses to the controls or the amphotericin emulsions over the timescale and concentrations studied. The response to Fungizone was rapid and monolayer integrity was retained at only the lowest concentrations (1–5 $\mu g\ mL^{-1}$).

The results demonstrate that the emulsion formulation of amphotericin B has a low toxicity to kidney cells grown in monolayer culture. Since nephrotoxicity is a major clinical side-effect occurring with the conventional amphotericin formulations, the emulsion system may have a significant clinical advantage.

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