Effect of free versus liposomal-complexed pentamidine isethionate on biological characteristics of *Acanthamoeba castellanii* *in vitro*

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Acanthamoeba is an opportunistic protozoan pathogen that can cause blinding keratitis and a rare but fatal encephalitis involving the central nervous system with a very poor prognosis. This is due to limited availability of effective anti-acanthamoebic drugs. Here, we tested whether the use of liposomes can improve the potency of pentamidine isethionate, an anti-amoebic compound. The liposomes consisted of L- α -phosphatidylcholine and cholesterol or ergosterol in a molar ratio of 1 : 5. Pentamidine isethionate was incorporated to achieve a final drug to lipid ratio of 1 : 5. At a drug concentration of 10 $\mu\text{g ml}^{-1}$, the liposomal drug was >12 times more effective than the free drug at preventing *Acanthamoeba* binding to human cells and significantly more effective in reducing parasite-mediated human cell cytopathogenicity, compared with the drug alone. Both the free and liposomal drug blocked *Acanthamoeba* encystation.

INTRODUCTION

The protozoan pathogen *Acanthamoeba* can cause two recognized diseases: a sight-threatening keratitis and a fatal granulomatous encephalitis involving the central nervous system (Marciano-Cabral & Cabral, 2003; Khan, 2006; Visvesvara *et al.*, 2007). Despite advances in antimicrobial chemotherapy and supportive care, the prognosis for *Acanthamoeba* infections, in particular for encephalitis, remains extremely poor. In part, this is due to our incomplete understanding of the pathogenesis and pathophysiology of *Acanthamoeba* infections together with a lack of awareness leading to delayed chemotherapy, limited efficacy of available drugs at the recommended levels, or the development of amoebae resistance to drugs during the course of therapy (Ficker *et al.*, 1990; Turner *et al.*, 2000), and the ability of amoebae to transform into dormant cyst forms (reviewed by Marciano-Cabral & Cabral, 2003; Khan, 2006; Visvesvara *et al.*, 2007). It is therefore desirable to find new therapeutic approaches as well as modify known anti-amoebic drugs to enhance their antimicrobial activities. The use of liposomes for drug delivery specific to parasites may hold promise in the treatment of *Acanthamoeba* infections. Because liposomes mimic the bio-membranes in terms of structure and bio-behaviour and protect the encapsulated drug in the biological milieu,

they are potential carriers for localized drug delivery. For example, liposomal amphotericin and liposomal arsenic enhanced the potency of drugs against *Leishmania* spp. and *Trypanosoma* spp., respectively (Paul *et al.*, 1997; Antimisiaris *et al.*, 2003). The overall aim of this study was to determine a possible therapeutic value of free versus liposomal-complexed anti-amoebic drug pentamidine isethionate using an *in vitro* assay.

METHODS

Culture of *Acanthamoeba*. A clinical isolate of *Acanthamoeba castellanii* belonging to the T4 genotype was obtained from the American Type Culture Collection (ATCC 50492). *Acanthamoeba* was routinely grown without shaking in 10 ml PYG medium [0.75 % (w/v) proteose peptone, 0.75 % (w/v) yeast extract and 1.5 % (w/v) glucose] in T-75 tissue culture flasks as previously described (Khan, 2001).

Culture of human brain microvascular endothelial cells (HBMECs). For adhesion and cytopathogenicity assays, primary HBMECs were isolated from human tissue and grown in RPMI 1640 containing 10 % fetal bovine serum, 10 % NuSerum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U ml^{-1}), streptomycin (100 U ml^{-1}), non-essential amino acids and vitamins as previously described (Stins *et al.*, 1997; Alsam *et al.*, 2003). All chemicals were purchased from Sigma Laboratories, unless otherwise stated. Stock solutions of the anti-amoebic drug pentamidine isethionate, dissolved in distilled water (25 mg ml^{-1}), were prepared and maintained at $-20\text{ }^{\circ}\text{C}$ until required.

Abbreviation: HBMECs, human brain microvascular endothelial cells.

Preparation of liposomal pentamidine isethionate. A drug to lipid ratio of 1:5, which was found to be suitable for stabilizing the drug in the liposomal suspension, was used (other ratios were found to be insoluble). The presence of ergosterol in the lipid mixture was ascertained by preparing liposomes using lipid mixtures containing 25% ergosterol, and comparing with liposomes containing 25% cholesterol as previously described (Ning *et al.*, 2005). Liposomes were prepared using the dehydration–rehydration method (Ning *et al.*, 2005). Briefly, in a typical experiment, 1.94 mg cholesterol or 1.99 mg ergosterol, 11.7 mg 1- α -phosphatidylcholine from egg yolk (EYPC) and 2.37 mg pentamidine isethionate were dissolved in 5 ml of a mixture of 1:1 dichloromethane/methanol. The solution was transferred to a round-bottomed flask and the bulk of the solvent was removed under reduced pressure using a rotary evaporator. Traces of solvent were removed under high vacuum for 12 h. Next, 5 ml RPMI 1640 buffer was added and the lipid–drug mixture was suspended by vortexing (~3 min), sonicated for 20 min (Ultrasonic homogenizer 4710 series) and stored at 4 °C. The sample thus generated contained 4 mM lipids (3 mM EYPC and 1 mM sterol) and 800 μ M pentamidine isethionate, equivalent to 474 μ g ml⁻¹.

Adhesion, cytopathogenicity and encystment assays. Adhesion assays were performed as previously described (Sissons *et al.*, 2005). Briefly, *Acanthamoeba* [5×10^5 amoebae (0.5 ml)⁻¹ per well] was incubated with HBMEC monolayers (grown to confluency in 24-well plates) in RPMI 1640 containing 2 mM glutamine, 1 mM sodium pyruvate and non-essential amino acids. The plates were incubated at 37 °C in a 5% CO₂ incubator for 60 min. The numbers of unbound amoebae in the supernatants were determined by haemocytometer counting. The percentage of bound amoebae was calculated as follows: no. of unbound amoebae/total number of amoebae $\times$ 100 = % unbound amoebae. The numbers of bound amoebae were deduced as follows: % unbound amoebae–100 = % bound amoebae. To determine the effects of drugs, amoebae were treated with various concentrations of pentamidine (2 μ g ml⁻¹ and 10 μ g ml⁻¹) or liposomal pentamidine (2 μ g ml⁻¹ and 10 μ g ml⁻¹ pentamidine associated with liposomes) for 45 min. Following this incubation, amoebae plus inhibitors were transferred to HBMEC monolayers for adhesion assays as described above.

Cytopathogenicity assays were performed using a cytotoxicity detection kit (Roche Applied Science) (Sissons *et al.*, 2005). Briefly, amoebae in the presence or absence of drugs were added to HBMECs as described for adhesion assays. The plates were incubated at 37 °C in a 5% CO₂ incubator and periodically observed for cytopathic effects for up to 24 h. At the end of this incubation period, supernatants were collected and cytopathogenicity was determined by measuring lactate dehydrogenase (LDH) release. LDH is a cytoplasmic enzyme and its release in the supernatant indicates host cell damage. It is thus a useful measure to estimate cell death. The cell supernatant containing LDH catalyses the conversion of lactate to pyruvate, generating NADH and H⁺. In the second step, the catalyst (diaphorase) transfers H and H⁺ from NADH and H⁺ to the tetrazolium salt *p*-iodonitrotetrazolium violet, which is reduced to formazan (dye), and the A₄₉₂ is read. Thus an increase in formazan formation is directly related to an increase in the release of LDH and indicates an increase in damaged cells. The percentage LDH was determined as follows: (sample value–control value)/(total LDH release–control value) $\times$ 100 = % cytotoxicity. Control values were obtained from HBMECs incubated in RPMI 1640 alone. Total LDH release was determined from HBMECs treated with 5% Triton X-100 for 60 min at 37 °C.

Encystment was induced by incubating *Acanthamoeba* (>95% trophozoites, at a cell density of 2×10^5 ml⁻¹) without nutrients in RPMI 1640 at 37 °C for 48 h. Next, cells were counted using a haemocytometer, followed by the addition of sodium dodecyl sulfate

(final concentration 0.5%) to lyse any remaining trophozoites. The counts were performed microscopically using a haemocytometer, both prior to and subsequent to the addition of SDS. Trophozoites are SDS-sensitive and are lysed immediately upon addition, while cysts remain intact to be counted. To quantify encystment, the percentage of *Acanthamoeba* transforming into cysts was determined as follows: no. of amoebae (post-SDS treatment)/no. of amoebae (pre-SDS treatment) $\times$ 100 = % encystment. Data are presented as mean $\pm$ standard error.

RESULTS AND DISCUSSION

Liposomal pentamidine isethionate inhibited *Acanthamoeba* binding to human cells

The effect of pentamidine isethionate, liposomal cholesterol-pentamidine or liposomal ergosterol-pentamidine and controls was determined on parasite binding to human cells, encystation, and *Acanthamoeba*-mediated host cell death. Adhesion assays were performed by incubating amoebae with HBMECs in the presence or absence of various concentrations of pentamidine (2 μ g ml⁻¹ and 10 μ g ml⁻¹) or liposomal pentamidine (2 μ g ml⁻¹ and 10 μ g ml⁻¹ pentamidine associated with liposomes). At 10 μ g ml⁻¹, the liposomal drug was >12 times more effective than the free drug at preventing *Acanthamoeba* binding to human cells (Fig. 1). Both liposomal cholesterol-pentamidine and liposomal ergosterol-pentamidine showed significant inhibitory effects on parasite binding to human cells, compared with amoebae alone ($P < 0.05$, using paired *t*-test; one-tailed distribution) (Fig. 1). However, at 10 μ g ml⁻¹ there was no significant difference between liposomal cholesterol-pentamidine and liposomal ergosterol-pentamidine ($P > 0.05$, using paired *t*-test; one-tailed distribution) (Fig. 1). In controls, pentamidine isethionate alone, ergosterol alone or cholesterol alone had no effects on amoebae adhesion to the host cells. However, liposomes alone at 10 μ g ml⁻¹ also showed amoebae adhesion to the HBMECs (Fig. 1). These findings suggest that liposomes (but not drug alone) interfere with the amoeba adhesins and/or the expression of adhesins on the surface of amoeba, resulting in reduced binding. In contrast, pentamidine is known to target intracellular molecules (Alizadeh *et al.*, 1997) and has no effect on amoeba adhesins, which may explain its failure to block amoebae binding to HBMECs. Of note, liposomes alone had no amoebicidal or amoebistatic effects, as determined by trypan blue staining and amoebae cultivation (data not shown).

Liposomal pentamidine inhibited host cell cytopathogenicity

Next we determined the effects of liposomal drug on *Acanthamoeba*-mediated host cell death using cytopathogenicity assays. In the absence of drug, *Acanthamoeba* produced severe host cell cytopathogenicity (up to 70% cell death) within 24 h (Fig. 2). Drug alone significantly

Fig. 1. Liposomal pentamidine inhibits *Acanthamoeba* binding to human cells. To investigate the effects of liposomal pentamidine isethionate on *Acanthamoeba* binding to host cells, adhesion assays were performed. At 10 µg ml⁻¹, the liposomal drug was >12 times more effective than the free drug (<10 % and 56 % binding, respectively) at preventing *Acanthamoeba* binding to human cells. Both liposomal cholesterol-pentamidine isethionate and liposomal ergosterol-pentamidine isethionate showed significant inhibitory effects on parasite binding to human cells, as compared with the drug alone or liposome alone ($P < 0.05$, using paired t -test; one-tailed distribution). However, at 10 µg ml⁻¹ there was no significant difference between liposomal cholesterol-pentamidine and liposomal ergosterol-pentamidine ($P > 0.05$, using paired t -test; one-tailed distribution). In controls, pentamidine isethionate alone, ergosterol alone or cholesterol alone had no effects on amoebae adhesion to the host cells. Data are presented as the mean $\pm$ standard error of three independent experiments.

reduced *Acanthamoeba*-mediated host cell cytotoxicity, from 70 % $\pm$ 7.5 in the absence of pentamidine to 20 % $\pm$ 2.1 in the presence of pentamidine (Fig. 2). Interestingly, the liposomal drug significantly enhanced

the inhibitory effects of pentamidine to less than 10 % ($P < 0.05$, using paired t -test; one-tailed distribution) (Fig. 2). However, at 10 µg ml⁻¹ there was no significant difference between liposomal cholesterol-pentamidine and liposomal

Fig. 2. Liposomal pentamidine isethionate inhibits host cell cytopathogenicity. To determine the effects of liposomal pentamidine isethionate on *Acanthamoeba*-mediated host cell death, cytopathogenicity assays were performed. In the absence of pentamidine isethionate, *Acanthamoeba* produced severe host cell death within 24 h. Pentamidine isethionate alone significantly reduced *Acanthamoeba*-mediated host cell cytopathogenicity ($P < 0.05$, using paired t -test; one-tailed distribution), while liposomal pentamidine isethionate significantly further enhanced its inhibitory effects ($P < 0.05$, using paired t -test; one-tailed distribution). Data are presented as the mean $\pm$ standard error of three independent experiments. Of note, drug alone or liposomes alone had no significant cytotoxic effects on HBMECs at the concentrations tested (data not shown).

Fig. 3. Both free pentamidine isethionate and liposomal pentamidine isethionate inhibit *Acanthamoeba* encystation. To investigate whether liposomal pentamidine isethionate affects *Acanthamoeba* encystation, encystment assays were performed. In the absence of pentamidine isethionate, there was approximately 50% *Acanthamoeba* encystment. At 10 µg ml⁻¹, pentamidine isethionate significantly reduced encystation in *Acanthamoeba* compared with amoebae alone ($P < 0.05$, using paired t -test; one-tailed distribution). However, there was no significant difference between liposomal pentamidine isethionate or pentamidine isethionate alone ($P > 0.05$, using paired t -test; one-tailed distribution). Data are presented as the mean $\pm$ standard error of three independent experiments.

ergosterol-pentamidine ($P > 0.05$, using paired t -test; one-tailed distribution) (Fig. 2). Of note, drug alone or liposomes alone had no significant cytotoxic effects on HBMECs at the concentrations tested (data not shown).

Both free and liposomal pentamidine isethionate inhibited *Acanthamoeba* encystation

To determine the effects of drugs on parasite differentiation, encystment assays were performed by incubating *Acanthamoeba* in nutrient-free medium. When incubated alone, there was approximately 50% *Acanthamoeba* encystment (Fig. 3). At 10 µg ml⁻¹, pentamidine reduced *Acanthamoeba* encystment to 12.5% $\pm$ 3.5. Although there were small differences between drug alone, ergosterol-drug and cholesterol-drug at 2 µg ml⁻¹, these differences were not significant ($P > 0.05$, using paired t -test; one-tailed distribution) (Fig. 3). It was surprising to observe no additive effect of liposomal drug on encystation and studies are in progress to address this issue.

In conclusion, it is shown that liposomal pentamidine isethionate was more active than pentamidine isethionate alone against *Acanthamoeba* spp. Future studies will test the liposomal drug against various *Acanthamoeba* isolates of different genotypes.

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