Cytokines in mice treated with amphotericin B-intralipid

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Amphotericin B (AMB) intralipid (IL) admixtures (AMB-IL) are composed of components approved for clinical use and are commercially available at low cost. They are stable and exhibit in-vitro and in-vivo efficacy against Candida infections, as well as resulting in significantly reduced toxicity in comparison with that of conventionally administered amphotericin B. We examined the production of cytokines in uninfected mice treated with AMB or AMB-IL, as evaluated by expression of mRNA corresponding to the cytokines. Expression was measured by intensity of bands in comparison to the intensity of β-actin control bands, with the latter assigned an arbitrary standard value of 100% and other bands measured in relative percentages. We found that both in naïve and compromised mice, AMB treatment caused significantly greater production of the pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) than was seen in animals treated with AMB-IL or with another lipid AMB formulation, AmBisome. We hypothesize that the superior tolerance for the AMB-IL admixtures, as compared with conventional AMB, might derive from the reduced expression of the pro-inflammatory cytokines. TNF-α and IL-1β, which mediate many potentially adverse pathophysiological events similar to those seen as side-effects of AMB usage.

Keywords amphotericin B, cytokines, Intralipid, mice

Introduction

In a previous study [1], we developed and characterized amphotericin B (AMB) intralipid (IL) admixtures (AMB-IL). AMB-IL admixtures were prepared by vigorous overnight agitation of Fungizone® brand (Bristol-Meyers Squibb, Dublin, Ireland) amphotericin B with lipid emulsions. They were characterized by determination of the size of the emulsion particles, and were tested for their stability for 1 month at room temperature and at 4°C, their in-vitro antifungal activity against various Candida species and their toxicity to red blood cells (RBC), as evaluated by haemolytic activity and AMB-induced K⁺ leakage [1].

Our AMB-IL admixtures had several important advantages. First, they were composed of components already approved for clinical use and commercially available at low cost. In addition, they proved to be stable and to exhibit in-vitro antifungal efficacy and, in comparison with conventionally administered amphotericin B, significantly reduced in-vitro toxicity. After obtaining these promising data, we evaluated the in-vivo efficacy of AMB-IL admixtures in comparison to conventional AMB in both normal and compromised mice [2,3]. We showed that AMB-IL, both at low (0.4 mg/kg/day × 5) and high concentrations (1–2 mg/kg/day × 5), was more effective than conventional AMB and significantly increased the survival rate. It affected the course of infection in comparison to that seen in untreated controls by prolonging the survival time of the animals that did succumb to the fungal challenge. We also assessed the toxicity in vivo of AMB-IL versus AMB by evaluating the dose tolerated by the mice, as measured by the lethal dose (LD). It was found that the mice tolerated AMB-IL at significantly higher doses than they did AMB [2].

To understand the mechanism by which AMB-IL admixtures reduce the toxicity of AMB, we also...
investigated the biodistribution of AMB in blood and tissues in mice treated with AMB-IL in comparison to Fungizone and AmBisome (commercially available liposomal AMB; NeXstar Pharmaceuticals, Blackrock, Ireland) [4]. We found that the levels of AMB in the blood of mice injected with AMB-IL were similar to those in mice treated with AmBisome and consistently higher than in mice treated with Fungizone. The highest AMB concentrations after AMB-IL and AmBisome administration were present in the organs of the reticuloendothelial system (RES) (i.e. the liver and spleen), with lesser amounts in the kidneys and lungs, and with minimal amounts in the heart [4].

As is widely known, use of AMB is limited by severe side-effects such as fever, chills, and hypotension [5–7]. It has been hypothesized that these adverse effects may be mediated through induction of proinflammatory cytokines [8,9]. Arning et al. [10] measured plasma levels of proinflammatory cytokines in leukaemic patients and associated the higher levels of these substances with acute AMB toxicity. In the present study we compared the influence of AMB therapy on expression of different cytokines in animals treated with conventional AMB, AmBisome or AMB-IL.

Material and methods

Antifungal compounds

Fungizone® is a yellow powder containing a ratio of 50000 units (50 mg) AMB to 41 mg sodium desoxycholate. AMB for intravenous (i.v.) injection was prepared according to the manufacturers’ instructions and diluted with 5% D-glucose prior to use in injections.

AmBisome was prepared according to the manufacturers’ instructions and was also diluted with 5% D-glucose prior to use in injections.

Fat emulsion

We used 20% Intralipid™, a fat emulsion for i.v. use (Kabi Pharmacia, Stockholm, Sweden).

Preparation of AMB-IL admixtures

A stock solution of standard Fungizone® (5 mg/ml) was prepared in 10 ml 5% D-glucose, and AMB-IL was prepared by making a 25-fold dilution of this stock solution in 20% Intralipid™ [1]. The final concentration of AMB in these preparations was 0.2 mg/ml. These preparations were agitated vigorously at 24°C for 18 h on an Orbit Environmental Shaker (Lab Line; New Brunswick Scientific, Edison, NJ, USA) (orbital diameter = 4 cm) at 280 r.p.m.

Animals

Two experimental animal models were used: uninfected, non-compromised, naïve animals [2] and uninfected, cyclophosphamide (CY) compromised mice. The latter received a single intraperitoneal (i.p.) injection of 200 mg CY per kg [3]. Female 4-week-old ICR mice were used. Animals were kept under conventional conditions and were given food and water ad libitum.

The Ethics Committee of the Faculty of Medicine of Tel Aviv University granted permission for the animal experiments described in the work. All AMB preparations were administered i.v. into the tail vein at doses of 1 mg/kg.

Total RNA isolation

The normal and compromised mice were killed 48 h after drug administration and the spleens were removed and immediately frozen in liquid nitrogen and stored at −70°C until further processing could be done. Total RNA were isolated from spleen samples using TRI reagent® (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s recommended protocol. Tissue explants were homogenized with TRI reagent solution (1 ml per 50–100 mg of tissue), mixed with an adjusted volume of chloroform (Merck, Darmstadt, Germany) (0.2 ml per 1 ml of initial TRI reagent), and then centrifuged for 15 min at 13000 g. For further RNA precipitation, the upper aqueous phase was transferred and mixed with isopropanol (Merck) at 0.5 ml isopropanol per 1 ml initial TRI reagent, and then centrifuged for 15 min at 13000 g. The pellet was washed with 75% ethanol (Merck), centrifuged for 5 min at 10500 g, air-dried for 15 min and then dissolved in 100 µl diethyl pyrocarbonate (DEPC)-treated water (Biological Industry, Beit Ha-Emek, Israel), followed by incubation at 60°C for 10–15 min. The final product was stored on ice.

RNA concentrations in the samples were determined by measuring the optical density (OD) at 260 nm (1 OD unit at 260 nm equals 40 µg RNA per ml). Purity of the RNA was determined by measuring the ratio of OD values at 260 and 280 nm. Only samples with 260:280 OD ratios of 1.6–2.0 were included in the analysis. The samples were stored at −70°C for later use.

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RT-PCR system

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the mRNA was performed using a one-step RT-PCR system (Titan One Tube RT-PCR System; Roche Molecular Biochemicals, Mannheim, Germany) according to the protocol recommended by the manufacturer. Samples consisting of 1 μg total RNA were amplified using specific mouse cytokine primers (Bio Technology General, Rehovot, Israel) for the following cytokines: TNF-α, IL-1β, IL-2, and IL-6. β-actin was amplified as a control. Thermocycling (Table 1) was performed in a Perkin Elmer GeneAmp 9600 Thermocycler (Biometra, Göttingen, Germany), as recommended in the protocol for the Titan One Tube RT-PCR System, and was adjusted for the annealing time of the primers used.

Separation of the PCR products

After thermocycling, the samples were analysed on a 2% agarose gel (Gibco Lita Technologies, Paisley, UK) containing ethidium bromide 10 ng/ml (Sigma, St Louis, MO, USA), using the dye bromophenol blue as the front-runner. A DNA molecular size marker ladder was used (φX 174 DNA/BsuRI Marker; Fermentas, Vilnius, Lithuania). The bands were photographed and further analysed semiquantitatively and qualitatively in comparison to rat β-actin expression, a control based on a perpetually expressed housekeeping gene. To compare results we used the TINA program (Raytest Isotopenmessgeraete, Straubenhardt, Germany) based on determination of intensity of bands of the image, using intensity of β-actin expression as a control taken to represent 100% expression. The intensity of bands was measured in photo-stimulated luminescence (PSL) units as defined by the instrument manufacturer.

Table 1  Thermocycling conditions used in PCR for cytokine cDNA detection

<table>
<thead>
<tr>
<th>Procedure temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
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<tbody>
<tr>
<td>Reverse transcription at 60</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturate template at 94</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation at 94</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing at 56</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Elongation at 68</td>
<td>2 min × 10</td>
<td></td>
</tr>
<tr>
<td>Denaturation at 94</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing at 56</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Elongation at 68</td>
<td>2 min* × 25</td>
<td></td>
</tr>
<tr>
<td>Prolonged elongation at 68</td>
<td>7 min</td>
<td>1</td>
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<tr>
<td>4</td>
<td>Pause</td>
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</tbody>
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*Elongation at 68°C for 2 min plus cycle elongation for 5 s for each cycle

Statistical analysis

Data were statistically analysed using Student’s t-test and significance was attributed to P < 0.05.

Results

Total RNA was extracted from spleens and analysed on a 2% agarose gel. We observed no expression of cytokines in untreated control mice, while β-actin was expressed at moderate levels (data not shown). In contrast, treatment with AMB preparations in normal mice stimulated the production of cytokine mRNA. The levels of IL-1β expression following AMB-IL or AmBisome treatment were 33–36% of actin control levels, as compared to 52% following AMB treatment (Fig. 1). Differences were statistically significant at P < 0.05. TNF-α expression decreased in AMB-IL treated naïve mice to 22% from 30% in AMB treated animals (P < 0.05). There was no TNF-α expression noted in AmBisome treated mice. No IL-2 or IL-6 mRNA production was identified.

In animals compromised by CY treatment, controls not exposed to AMB showed no cytokine mRNA expression; only β-actin expression was seen, as in normal animals (data not shown). Compromised animals treated with AMB-IL showed no expression of TNF-α, but a 12.5% level was seen in AMB-treated mice (Fig. 2). IL-1β expression in AMB-treated mice was at a 42% level, but decreased to 22% in AMB-IL-treated mice (P < 0.05).

Discussion

During the last 30 years there has been a dramatic increase in the number of patients with serious fungal infections [11–14]. Invasive fungal infections continue to be a major source of morbidity and mortality in compromised and immunosuppressed hosts [15,16,17]. The commonest choice of treatment is AMB [6,18]. The activity of AMB is based on its affinity to bind to the fungal membrane sterol ergosterol, thereby changing the permeability of the fungal cell membrane and leading to leakage of cell contents, culminating in fungal death [5,8,18]. In addition, AMB binds, although to a lesser extent, to the cholesterol in human membranes, leading to the various toxic effects of this drug [19,20].

The mechanism by which AMB-IL admixtures reduce the toxicity of AMB without alteration of its antifungal activity is not clear. It is likely that AMB, diluted in IL, is bound to oil components of the fat emulsions.
In the present study, animals treated with lipid formulations of AMB (AMB-IL or AmBisome) had lower expression of pro-inflammatory cytokines than was seen with mice treated with conventional AMB. Levels of cytokines in compromised mice were lower than those seen in naïve animals, possibly due to the immunosuppressed state of the former. These results support the hypothesis that the toxicity of AMB is correlated with increased induction of pro-inflammatory cytokines, as proposed by several investigators [10,21]. Based on our study results, we suggest that the improvement in AMB tolerance brought about by preparation of the drug as AMB-IL is indeed mediated by the reduction in expression of pro-inflammatory TNF-α and IL-1β.

TNF-α and IL-1β are known to mediate many pathophysiological events, including fever, chills and multiple organ failure [9], that are connected with adverse responses to AMB. TNF-α, principally produced by activated macrophages and polymorphonuclear cells (PMN), induces various inflammatory responses such as fever, enhanced blood coagulation, and inflammatory tissue destruction, ultimately resulting in a toxic effect on the host [8,9]. The immunopharmacological effects of AMB, including the immunologically mediated component of its antifungal activity as well as its toxic effects, may be regulated by such inflammatory cytokines produced in the host [22,23]. In-vitro studies support the proposition that AMB-related toxicity is due to drug-induced release of TNF-α from macrophages [24].

Our previous research showed that AMB-IL is stable, inexpensive and effective both in vitro and in vivo [1–3]. In relation to ease of management and reduction of
toxicity, our AMB-IL admixture showed promising results [2–4]. Disclosure by the present study of a mechanism bringing about this reduced toxicity, and moreover of a mechanism well correlated with results of various other studies on cytokines and AMB, helps to demonstrate the reliability of AMB-IL as a potentially valuable therapeutic tool.

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References


Fig. 2 Expression of cytokines in cyclophosphamide-immunocompromised mice treated with various AMB preparations.