Role of Macrophages in the Candidacidal Activity of Liposomal Amphotericin B

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The role of macrophage activation in the candidacidal activity of liposome-incorporated (L) amphotericin B was investigated. Macrophages treated with L-amphotericin B killed Candida albicans more effectively than did macrophages treated with free (F) amphotericin B. However, macrophages treated with neither F- nor L-amphotericin B killed amphotericin B-resistant Candida tropicalis. In vivo stimulation of macrophages by intraperitoneal administration of thioglycolate, Freund’s complete adjuvant, or heat-killed C. albicans followed by in vitro treatment with F- or L-amphotericin B, did not enhance their candidacidal activity. Intravenous administration of F- or L-amphotericin B did not augment the candidacidal activity of macrophages sensitized in vivo; however, sensitized macrophages showed enhanced killing compared with resident unstimulated cells. These studies suggest that macrophage-mediated enhancement of C. albicans killing may be due to uptake, transport, and delivery of L-amphotericin B to infected sites rather than to macrophage activation.

Despite the advent of newer agents, amphotericin B remains the mainstay of antifungal chemotherapy, although its toxicity has limited its clinical use. Today, however, liposome incorporation of amphotericin B is a well-accepted means of reducing the drug’s toxic effects. Earlier studies by us [1] and others [2, 3] demonstrated that liposome encapsulation not only reduces the toxicity but also maintains or increases the efficacy of amphotericin B, thereby significantly improving its therapeutic index. In vivo studies on liposome-encapsulated (L) amphotericin B efficacy against diseases such as aspergillosis, candidiasis, cryptococcosis, histoplasmosis, and leishmaniasis have been reported, and clinical trials have shown L-amphotericin B to be therapeutically effective against disseminated fungal infections [1–4]. The great interest in the drug’s therapeutic activity [3] motivated us to study the mechanisms of action responsible for the improved therapeutic index of L-amphotericin B.

In the present study, we investigated the effect on the candidacidal activity of macrophages activated in vitro and in vivo by free (F) or L-amphotericin B.

Materials and Methods

Drugs, lipids, and chemicals. Pure (deoxycholate-free) amphotericin B was obtained from E. R. Squibb & Sons (Princeton, NJ). Fungizone was purchased from the University of Texas M. D. Anderson Cancer Center pharmacy. Chromatographically pure di-myristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were from Avanti Polar Lipids (Birmingham, AL). 3H-glucose was from NEN Research Products (Wilmington, DE). Thioglycollate broth (TG) was purchased from Difco (Detroit), Freund’s complete adjuvant (FCA) and fetal calf serum were from Sigma (St. Louis), and RPMI was from Cellgro/ Mediatech (Washington, DC). All other reagents and chemicals were of analytical grade.

Animals. Male Stoner mice were purchased from the University of Texas Science Park (Bastrop, TX). Groups of mice were injected with TG, Candida albicans heat-killed in boiling water for 10 min, or FCA, and peritoneal exudate cells were collected after 2, 15, and 21 days, respectively.

Preparation of macrophage monolayers. Peritoneal exudate cells were obtained from both control and treated mice as described elsewhere [5] and plated at 1.5 × 10^6 or 1.5 × 10^5 cells/well in 24- or 96-well plates, respectively. Monolayers were prepared by washing the cells twice with complete RPMI medium (containing 1% fresh mouse serum, 1% penicillin-streptomycin, and 1% HEPES) to remove nonadherent cells. The monolayers were >98% macrophages, as detected by nonspecific esterase staining [5].

Preparation of liposomes. Multilamellar vesicles composed of DMPC:DMPG (7:3 M ratio) containing amphotericin B were prepared by the rotary evaporator method as previously described [1]. The liposome suspension was centrifuged at 48,000 g for 60 min, and the amount of drug in liposomes was calculated by dissolving an aliquot in methanol and measuring the absorbance at 405 nm.

Sensitization of macrophages in vivo. Groups of mice were injected intraperitoneally with 0.5 mL of TG (prepared a day before), 10^6 heat-killed C. albicans [6], or a 0.5-mL suspension of FCA per mouse. Peritoneal cells were harvested on day 2, 15, or 21, respectively, from the 3 groups. The cells were treated in vitro with various concentrations of F- or L-amphotericin B and evaluated for their candidacidal activity. For studying the effect of amphotericin B in vivo, animals in the TG-, C. albicans-, and FCA-treated groups were injected intravenously 2, 15, or 21 days later, respectively, with 0.8 mg/kg F- or L-amphotericin B. Three
mice in each group did not receive amphotericin B and served as controls to obtain untreated resident macrophages. Peritoneal macrophages were obtained from all mice 1 day after amphotericin B administration and assessed for candidacidal activity.

Assay of candidacidal activity. Macrophage monolayers in 96-well plates were incubated overnight with or without various concentrations of F- or L-amphotericin B. Cells were washed with fresh medium and exposed overnight to *C. albicans* at a ratio of 200:1. The medium was discarded by inverting the plate, and incorporation of ^3^H-glucose (0.25 μCi/well) by *Candida* species was measured as described previously [7]. Incorporated radioactivity was expressed as the percent counts per minute taken up by treated cells, compared with 100% taken up by control untreated cells.

Scanning electron microscopy. Peritoneal exudate cells were plated (1.5 × 10^6^ cells/well) in 24-well plates containing cover slips at the bottom of each well. After a 2-h incubation at 37°C, nonadherent cells were removed, and monolayers were incubated overnight with 2 μg/mL F-amphotericin B or 5 μg/mL L-amphotericin B; a set of triplicate wells with no amphotericin B served as controls. Cells were then washed twice and cocultured with 5 × 10^6^ *Candida* cells/well. After overnight incubation, infected monolayers were washed with 0.125 M sodium cacodylate buffer (pH 7.3, 310 mOsm, at 37°C) and fixed with 1 mL of Karnofsky’s fixative (pH 7.5) for at least 30 min. Cells were then rinsed three times for 3 min each with 0.125 M sodium cacodylate buffer and postfixed at room temperature with 2% OsO₄ in cacodylate buffer for 30 min. After three more rinses in cacodylate buffer, the cells were dehydrated in a series of graded concentrations of ethanol and transferred to refrigerant 113 for critical-point dehydration. The dehydrated cells were mounted onto stubs and sputter-coated with 200 Å of gold-palladium (80:20) in a Hummer VI (Technics, Springfield, VA) and examined using a scanning microscope (model S520; Hitachi Scientific Instruments, Mountain View, CA).

Results

Role of macrophages in candidacidal activity. The candidacidal activity of L-amphotericin B by cells cultured in fresh mouse serum was much higher than that of those cultured in fetal calf serum; therefore, further experiments were done using fresh mouse serum containing media. Macrophages treated with L-amphotericin B showed no uptake of radio-labeled glucose at 1.0 μg/mL, suggesting 100% killing of *C. albicans*. In contrast, cells treated with free drug did not kill *C. albicans*, even at concentrations up to 2.0 μg/mL.

These results are supported by scanning electron micrographs (figure 1). Although cells treated with 2 μg/mL F-amphotericin B showed significant growth inhibition of *Candida* species compared with growth in the control untreated cultures, treatment with the equivalent concentration of liposomal drug completely killed *Candida* species in these cultures. Similar results were observed with 5 μg/mL F- or L-amphotericin B.

Role of macrophage activation in candidacidal activity in vitro. Although macrophages treated with F- or L-amphotericin B showed differential but significant candidacidal activity against *C. albicans*, they were unable to kill *C. tropicalis*, a strain resistant to amphotericin B, even at concentrations up to 10 μg/mL. These results suggest that neither F- nor L-amphotericin B could activate macrophages in vitro to kill *Candida* species (figure 2A);

Figure 1. Scanning electron micrographs of treated and untreated macrophage monolayers exposed to *Candida albicans*. A, Controls show extensive candida growth and no macrophages. Original magnification, ×1470. B, Monolayers treated with free amphotericin B (2 μg/mL) show reduced candida growth, but some surviving candida cells can be seen growing out of macrophages. Original magnification, ×1275. C, Monolayers treated with liposomal amphotericin B (2 μg/mL) show no candida growth. Original magnification, ×1280.
killed *C. albicans* cells killed 15%–25% of *C. albicans*. However, after in vitro treatment with L-amphotericin B (2 μg/mL), these cells could kill 80%–90% of the *C. albicans*, while cells treated with the equivalent concentration of free drug showed only 5%–30% candidacidal activity. None of the in vivo stimulating agents, however, could sensitize or activate macrophages, with or without amphotericin B, to kill *C. tropicalis*.

We also examined the effect of in vivo amphotericin B treatment as an activating signal for macrophages primed with TG, FCA, and heat-killed *C. albicans* as described in Materials and Methods. Macrophages obtained from these mice were tested for their candidacidal activity. Resident control macrophages (from untreated mice) killed only 4% of *Candida* species, whereas cells from all the primed groups showed 40%–50% killing, irrespective of treatment with F- or L-amphotericin B. It was interesting to note that macrophages from control mice injected with F- or L-amphotericin B showed enhanced killing of *Candida* species. However, treatment of primed mice with both F- and L-amphotericin B did not augment their macrophage candidacidal activity; a higher dose of L-amphotericin B (4 mg/kg) was also ineffective (data not shown).

**Discussion**

The results obtained demonstrate the enhanced ability of macrophages pretreated with L-amphotericin B to kill *Candida* species compared with those treated with F-amphotericin B. This may, partially, be due to the higher uptake and retention of L-amphotericin B by macrophages and slow release of drug from the cells leading to enhanced candidacidal activity, as shown in figure 1. Macrophage cultures treated with free drug showed growth of *Candida* species; some candidal hyphae seemed to grow out from the macrophages, which probably did not contain enough drug to kill the *Candida* species. In contrast, macrophage cultures treated with an equivalent concentration of L-amphotericin B did not show any live *Candida* species. In vivo, however, the dose-limiting toxicity of amphotericin B and low achievable serum levels minimize the effect of F-amphotericin B. Also, differences in susceptibility of various strains as well as the extent of infection may affect the outcome of therapy. Nonetheless, in an in vivo situation, macrophages may serve as secondary carriers of the liposomal drug, delivering high amounts of amphotericin B to sites of infection and inflammation [8]. A recent study [9] showed that the uptake of *C. albicans* blastospores treated with L-amphotericin B was enhanced compared with uptake by those treated with fungizone; however, killing of *Candida* species was not affected.

Since amphotericin B has been reported to activate macrophages [1, 3, 10, 11], we investigated the effect of F- and L-amphotericin B on macrophage activation and candidacidal...
activity. Our studies suggested that macrophages treated with amphotericin B in vitro could not be activated to kill amphotericin B–resistant C. tropicalis (figure 2A); hence we inferred that amphotericin B could not activate macrophages in vitro. We therefore stimulated mice with TG, a sterile inflammatory signal; FCA, a bacterial inflammatory signal; and heat-killed C. albicans, the infective signal itself [6]. Treatment with amphotericin B of macrophages obtained from these mice also failed to activate them to a candidacidal stage, as revealed by negligible killing of amphotericin B–resistant C. tropicalis (figure 2B). Furthermore, priming the mice with both primary (TG, FCA, heat-killed C. albicans) and secondary (amphotericin B) inflammatory signals in vivo was not sufficient to activate macrophages and enhance their candidacidal activity (data not shown).

Expression of microbicidal mechanisms by macrophages depends on their level of activation. Although our more recent studies show that activation of macrophages with a combination of interferon-γ (100 U/mL) and lipopolysaccharide (10 ng/mL) induced substantial amounts of nitric oxide (0.3 nmol/10^4 cells), it was ineffective to kill Candida species and to synergize with candidacidal effects of amphotericin B (data not shown). We observed that F– but not L–amphotericin B enhanced the nitric oxide production by interferon-γ–treated macrophages; however, candidacidal activity was unaffected. Although superoxide anions and TNF–α have been implicated in antifungal activity [12], the role of nitric oxide is controversial [13, 14]. These results suggested to us that macrophage activation may not be a major mechanism for killing Candida species. It is likely that neutrophils may have higher candidacidal activity than do macrophages and may have this capability indirectly by producing myeloperoxidase, which activates macrophages to exhibit candidacidal activity [15]. We therefore conclude that the enhanced candidacidal effect of amphotericin B in macrophages is mediated through drug delivery rather than macrophage activation.

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References