Efficacy of liposomal amphotericin B combined with interferon-gamma or granulocyte-macrophage colony-stimulating factor for treatment of systemic zygomycosis in mice.

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ABSTRACT

Granulocyte-macrophage colony-stimulating factor enhanced the efficacy of liposomal amphotericin B (LAMB), in a murine model of disseminated infection by *Rhizopus oryzae*, significantly prolonging the survival and reducing the tissue burden. The use of interferon-gamma alone was ineffective and combined with LAMB did not improve the results obtained with LAMB alone.
Zygomycosis is a frequently lethal invasive infection (2). The standard therapy for zygomycosis is not yet resolved (2). Historically, conventional amphotericin B (AMB) was the drug of choice for invasive zygomycosis, but its use is limited by its potential toxicity. The lipid formulation of amphotericin B (LAMB) allows higher doses to be administered due to its low toxicity and represents first-line therapy (17). In murine zygomycosis, LAMB has showed efficacy and has been even better than AMB deoxycholate (11, 12). The efficacy of posaconazole (POS) is controversial as some clinical data show good results (9, 20) but some authors have demonstrated that this drug is poorly active against *Rhizopus oryzae* the most common species causing zygomycosis (13, 16). Since the mortality rate is often high in disseminated zygomycosis, despite aggressive therapy, new strategies for the treatment of this infection are urgently needed (5).

Cytokines are critical components of the functional host defences promoting activation and recruitment of granulocyte and mononuclear phagocyte effector cells (18). Over the last decade, the usefulness of these compounds as adjunctive agents in antifungal therapy has been evaluated in the treatment of severe fungal infections (2, 10). In particular, interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have showed efficacy as adjunctive agents in the treatment of experimental cryptococcosis and histoplasmosis in mice (3, 4) and also in several clinical cases of zygomycosis (1, 5, 8, 14). Moreover these cytokines have shown an increase in *in vitro* polymorphonuclear leucocytes-induced hyphal damage of *R. oryzae* (6).

In this study, we have evaluated the effects of IFN-γ and GM-CSF, alone and combined with LAMB, in a murine model of infection by *R. oryzae*. 
Two clinical isolates of *Rhizopus oryzae*, FMR 6485 and FMR 8542, were used in this study. The isolates were cultured on potato dextrose agar (PDA) at 35°C. The AMB MICs were identical for both strains (0.5 µg/ml) (16).

Male OF1 mice were used in this study. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare Committee. Animals were immunodepressed one day prior to infection by administering a single dose intraperitoneally (i.p.) of 200 mg of cyclophosphamide per kg plus a single dose intravenously (i.v.) of 150 mg of 5-fluorouracil per kg. Mice were challenged with $0.2 \times 10^5$ CFU/animal. Ten mice were used for survival studies and ten for tissue burden studies, with the latter being identified before the study started. All mice received ceftazidime (5 mg/day subcutaneously) from days 1 to 7 after infection.

LAMB was administered i.v at doses of 5 or 10 mg/kg of body weight/dose once daily. Human recombinant IFN-γ (GenScript Corporation, USA) was administered i.v. at a dose of $10^5$ U once daily (3); human recombinant GM-CSF (GenScript Corporation USA) was administered subcutaneously (s.c.) at a dose of 5 µg/kg/day once daily (19). The different groups were treated as follows: LAMB at 5 or 10 mg/kg of body weight i.v.; IFN-γ at $10^5$ U i.v.; GM-CSF at 5 µg/kg/day s.c.; LAMB at 10 mg/kg i.v. plus IFN-γ at $10^5$ U i.v.; and LAMB 10 mg/kg i.v. plus GM-CSF at 5 µg/kg/day s.c.

All treatments began 24 h after challenge, and the therapies lasted for 7 days (16). Survival of mice was evaluated daily for 30 days. For tissue burden studies mice were sacrificed on day 4 post-infection. Kidneys and brains were removed aseptically and were homogenized in 1 ml of sterile saline; care was taken to minimize tissue trauma. Serial 10-fold dilutions of the homogenates were plated.
on PDA and incubated 18-24 h at 35°C. Mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log rank test. Colony counts in tissue burden studies were analyzed by the Kruskal-Wallis test. When the results of this test were significant, we used the Man-Whitney U test to compare treatment pairs. The Bonferroni correction was used to avoid an increase in the type I error due to multiple comparisons. When p<0.05, the observed differences were statistically declared significant.

For both strains the two doses of LAMB and the combination of LAMB with IFN-γ or GM-CSF significantly prolonged survival with respect to control, and the groups treated only with IFN-γ or GM-CSF (Fig. 1). LAMB combined with GM-CSF was able to prolong survival with respect to the group treated with LAMB at 10 mg/kg for the strain FMR 8542. The combination of LAMB with IFN-γ showed similar efficacy as the monotherapy with LAMB at 10 mg/kg, with no differences between them. Neither of the two cytokines prolonged survival.

All the treatments with the exception of IFN-γ, significantly reduced the fungal load in brain and kidney in comparison to the control group for both strains. In addition, GM-CSF and IFN-γ + LAMB failed to reduce tissue burden in kidney for the strain FMR 6485 (Figure 2). The combination of LAMB at 10 mg/kg with GM-CSF was the most effective treatment, reducing the fungal load in brain and kidney tissues, although for the strain FMR 8542 only in brain.

AMB has showed similar efficacy to a previous study (16) that proved the reproducibility of this murine model.

LAMB in general shows efficacy in zygomycosis treatment but in many cases the patients die. In a recent review of 120 cases of zygomycosis in patients with haematological malignances, LAMB was associated with a 67% survival rate,
compared to 39 % survival rate of AMB deoxycholate (7). In severe zygomycosis, correction of metabolic disturbances and reversal of immunosuppression is as essential as the other therapeutic measures for successful management (2). For this reason we have assessed the efficacy of cytokines as adjuvant agents for returning the host immune response and tried to restore the host’s defenses. In our model only the combination of LAMB with GM-CSF was significantly better than the treatment with LAMB alone. These results agree with a few clinical cases of zygomycosis in which GM-CSF was administered successfully as an adjunctive therapy of AMB or its lipid formulation (5, 8, 14).

These studies suggest a potential use of GM-CSF as an immunomodulator for improving the benefits of the therapy with LAMB against zygomycosis.

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REFERENCES


FIG. 1. Cumulative mortality of mice infected with *Rhizopus oryzae* FMR 6485 (A) or *R. oryzae* FMR 8542 (B) and treated with LAMB, GM-CSF and IFN-γ. \(^{a}P < 0.05\) versus control, IFN-γ (100000 U) and GM-CSF (5 µg/kg), \(^{b}P < 0.05\) versus LAMB (5 mg/kg) and \(^{c}P < 0.05\) versus LAMB (10 mg/kg). LAMB, liposomal amphotericin B; IFN-γ, interferon-gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor.
**FIG. 2.** Effects of the antifungal treatment on colony counts of the *Rhizopus oryzae* strains FMR 6485 (A) and FMR 8542 (B) in brain and kidney of mice. \(^a\)P < 0.002 versus control; \(^b\)P < 0.002 versus LAMB (5 mg/kg); \(^c\)P < 0.002 versus LAMB (10 mg/kg); \(^d\)P < 0.002 versus IFN \(\gamma\) (100000 U); \(^e\)P < 0.002 versus GM-CSF (5 μg/kg); \(^f\)P < 0.002 versus LAMB 10+ IFN \(\gamma\). Horizontal lines indicate mean values. LAMB, liposomal amphotericin B; IFN \(\gamma\), interferon-gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor.