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Liposomal amphotericin B and granulocyte colony-stimulating factor therapy in a murine model of invasive infection by *Scedosporium prolificans*

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We established a reproducible lethal disseminated infection by the opportunistic fungus *Scedosporium prolificans* in an immunosuppressed murine model. We compared the effectiveness of the combined administration of liposomal amphotericin B (LAMB) and granulocyte colony-stimulating factor (G-CSF) with that of either agent alone and with that of amphotericin B deoxycholate (AMB). LAMB + G-CSF and LAMB treatments improved survival significantly with respect to the untreated control. The mean survival times of these three groups were 13.2, 9.1 and 7.9 days, respectively. Culture results in terms of colony counts for samples of deep organs were lower in mice treated with the combined therapy, although differences were not significant. Combined LAMB + G-CSF therapy could be a promising approach for the treatment of disseminated infections of *S. prolificans*, although further studies are required to determine the most appropriate doses.

Introduction

Invasive fungal infections are serious complications for immunocompromised patients, especially those suffering haematological malignancies. Numerous moulds are responsible for these opportunistic infections,[1] although *Scedosporium prolificans*[2] causes a lot of concern due to its high virulence and *in vitro* and clinical resistance to practically all available antifungal drugs. As antifungal therapies are not effective, recovery from neutropenia has been considered the most important prerequisite for resolving the infections caused by this fungus. Recent studies have demonstrated *in vitro* resistance of *S. prolificans* to amphotericin B,[2–5] but this drug, alone or combined with other antifungal agents, is still the most frequently used.[6] Liposomal amphotericin B (LAMB), alone or combined with granulocyte colony-stimulating factor (G-CSF), has been tried in several cases with some success,[4,5] although studies in animals have not been performed, and there is insufficient clinical published data to recommend this therapy. In this study we have investigated the activity of LAMB alone and combined with G-CSF in a murine model of disseminated infection by *S. prolificans* and the results have been compared with those obtained with amphotericin B deoxycholate (AMB) therapy.

Materials and methods

Strain

S. prolificans FMR 3569 isolated from a patient with disseminated infection in Spain, was cultured on potato dextrose agar (PDA) plates for 7–10 days at 30°C. The inocula were prepared by flooding the surface of the agar plate with saline solution, scraping the sporulating mycelium with a culture loop and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were then filtered once through a sterile gauze to remove

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hyphae. The numbers of conidia in the suspensions were counted with a haemocytometer and verified by plating dilutions of the suspensions on Sabouraud dextrose agar (SDA).

Animals

OF1 male mice (Charles River, Criffa S.A., Barcelona, Spain) with a mean weight of 30 g were used. Animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare Committee.

Drugs

AMB (purchased as Fungizone; Squib Industria Farmacéutica, S.A., Barcelona, Spain), LAMB (provided as AmBisome by NeXstar Farmacéutica S.A., Madrid, Spain) and recombinant human G-CSF (purchased as Granulokine; Laboratorios Pensa, Barcelona, Spain) were used. The three compounds were diluted in 5% dextrose solution to reach the desired concentrations.

Immunosuppression

Mice were immunosuppressed with a single dose of cyclophosphamide 200 mg/kg ip (purchased as Genoxal; Laboratorios Funk S.A., Barcelona, Spain) and with 5-fluorouracil 150 mg/kg iv (purchased as Fluoro-uracil; Productos Roche S.A., Madrid, Spain) on the day of infection.

Infection

In a series of pilot experiments, 0.2 mL of four conidial suspensions (2.5×10^4, 2.5×10^5, 2.5×10^6 and 2.5×10^7 conidia/mL) were injected via a lateral tail vein of mice to determine (i) the inoculum that provoked a lethal infection that resulted in 100% mortality by days 10–12 for the survival study, and (ii) the inoculum that provoked a weaker infection, with a 50% survival by day 10, for the tissue burden studies.

Treatment

For the survival study 150 mice were infected with 0.2 mL of a suspension of *S. prolificans* 2.5×10^5 conidia/mL. The inoculated mice were randomly assigned to five treatment groups of 30 animals. Each group received a different treatment, i.e., AMB, LAMB, G-CSF and LAMB + G-CSF, the fifth being the untreated control group, which received 5% dextrose solution iv. Therapy with AMB (1.5 mg/kg/day, ip) and LAMB (10 mg/kg/day, iv) began 1 day after infection and lasted for 10 days. AMB and LAMB were administered by different routes because AMB is toxic for mice when given iv at doses higher than 1 mg/kg/day.[7] Therapy with G-CSF (300 μg/kg/day, ip) began 3 days before infection and lasted for 8 days (from day −3 to day +5). Mortality was recorded daily for 30 days.

For the tissue burden study three groups of 10 animals were infected with 0.2 mL of *S. prolificans* at 2.5×10^4 conidia/mL, which were treated with LAMB + G-CSF, AMB or 5% glucose, respectively. The treatment regimes were identical to those in the survival study. On day 11 after challenge the surviving mice (approximately four to six per group) were anaesthetized with halothane (Fluothane; Zeneca Farma, S.A., Pontevedra, Spain) and humanely killed by cervical dislocation. The brain, lungs and kidneys were aseptically removed, and half of each organ was weighed and homogenized in 1 mL of sterile saline solution. This produced a minimal threshold of *c.* 2–10 cfu/organ. Six 10-fold serial dilutions of this homogenate were made with sterile saline. Volumes of 0.1 and 0.5 mL of the homogenate and 0.1 mL of each dilution were inoculated on to plates of SDA and incubated at 30°C for 3 days. Colonies were counted and plates with <200 colonies were used. The other halves of the organs were fixed in 10% neutral buffered formaldehyde for 10 days, embedded in paraffin wax and automatically processed. Sections (3 μm in thickness) of the embedded tissues were stained with haematoxylin–eosin, periodic acid Schiff (PAS) and methenamine silver (Grocott) for light microscopy observations.

Statistics

Mean survival time (MST) was estimated by the Kaplan–Meier method and compared among groups using the log-rank test. Colony counts in tissue burden studies were analysed by a parametric method that consisted of an analysis of variance. Calculations were performed using SPSS for Windows version 9.0.

Results

Immunosuppressed mice inoculated with *S. prolificans* developed systemic infection characterized by weight loss, lethargy, change in fur consistency and neurological abnormalities such as ataxia and stiff neck. Preliminary studies testing different infection doses of this fungus showed that survival correlated with inoculum size (Figure). All mice infected with 2.5×10^7, 2.5×10^6 and 2.5×10^5 conidia/mL had died within 8, 8 and 11 days, respectively. There was a 20% survival in the group infected with an inoculum of 2.5×10^4 conidia/mL after 30 days. Therefore we chose inocula of 2.5×10^5 and 2.5×10^4 conidia/mL for survival and tissue burden studies, respectively.

The efficacy of the different treatments is shown in Table 1. LAMB and LAMB + G-CSF treatments improved survival significantly with respect to the control (MST =

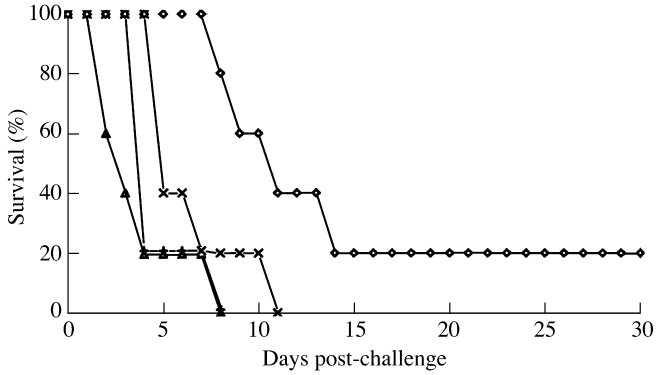

Figure. Survival after intravenous infection of groups of five mice with conidia of *S. prolificans*. Single doses of cyclophosphamide and 5-fluorouracil were administered on the day of infection. Mice were infected with 0.2 mL of four different inocula: ◇, 2.5×10^4 conidia/mL; ×, 2.5×10^5 conidia/mL; +, 2.5×10^6 conidia/mL; ▲, 2.5×10^7 conidia/mL.

7.93 days), showing a MST of 9.07 and 13.20 days, respectively. There was 20% survival (six mice of 30) in the combined therapy group and the MST of this group was significantly longer than that of the AMB group ($P = 0.0377$) and the G-CSF group ($P = 0.0004$), although it was not significantly different from that of the LAMB group ($P = 0.2995$). The MST of the G-CSF treatment group (7.47 days) was significantly shorter ($P = 0.0211$) than that of the control group.

The results of the tissue burden studies are shown in Table 2. The combined therapy reduced tissue counts, although not significantly with respect to untreated controls. Histological examination showed that the kidney was the organ most affected in all the groups. All autopsied mice had abscesses, with variable amounts of fungal elements (hyphae with or without conidia) and inflammatory response by polymorphonuclear leucocytes (PMNs). In general animals from both the control group and from the AMB group showed more diffuse abscesses with large amounts of fungal elements and a very low inflammatory response, as their immunosuppression had not resolved. Those of the combined therapy group had smaller abscesses with less fungal elements and a higher infiltration by PMNs and showed a comparatively stronger inflammatory response.

Discussion

We established a reproducible lethal disseminated infection by *S. prolificans* in mice immunosuppressed with a combination of 5-fluorouracil and cyclophosphamide. The administration of these drugs at 150 and 200 mg/kg, respectively, had previously been indicated as appropriate to make mice neutropenic (blood PMN $< 100/\mu L$) for at least 10 days.[8] This study confirmed the high virulence of this species for immunosuppressed mice, because all the mice in the untreated control group died during the survival study. The high virulence of the strain of *S. prolificans* tested had also been demonstrated previously by Cano *et al.*[9] Our results correlate with clinical outcome in immunocompromised patients, who almost always die if the neutropenia is not resolved.[6] Recovery of the phagocytic mechanisms in the form of rising neutrophil counts is a basic requirement for success of antifungal chemotherapy in these infections.

The combination of LAMB and G-CSF was clearly the most effective treatment. This therapy regime allowed the longest MST and the best survival rate. The combination of these two agents could provide important advantages for the treatment of refractory infections. The lipid vehicle of LAMB reduces toxicity and allows administration of higher doses, which results in higher concentrations of AMB in serum than treatment with conventional AMB.[7] In addition, a more optimal tissue distribution is reached by LAMB, resulting in a higher concentration of the drug in reticuloendothelial tissues heavily involved in fungal infections.[10] Clinical studies have demonstrated the efficacy of

Table 1. Survival of mice infected with *S. prolificans* and treated with AMB, LAMB, G-CSF or LAMB+G-CSF

Group[a]	No. of deaths/no. of animals tested	Survival (days)[b]	P[c]
Control	30/30	7.9 (7.5–8.4)	–
AMB	30/30	9.6 (7.7–11.5)	0.273
LAMB	30/30	9.1 (8.1–10.0)	0.022
G-CSF	29/30	7.5 (5.9–9.3)	0.021
LAMB+G-CSF	24/30	13.2 (9.8–16.6)	0.035

[a]AMB, amphotericin B deoxycholate; LAMB, liposomal amphotericin B; G-CSF, granulocyte colony-stimulating factor.
[b]Kaplan–Meier estimate of mean survival in days. Values in parentheses are the 95% confidence intervals.
[c]For comparison with the control group by the log-rank test.

Table 2. Effects of treatment on colony counts of *S. prolificans* in kidney, brain and lungs

Group[a]	Colony counts (mean $\log_{10}$ cfu/g of organ weight)		
	kidney	brain	lung
Control	5.46 (3.90–6.98)[b]	4.21 (3.09–5.33)	4.11 (1.36–6.85)
AMB	4.96 (0.63–9.28)	3.90 (2.52–5.28)	3.77 (0.02–7.51)
LAMB + G-CSF	4.46 (2.86–6.06)	3.35 (1.92–4.78)	2.54 (1.94–3.14)

[a]AMB, amphotericin B deoxycholate; LAMB, liposomal amphotericin B; G-CSF, granulocyte colony-stimulating factor.
[b]Values in parentheses are 95% confidence intervals.

this compound for empirical antifungal therapy in neutropenic patients.[11–13] G-CSF could contribute to recovery from the infection by increasing the number of differentiated granulocytes and their phagocytic activity.[14,15] Our study seems to confirm this because histological examination revealed a greater inflammatory response by PMNs and a lower number of fungal cells in the LAMB + G-CSF-treated group than in the other groups. Graybill *et al.*[16] reported no effect of G-CSF alone on survival of experimental cryptococcal meningitis, in agreement with Clemons *et al.*[17] in their study of orogastrointestinal candidosis. However, the combination of G-CSF with antifungal drugs showed an additive effect in experimental murine aspergillosis.[8]

Treatment with AMB was ineffective in our study. In contrast, treatment with LAMB alone improved the MST compared with the control group, although all the animals died before the end of the testing. This latter compound has been used in several human infections caused by *S. prolificans* and in one of only two cases of disseminated infection that have been resolved.[4,5] In a previous trial (data not published) the strain used in this study clearly showed *in vitro* resistance to AMB (MIC $>$ 16 mg/L) by the method adapted from the National Committee for Clinical Laboratory Standards for moulds.[18] This agrees with other *in vitro* studies that have shown high resistance of *S. prolificans* to AMB. [4,6]

Our experimental results, although still preliminary, are promising for future treatment of *S. prolificans* infections in neutropenic patients. It is even possible that by increasing the LAMB doses and/or making the cytokine regimen more suitable, better results can be obtained. Some of the new antifungals are also promising candidates for new therapeutic strategies. For instance, the new triazole UR-9825 (Uriach, Barcelona, Spain) has showed excellent *in vitro* results.[2] Synergy of several antifungal drugs against clinical isolates of this species has also been documented *in vitro*.[19,20] Nevertheless, these findings need to be viewed with caution and before using these compounds in the clinical setting, proof of their efficacy in adequate animal models is required.

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