



Modulatory Effect of Voriconazole on the Production of Proinflammatory Cytokines in Experimental Cryptococcosis in Mice with Severe Combined Immunodeficiency

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Abstract

Introduction: Cryptococcosis is a subacute or chronic disease. For many years, amphotericin B has been used in severe fungal infections. The triazole voriconazole is the antifungal with high bioavailability, a large distribution volume and excellent penetration of the central nervous system (CNS). 1.2 Objective: The purpose of the study was to evaluate the production of cytokines in the lungs during an experimental infection caused by *C. neoformans* in murine model (SCID) that was treated with amphotericin B and voriconazole.

Materials and Methods: After intravenous inoculation with 3.0×10^5 viable yeast cells, the animals were treated with amphotericin B and voriconazole. The daily treatments began 24 hours after inoculation and lasted 15 days. We evaluated the survival curve and measured the levels of TNF- α , IL-6 and IL-10.

Results: For all treatments, there was a significant increase in survival compared to the untreated group of animals and the group treated with voriconazole (maximum concentration). The levels of TNF- α were significantly lower in the groups treated with voriconazole (maximum concentration) and amphotericin B (minimum concentration).

Conclusion: Under the conditions studied, we can suggest that the production of cytokines mediated by amphotericin B and voriconazole is dependent on the concentration administered.

Keywords: Cryptococcosis; Amphotericin B; TNF- α , IL-6; IL-10 and Voriconazole; *C. neoformans*

Introduction

Cryptococcus neoformans variety *neoformans* is a pathogenic opportunistic yeast commonly found in the excreta of pigeons and other birds [1]. The yeast is responsible for subacute or chronic systemic mycoses termed "cryptococcosis" [2]. The diseases are caused by inhaling infective particles of yeast, which remain in the lungs and cause pulmonary cryptococcosis. *C. neoformans* spreads to other organs, mainly the central nervous system (CNS), through the bloodstream [2]. Mice with severe combined immunodeficiency (SCID) are more susceptible to systemic experimental cryptococcosis and can be a good model to study the immunologic response and therapeutics [3]. SCID mice are highly susceptible to infections due to their lack of B and T cells that are integral to the immune clearance of infections [4]. Tumor necrosis factor (TNF- α) is an important marker in cryptococcosis in both humans and the murine model and is a pro-inflammatory cytokines with various biological functions, including increasing the modulating the expression of other cytokines, such as IL-1 and IL-6, that are secreted by macrophages, neutrophils and T cells [5,6]. The increased levels of IL-6 occur within three days of treatment with amphotericin B in cryptococcosis [7].

Amphotericin B, fluconazole and fluorocytosine are common antifungal agents used for treating cryptococcosis [8]. For more than 30 years, amphotericin B has been used in severe fungal infections, but limited by significant

nephrotoxicity [9,10]. Fluconazole is used to treat cerebral cryptococcosis, but long-term indiscriminate use can result in resistance to this drug [11,12]. Voriconazole is a triazole with high bioavailability, a large distribution volume, excellent penetration of the CNS, excellent in vitro activity against *C. neoformans* and good concentrations in the serum [11,13].

The objective of this study is to evaluate the proinflammatory cytokines production in the lungs in an experimental infection by *C. neoformans* in SCID a murine model treated with amphotericin B and voriconazole.

Materials and Methods

Cryptococcus Neoformans Strain

The studies were performed using the *C. neoformans* strain ATCC 90112 (serotype A). This strain was maintained in tubes containing Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) and glycerol at -20°C in the Laboratory of Pathogenic Yeasts, Department of Microbiology, Institute of Biomedical Sciences of São Paulo University, São Paulo, Brazil [14].

Animal Model and Experimental Cryptococcosis

Forty-five SCID mice with a mean weight of 20g were obtained from the Animal Center, which was responsible for breeding isogenic animals at the Institute for Energy and Nuclear Research, São Paulo, Brazil. These mice were housed in microisolator cages, provided with sterile food and water and randomly distributed into six groups. The strain (ATCC 90112) was cultivated in YPD medium (1% yeast extract (Difco), 1% Bacto Peptone (Difco) and 2% dextrose (Sigma-Aldrich, Milwaukee, WI, USA) for 18 h at 30°C; the cells were collected by centrifugation, washed twice in a phosphate buffer solution (PBS) and resuspended at the inoculation concentration. Five groups were inoculated intravenously with 100 µL of the suspension containing 3.0×10^5 viable yeast cells. Among these groups (with 5 animals each) four groups were treated, and one group (n=10) was not treated. The untreated group served as the positive control, and one group (n=10) was inoculated with PBS, serving as the negative control. Animal handling and treatments observed the ethical principles of the Brazilian college of animal experimentation (COBEA), [14].

Treatments

The daily treatments began 1 day after the initial inoculation and lasted 15 days. The animals were inoculated intraperitoneally with 0.1 mL of amphotericin B (0.75 or 1.5 mg/kg/day) (Fungizone, Bristol-Meyers, Squibb S.p.A., Sermoneta, Italy), and 0.1 mL of voriconazole (20.0 or 40.0 mg/kg/day) (Vfend® IV) (Pfizer Inc, New York, NY, USA), [14-16]. At the end of study period (50 days), all animals that survived were euthanized in a CO₂ chamber. The dead and surviving mice were evaluated by survival curves and cytokine levels (TNF- α , IL-6 and IL-10) in lungs homogenates.

Removal and Homogenization of lungs

The lung were aseptically removed, weighed and homogenized in a solution containing 1.0 mL of phosphate buffer solution (PBS) supplemented with 0.05% Tween 20, 1% protease inhibitor (Sigma-Aldrich) and 1% phenylmethylsulfonyl fluoride (PMSF-1mM) (Sigma-Aldrich). They were centrifuged for 5 minutes at 14000 rpm and homogenates of the organ that were not used immediately were stored at -80°C for later use.

Levels of TNF- α , IL-6 and IL-10 in lungs homogenates.

The inflammatory mediators TNF- α , IL-6 and IL-10 were measured by Enzyme-linked Immunosorbent assay (ELISA) using the following kits, which were purchased from eBioscience and used according to the manufacturer's instructions: Mouse IL-6 (Interleukin-6) ELISA Ready-SET-Go, Mouse IL-10 (Interleukin-10) ELISA Ready-SET-Go and Mouse Cytokines ELISA Ready-SET-Go.

Statistical Analysis

The mean survival times were estimated by the Kaplan-Meier method and compared among groups by using the log-rank test. The data obtained in relation to the levels of TNF- α , IL-6 and IL-10 in the lungs homogenates were analyzed by with the Mann-Whitney test. The correlation was performed using the Pearson correlation test. All the statistical tests were performed using the software GraphPad Prisma 5 (GraphPad Prism™, Version 5.0, and GraphPad Software Incorporated). Differences were considered significant when $p < 0.05$.

Results

Survival

With the exception of the group treated with voriconazole (40.0 mg/kg/day), all the other treatments significantly increased the survival of animals ($p < 0.05$) when compared to the untreated group (11 days) (Figure 1). In the groups treated with 1.5 or 0.75 mg/kg/day amphotericin B, the survival times were 14 and 13 days, respectively. The survival of the group treated with voriconazole (20.0 mg/kg/day) was 15 days (Figure 1).

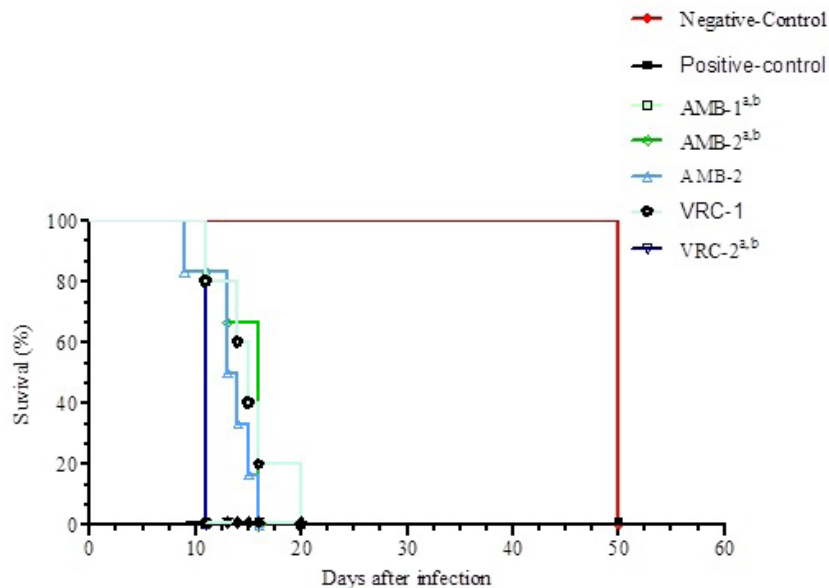


Figure 1: Cumulative mortality of mice treated at 15 days with amphotericin B AMB -1 (1.5 mg/kg/day), AMB-2 (0.75 mg/kg/day); voriconazole VRC-1 (40.0 mg/kg/day) and VRC-2 (20 mg/kg/day). Mean survival time were estimate by the kaplan-Meier method and compared among groups by using the log-rank test. Differences were considered significant when $p < 0.05$
^a $P < 0.05$ versus Untreated;
^b $P < 0.05$ versus VRC-1

Proinflammatory cytokines production Tumor necrosis factor (TNF- α)

The level of TNF- α production was significantly higher in the groups treated with amphotericin B (1.5 mg/kg/day; mean 13.347 pg/mL) and voriconazole (20.0 mg/kg/day; mean 6.848 pg/mL), when compared to the untreated group (mean 1.190 pg/mL), the group treated with amphotericin B (0.75 mg/kg/day; mean 2.013 pg/mL) and the group treated with voriconazole (40.0 mg/kg/day; mean 1.783 pg/mL) (Figure 2). There was no significant difference in the levels of cytokine production between the groups treated with amphotericin B (0.75 mg/kg/day) and voriconazole (40.0 mg/kg/day) (Figure 2).

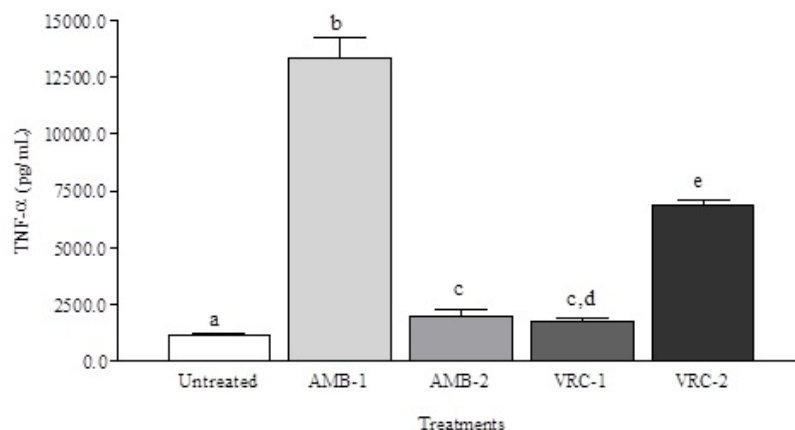


Figure 2: TNF- α level the lung homogenate in murine model with severe combined immunodeficiency (SCID), inoculated in travenously with 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112-serotype A). Evaluated after treatments by 15days with AMB-1, (amphotericin B (1.5 mg/kg/day), AMB-2, (amphotericin B 0.75 mg/kg/day), VRC-, (voriconazole mg/kg/day) and VRC-2 (voriconazole 20.0mg/kg/day. Different letters indicate statistical significant difference for $p < 0.05$ (Mann-Whitney test).

IL-6 (Interleukin 6)

The levels of IL-6 production were significantly higher ($p < 0.05$) in the group treated with amphotericin B (1.5 mg/kg/day; mean 3.574 pg/mL) and voriconazole (20.0 mg/kg/day; mean 4.661 pg/mL), when compared to the untreated group and all other treatments (Figure 3). The levels were significantly decreased ($p < 0.05$) in the groups treated with voriconazole (40.0 mg/kg/day; mean 526.1 pg/mL), when compared with all other treatments (Figure 3). In the groups treated with voriconazole (40.0 mg/kg/day) and voriconazole (20.0 mg/kg/day), the correlations between TNF- α and IL-6 were strongly negative ($r = -0.99$ and $r = -0.99$, respectively).

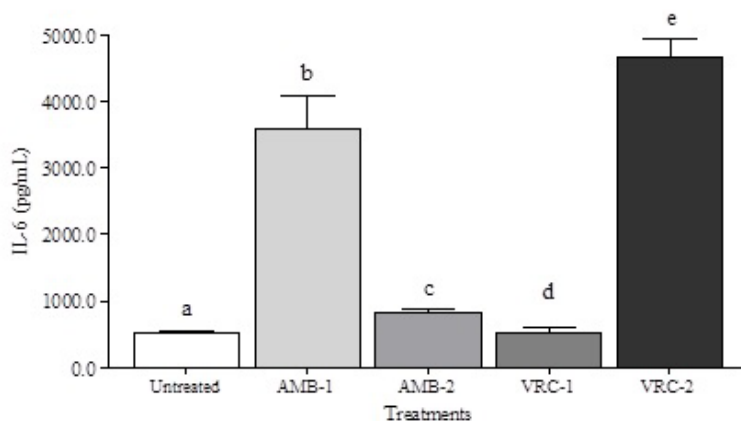


Figure 3: IL-6 Level the lung homogenate in murine model with Severe Combined Immunodeficiency (SCID), inoculated intravenously with 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112 – serotype A). Evaluated after treatments by 15 days with AMB-1, (amphotericin B 1.5 mg/kg/day), AMB-2, (amphotericin B 0.75 mg/kg/day), VRC-1, (voriconazole 40.0 mg/kg/day). Different letters indicate statistical significant difference for $p < 0.05$ (Mann-Whitney test)

Interleukin 10 production

The levels of IL-10 production were significantly higher ($p < 0.05$) in the groups treated with amphotericin B (1.5 mg/kg/day; mean 13.594 pg/mL) and voriconazole (20.0 mg/kg/day; mean 7.908 pg/mL) when compared to the untreated group and all others treatments (Figure 4). No significant difference was observed ($p < 0.05$) between the group treated with amphotericin B (0.75 mg/kg/day; mean 3.398 pg/mL) and the group treated with voriconazole (40.0 mg/kg/day; mean 3.477 pg/mL), (Figure 4). In the group treated with voriconazole (20.0 mg/kg/day), the correlation between TNF- α and IL-10, IL-6 and IL-10 was strongly positive ($r = 0.94$) and strongly negative, respectively.

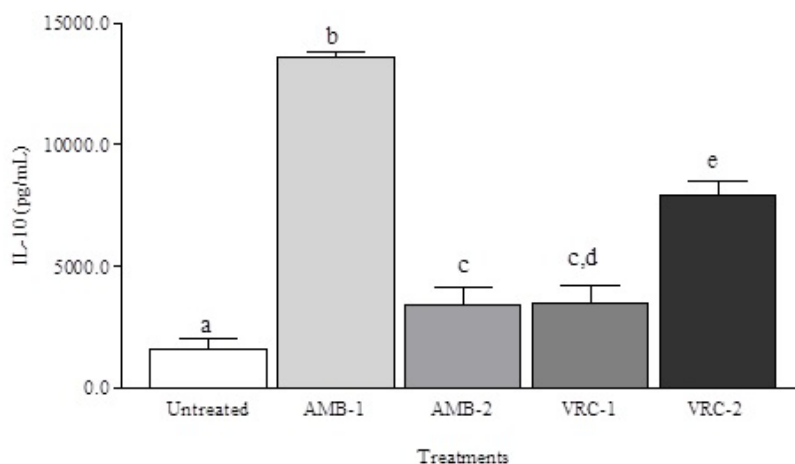


Figure 4: IL-10 level the lung homogenate in murine model with serve Combined immunodeficiency (SCID), inoculated in travenously with 3.0×10^5 viable cells of *C. neoformans* (ATCC 90112-serotype A). Evaluated after treatments by 15 days with AMB-1, amphotericin B (1.5 mg/kg/day); AMB-2, amphotericin B (0.75 mg/kg/day); VRC-1, voriconazole (40.0 mg/kg/day) and VRC-2, voriconazole (2.0 mg/kg/day). Different letters in dicate statistical significant difference for $p < 0.05$ (Mann-Whitney test)

There was no significant difference ($p < 0.05$) between the levels of TNF- α and IL10 production in groups treated with amphotericin B (1.5 mg/kg/day) and voriconazole (20.0 mg/kg/day). In all of the groups from this study, treated and untreated, the production of IL-6 was significantly lower ($p < 0.05$) than the production of TNF- α . In the group of untreated animals, as well as in the groups treated with amphotericin B (0.75 mg/kg/day) and voriconazole (40.0 mg/

kg/day), the levels of production were significantly higher ($p < 0.05$), when compared with the levels of production of TNF- α and IL-6.

Discussion

The model of cryptococcosis in SCID mice can be useful in the immunologic and therapeutic study of diseases in immuno deficient hosts [3]. Amphotericin B has been successfully used for over 30 years to treat cryptococcosis; however, its toxicity and the increase in strains resistant to fluconazole have stimulated the study of appropriate choice of treatments [16]. In this study, the effects of treatments with amphotericin B and voriconazole on the proinflammatory cytokines production to systemic cryptococcosis in SCID mice were evaluated. TNF- α is a proinflammatory cytokine that provides important protection against lungs infections caused by *C. neoformans* [17]. As observed in this study, the administration of high dose of amphotericin B and lower concentrations of voriconazole resulted in a significant increase in the level of cytokines produced. The presence of an inflammatory process represents a fundamental role in restricting *C. neoformans* at the site of infection [18]. Amphotericin B has been shown to induce Cytokines production in human and murine monocytes [19]. The production of proinflammatory cytokines mediated by amphotericin B depends on the concentration administered. In this study, we found that administering this drug at a low concentration caused a low yield of cytokine. Several studies have reported immunomodulatory effects of voriconazole in aspergillosis; however, no work has been performed in cryptococcosis [20]. In this study the treatment with voriconazole (maximum concentration) resulted in low levels of cytokines. IL-6 has a protective role in the immune response against *C. neoformans* is involved in the differentiation of monocytes into macrophages and is responsible for reducing the production of cytokines [21,22]. The production of IL-6 was significantly lower than the production of cytokines in all treatments, and was significantly higher in the group of animals treated with the maximum concentration of amphotericin B and in the group of animals treated with the minimal dose of voriconazole. In the groups treated with voriconazole at both maximal and minimal concentrations, the production of IL-6 was responsible for the reduced production of TNF- α ; the statistical correlation of this cytokine and IL-6 showed a strong negative correlation. IL-10 is produced mainly by macrophages, dendritic cells, B lymphocytes and T. Usually participates as an anti-inflammatory and immunosuppressive cytokine, reducing chronic inflammation therefore it is able to restrict the inflammatory response [23-26]. The production of IL-10 was significantly higher in the groups treated with amphotericin B (maximum concentration) and voriconazole (minimum concentration). IL-10 is normally produced during the inflammatory response as a regulatory mechanism to prevent exuberant inflammation [27]. The high production of IL-10 in these treatment groups was not able to regulate inflammation, and it was found that there was no significant difference between the production of this cytokine and TNF- α . In the group treated with amphotericin B (minimum concentration) and in the group treated with voriconazole (maximum concentration), the production of IL-10 was significantly higher than the production of cytokines [28-30].

Conclusion

In conclusion, under the conditions studied, we can suggest that the production of proinflammatory cytokines mediated by amphotericin B and voriconazole is dependent on the concentration administered. Treatment with voriconazole (maximum concentration) may have been responsible for modulating the production of these cytokines in this model. Future studies should be performed on these drugs to find a concentration that is more effective in the treatment of cryptococcosis in this model, as well as on the kinetics of voriconazole should be performed to verify the most effective dose in the treatment of the disease should be performed on these drugs to find a concentration that is more effective in the treatment of cryptococcosis in this model.

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Conflict of Interest Statement

There is no conflict of interest in the present study.

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