Antibodies Against Sporothrix schenckii Enhance TNF-a Production and Killing by Macrophages
Antibodies Against *Sporothrix schenckii* Enhance TNF-α Production and Killing by Macrophages

D. de Lima Franco*, R. C. Nascimento*†, K. S. Ferreira‡ & S. R. Almeida*

*Abstract*

Sporotrichosis is a chronic granulomatous mycosis caused by the dimorphic fungus *Sporothrix schenckii*. The immunological mechanisms involved in the prevention and control of sporotrichosis suggest that cell-mediated immunity plays an important role in protecting the host against *S. schenckii*. Nonetheless, recent data strongly support the existence of protective Abs against this pathogenic fungus. In a previous study, we showed that passive Ab therapy led to a significant reduction in the number of colony forming unit in the organs of mice when the MAb was injected before and during *S. schenckii* infection. The ability of opsonization to enhance macrophage damage to *S. schenckii* and subsequent cytokine production was investigated in this work. Here we show that the fungicidal characteristics of macrophages are increased when the fungus is phagocytosed in the presence of inactivated serum from mice infected with *S. schenckii* or mAb anti-gp70. Additionally, we show an increase in the levels of pro-inflammatory cytokines such as TNF-α and IL-1β. This study provides additional support for the importance of antibodies in protecting against *S. schenckii* and concludes that opsonization is an important process to increase TNF-α production and fungus killing by macrophages in experimental sporotrichosis.

**Introduction**

Sporotrichosis is a chronic granulomatous mycosis caused by the dimorphic fungus *Sporothrix schenckii*, which is widely distributed in nature and exists in a saprophytic mycelial form on plant debris and soil. This disease is caused by traumatic inoculation of the dimorphic fungus *S. schenckii* [1]. In the past, infections were limited to the cutaneous forms of the disease. Recently, occurrences of more severe clinical forms of this mycosis have been described, especially among immunocompromised individuals [2, 3].

The immunological mechanisms involved in the prevention and control of sporotrichosis suggest that cell-mediated immunity plays an important role in protecting the host against *S. schenckii* [4]. In experimental infections, athymic nude mice are more susceptible to sporotrichosis, and acquired immunity against *S. schenckii* is mainly mediated by T cell-activated macrophages [5, 6].

In contrast, the role of the humoral immune response in protection against this fungus has not been studied in detail. Although many studies have investigated antibody responses in fungal infections, critical features, such as fine antibody specificity or antibody isotype, have seldom been examined. Moreover, because many different clinical and experimental settings have been utilized, no general conclusion has been reached [7]. Nonetheless, recent data strongly support the existence of protective Abs against pathogenic fungi [8–10].

In a previous study, we showed that antigens secreted by *S. schenckii* induce a specific humoral response in infected animals, mainly against the 70 kDa molecule, indicating the possible participation of specific antibodies against this molecule in the control of infection [11]. Our group produced an IgG1 mAb (monoclonal antibody) against a 70-kDa glycoprotein of *S. schenckii*. Our results showed a significant reduction in the number of colony forming unit (CFU) in the organs of mice when the mAb was injected before and during *S. schenckii* infection. Similar results were observed when T cell-deficient mice were used. Moreover, in a second treatment schedule, the mAb was injected after infection was established, and we observed a significant reduction in CFU [12]. However, the mechanism associated with the protection was unclear.

The goal of the current study was to determine whether opsonization of *S. schenckii* affects the ability of macrophages to clear this organism and/or stimulates cytokine production.
Materials and methods

Fungal strains. Sporothrix schenckii strain M-64, previously isolated from a human case of sporotrichosis, was obtained from the Department of Dermatology of the Medical School, University of Sao Paulo. Yeast forms of S. schenckii were grown (3 days, 37°C) in brain-heart infusion agar (Oxoid, Hampshire, England).

Animals. Female BALB/c mice, 8–12 weeks old, provided by the animal facilities of the University of Sao Paulo (Sao Paulo, SP, Brazil) were used throughout this study. The local Animal Care and Use Committee approved the experimental protocols.

Animal infection. Groups of six BALB/c mice were infected intraperitoneally with $5 \times 10^6$ yeast forms of S. schenckii suspended in PBS (2.65 g Na$_2$HPO$_4$, 0.35 g NaH$_2$PO$_4$, 0.05 g NaCl in 1 l of distilled water). Blood samples were collected from individual mice at 14 days after infection, and the serum was inactivated and stored at −20°C until use. As a negative control the mice were infected with only PBS.

Phagocytosis assay. For phagocytic assays, J774.16 cells were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (R10), plated on a 24-well tissue culture plate containing round glass cover slips and incubated overnight at 37°C in a 5% CO$_2$ atmosphere. The adherent monolayers were co-cultured with S. schenckii yeast cells. Before the co-culture, yeast cells were incubated (1 h, 37°C) with heat-inactivated serum (56°C, 15 min) diluted at 1:50, 1:100, 1:200 or 1:400 in PBS or mAb P6E7 (50 μg/ml) with agitation. After being washed to remove unbound antibody, macrophages were challenged by 24 h with S. schenckii yeast cells (parasite-to-cell ratio, 3:1). The slides were stained with Giemsa (Instant Prov Kit; Newprov, Curitiba, Parana, Brazil), and the phagocytosis index (PI) was calculated. An average of 200 macrophages were counted to determine the PI, calculated as the per cent of phagocytic cells multiplied by the mean number of internalized particles [13]. Phagocytosis was confirmed by transmission electron microscopy, which showed the internalization of yeast cells by macrophages. The antifungal efficacy of the J774.16 cells was determined by counting the CFU of S. schenckii after co-culturing yeasts and macrophages. Briefly, macrophages were mixed with yeast cells previously opsonized as described above, and the mixtures were incubated for 24 or 72 h at 37°C. The contents of each well were then removed (stored at −80°C for cytokine measurement), and the cells were lysed by the addition of 200 μl of sterile water and incubated for 15 min. Supernatants and lysates were pooled and centrifuged, while the pellet was resuspended in 200 μl of PBS and then diluted 1:20 in PBS. Five hundred microlitres of this suspension was plated in Sabouraud dextrose agar. The number of colonies per plate was counted after 10 days of incubation at 30°C. Controls were performed using fungi alone or fungi incubated with serum from non-infected animals. As control of mAb P6E7 was used an irrelevant monoclonal antibody (MAb anti-carcinoembryonic antigen-CEA 1.H2, IgG1, κ light chain). Contamination of the cultures with lipopolysaccharide (LPS) was monitored using the Limulus amebocyte lysate test (Endosafe LAL; Charles River, Charleston, SC, USA). Maximal concentration of LPS detected in the cultures was always <0.2 ng LPS/ml.

Cytokine measurements. The levels of cytokines in the supernatants of macrophage cultures were determined by sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions. Macrophages were incubated with yeast as described, and after 24 h, the supernatants were harvested and stored at −70°C for cytokine quantification. Cytokines were quantified in the supernatants by ELISA (Pharmingen, San Diego, CA, USA) according to the manufacturer’s instructions. Cytokine activity was determined using standard curves with serial dilutions of mouse recombinant cytokines.

Transmission electron microscopy. The phagocytosis assay was performed as described above. After being cultured, the macrophages were collected with a cell scraper and were fixed in osmium tetroxide 1% in cacodylate buffer 0.1M (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 min. Dehydration was achieved using various percentages of ethyl alcohol (MERCK, Whitehouse, NJ, USA). Propylene oxide (Electron Microscopy Sciences) was used as a solvent for the gradual addition of Araldite resin. After embedding, samples were maintained in a vacuum for 4 h and polymerized at 56°C for 48 h. Embedded specimens were cut (model 702501, Reichert Ultracut; Leica, Solms, Germany) with glass knives (Reichert-Jung knifemaker type 705202). Sections 90–95-nm thick were placed onto 300 mesh copper or nickel grids and stained with a 2% aqueous solution of uranyl acetate (Electron Microscopy Sciences) for 8 min. These preparations were subsequently stained with lead citrate in distilled water (Electron Microscopy Sciences) for 4 min. The grids were examined and cells photographed in a JEM 1200 EX II electron microscope (JEOL Ltd., Tokyo, Japan).

Statistical analysis. Statistical comparisons were made by analysis of variance (ANOVA) and by the Tukey-Kramer post-test. All values are reported as the mean ± standard error of the means.

Results

Phagocytosis of yeast form of S. schenckii opsonized with inactivated serum

In this work, we first investigated whether macrophages would internalize opsonized S. schenckii. For this purpose, we determined the phagocytic index of macrophages...
co-cultured with *S. schenckii* previously incubated with serum. We found that the phagocytosis of *S. schenckii* by macrophages in the presence of serum from infected mice was higher than serum from non-infected mice (control). A significant increase in phagocytosis was observed in the presence of serum diluted at 1:50, 1:100 and 1:200 (Fig. 1A). Electronic microscopy was used to demonstrate the uptake of opsonized yeast (Fig. 1B).

*Sporothrix schenckii* viability test

We were interested in determining the persistence of *S. schenckii* after its uptake by the macrophages. Therefore, we analysed the fungicidal capacity of the macrophages and compared the opsonized yeast with normal (control) or immune heat serum. In this experiment, we used serum diluted at 1:200, as dilutions at 1:50 or 1:100 resulted in similar results as those observed above. A significant decrease in the number of viable yeast cells after 72 h of incubation was observed in yeast opsonized with serum (Fig. 2).

Cytokine production

The next step was to determine the cytokine production by infected macrophages. We found that phagocytosis of yeast opsonized with serum induced an increase in the levels of TNF-α, IL-1β, IL-6 and IL-10 compared to non-opsonized yeast or opsonized with normal serum (Fig. 3). Similar results were observed when the yeasts were opsonized with mAb P6E7 (Fig. 4).

Viability of *S. schenckii* yeast opsonized with mAb P6E7

To verify whether mAb P6E7 against gp70 from *S. schenckii* could regulate the killing as observed above, we performed a CFU assay. We observed that yeast opsonized with mAb induced an increase in the death of *S. schenckii* after 72 h of interaction (Fig. 5).

Discussion

The protective role of antibodies against some fungi, such as *Cryptococcus neoformans*, is well established [14]. An important mechanism of protection mediated by antibodies is the opsonization of the microorganism by the Fc gamma receptor (FcγR) [15]. FcγR mediates several macrophage functions, including stimulating cytokine secretion, activating respiratory burst, phagocytosis and antibody-dependent cellular cytotoxicity [16, 17]. Phagocytes that bind bacteria via FcγR demonstrate increases in oxidative burst and cytokine production [18, 19].
The binding of antibody-opsonized organisms by phagocytes causes crosslinking of phagocyte receptors for IgG (FcγR), resulting in a complex array of intracellular signals [21].

The ability of antibody opsonization to enhance macrophage damage to S. schenckii, and the subsequent cytokine production, has not been analyzed. In the present study, we have shown that the fungicidal ability of macrophages is increased when the fungus is phagocytosed in presence of immune-inactivated serum or mAb P6E7. However, we observed that macrophages need, at least, 72 h to efficiently kill the yeast. This mechanism was more evident when the yeast was opsonized with mAb. Maybe the activation of macrophages by mAb P6E7-opsonized yeast is late when compared with immune serum-opsonized yeast. This result could indicate that other factors in the immune serum, apart from anti-gp70, could participate in macrophage activation. It is well documented that the activating FcγR plays an important role in activating the production of pro-inflammatory components. Stimulation of macrophages via FcγR induces TNF-α secretion. Wijngaarden et al. [22] showed that treatment of rheumatoid arthritis patients with anti-TNF-α induced a down-regulation of the activating FcγR on monocytes. Pro-inflammatory cytokines were analysed in our study, and our results showed a high production of TNF-α and IL-1β when the fungus was opsonized by serum or mAb P6E7. The production of the anti-inflammatory cytokine IL-10 by macrophages was lower when compared with TNF-α or IL-1β. The production of TNF-α and IL-1β by macrophages incubated with mAb P6E7-opsonized yeast was lower than immune serum. TNF-α is a multifunctional cytokine that plays an important role in inflammatory responses. Some studies have shown that TNF-α is an important anti-inflammatory cytokine involved in the control of sporotrichosis [23, 24]. Gottlieb et al. [25] reported a case of disseminated sporotrichosis in a patient treated with TNF-α antagonists, presumably for inflammatory arthritis. This case illustrates the importance of TNF-α in the control of sporotrichosis. It is tempting to speculate that Ab-mediated phagocytosis may be essential to macrophage killing, the production of TNF-α and consequently, the control of sporotrichosis.

Characterizing the mechanism of interactions during Ab-mediated phagocytosis is important for understanding the role of Ab generated during the host immune response and assessing the mechanism of action of passive Ab therapy, by which treatment with specific mAbs can improve disease.
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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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