Therapeutic vaccine using a monoclonal antibody against a 70-kDa glycoprotein in mice infected with highly virulent *Sporothrix schenckii* and *Sporothrix brasiliensis*

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Abstract

Sporotrichosis is a chronic granulomatous mycosis caused by the dimorphic fungi that comprise the *Sporothrix* complex. The latter are widely distributed in nature, developing a saprophytic mycelial form on plant debris and soil. Formerly, the *S. schenckii* species was thought to be the only species capable of causing sporotrichosis. However, in recent years, the existence of a group of highly genotypically and phenotypically variable species has been reported as etiologic agents of this mycosis. Recently, it has become important to study aspects such as virulence and the immune response against key members of the *Sporothrix* complex and to observe the presence of glycoprotein (gp) 70 and efficacy of the P6E7 monoclonal antibody against more virulent strains. The data presented here demonstrate that the strain isolated from a case of feline sporotrichosis, that is, strain 5110 (American Type Culture Collection MYA-4823) is the most virulent and the only one able to secrete gp70. This glycoprotein is apparently an important factor in the virulence of *Sporothrix* spp. because treatment with MAb P6E7 resulted in the reduction of fungal burden in the analyzed organs. Additional studies of the role of gp70 in modulating the immune response of the host are needed to understand the pathology of sporotrichosis.

Key words: *Sporothrix*, monoclonal antibody, virulence, therapeutic vaccine, sporotrichosis.

Introduction

Sporotrichosis is a subcutaneous mycosis that affects humans and animals and frequently involves the lymph system. *Sporothrix* spp. include thermally dimorphic fungi with a saprophytic form that is mycelial and yeast like at elevated temperatures, as in host tissue [1,2].
Sporotrichosis has a worldwide distribution but is reported more frequently in countries with tropical and subtropical climates, such as Mexico, Brazil, Peru, and Uruguay [3]. Some activities such as gardening, agriculture, horticulture, fishing, hunting, and mining, which facilitate exposure to the fungus, are associated with this mycosis [4,5]. Due to evidence for zoonotic transmission, veterinarians, veterinary technicians, and cat owners are considered a new risk category for acquiring the disease [4]. The pathogenesis of this disease is related to traumatic inoculation of conidia or hyphae into the skin [6].

Formerly, S. schenckii was the only species considered to be the etiologic agent of sporotrichosis. However, in recent years, a group of highly genotypically and phenotypically variable species was recognized as the Sporothrix complex and has been described as the cause of this mycosis [7–9]. This genotypic variability was observed after the outbreak of sporotrichosis in the state of Rio de Janeiro [10]. Thus, the following four new species have been described in the Sporothrix complex: S. globosa, S. brasiliensis, S. mexicana, and S. luriei.

We demonstrated that mice infected with the M-64 S. schenckii strain produced immunoglobulin (Ig) G1 antibodies against a 70-kDa glycoprotein (gp) 70, which indicated that it could be a major antigenic component of S. schenckii, which participates in modulation of the immune response [11]. We produced a monoclonal antibody against gp70, termed P6E7, in our laboratory and demonstrated it to be protective experimental sporotrichosis. A decrease in the fungal load in the spleen and liver, as well as prevention of the development of more severe forms of the disease, was observed in mice treated with MAb P6E7 [12].

Virulence and immune responses are crucial in the development and severity of sporotrichosis. With the recent outbreaks in urban areas, the emergence of feline zoonotic transmission and prevalence of S. brasiliensis in the disease cases created a new paradigm of the disease. This increased the importance of studying aspects such as virulence and the immune response against key members of the Sporothrix complex, as well as observing the presence of gp70 and efficacy of the P6E7 monoclonal antibody against more virulent strains. Teixeira et al. [13] showed a significant difference in the virulence between strains of S. brasiliensis and S. schenckii. Moreover, Castro et al. demonstrated a different concentration of gp70 in the cell wall of S. brasiliensis and S. schenckii [14]. Considering these recently published data, our objective was to determine whether the P6E7 monoclonal antibody could be effective regardless of the species of fungus and the amount of gp70 in the cell wall.

Material and methods

Fungus and culture conditions

Sporothrix schenckii strain 1099–18 (American Type Culture Collection [ATCC] MYA-4821), S. schenckii strain 15383 (ATCC MYA-4820), S. brasiliensis strain 17943 (ATCC MYA-4824) recovered from human cases and S. brasiliensis strain 5110 (ATCC MYA-4823) isolated from a case of feline sporotrichosis were used in the study. All strains were maintained in the Mycology Collection of the Laboratory of Clinical Mycology, Faculty of Pharmaceutical Sciences, University of São Paulo. The yeast phase was grown on brain heart infusion (BHI) agar (HIMEDIA, Mumbai, India) at 37°C, with samples taken every 15 days.

Production of exoantigen

The yeast cells of each strain of Sporothrix spp. were grown in Erlenmeyer flasks containing 400 ml of liquid YGC medium (yeast nitrogen–glucose casamino acids) for 7 days with continuous stirring at 8.9 g at 37°C as previously described. After this period, the proteins were measured using the Bradford reagent (Sigma, Saint Louis, MO, USA); BSA served as the standard (Sigma, Saint Louis, MO, USA). The manufacturers’ suggested procedures were followed in all instances.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins released by strains of Sporothrix

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel for electrophoresis was prepared upright in a mini gel electrophoresis apparatus (Bio-Rad mini PROTEAN), which consisted of a linear separating gel of 12% polyacrylamide and 0.75-mm–thick, 3% polyacrylamide stacking gel. The soluble antigen fractions were applied onto a vertical slab gel apparatus and electrophoresed (40 mA, 1 h). The gels were then stained with silver nitrate or processed for immunoblotting.

Experimental infection with strains of Sporothrix

Eight- to 12-week-old BALB/c mice were obtained from the Animal House Production and Experimentation facility of the Faculty of Pharmaceutical Sciences and Institute of Chemistry, University of São Paulo. The local animal care and use committee approved the experimental protocols. The animals were reared in pathogen-free conditions. Strains of Sporothrix spp. were grown on BHI agar at 37°C for 5 days and then suspended in 5 ml of sterile phosphate-buffered saline (PBS) to determine cell counts.
chamber. The samples were then diluted 1:1 with trypan blue dye to check cell viability. Four groups of 20 mice were infected intraperitoneally with a 100-µl inoculum of 5 × 10⁶ yeast cells for each Sporothrix spp. test, while another group received 100 µl of sterile PBS to serve as the control.

On days 7, 14, and 21 post-infection, five mice from each group were anesthetized and euthanized by intracardiac puncture. Serum samples were obtained. The spleen and liver of these animals were removed to assess infection by counting colony-forming units (CFUs) and measuring cytokine levels (interleukin IL-4, interferon-gamma IFN-γ) and IL-10).

**Determination of colony-forming units**

The liver and spleen from each animal were placed in sterile Petri dishes and immediately weighed. Each organ was homogenized mechanically in 2 ml of sterile PBS; dilutions were prepared from this solution and subsequently plated on BHI agar plates. The plates were maintained at 30 °C for 7 days; then colony counts were taken to assess the CFU values to use in the calculations of the average CFU per gram of organ.

**Determination of cytokines**

The IL-4, IL-10, and IFN-γ concentrations were determined using enzyme-linked immunosorbent assay (ELISA). For these tests, the macerated spleens and livers were centrifuged for 10 min at 3500 rpm; the pellet was discarded; and the supernatant was stored at −20 °C until use. ELISA was performed using a commercial kit specific for each cytokine (R & D Systems, Mineapolis, MN, USA), following the manufacturer’s instructions.

**Determination of total IgG**

The levels of total IgG against exoantigens of each strain were determined using ELISA with Sporothrix diluted in carbonate buffer at a concentration of 4 mg/ml. The plates were incubated overnight at room temperature, after which three washings with PBS-T (PBS + 0.05% Tween 20), followed by the addition of 200 µl of blocking solution (PBS + 5% milk + 2.5% BSA) were performed; the plates were then incubated for 2 h at 37 °C. Subsequently, the solution was discarded, and sera diluted at a ratio of 1:50 in PBS–milk (PBS + 5% milk) from infected mice were added. The plates were then incubated for 1 h at room temperature and washed three times with PBS-T. Then 50 µl of conjugated antibody that was previously titrated with antimus IgG peroxidase and diluted in blocking solution was added. The plates were incubated at room temperature for 1 h. After incubation, the plates were washed three times with PBS-T, 100 µl/well development solution was added (1 mg/ml o-phenylenediamine in phosphate citrate buffer; Na₂HPO₄ · 12H₂O, 3.68 g; 1.16 g citric acid; 200 ml H₂O₂; pH adjusted to 5.3), and the plates were incubated for 15 min in the dark. The reaction was stopped with 4N H₂SO₄. The reading was performed using an ELISA reader with a 492-nm filter. The optical density readings of the sera were compared at different times of infection.

**Western blot analysis**

After electrophoresis, the proteins were transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using the semi-dry Blotter Enhanced Chemiluminescence (ECL) unit (Amersham Biosciences, Piscataway, NJ, USA). The membranes were then washed three times with PBS-T for 10 min and incubated with the primary antibody (sera from mice infected with strains of Sporothrix spp., sera from uninfected mice, and the monoclonal antibody P6E7). The primary antibodies in 5 ml of diluted 1:200 PBS-T were incubated with the membranes overnight under gentle agitation at 4 °C. After reaction with the primary antibody, the membranes were washed three times with PBS-T for 10 min and then incubated with anti-mouse Ig linked to horseradish peroxidase (BioRad) that was diluted at 1:5000 in PBS-T for 2 h at room temperature under gentle agitation. After this step, the ECL substrate solution Novex (Invitrogen, Carlsbad, CA, USA) was added for 1 min. In a dark room, the nitrocellulose membrane was incubated for 2 min with Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, UK), revealed, washed, and fixed for visualization of the bands.

**Prophylactic treatment**

The mice were intraperitoneally inoculated with different strains of Sporothrix spp. On day 3 and day 10 post-infection, the mice received 100 µg of monoclonal antibody P6E7 intraperitoneally. After 7, 14, and 21 days of infection, the mice were sacrificed, and the spleens and livers were removed to evaluate infection using CFU.

**Results**

**Profile of electrophoretic exoantigen strains of Sporothrix sp.**

Components of the exoantigens were analyzed on a 10% SDS-PAGE, demonstrating that the protein profiles of the exoantigen strains of Sporothrix spp. ranged from 250 to 20 kDa (Fig. 1). Sporothrix schenckii strain 15383 had high expression of proteins between 70 kDa and 50 kDa,
Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of different strains of *Sporothrix*. Line 1 shows the profile of electrophoretic proteins secreted by the yeast cells in *Sporothrix* strains. Line 2 shows the molecular weight standard. The SDS-PAGE gel was silver stained.

whereas strain 1099–18 had high expression of proteins between 150 kDa and 100 kDa. For *S. brasiliensis* 5110 strain, the majority of bands were between 100 kDa and 70 kDa. Strain 17943 had a wide variety of exoantigens when compared with the other strains; the majority of bands were between 100 kDa and 70 kDa.

**Detection of antigenic components in exoantigen strains of *Sporothrix* spp.**

The serum obtained from mice on days 7, 14, and 21 post-infection with different strains of *Sporothrix* spp. were incubated with exoantigens of related strains to detect antigenic components. Figure 2 shows that sera from infected mice recognized a band between 100 kDa and 70 kDa for all analyzed exoantigens. This reaction was more evident on days 14 and 21 post-infection.

**Total IgG detection by ELISA in the sera of infected mice**

Antibodies to *Sporothrix* spp. in the sera of infected mice were determined as total IgG through using ELISA plates sensitized with exoantigens (Fig. 3). While the level of IgG was low 7 days after infection, IgG against exoantigens increased significantly 14 days after infection and was maintained at a high level for at least 21 days following infection. We also observed that mice infected with *S. brasiliensis* 17943 produced more IgG than the other strains.

**Detection of cytokines in the spleen and liver homogenates**

Figure 4 shows that mice infected with *S. schenckii* produced a mixed pattern of T helper (Th) 1/Th2 cytokines. Additionally, IFN-γ, IL-4, and IL-10 were produced in the spleens and livers of mice infected with both strains of *S. schenckii*. However, we could not detect cytokine production in the organs of mice infected with *S. brasiliensis* strains.

**Counting of CFU in organs infected with strains of *Sporothrix* sp.**

Figure 5 shows the fungal burden in the livers and spleens of animals infected with different strains of *Sporothrix*. The results are expressed as log 10 of the base per gram of organ. The fungal burden with all strains of *Sporothrix* was high. A small decrease in the fungal burden in mice infected with strain 1099–18 was observed on day 14 post-infection. However, during most of the infection period, the burden remained constant, suggesting that all strains induced a chronic course of the disease.

**Presence of gp70 in exoantigen of different strains of *Sporothrix* sp. using MAb P6E7**

Figure 6 shows the presence of gp70 in the exoantigens of different strains of *Sporothrix* spp.; Western blot analysis
Figure 2. Western blot analysis with serum from infected mice against *Sporothrix* strains. Serum was collected from BALB/c mice (n = 5 per group) on days 7, 14, and 21 post-infection with $5 \times 10^6$ yeast of *Sporothrix* strains. The serum control (n = 5 per group) was collected 21 days after inoculation of sterile phosphate-buffered saline in BALB/c mice. Representative of three independent experiments.

Figure 3. Total antibody levels of infected mice with *Sporothrix* sp. Serum was collected from BALB/c mice (n = 5 per group) on days 7, 14, and 21 post-infection with $5 \times 10^6$ yeast of *Sporothrix* strains. The serum control (n = 5 per group) was collected 21 days after inoculation of sterile phosphate-buffered saline in BALB/c mice. Representative of three independent experiments. The enzyme-linked immunosorbent assay was performed using the sera against their respective exoantigens. Representative of three independent experiments. The results represent the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance and multiple comparisons by the Tukey test. The levels of significance were as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. IgG, immunoglobulin G; OD, optical density; PBS, phosphate-buffered saline. Therapeutic immunization with P6E7 of mice infected with different strains of *Sporothrix* sp.

Reduced fungal burden in the spleens of all mice infected with *Sporothrix* spp. was observed, especially during the

with the monoclonal antibody P6E7 was used for this analysis. The gp70 was only present in the exoantigens of *S. brasiliensis* strain 5110; *S. schenckii* strain M-64 was used as a positive control.
Figure 4. Cytokine production by spleen and liver of infected mice. The cytokines were determined using enzyme-linked immunosorbent assay in the homogenates of spleen and liver of BALB/c mice (n = 5 per group) that were infected with 5 × 10^6 yeast Sporothrix strains. The control group (n = 5 per group) received sterile phosphate-buffered saline and was sacrificed on the same days as those in the infection group. Representative of three independent experiments. The results represent the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance and multiple comparisons by the Tukey test. The levels of significance were as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. IFN-γ, interferon-gamma; IL, interleukin; PBS, phosphate-buffered saline.

early stages of infection (Fig. 7). Relapse occurred during the final period of the infection, mainly when the mice were infected with strain 5110. In the liver, a significant reduction in the fungal load was observed after 14 and 21 days of infection. However, the reduction in the fungal burden of strain 1099–18 was not significant.

Statistics
Statistical comparisons were made using analysis of variance (ANOVA) and the Tukey–Kramer test. All values were reported as the mean ± the standard error of the mean.

Discussion
It has been demonstrated that isolates recovered from specimens of different clinical forms of sporotrichosis have distinct virulence patterns in experimental infections [13]. We reported that mice infected with a low-virulent strain of S. schenckii, M-64, produced a mixed immune response in which sera from infected mice reacted only with a 70-kDa glycoprotein (gp70) [11]. Here, we used two high-virulent strains of S. schenckii (1099–18 and 15383) and two of S. brasiliensis (5110 and 17943).

Analysis of the profile of proteins secreted by strains of Sporothrix spp. showed that the strains had different profiles. Using Western blot, it was observed that during the course of the infection, the sera from infected mice reacted with antigenic components ranging in size from 50 kDa to 100 kDa.

In the present study, Western blot analysis, conducted using MAb P6E7, showed the presence of gp70 only in the exoantigen of the most virulent strain, S. brasiliensis 5110. In contrast, Castro et al. found gp70 to be present in the cell wall of S. schenckii strains 1099–18 and 15383 and S. brasiliensis strain 17943; a low presence was noted on the cell surface of the highly virulent S. brasiliensis 5110 strain [14]. These results suggest that the secretion of gp70 is dependent on the strain of Sporothrix and could be a reflection of its virulence status.

Analysis of the fungal load in the spleens and livers of mice infected with Sporothrix spp. showed that all strains...
caused chronic evolution of the disease in both organs. It is important to note that the strains in the present study are more virulent than *S. schenckii* strain M-64, which was previously studied in our laboratory because infection with this strain showed a significantly reduced fungal burden after 14 days of infection that remained low until day 28 [11].

In the experimental sporotrichosis model developed in this study, we observed a mixed pattern of response because cytokines characteristic of the two types of response, Th1 and Th2, were produced. A mixed cytokine response was shown to control infection by *S. schenckii* strain M-64 [11]. However, this was not observed for the two virulent strains analyzed in this study.

Recent studies have demonstrated that specific antibodies induce protection against fungal infections [15]. Our laboratory group observed that treatment with MAb P6E7 before, during, and after infection caused a decrease in fungal load in the spleens and livers of mice infected with *S. schenckii* strain M-64. This protection also occurred in nude mice after both treatments, and an increase in IFN-γ occurred in both organs [12].

The results presented here demonstrate that MAb P6E7 was able to reduce the fungal burden in mice infected with high-virulent strains. Reduced fungal burden in the spleen resulted in better prognosis, especially in mice infected with *S. schenckii* strain 1099–18; however, this was not observed in the mice 21 days after infection. However, we found a significant decrease in the fungal load in the liver only in the early stage of infection. Most likely, treatment with a higher dose or more doses of MAb P6E7 could promote better infection control.

The data presented in this study demonstrate that the strain isolated from a case of feline sporotrichosis is the most virulent strain and is the only one able to secrete gp70. This glycoprotein is apparently an important
factor in the virulence of *Sporothrix* spp. because MAb P6E7 reduced the fungal burden in the analyzed organs. However, further studies regarding the role of gp70 in modulating the immune response of the host and the main involvement of pattern recognition receptor (PRR) in the recognition of *S. brasiliensis* and *S. schenckii* are needed to understand the pathology of sporotrichosis.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**


