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Comparison of virulence of different *Sporothrix schenckii* clinical isolates using experimental murine model

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The virulence of two strains of *Sporothrix schenckii* isolated from patients with lymphocutaneous or disseminated sporotrichosis were examined in BALB/c mice (Group 1 and 2, respectively). The mice were inoculated subcutaneously into the left hind footpad with 4×10^6 *S. schenckii* yeast cells in order to evaluate (i) the development of cutaneous lesions, (ii) signs of inactivity, (iii) weight loss, (iv) survival rates, (v) number of viable yeast cells in the lungs and spleen, (vi) splenic index, (vii) extent of organ lesions, and (viii) immunological responses. Comparison of the two groups showed more severe disease in Group 2 mice that developed significant weight and hair loss associated with inactivity and left hind footpad lesions that extended close to the testicular area. The histopathology and large number of viable microorganisms isolated from the spleen confirmed the higher invasive ability of this strain. Moreover, a decrease of an *in vitro* specific lymphoproliferative response and IFN-gamma production were observed over time in Group 2 mice. As a result, at the end of the experiment, the *S. schenckii*-antigen (Ss-Ag) response was considered negative with a stimulation index (SI) = 2. In contrast, Group 1 mice presented a positive response to Ss-Ag (SI = 14.1). These results confirm the existence of different virulence profiles in *S. schenckii* strains. In addition, the use of subcutaneous inoculation as a suitable route for verification of the pathogenicity of this fungus in the murine model was confirmed.

Keywords *Sporothrix schenckii*, virulence, human isolates, experimental inoculation, immune response, sporotrichosis

Introduction

The dimorphic fungus *Sporothrix schenckii* is the causal agent of sporotrichosis, mycosis widely distributed all over the world. The disease is most commonly seen as a chronic infection of the cutaneous and subcutaneous tissues, characterized by the development of lymphatic nodules in humans and in some animals. The fungus exhibits both a hyphal saprophytic phase and a yeast parasitic phase which can be seen when the organism is grown, at 25°C and 37°C, respectively [1].

In Brazil, the incidence of sporotrichosis has gradually increased in humans. In addition, veterinarians and personal who take care of cats are at highest risk. Since 1998, the Zoonosis Service and the Infectious Dermatology Outpatient Clinic of the Evandro Chagas Research Institute – Fiocruz have been following-up a sporotrichosis zoonotic epidemic involving cats, dogs and humans in Rio de Janeiro, Brazil [2,3]. The cases reported in Rio de Janeiro, most of them from underprivileged areas around the periphery of the city, probably represent only a part of the problem and suggest that this outbreak in humans, mostly originating from feline sporotrichosis, may have started insidiously prior to 1998 [4].

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Infected individuals can present a spectrum of clinical forms ranging from a limited fixed lesion to a severe disseminated disease [4–6]. The most common presentation takes the form of cutaneous ulcers associated with regional lymphatic involvement, referred to as the lymphocutaneous form [5–7]. However, the precise mechanism that determines the outcome of the infection and the clinical forms is still unknown. One hypothesis is that strains of the fungus differ in their virulence. [8,9]. Nevertheless, little is known about the factors contributing to the virulence of *S. schenckii*, but some factors have emerged from investigations of thermotolerance, extracellular enzymes and polysaccharides [10–12]. Moreover, the immunological mechanisms involved in the prevention and control of sporotrichosis are not fully understood [13]. Studies have suggested that cell-mediated immunity plays an important role in the protection of the host against this fungus [8,14–16]. In this context, previously published papers have pointed out the correlation between immunodeficiency and severe disease [17–20]. Thus, the clinical presentation of sporotrichosis could be a result of an interplay between factors involving both the mammalian host and the fungal strain.

The objective of the present study was to compare with a murine model, the virulence of two *S. schenckii* strains isolated from patients presenting disseminated and lymphocutaneous sporotrichosis.

Materials and methods

Strains

Two *S. schenckii* strains, i.e., Ss 22932 from a patient with the lymphocutaneous form of the disease and Ss 22582 recovered from an individual with disseminated sporotrichosis were kindly provided by Dr Zancopé-Oliveira from the Evandro Chagas Clinical Research Institute and used throughout the study.

Mice

Male BALB/c mice, weighing approximately 23 g, were used in accord with requirements of the FIOCRUZ Ethics Commission for the Use of Laboratory Animals. Thirty mice were inoculated with *S. schenckii* strains Ss 22932 (Group 1) another 30 with Ss 22582 (Group 2). Fifteen from each group were used in studies to determine colony forming units (CFU) and histopathological investigations to investigate signs of disease and evaluate mortality. Another 15 mice of each groups were used in the immunological studies. The mice received food and filtered water *ad libitum*.

Culture conditions and inoculation

Sporothrix schenckii strains were cultured in brain–heart infusion (BHI) (Gibco, USA) broth at 37°C with shaking at 120 oscillations/min. After three passages of seven days each, the yeast cells were washed with phosphate buffer solution (PBS; pH 7.2), counted with a Neubauer chamber and their viability determined using the fluorescein diacetate-ethidium bromide stain [21]. Each mouse was inoculated subcutaneously into the left hind footpad with 4×10^6 yeast cells (85% to 87% viability) in 0.02 ml of sterile PBS. One group of 15 mice was similarly inoculated with PBS without yeast cells to be used as a control.

Euthanasia, CFU and histopathology

Three mice each from Groups 1 and 2 were weighed, underwent euthanasia by prolonged CO_2 exposure and necropsied at 14, 21, 28, 90 and 97 days after inoculation. The spleen, lungs and liver were removed aseptically. The left hind footpad was also removed for histopathology studies. The spleen and always the right lung were weighed and homogenized separately in sterile PBS to determine the number of viable fungal cells in each of these organs. The suspensions from each organ was adjusted to 30 mg of tissue/ml and 100 µl samples of the homogenate were transferred to each of four Petri dishes containing Mycosel agar (Difco, USA), incubated at 37°C for 10 days and the number of colonies on each plate were counted. The left lung, liver and footpad were fixed in 10% formalin, embedded in paraffin, cut into thin sections and stained with hematoxylin-eosin.

Splenic index

The spleen and body weight ratios of each infected mouse and of control mice were determined. The ratios of relative weight of spleens from infected mice were expressed as units in relation to the control. The mean value for the relative weight of spleens in each control group of mice was considered to be equal to one unit [22].

In vitro *detection of a specific immune response*

In vitro *proliferative response assay.*

a. *S. schenckii antigen preparation (Ss-Ag)*. The yeast phase of the *S. schenckii* strain 17629, kindly provided by Dr Zancopé-Oliveira, was cultured in BHI under the same conditions as described above. The yeasts were washed three times in sterile PBS buffer, adjusted to 10^8 cells/ml in PBS and disrupted

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by 10 repeated cycles of freezing and thawing, followed by ultra-sonication (Ultra-Lip Labsonic Systems, lab-Line, USA). Samples were stored at −20°C until used in experimental studies.

b. *Primary lymphoproliferative response (LPR).* Spleen (SP) and draining lymph node (LN) from normal non-infected (control) or *S. schenckii*-infected mice (Groups 1 and 2) were collected at 15, 28 and 90 days post-infection and processed separately. Recovered mononuclear cells from SP or LN were adjusted to 4×10^6 cells/ml in complete medium (RPMI 1640 medium supplemented with 10 mM HEPES, 0.04 mM 2-ME, 0.2 g/ml L-glutamine, 1 mM sodium pyruvate [Gibco, USA], 200 U/ml of penicillin, 200 μg/ml of streptomycin and 10% fetal calf serum [Hyclone, USA]). One hundred microliters of each cell suspension (SP or LN from each group) were each distributed into three wells of a 96 flat bottom microtiter plates (TPP, Switzerland) and then stimulated with either Concanavalin A (2 μg/ml ConA- Sigma, USA) as mitogen or Ss-Ag (the equivalent of 10^6 disrupted yeasts/well). In addition, cells of each suspension were cultured in complete medium as negative control (back ground – BG). The cells were left in culture at 37°C for 4 days in a humidified 5% CO_2-enriched atmospheric air incubator (Napco, USA). To measure the proliferation, 1 μCi of 3H- thymidine (Amersham, UK) was added to each well 16 h before harvesting. Results were expressed as stimulation indexes (SI). The latter was calculated as the ratio between the mean counts of each stimulated samples and the mean counts of the same sample without stimulation. The threshold of positive was the mean SI plus 2 times the standard deviation of control non-infected mice. The proliferative response were considered positive if the SI was higher than 2.5.

Cytokines assay. The IFN-γ secretion from stimulated cells was assayed by ELISA in the supernatants from 90-h-old *in vitro* culture (the 90 h were chosen after a preliminary experiment in which different time periods were tested using the same experimental conditions). Briefly, microtiter plate (MaxiSorp- Nunc, Denmark) wells were coated with purified rat-anti mouse IFN-γ or – IL-4 capture antibody (Pharmingen, USA) and incubated at 4°C overnight followed by washing and blocking steps. Supernatants or recombinant cytokine as standard curve (Pharmingen) were then added to the duplicated wells. To determine the cytokine concentration, sequential steps of washes and incubations with biotinylated rat anti mouse IFN-γ or – IL-4 and streptavidin-peroxidase conjugate were performed. The peroxidase-OPD reaction (Sigma) and the OD compared with the standard curve were determined with the results expressed in pg/ml of each cytokine.

Surface phenotype of responding cells. Surface phenotype of the cells recovered from cultures stimulated with Ss-Ag were analyzed by flow cytometry using monoclonal antibodies anti-mouse $CD4^+$ -FITC and anti-mouse $CD8^+$ RD (Pharmingen), as previously described [23].

Statistical analysis. Comparisons between groups for the CFU and weight loss experiments were analyzed by the ANOVA test, statistical significance set to be $P < 0.05$. For the immunological experiments and splenic index the non-parametrical tests Mann-Whitney and Spearman Correlation were used based on the calculation of the arithmetic means and standard errors of the means.

Results

Observation of mice after S. schenckii inoculation

Signs of mice inactivity and weight loss (Table 1) were observed in a time course study on the development of cutaneous lesions. No statistical significance differences in weight loss were observed between the Groups 1 and 2 mice. There was an initial weight loss at day 14 post inoculation in mice of both groups, probably due to inoculation stress. Subsequent increases in weight from 21 until 28 days were seen in both groups suggesting an adaptation period of the fungus to the host. However, a weight loss was noted in Group 2 mice at days 90 and 97 probably as a result of more severe infections. At 11 days post-inoculation, a nodule followed by ulceration was detected at the site of inoculation in both groups. Within 30 days of infection, Group 2 mice started to lose weight and hair, in association with signs of inactivity. The nodules in these mice suppurated 13 days after inoculation, reaching the thigh and testicular areas on the 47th day post-inoculation. In contrast, Group 1 mice presented only edema in the left hind footpad with nodules that ulcerated, but which regressed on the 45th day post-inoculation.

Survival of mice inoculated with S. schenckii

The survival rates of mice inoculated with *S. schenckii* strains are shown in Fig. 1. There were no deaths in Group 1 mice during 100 days of observation. In contrast, severe infections were found in Group 2 mice with 20% mortality at 90 days post-inoculation. The remaining mice in this group showed important signs of systemic disease (greater weight loss, immobility and

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Table 1 Weight variation (g) of mice after inoculation with *Sporotrhix schenckii* strains.

	Days after inoculation				
Strains	14	21	28	90	97
22582	↓3.33±2.91	↑3.65±0.21	↑4.60±0.28	↓4.00±0.42	↓4.00±1.27
22932	↓1.63±1.20	↑2.10±0.14	↑3.75±5.65	↑5.00±1.55	↑5.05±0.35
Control	↑3.80±1.10	↑2.00±1.00	↑2.00±0.40	↑2.00±1.41	↑4.00±1.69

↑ = increase of weight.
↓ = loss of weight.
$P < 0.05$.

tachypnea) when euthanized. This survival difference was not statistical significant due to the small number of dead mice.

Colony forming unit or fungal cells recuperated from spleen (CFU)

The number of viable microorganisms in the spleens 90 and 97 days after inoculation was significantly lower ($P < 0.05$) in Group 1mice as compared to those in Group 2 mice (Fig. 2). These results indicate a higher invasive capacity of the Ss 22582 strain. The number of cells recovered from the lungs of Group 1 mice was higher when compared to Group 2 mice but was lower than the number of fungal cells recovered from spleen demonstrating a limited proliferation of both strains in the lungs of the animals. In contrast there were no statistical significant differences in recovery of viable cells between the two groups at 14, 21 and 28 post-inoculation. The latter results could possibly be explained by the fact that as the mice were inoculated by subcutaneous route, there would be a period of time in which the fungus would adapt to the host, initiate adhesion mechanisms and then disseminate to internal organs.

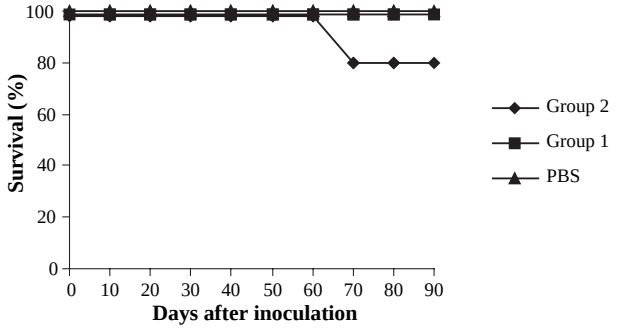

Fig. 1 Survival of mice following subcutaneous inoculation of 4×10^6 conidia of *Sporothrix schenckii*. The survival rates of inoculated mice from both groups (Group 1 – mice inoculated with strain Ss 22932 and Group 2 – mice inoculated with strain Ss 22582) differed from day 60 post-inoculation, with 20% mortality in Group 2 at the end of experiment. The data represents the survival rates for 10 animals/mouse strain. Control mice (100% survival) were inoculated with PBS.

Histological studies

Lesions in the pulmonary parenchyma, with mononuclear cell infiltrate without granulomas were found in Group 2 mice. A significant inflammatory reaction with the proliferation of connective tissue was noted in their footpads. Lesions were observed in the livers of the mice, e.g., inflammatory infiltrates, encapsulated abscesses (data not shown) within 90 days post-inoculation (Fig. 3). Group 1 mice showed granulomatous lesions in the liver and pulmonary parenchyma, but the lesions were less extensive than those seen in the Group 2 mice. The footpads of the mice in Group 1 also presented an inflammatory reaction with the proliferation of conjunctive tissue, but it was practically undetectable on day 97 post-inoculation.

Splenic index

The mean of splenic index values revealed the presence of splenomegaly in both groups (Table 2). However, Group 2 mice showed a significant splenomegaly ($P = 0.02$) as compared with Group 1 mice, with splenic index values of 14.2 and 3.5, respectively. While the splenic index of Group 2 mice showed a continuous increase, the splenic index values of Group 1 mice decreased with disease evolution. The relative weight of control group spleens was considered one unit.

In vitro *detection of a specific immune response*

The spleen and lymph nodes of each mouse were analyzed separately. The results illustrated in Fig. 4A and 4B are the arithmetic means and standard errors of four mice per group/time point. Each time point corresponded to the same time of which CFU analysis was conducted in order to verify the fungal load. The microscopic examination of the cultures

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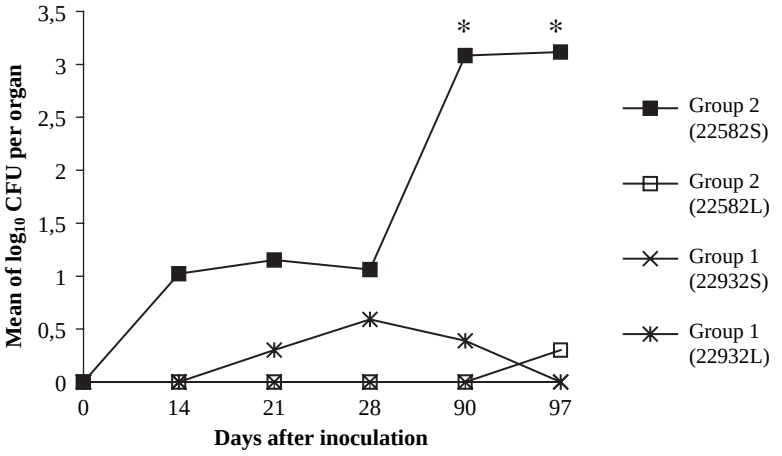

Fig. 2 Virulence of *Sporothrix schenckii* strains. Groups of 15 mice were infected subcutaneously with 4×10^6 conidia. Each point represents the mean number of colony forming units recovered from spleens (S) and lungs (L) from three mice sacrificed at predetermined times of 14, 21, 28, 90, 97 days after inoculation. (**x**) = Group 1mice (Ss 22932S); (*) = Group 1 mice (Ss 22932L) (■) = Group 2 mice (Ss 22582S); (□) = Group 2 mice (Ss 22582L), $P < 0.05$.

revealed a few fungal cells during the four days of *in vitro* cultivation. The lymphoproliferative response (LPR) to ConA was positive in all groups form the 15th and 28th day post inoculation. In contrast, at day 90 the ConA response decreased in all groups and was abolished in spleens of Group 2 mice (data not shown). It was possible to detect a time and/or organ-related specific *in vitro* stimulation with Ss-Ag in both infected groups of mice (Fig. 4A). The control group did not show any Ss-Ag specific response at all points studied (Fig. 4A). A positive and specific response to Ss-Ag was obtained in either SP or LN cells from both infected groups at days 15 and 28, but, as the mice were infected subcutaneously into the footpad, an expressive and contrasting proliferative response was detected in the LN cells when compared with those obtained from SP cells. When LN cell responses of the two groups were compared, no differences were observed on the 15th day (Group 1 mice-LN-SI = 16.3. $\pm$4.46 and Group 2 mice-LN-SI = 13.17 $\pm$ 1.9 $P > 0.05$) However, at day 28 Group 1 mice showed an important difference in the SI as compared with Group 2 mice (Group 1 mice-LN-SI = 16.92 $\pm$ 4.7 and Group 2 mice-LN-SI = 4.62 $\pm$ 0.7 $P = 0.02$). Nevertheless, LN cells from Group 2 mice showed a decrease in the Ss-Ag response at day 28 when compared with day 15 ($P = 0.028$). In contrast, LN cells from Group 1 mice showed a similar LPR during the same period (Fig. 4A). At day 90, a general decrease in Ss-Ag specific response was observed (Fig. 4A). However, no statistical differences were verified when LN cells from Group 1 mice were analyzed (day 15 $\times$ day 28 $\times$ day 90 $P > 0.05$). In contrast, LN cells from Group 2 mice showed a signifcant decrease in the capacity to specifically respond to the Ss-Ag (day 90 SI = 2 $\pm$ 0.3). In fact, as the cut off of the experiment there was an SI = 2.5 whereas at this time point the *in vitro* proliferation of cells from Group 2 mice were considered non specific (Fig. 4A).The Ss-Ag proliferative response observed in the two groups was significantly different at this time point ($P = 0.02$). In the cultures in which specific responses were detected, the proliferating cells were predominantly from $CD4^+$ phenotype, ranging from 53 to 66% of total cells.

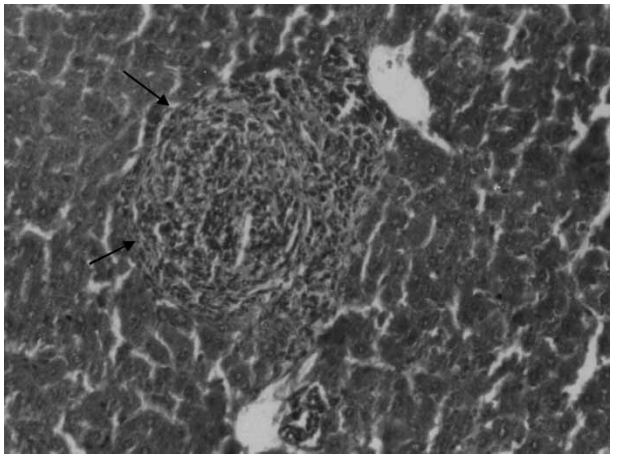

Fig. 3 Liver section from a BALB/c mouse inoculated with *Sporotrhix schenckii* Ss 22852 strain stained with H&E. Encapsulated abscess can be seen in the hepatic tissue (→); Original magnification: $\times$200.

The analysis of the IFN-γ secretion was performed by ELISA with the supernatants from the *in vitro* cultures at 15 and 28 days post infection (Fig. 4B). The amount of IFN-γ detected in the control group was 0.47 ng/ml at day 15 and close to zero at day 28. There were no differences in the results when the Group 1 and 2 mice were compared at day 15 post inoculation (Group 1 mice = 4.7 $\pm$ 3.2 ng/ml and Group 2 mice = 5.2 $\pm$ 3.4 ng/ml). In contrast, at 28 day post-infection, a dichotomy was found in IFN-γ production with regard

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Table 2 Splenic index of mice after inoculation with *Sporotrhix schenckii* strains.

Strains	Days after inoculation					M	SEM
	14	21	28	90	97		
22932	4.4	nd	3.5	3.6	2.5	3.5	0.389
22582	13.6	16.5	nd	12.6	14.2	14.2	0.827

The spleen and body weight ratios of each infected mouse and of control mice were determined. The ratios of relative weight of spleens from infected mice were expressed as units in relation to the control. $P = 0.02$.
Control group = mean value for the relative weight of spleens was considered to be equal to one unit.
M = mean; SEM = minimal deviation; nd = not done.

to the LN cell responses from the two groups. Greater IFN-γ production was detected in the LN cells supernatants from Group 1 mice (3.8 ± 2.2 ng/ml) as compared with LN cells from Group 2 mice (1.9 ± 1.8 ng/ml). However, as there was an intrinsic variation inside each group, the differences were not considered significant ($P = 0.05$, Fig. 4B). Thus, compared with Group 1 mice, Group 2 mice showed signs of immunosuppression of Ss-Ag specific T cell response, beginning at day 28 post-infection and reaching maximum value at day 90 post-infection at which time no Ss-Ag specific response was detected.

Discussion

Evidence presented here indicates differences in virulence scores of two different of *S. schenchii* isolates recovered from patients with active sporotrichosis. According to the medical records, patients from the same geographic area can develop different clinical presentations that involve both fungal and host factors. In the present epidemics in Rio de Janeiro, most of the patients were suspected of being infected through cutaneous injuries caused by scratches or bites from domestic cats, with more than 70% of them presenting

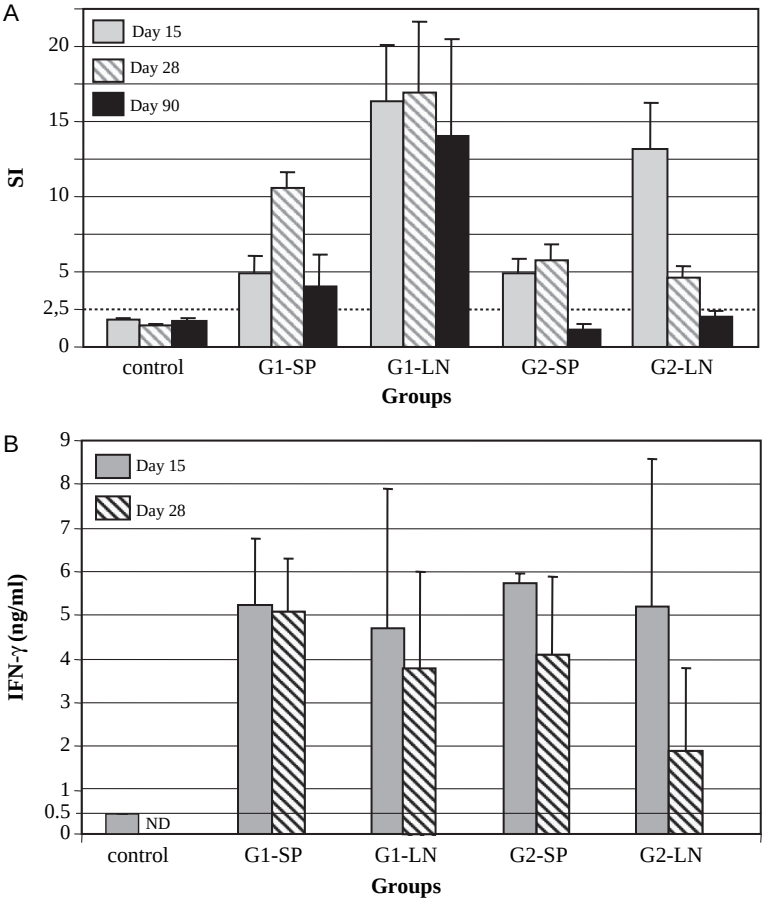

Fig. 4 (A) *In vitro* lymphoproliferative response to *Sporotrhix schenckii* antigen (Ss-Ag). Lymph node (LN) or spleen (SP) cells were stimulated separately as described in material and methods. Mice from control (non-infected) group, infected (Ss 22932) Group 1 mice (G1) and infected (Ss 22582) Group 2 (G2). The results of each group were expressed as the mean of stimulation indexes (SI) ± SEM. SI was calculated by the ratio between the mean counts of stimulated and non-stimulated wells of each group. SI ≥ 2.5 was considered as positive. Proliferative response of LN cells from G2 at day 28 of culture was significantly diminished when compared with cells from day 15 (G2-LN 15 days × G2-LN 28 days, $P = 0.028$) as well as cells collected from Group 1 mice at day 28 of infection (G1-LN 28 days × G2-LN 28 days, $P = 0.02$); (B)- IFN-γ secretion from stimulated (Ss-Ag) cultures according to the groups (control, Group 1 and Group 2 mice). The results are expressed in ng/ml ± SEM; G = group; LN = lymph node cells; SP = spleen cells; ND = not detected. Four mice were tested separately for each time point. Day 15 = gray bars, day 28 = hatched bars, day 90 = black bars.

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with the lymphocutaneous form of the disease [4,6,7]. Two different isolates from patients with sporotrichosis were studied in order to verify the correlation of human disease and fungal virulence in a murine model. The first, Ss 22932, was recovered from a patient with a typical lymphocutaneous form of sporotrichosis, which regressed after treatment. In contrast, the second isolate, Ss 22582, came from a patient with a severe and disseminated form of the disease that involved skin and bone lesions. In addition, the latter patient was found to be coinfected with *Nocardia* spp., which may have affected the evolution of the disease. Simultaneous isolation of *S. schenckii* and *Nocardia* spp. from lesions is uncommon, but it has been previously described in the literature [24]. Many authors have used the murine model to study the virulence of *S. schenckii* strains [8,9,25]. Staib *et al*. [26], while studying the pathogenicity of 16 strains of *S. schenckii* in a murine model by intramuscular inoculation, verified that all strains from human or animal sources caused infection, suggesting that intramuscular inoculation in mice might prove to be a suitable technique for the verification of *S. schenckii* pathogenicity. On the other hand, it has been found that even when using the same strain, the degree of virulence can vary according to the route of infection [8]. These authors showed that the intravenous route favored systemic infection when compared with the subcutaneous route of inoculation. In the present study, this variable was minimized by the use of the same route of infection. Nevertheless, the subcutaneous route was chosen in order to mimic natural infection produced by cat bites or scratches.

Studies have demonstrated that isolates from different lesions show different virulence profiles when inoculated in experimental animals. One of the characteristics that determine the degree of virulence is thermotolerance, defined as the capacity of fungi to grow at 37°C [27]. Kwon-Chung [28] demonstrated that clinical isolates of *S. schenckii* from lymphocutaneous lesions grew at higher temperatures and showed more evidence of multiplication in the internal organs of mice than strains isolated from fixed cutaneous sporotrichosis. In this context, both strains studied here disseminated to internal organs but they showed differing potentials to invade tissues after subcutaneous inoculation.

Factors other than thermotolerance have been described as participating in the establishment of the infection. Recently, Langfelder *et al*. [29] reviewed the biosynthesis of melanin/melanin-like substances by different fungi and the possible role of these cellular components in their pathogenicity. Furthermore, Moris-Jonis *et al*. [30] published a paper showing the synthesis of a melanin-like compound by *S. schenckii* and suggested a role for this compound during mammalian infection. Moreover, the presence of fungal cell wall components that are involved in *S. schenckii* adhesion to fibronectin has been pointed out as factors affecting infection [11], as well as the organism's capacity to adhere to and be internalized by endothelial cells during *in vitro* infection of [31]. Thus, the capacity of *S. schenckii* strains to maintain an infection are the result of different interactions between fungal and host cells.

In this study, it was possible to determine the virulence of the strains after the subcutaneous inoculation of BALB/c mice. The invasive capacity of the Ss 22392 strain was lower than the Ss 22582 strain. In addition, the Ss 22392 strain caused weak inflammatory reaction in the organs studied, as well as at the site of inoculation and was unable to kill the mice. Moreover, the lesions in the liver and pulmonary parenchyma were less extensive than that caused by the Ss 22582 strain. Other results that contributed to our conclusion that the Ss 22582 strain was more virulent were the greater splenomegaly seen in the mice and the number of cells isolated from the spleen.

In addition to the fungus virulence factors, the host could also influence the outcome of the disease. Some results have highlighted the role of the immune response during infection in both murine models and human patients. Despite the detection of a specific humoral immune response during active disease [13], previous studies have pointed out the importance of the cellular immune response in determining the severity of sporotrichosis. In the early 1980s [17,32], the course of murine sporotrichosis in immunodeficient mice was studied and compared with their immunocompetent littermates. The nude mice showed more severe disease but thymus reconstitution in these animals produced a significant degree of resistance to the infection [17]. Subsequent investigations confirmed these data and in addition, described the function of macrophages in infection [14,16]. More recently, studies demonstrated that the depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, could increase the severity of the infection in a murine model [33]. The present results showed a correlation between the degree of specific cellular immune response and the severity of the disease. Observations revealed a time related decrease in the *in vitro* specific lymphoproliferative response of the BALB/c mice infected with the Ss 22582 strain. The isolate was also found to have a greater capacity for dissemination throughout the tegumentary and visceral tissues, involving both its invading and proliferating capacity. In fact, at day 90 there was no cellular

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immune response for either *S. schenckii* antigens or ConA mitogen. The correlation between immunodeficiency and disease severity has been already suggested. For example, the course of sporotrichosis in patients co-infected with HIV is often more severe [18,19]. Recently, Kajiwara *et al*. [20] demonstrated that mice with chronic granulomatous disease (CGD) were more susceptible to sporotrichosis than the wild-type controls. As this genetic defect impaired the function of NADPH oxidase, the results suggested the importance of neutrophils and macrophages in controlling the infection. The Ss 22582 strain was initially isolated from a patient co-infected with *S. schenckii* and *Nocardia* spp. The severity of the clinical case was first correlated with either the presence of this co-infection or to an immune defect inherent to the patient. However, tests to verify the efficiency of T cells, macrophage and neutrophils of the patient did not reveal any functional inactivity of these cells. In addition, this patient was HIV, HTLV and HCV negative. Moreover, the present results demonstrated that this strain caused a more severe infection when compared with the second test isolate recovered from an individual with a typical case of lymphocutaneous sporotrichosis. Therefore, the data indicate that Ss 22582 could create a severe infection in even immunocompetent hosts. The mice used during this study were immunocompetent, as demonstrated by the test of the control litter mates (data not shown). In this regard, mice infected with the Ss 22932 strain showed a weak and transient decrease in the *in vitro* specific cellular immune response during the infection. However, these differences were not significant. As can be observed in Fig. 4A, the lymph node cells retained the capacity to respond *in vitro* to antigenic stimulus. In contrast, mice infected with the Ss 22582 strain were immunosuppressed by day 90 post-infection. Early events in an immune response stimulate the production of cytokines that direct the subsequent development of T cells with discrete patterns of cytokine production. These events are dictated by the type of antigen/microorganism administered to a host, as well as dose, route of immunization and mouse strain. In the murine model of paracoccidioidomycosis different responses are induced when different routes of infection were employed [8,34,35]. Recently, Arruda *et al*. [34] discussed the role of different routes of infection in the development of or protection from paracoccidioiomycosis in the murine model. Differences can also be found using different strain of mice [35]. In our study we decide to use SC inoculation as it mirrors the route of human infection and found that it induced a strong specific immune response in the cells recovered from the draining lymph nodes. This finding was also observed in the murine model of *Leishmania major* infection [36–38]. Thus, the differences observed in the proliferative response of cells collected from draining lymph nodes and spleens could reflect the involvement of particular antigen presenting cells. In addition, the results could be explained by the production of particular cytokines by either accessory cells or T cells which play an important role in the development of Th cells producing arrays of cytokine. These findings suggest that virulence could interfere with the immune function. At this point in the study, it is not possible to explain the role of fungal cells in modulating the cellular immune response. Further experiments are required to fully clarify this effect.

The results presented here suggest a multifactorial involvement of both fungal compounds and host responses in determining the course of *S. schenckii* infection in mammalians, while considering that in the present epidemic in Rio de Janeiro, cats are being reported as an important infection source [4,39]. It is possible to infer that in the same endemic area, strains with different degrees of virulence can be co-circulating in the population. It is also important to consider the possibility of biological filters, wherein cats, and possibly even patients, select different fungus clones during the infection. Further studies are required to fully clarify the mechanisms involved in sporotrichosis to elucidate the control of the severity of lesions and to effect of a cure.

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