

# Adhesion of the human pathogen *Sporothrix schenckii* to several extracellular matrix proteins

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## Abstract

The pathogenic fungus *Sporothrix schenckii* is the causative agent of sporotrichosis. This subcutaneous mycosis may disseminate in immunocompromised individuals and also affect several internal organs and tissues, most commonly the bone, joints and lung. Since adhesion is the first step involved with the dissemination of pathogens in the host, we have studied the interaction between *S. schenckii* and several extracellular matrix (ECM) proteins. The binding of two morphological phases of *S. schenckii*, yeast cells and conidia, to immobilized type II collagen, laminin, fibronectin, fibrinogen and thrombospondin was investigated. Poly (2-hydroxyethyl methacrylate) (poly-HEMA) was used as the negative control. Cell adhesion was assessed by ELISA with a rabbit anti-*S. schenckii* antiserum. The results indicate that both morphological phases of this fungus can bind significantly to type II collagen, fibronectin and laminin in comparison to the binding observed with BSA (used as blocking agent). The adhesion rate observed with the ECM proteins (type II collagen, fibronectin and laminin) was statistically significant ( $P < 0.05$ ) when compared to the adhesion obtained with BSA. No significant binding of conidia was observed to either fibrinogen or thrombospondin, but yeast cells did bind to the fibrinogen. Our results indicate that *S. schenckii* can bind to fibronectin, laminin and type II collagen and also show differences in binding capacity according to the morphological form of the fungus.

## Key words

- *Sporothrix schenckii*
- Fibronectin
- Laminin
- Type II collagen
- Adhesion

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## Introduction

Sporotrichosis is a deep mycosis caused by the traumatic implantation of the dimorphic pathogenic fungus, *Sporothrix schenckii*. This disease has several clinical forms, the most frequent being the cutaneous/subcutaneous one, with associated lymphangitis and

lymph node enlargement (1). Systemic spread may affect the bone, central nervous system and lung, and has been associated mainly with immunocompromised individuals (1-5). These systemic forms of the disease can follow a pulmonary infection by conidia inhalation (6) or dissemination from a cutaneous/subcutaneous lesion (2). Recently, spo-

rotrichosis has been reported to be an important mycosis in HIV<sup>+</sup> individuals (5). In these patients, osteoarthritis and the disseminated form of sporotrichosis are frequently observed (3,4).

The dimorphism of *S. schenckii* is determined by temperature. At 37°C this fungus differentiates to the yeast form. This morphology is present in cutaneous lesions. The mycelial phase is the saprophytic form of *S. schenckii* and is obtained *in vitro* by cultivation at 28°C.

The adherence of a pathogen to host tissues is believed to be an essential step in infectious diseases leading to the dissemination of the microorganism. The most important barriers to be overcome are the endothelium and basement membranes. Little is known about the adhesion and dissemination of pathogenic fungi inside the host. Entactin, fibronectin (FN), laminin, type I collagen and type IV collagen were reported to be involved in the adhesion of some pathogenic fungi to the extracellular matrix (ECM) (7-9). Differences in the binding capacity of *Candida albicans* were correlated with growth conditions and with the fungal isolate (10). Virulent strains have enhanced binding capacity to fibronectin when compared to avirulent strains (10). An association between binding capacity to ECM proteins and fungus morphological phase was also reported. Conidia of *Aspergillus fumigatus* bind to fibronectin but the mycelia do not (11). On the other hand, no differences in the ability to bind to entactin were observed between the yeast and hyphal morphologies of *Candida albicans* (7).

Fungal cell wall components involved in the adhesion process of these pathogens such as cell wall proteins (integrin-like proteins) and mannoproteins were described (12). *S. schenckii* expresses on its cell wall a glycopeptide component, peptido-rhamnomannan (13). This glycopeptide possesses the main antigenic epitopes present on the cell surface

of *S. schenckii* and also binds to the lectin Concanavalin A by means of the O-glycosidically linked oligosaccharides (14,15). Differences in cell wall composition of both conidia and yeast cells were observed in *S. schenckii* and are related to the virulence and pathogenesis of this microorganism (16). It is not known whether the cell surface of *S. schenckii* contains adhesins that could mediate host attachment and invasion.

The present investigation is a preliminary study carried out to determine the binding capacity of *S. schenckii* to several extracellular matrix proteins.

## Material and Methods

### Material

Poly(2-hydroxyethyl methacrylate) (poly-HEMA), and mouse and human laminin were from Sigma Chemical Co., St. Louis, MO, USA; goat anti-rabbit peroxidase conjugate was from Gibco Life Technologies do Brazil (São Paulo, SP, Brazil); an antiserum against yeast cells of *S. schenckii* was raised in rabbits as previously described (15).

### Microorganism and growth conditions

*Sporothrix schenckii* strain 1099-18 was used throughout this study. This strain was originally obtained from the Mycology Section, Department of Dermatology, Columbia University, New York, USA. Yeast forms of *S. schenckii* were grown in brain heart infusion broth (BHI, Difco, Detroit, MI, USA) at 37°C in rotary shaker (150 rpm). After 7 days in culture the cells were harvested and washed with sterile 50 mM phosphate buffered saline (PBS), pH 7.2. The mycelial phase of *S. schenckii* was grown in Sabouraud broth (2% glucose, 0.5% yeast extract and 1% peptone) at 25°C for 7 days. Conidia were separated by gauze filtration and washed with sterile PBS. The numbers of yeast cells and conidia were determined with a Neubauer

chamber. The cell suspension was adjusted to  $10^8$  cells/ml.

#### **Isolation of plasma fibronectin and human platelet thrombospondin**

Plasma fibronectin was freshly prepared by gelatin-Sepharose chromatography according to Vuento and Vaheri (17). Thrombospondin (TSP) was obtained from thrombin-activated platelet supernatants as previously described (18). Briefly, supernatants were submitted to heparin-Sepharose chromatography and bound proteins were eluted with growing NaCl concentrations in the running buffer. The fraction eluted with 0.55 M NaCl was concentrated and applied to a Sepharose CL4B column. TSP was eluted in the void volume.

#### **Cartilage collagen purification**

Type II collagen was purified from xiphoid cartilage of chicks by a modification of the method of Trentham et al. (19). The cartilage was pulverized with liquid nitrogen and incubated with 20 volumes (w/v) of a 50 mM Tris/2 M  $MgCl_2$  solution, pH 7.4, at 4°C for 18 h with constant shaking. The extract was then centrifuged at 25,000 *g* for 15 min and the precipitate was washed 3 times with distilled water. The residue was then suspended in 3 volumes of 0.5 M acetic acid and the pH was adjusted to 2.5 with formic acid. Pepsin was added to the suspension (1 g/30 g cartilage) followed by incubation at 4°C for 48 h, with constant gentle shaking. After this period, the undigested residue was separated by centrifugation at 25,000 *g* for 15 min and washed 3 times with 0.5 M acetic acid under the same conditions. The supernatants from the extraction and washes were pooled and dialyzed twice, first against distilled water for 12 h and then against 50 mM Tris/0.2 M NaCl, pH 7.6, for 24 h, followed by centrifugation. The extract was diluted 5-fold in dialysis buffer and then passed through a

DEAE-cellulose column previously equilibrated with the same buffer. Non-binding protein (collagen) was eluted and precipitated by the addition of NaCl to a final concentration of 3 M. After centrifugation at 20,000 *g* for 10 min, the precipitate was dissolved in 0.5 M acetic acid and exhaustively dialyzed against 10 mM  $Na_2HPO_4$ . The precipitate isolated by centrifugation was dissolved in acetic acid and then submitted to another precipitation with 1 M NaCl. Finally, the collagen was solubilized and dialyzed against acetic acid at 4°C and lyophilized.

#### **Polyacrylamide gel electrophoresis**

Purity of type II collagen, FN and TSP preparations was characterized by SDS-polyacrylamide gel discontinuous electrophoresis (SDS-PAGE) using the method of Laemmli (20). The proteins were silver stained by the method of Morrissey (21).

#### **ELISA**

The adhesion of *S. schenckii* cells to the ECM proteins was evaluated by ELISA with the immobilized proteins. Wells of polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 100  $\mu$ l of TSP, FN, laminin, fibrinogen or type II collagen (CII) (10  $\mu$ g/ml in 0.2 M bicarbonate buffer, pH 9.4) by passive adsorption overnight at 4°C. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-Tween). Non-specific binding was blocked by incubating the plates for 2 h at 37°C with 1% BSA in 50 mM PBS, pH 7.4. After a further PBS-Tween washing step,  $10^7$  yeast cells or conidia were added per well followed by incubation for 1 h at 37°C. The plates were then washed in order to remove unbound cells and 100  $\mu$ l of a rabbit anti-*S. schenckii* antiserum (1:500) was added to each well. The plates were incubated for 1 h at 37°C, washed and then incubated with a

Figure 1 - Adhesion of *S. schenckii* yeast cells (A) and conidia (B) to immobilized fibronectin, thrombospondin and fibrinogen at 10 µg/ml compared to adhesion to BSA. Values were subtracted from the negative control (poly-HEMA). *S. schenckii* adhesion ( $10^7$  cells/well) was assessed by ELISA with an anti-*S. schenckii* antiserum \* $P < 0.05$  compared to BSA (Student *t*-test).

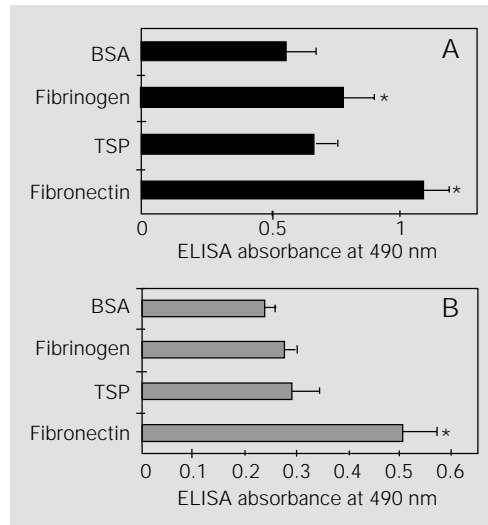
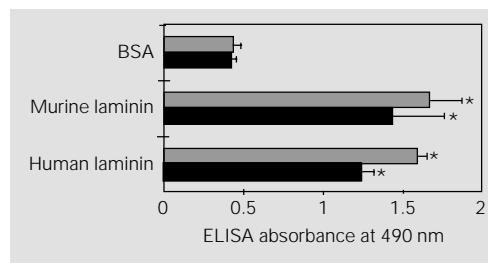


Figure 2 - Binding of *S. schenckii* conidia (gray bars) and yeast cells (black bars) to immobilized murine and human laminins at 10 µg/ml compared to adhesion to BSA. Values were subtracted from the negative control (poly-HEMA). *S. schenckii* adhesion ( $10^7$  cells/well) was assessed by ELISA with an anti-*S. schenckii* antiserum. \* $P < 0.05$  compared to BSA (Student *t*-test).



goat anti-rabbit IgG peroxidase-linked conjugate (1:4000 in PBS-Tween). The plates were washed with PBS-Tween and the reaction was developed with the substrate *o*-phenylenediamine (OPD) (0.5 mg/ml and 0.005%  $H_2O_2$  in 0.01 M sodium citrate buffer, pH 5.6). The reaction was stopped after 5 min with 0.2 M  $H_2SO_4$ , and the  $A_{490}$  was measured using an automated reader (BioRad ELISA Reader, Hercules, CA, USA). Each experiment was done in triplicate and the results correspond to a typical experiment from at least three independent repeats.

#### Adhesion index

The differential binding capacity between the yeast phase and conidia of *S. schenckii* was determined by the application of the formula presented below, in order to estab-

lish the relative adhesion index for FN and laminin:  $A_{490}$  ECM protein/ $A_{490}$  BSA -  $A_{490}$  poly-HEMA

#### Statistical analysis

Values are reported as mean  $\pm$  standard deviation. The unpaired Student *t*-test was used to compare differences between the binding capacity to extracellular matrix proteins and the binding to BSA, as well as to determine differences associated with the morphological phase of the fungus.  $P < 0.05$  was considered significant.

## Results

### Adhesion of *S. schenckii* to FN, TSP and fibrinogen

In order to evaluate the adhesion of *S. schenckii* yeast cells and conidia to the extracellular matrix proteins, fibronectin, thrombospondin and collagen, these proteins were immobilized on ELISA plates and incubated with  $10^7$  cells of each morphological phase of this fungus. The binding was measured as described in Methods and expressed by the absorbance at 490 nm. Figure 1 (A and B) represents a typical experiment from at least three independent experiments. As observed in Figure 1, both morphological phases significantly bound to fibronectin, as compared to the binding observed with BSA ( $P < 0.05$ ). No significant adhesion was observed for conidia assayed with TSP or fibrinogen (Figure 1B). On the other hand, *S. schenckii* yeast cells showed a significant extent of adhesion to fibrinogen ( $P < 0.05$ ) but not to TSP (Figure 1A).

### Adhesion of *S. schenckii* to murine and human laminin

Both the yeast phase and conidia of *S. schenckii* bound to laminin from different sources (Figure 2). No differences were ob-

served in the rate of adhesion to human and murine laminins.

### Comparison of the adhesion index for laminin and FN

Since the absolute adhesion rates varied between experiments, although adhesion to fibronectin and laminin was always significant, we determined the relative adhesion index as described in Methods. Figure 3 shows that the adhesion rate to laminin and FN is almost similar. However, differences according to the fungal morphological phase were observed only for FN.

### Binding of *S. schenckii* to type II collagen

Type II collagen was purified as described in Methods. The purification of chick type II collagen by this procedure yielded  $35.5 \pm 16$  mg CII/g cartilage. The degree of purification of the CII preparation was evaluated by SDS-PAGE (Figure 4). A main 156-kDa protein band corresponding to the alpha chains of chick type II collagen was observed. The purified CII showed a high degree of purity in comparison to the commercial patterns. *S. schenckii* conidia and yeast cells were able to significantly attach to type II collagen (Figure 5). Conidia displayed an increased adhesion to CII compared to yeast cells ( $P < 0.05$ ).

### Discussion

Sporotrichosis is a deep mycosis which may evolve to a systemic pathology in immunocompromised individuals. The yeast phase of *S. schenckii* is the morphology present in lesions and is thought to disseminate in the systemic disease. However, mycelial elements have already been described in histopathological specimens of lung from sporotrichosis patients (22). Conidia are also thought to infect humans by inhalation (6).

Primary adhesion of endothelial and/or

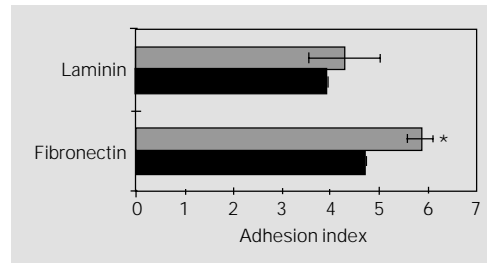


Figure 3 - Relative adhesion index of *S. schenckii* yeast phase (black bars) and conidia (gray bars) to immobilized fibronectin and laminin. The adhesion index was calculated using the formula:  $A_{490}$  to ECM protein/ $A_{490}$  to BSA -  $A_{490}$  with poly-HEMA (negative control). *S. schenckii* binding ( $10^7$  cells/well) was assessed by ELISA with an anti-*S. schenckii* antiserum. \* $P < 0.05$  compared to yeast cells (Student t-test).

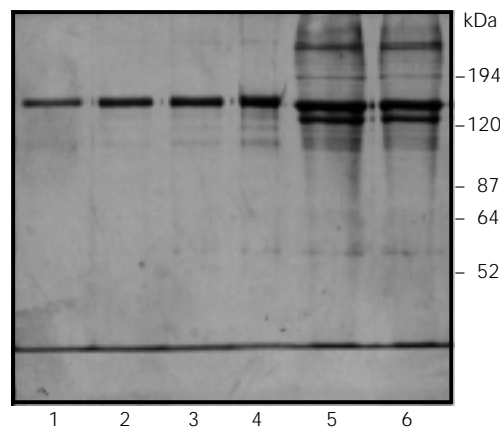


Figure 4 - Analysis of chick type II collagen by 7% SDS-polyacrylamide gel electrophoresis, with silver staining. Chicken CII: lane 1, 14 ng; lane 2, 20 ng; lane 3, 30 ng; lane 4, 60 ng and type VII collagen (Sigma); lane 5, 50 ng, and lane 6, 100 ng.

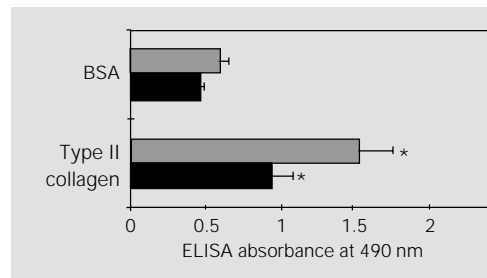


Figure 5 - Binding of *S. schenckii* conidia (gray bars) and yeast cells (black bars) to immobilized type II collagen at 10  $\mu\text{g/ml}$  in comparison with the adhesion observed to BSA. Values were subtracted from the negative control (poly-HEMA). *S. schenckii* adhesion ( $10^7$  cells/well) was assessed by ELISA with an anti-*S. schenckii* antiserum. \* $P < 0.05$  compared to BSA (Student t-test).

epithelial cells, as well as extracellular matrix components seem to be essential for successful host invasion by pathogens. In the present report we have investigated the binding capacity of two infective forms of *S. schenckii* (yeast form and conidia) to FN, laminin, type II collagen, TSP and fibrinogen. The binding to BSA observed here has already been described for other pathogenic fungi such as *Candida albicans* (23).

Our results show that *S. schenckii* cells can recognize three important ECM glycoproteins: fibronectin, laminin and type II

collagen. ECM-binding adhesins have been described for several microorganisms, including bacteria, protozoa and fungi. Most common adhesins recognize fibronectin and collagenous proteins (24). Type II collagen is present in hyaline cartilage, as well as in vitreous humor (25). In immunocompromised individuals, mainly in HIV<sup>+</sup> patients and alcoholics, the pulmonary and articular forms of sporotrichosis are the most frequent clinical manifestations (5,26). This fungus was also recently isolated from aqueous humor (27). Since this apparent tropism of *S. schenckii* may be associated with the ability to recognize specific ECM components, we investigated binding of this pathogen to type II collagen. Our results showed that conidia and, less markedly, yeast cells bind to type II collagen significantly more than to BSA. Interestingly, *S. schenckii* cells bound only slightly to type I and III collagens (data not shown). Fibronectin is a ubiquitous glycoprotein found in high concentrations in the extracellular matrix and body fluid of vertebrates. Binding to fibronectin may play a role in the invading ability of microorganisms.

In the present study, *S. schenckii* cells strongly recognized laminin from different sources. Laminin is a major component of basement membranes, which, together with endothelial and epithelial monolayers, constitute the main physiological barriers to be surmounted by invading organisms. However, data concerning the involvement of laminin as a target of fungal adhesins are

scarce. It has been suggested that the ability to bind basement membrane components is correlated with high virulence phenotypes. Vicentini et al. (28) have reported that the 43-kDa antigen binds to laminin, which leads to the enhancement of *Paracoccidioides brasiliensis* pathogenesis, by means of laminin recognition. Whether the laminin-binding capacity of *S. schenckii* is critical for the onset of systemic infections remains to be established.

Taken together, our data suggest that *S. schenckii* presents an adhesive behavior comparable to that of other opportunistic pathogens, since it is able to bind both to the basement membrane and to connective ECM proteins. Indeed, this is supported by the fact that this fungus also interacts with specific molecules on human endothelial cells *in vitro* (Figueiredo CC, Lima OC, Lopes-Bezerra LM and Morandi V, unpublished data).

The present report contributes new data to the understanding of the pathogenesis of sporotrichosis. The identification of *S. schenckii* cell surface adhesin(s), as already described for other pathogenic fungi (12,29), constitutes a future challenge that could lead to new antifungal therapies.

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