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Differential expression of sialylglycoconjugates and sialidase activity in distinct morphological stages of *Fonsecaea pedrosoi*

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Abstract The expression of sialoglycoconjugates in *Fonsecaea pedrosoi* conidia, mycelia, and sclerotic cells was analyzed using influenza A and C virus strains, sialidase treatment, and lectin binding. Conidium and mycelium whole cells were recognized by *Limax flavus* (LFA), *Maackia amurensis* (MAA), and *Sambucus nigra* (SNA) lectins, denoting the presence of surface sialoglycoconjugates containing α2,3- and α2,6-sialylgalactosyl sequences. Sialidase-treated conidia reacted more intensively with peanut agglutinin (PNA), confirming the occurrence of sialyl-galactosyl linkages. Conidial cells agglutinated in the presence of influenza A and C virus strains, which confirmed the results obtained from lectin-binding experiments and revealed the presence of sialoglycoconjugates bearing 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac_2) surface structures. Western blotting analysis with peroxidase-labeled LFA demonstrated the occurrence of sialylglycoproteins in protein extracts from conidia and mycelia, with molecular masses corresponding to 56 and 40 kDa. An additional band of 77 kDa was detected in conidial extracts, suggesting an association between sialic acid expression and morphogenesis. Synthesis of sialic acids was correlated with sialidase expression, since both conidial and mycelial morphological stages presented secreted and cell-associated enzyme activity. Sialoglycoconjugates were not detected in *F. pedrosoi* sclerotic cells from in vitro and in vivo sources, which also do not express sialidase activity. The surface sialyl residues in *F. pedrosoi* are apparently involved in the fungal interaction with immune effector cells, since sialidase-treated conidia were less resistant to phagocytosis by human neutrophils from healthy individuals. These findings suggest that sialic acid expression in *F. pedrosoi* varies according to the morphological transition and may protect infecting propagules against immune destruction by host cells.

Keywords *Fonsecaea pedrosoi* · Sialic acid · Sialidase · Lectins · Phagocytosis

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Introduction

Fonsecaea pedrosoi is the principal causative agent of chromoblastomycosis, a subcutaneous fungal disease occurring most frequently in tropical and subtropical areas (Silva et al. 1999; Hamza et al. 2003). Infection usually results from traumatic implantation of the fungus on subcutaneous tissues, producing initial lesions consisting of papules or nodules that become noticeably verrucous; the confluent verrucous lesions lead to plaque formation, which is very difficult to manage (Rivitti and Aoki 1999; Castro et al. 2003). *F. pedrosoi* dimorphism is particularly characterized by the presence of spherical, brownish-yellow cells with thick, deeply pigmented walls in the parasitic phase, which are most commonly known as sclerotic cells (McGinnis and Hilger 1987; Silva et al. 2002). The lack of physiological studies with tissue forms of *F. pedrosoi* was related to the difficulty of inducing sclerotic cells in vitro (Kwon-Chung and Bennett 1992; Mendoza et al. 1993). Recently, Silva et al. (2002) demonstrated ultrastructural and antigenic similarities between sclerotic cells obtained in vitro and those isolated from chromoblastomycosis lesions, validating studies in which these forms were obtained through stimulation with artificial inducers of differentiation (Alviano et al.1992, 2003).

F. pedrosoi, like other agents of subcutaneous and systemic mycoses, induces the influx of phagocytic cells, producing a mixed-granulomatous reaction in animal tissues. The interaction of *F. pedrosoi* with neutrophils (Rozen-

tal et al. 1996), epithelial cells (Farbiaz et al. 1992; Limongi et al. 2001), and macrophages (Farbiaz et al. 1990) has been described elsewhere, but the mechanisms by which *F. pedrosoi* escapes host defenses are unknown. However, since chromoblastomycosis is a chronic disease involving the continuous production of fungal-containing lesions, it can be concluded that cells of *F. pedrosoi* resist destruction by host defenses and persist in human tissues. The production of melanin by these fungal cells is a putative virulence factor contributing to resistance against host destruction (Alviano et al. 1991; Farbiarz et al. 1992).

Sialic acids, constituents of many glycoconjugates as terminal monosaccharides, are a structurally complex family of nine-carbon monosaccharides whose unique physicochemical properties confer biologically diverse activities to cell surfaces (Schauer and Kamerling 1997). Sialic acids have been detected in a number of microorganisms, including bacterial, fungal, and protozoan species (Angata and Varki 2002). In several microbial species, sialic acids are thought to function as anti-recognition molecules eluding host immune system mechanisms (Alviano et al. 1999; Angata and Varki 2002). In pathogenic fungi, a growing list of reports suggest the occurrence of sialic acids in *Cryptococcus neoformans* (Rodrigues et al. 1997, 2003), *Sporothrix schenckii* (Alviano et al. 1982; Oda et al. 1983), *F. pedrosoi* (Souza et al. 1986), *Paracoccidioides brasiliensis* (Soares et al. 1998), *Candida albicans* (Soares et al. 2000), and *Aspergillus fumigatus* (Wasylnka et al. 2001). In all these fungal species, *N*-acetylneuraminic acid (Neu5Ac) was the major sialic acid derivative detected. In addition, the presence of *O*-acetylated derivatives was suggested only in *C. neoformans* by the binding of influenza C virus, which specifically recognizes 9-*O*-acetylated sialic acids, to intact cryptococcal yeasts (Rodrigues et al. 1997) and to a 67-kDa glycoprotein (Rodrigues et al. 2003).

In fungal cells, sialic acids seem to contribute to microbial pathogenesis; for instance, Neu5Ac expression on the conidial cell wall of the pathogen *A. fumigatus* was 3–20 times greater than that on the cell wall of the nonpathogenic species *A. auricomus*, *A. wentii*, and *A. ornatus* (Wasylnka et al. 2001), and this was correlated with the increased ability of *A. fumigatus* to bind to proteins of the basal lamina. In *S. schenckii* (Oda et al. 1983) and *C. neoformans* (Rodrigues et al. 1997), sialic acid units protect yeast cells against phagocytosis, which may represent an early mechanism of fungal defense against host destruction. Besides influencing interaction with the host, sialic acids may have additional functions in fungal cells. In *F. pedrosoi* (Alviano et al. 1982), they seem to play a structural role in the preservation of cell morphology. It is not clear whether fungal cells also synthesize sialidases, which hydrolyze the *O*-glycosidic linkages between the terminal sialic acids and the subterminal monosaccharides of free and glycoconjugate-bound oligosaccharides (Schauer and Kamerling 1997). Although these enzymes are apparently not present in species of dermatophytes (Krug et al. 1997), an early report described the occurrence of this enzyme in *S. schenckii* (Uchida et al. 1974). The expression of sialidase was also reported in the human pathogen *C. albicans* (Royal et al. 1984), but further studies contested this finding (Roggentin et al. 1999). The possible relevance of sialidase production in fungal infections is not known, but this enzymatic activity was shown to be of fundamental significance in viral, bacterial, and protozoan infections (Schauer and Kamerling 1997).

In previous studies, sialic acid components of *F. pedrosoi* conidia and mycelia were described (Souza et al. 1986). In the present work, we have used fluorescence microscopy, flow cytometry and Western blotting using sialic-acid-specific lectins to study the distribution of sialoglycoconjugates on the *F. pedrosoi* cell surface. The presence of these acidic sugars on *F. pedrosoi* sclerotic cells obtained in vivo and in vitro was also evaluated. In addition, the influence of surface sialic acids in interactions of human neutrophils with this fungus, and the sialidase activity of *F. pedrosoi* conidia, mycelia, and sclerotic cells were determined.

Materials and methods

Microorganism

A human isolate of *F. pedrosoi* (strain 5VLP) was used in the present work (Oliveira et al. 1973). Stock cultures were maintained in our laboratory with 6-month transfers to Sabouraud-dextrose-agar under mineral oil and storage at 4 °C. In vivo sclerotic cells were obtained directly from the superficial skin scrapings of human patients. Epidermal scraps were washed several times in deionized distilled water and incubated sequentially for 1 h at 37 °C in the presence of collagenase type IA (1 mg ml^{-1}) and then with deoxyribonuclease I (50 µg ml^{-1}). After the enzymatic treatments, the samples were washed twice in deionized distilled water, and the sclerotic cells were separated by centrifugation (5,000×*g*, 5 min) (Silva et al. 2002).

Growth conditions and fungal morphology

F. pedrosoi from stock cultures was inoculated in Butterfield and Jong medium (BFJ), pH 6.5 (Butterfield and Jong 1987). After 15 days at room temperature (28 °C), the mycelium was obtained by filtration. To induce conidium formation, the stock culture was inoculated in BJF medium, pH 5.5, at room temperature (28 °C) for 5 days under shaking. Conidia were isolated by filtration through gauze. Cultures containing the sclerotic cells were obtained after inoculating the 15-day mycelium in BFJ medium supplemented with 800 µM DL-propranolol, pH 2.7. Propranolol-containing cultures were grown for 45 days, at 37 °C under shaking, as described by Alviano et al. (1992).

Determination of sialic acids

Sialic acids from *F. pedrosoi* sclerotic cells were extracted and purified as described previously (Reuter and Schauer 1994; Rodrigues et al. 1997; Alviano et al. 1999). Briefly, sclerotic cells (10^{10}) were successively hydrolyzed in diluted formic and chloridric acids. The extracts were further purified by a combination of ultrafiltration and chromatographic techniques (Alviano et al. 1999). The presence of sialic acid was monitored by high-performance thin-layer chromatography (HPTLC), on silica plates with a solvent mixture consisting of *n*-propanol:1 M ammonia:water (6:2:1,v/v/v). Spots were visualized by reaction with resorcinol-HCl. Samples containing sialic acids were quantified colorimetri-

cally by the thiobarbituric acid method, as described by Warren (1959). As a positive control of sialic acid expression, mycelial and conidial forms of *F. pedrosoi* were used.

Enzyme treatment

F. pedrosoi mycelia (10 mg, wet weight), conidia (10^7 cells) and sclerotic cells (10^7) were washed twice in 0.01 M PBS (150 mM NaCl; 20 mM phosphate buffer, pH 7.2) and incubated for 2 h at 37 °C in the presence of *Vibrio cholerae* sialidase (0.2 U ml^{-1}), pH 5.0, in the presence of 2 mM $CaCl_2$. After incubation, cells were washed in PBS and used for experiments. To determine the influence of sialidase treatment on fungal viability, control cells or sialidase-treated fungi were suspended in 50 µl PBS and mixed with the same volume of 0.04% trypan blue in PBS. No difference in cell viability was observed when sialidase-treated and -untreated fungal cells were compared.

Lectin binding

For fluorescence microscopy and fluorocytometric analysis, untreated (control) or sialidase-treated fungal cells were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. Fixed cells (10^6 ml^{-1}) were rinsed in PBS and then incubated in the presence of fluorescein isothiocyanate (FITC)-labeled *Limax flavus* (LFA), *Sambucus nigra* (SNA), and *Maackia amurensis* (MAA) agglutinins, at 40 µg ml^{-1} for 1 h at room temperature. SNA and MAA specifically recognize α2,6 sialylgalactosyl and α2,3 sialylgalactosyl residues, respectively, while LFA recognizes Neu5Ac in any linkage. Control or sialidase-treated cells were also incubated with *Arachis hypogaea* (peanut) lectin (PNA), which has affinity for Galβ-1,3GalNAc. Control cells, which had not been incubated with lectins, were also prepared. Cells were washed in PBS and observed in a Zeiss epifluorescence microscope (Axioplan 2). Sclerotic cells and conidia were also screened by flow cytometry analysis (n=5,000) using a FACScalibur flow cytometer (Becton Dickinson) equipped with a 15 mW argon laser emitting at 488 nm. The data were run using listmode, which makes further analysis possible. Control cells were analyzed first in order to determine their auto-fluorescence and relative size.

Virus samples

Paired clonal M1/5 and M1/5 HS8 strains of influenza A/Memphis/102/72 virus and a standard sample of influenza C (Taylor/1233/47) virus with known affinity for α2,6-sialylgalactosyl, α2,3-sialylgalactosyl, and 9-*O*-acetyl-Neu5Ac (Neu5,9Ac_2), respectively (Couceiro et al. 1993; Rogers et al. 1986), were used as probes to detect these structures on the fungal cell surface. Purified virus samples were titrated by hemagglutination; 25 µl of each virus sample was diluted in 25 µl of 0.15 M PBS, pH 7.0, and 25 µl of a suspension of chicken erythrocytes at 0.5% was added. The agglutination titer was determined after incubation at 4 °C for 2 h, and the reciprocal of the highest dilution of virus responsible for complete agglutination was taken as the number of hemagglutination units in the sample. Each virus preparation was adjusted to 512 hemagglutination units/25 µl for use.

Virus binding

The agglutination of *F. pedrosoi* conidia and sclerotic cells by the virus particles was carried out in glass tubes at 4 °C for 2 h. To determine *O*-acetylesterase activity of the influenza C virus, the agglutination assay was carried out at 37 °C for 1 h. Equal volumes of cell suspension in PBS, pH 7.2, containing 10^6 cells ml^{-1} and the virus suspension were rapidly mixed. Cell agglutination was scored visually, after gently re-suspending settled cells, by observation in a phase-contrast microscope. The control was the supernatant fluid from uninfected embryonated chicken eggs (Couceiro et al. 1993).

Western blotting

F. pedrosoi conidia, mycelia, and sclerotic cells were mixed with glass beads and broken in a cell disrupter (type 853023/8; B. Braun Biotech International, Germany), in ten cycles of 2-min shaking periods alternating with cooling intervals of 2 min. The glass beads were removed, and the suspension centrifuged at 2,000×*g* for 10 min at 4 °C. The protein-enriched supernatants were quantified using Lowry's colorimetric assay (Lowry et al. 1951). Protein extracts (20 µg) were loaded onto 12% SDS polyacrylamide gels (Laemmli 1970), and electrophoresis was carried out at 4 °C, at 100 V, for 1 h. The molecular mass of sample polypeptides was calculated from the mobility of molecular mass standards (GIBCO BRL, Grand Island, N.Y., USA). Separated protein extracts were silver stained or transferred to nitrocellulose membranes. The membranes were blocked in a solution containing 150 mM NaCl, 10 mM Tris, pH 7.5; and 10% Tween 20 for 2 h at room temperature, followed by incubation with peroxidase-labeled LFA (EY Laboratories) at 1 µg ml^{-1}, for 1 h at room temperature. Control of specificity was obtained by incubating the membrane strips in the presence of 0.2 M sialyllactose. As previously reported, periodate oxidation was also done to confirm the specificity of lectin binding (Arduino et al. 1994). The membrane strips were treated with 10 mM $NaIO_4$, in 0.05 M sodium acetate buffer, pH 5.0, for 2 h at 4 °C. After several washings with distilled water, the oxidation product was reduced with 10 mg sodium borohydride in 0.2 M sodium borate buffer, pH 8.2, for 3 h at 4 °C and then washed with distilled water for further incubation with peroxidase-LFA. Fetuin was used to control the efficiency of the periodate oxidation reaction. For the visualization of reactive molecules, membranes were washed five times in the blocking solution followed by chemiluminescence detection or reaction with 0.5 mg diaminobenzidine (DAB) ml^{-1} in 1.5 M Tris-HCl buffer, pH 7.4, supplemented with 0.01% H_2O_2. The color development was stopped by immersing the membrane sheets in distilled water.

Sialidase activity

Sialidase activity was measured by a fluorometric method (Potier et al. 1979) using 2′-(4 -methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-Neu5Ac) as a substrate for hydrolysis by culture fluids or intact cells. For stimulation of sialidase activity, *F. pedrosoi* conidia and mycelia were grown in brain heart infusion medium supplemented with 20% fetal bovine serum, pH 5.5 and 6.5, respectively, for 5 days at 37 °C. The sclerotic cells were grown in the same medium supplemented with 800 µM DL-propranolol, pH 2.7, for 20 days at 37 °C. Conidia and sclerotic cells were maintained under shaking. Fungal suspensions or culture supernatants (25 µl) were mixed with 25 µl of a reaction mixture containing 0.1 mM 4-MU-Neu5Ac, 2 mM $CaCl_2$, and 0.1 M sodium phosphate buffer, pH 5.5. After incubation at 37 °C for 60 min, the reaction was stopped by adding 200 µl of 0.266 M glycine buffer, pH 10, containing 120 mM NaCl and 80 mM Na_2CO_3. The fluorescence of released 4-methylumbelliferone was determined using a fluorometer (Fluoroskan II version 6.3) with excitation at 355 nm and emission at 460 nm. The instrument was calibrated with 4-methylumbelliferone standard solutions. Cell-associated enzymatic activities were estimated as the level of hydrolysis of 4-MU-Neu5Ac by 1 mg of cell pellet (dry weight). Secretory activities were determined using a ten-fold concentrated supernatant from fungal cultures containing similar cell densities. One unit of enzymatic activity was expressed as 1 µmol Neu5Ac liberated per min of the assay. The sialidase standard curve was prepared with commercial *Clostridium perfringens* sialidase in the range of 0–300 mU ml^{-1}.

Phagocytosis assay

Phagocytosis assays were carried out by using 2′,7′-bis-(carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester (BCECF/AM), as described previously (Schnitzler et al. 1999). BCECF/AM is a nonfluorescent membrane-permeable ester that is converted by non-specific prokaryotic and eukaryotic intracellular esterases to the trapped fluorescent indicator BCECF. Briefly, conidia and sclerotic cells were incubated in saline solution containing BCECF/AM (final concentration 1 µmol^{-1}) for 30 min at 37 °C. Fungal cells were then extensively washed in PBS, counted in a Neubauer chamber, and 5×10^6 labeled conidia were incubated at 37 °C under rotation in 1 ml heparinized blood (10 IU ml^{-1}) obtained from healthy donors.

After 15, 30, and 60 min, 100-µl aliquots were taken and mixed with 0.9 ml of a lysis buffer (per liter: NH_4Cl 9 g, $KHCO_3$ 1 g, EDTA 37 mg,pH 7.3), which resulted in the elimination of erythrocytes. Washed sediments were resuspended in 500 µl PBS and analyzed immediately by flow cytometry using Cellquest software (Becton Dickinson, version 3.1.f), using settings as described previously (Schnitzler et al. 1999). Neutrophils were selected through the examination of two distinct parameter plots, using measurements of forward and side scatter. To ensure the intracellular location of fungal cells associated with neutrophils, representative samples used in the flow cytometry analyses were examined by epifluorescence interference contrast microscopy (Zeiss microscope-Axioplan 2).

Results

Sialoglycoconjugates in *F. pedrosoi*

Sialic acid analysis demonstrated that these acidic sugars were not detected in sclerotic cells under our experimental conditions. TLC of purified fungal extracts showed that, although Neu5Ac was detected in hydrolysates from conidial and mycelial cells, typical resorcinol-reactive bands from sclerotic forms were not observed (data not shown). Quantitative analysis using the method of Warren (1959) confirmed these data.

No fluorescent reactions were observed after microscopy of sclerotic cells, from in vivo or in vitro sources, pre-incubated with FITC-sialic acid-specific lectins (not shown). However, mycelial cells were recognized by FITC-LFA, -SNA, and -MAA (Fig. 1). Lectin binding was sensitive to pre-treatment of fungal cells with sialidase, supporting the specificity of these reactions. At the concentration of 10 µg/ml, LFA was the most reactive lectin, but, although less intensive, the recognition of mycelia by SNA and MAA indicated the occurrence of α2,6- and α2,3-sialylgalactosyl sequences, respectively. The expression of galactose-bound sialic acids was confirmed by the strong interaction of sialidase-treated mycelium with PNA (data not shown).

FITC-lectin binding to *F. pedrosoi* cells was analyzed quantitatively by flow cytometry of conidial and sclerotic forms (Fig. 2). As expected, FITC-LFA, -SNA, -MAA were poorly reactive with sclerotic cells. The interaction of sclerotic bodies with FITC-PNA was stronger, but unaffected by sialidase treatment. By contrast, conidial forms became fluorescent after reaction with these four lectins, which was partially reverted by sialidase treatment. Conidia were also strongly recognized by FITC-PNA, which

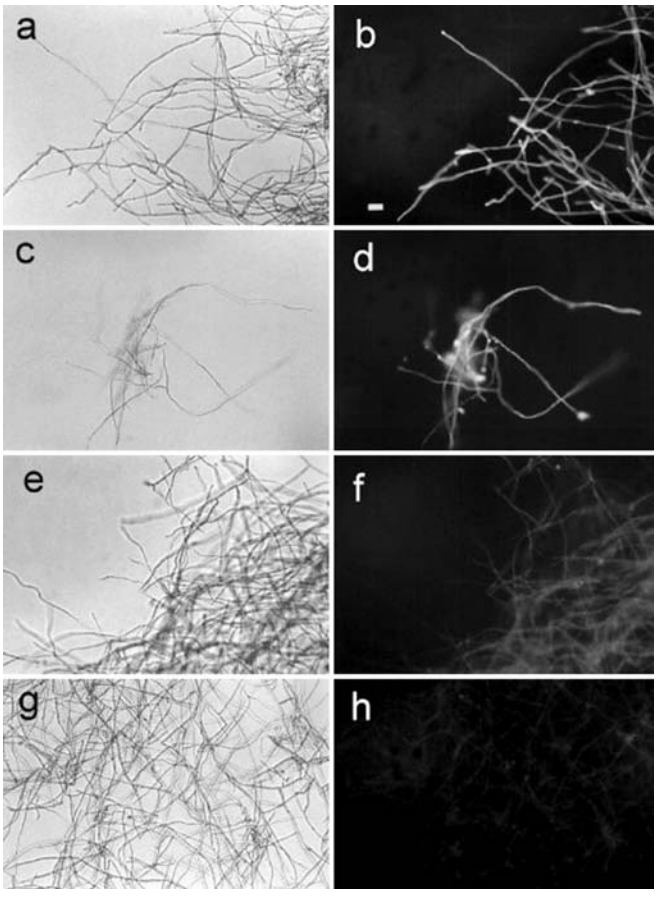

Fig. 1a–h Binding of FITC-lectins to *F. pedrosoi* mycelial forms. Experimental systems were analyzed under differential interferential contrast microscopy (**a, c, e**, **g**) and direct fluorescence (**b, d, f, h**). **b, d, f** Binding of FITC-LFA, -SNA and -MAA, respectively, to fungal cells. **h** Incubation of sialidase-treated fungi with FITC-LFA, which resulted in less intensive fluorescent reactions. This result was similar to those obtained after incubation of sialidase-treated mycelia with FITC-SNA and -MAA (data not shown). *Scale bar* 10 µm

may reflect the presence of β-galactosyl as constituents of cell wall polysaccharides and melanin (Alviano et al. 1991, 2003; Soares et al. 1995). PNA reacted even more intensively with sialidase-treated conidia, in accordance with the occurrence of galactose-bound sialic acids.

F. pedrosoi conidia agglutinated in the presence of influenza C virus, which is specific for 9-*O*-acetylneuraminic acid, at 4 °C (Table 1). Cell agglutination mediated by influenza C virus was inhibited when the cells were incubated at 37 °C, a temperature at which viral *O*-acetyl esterase is active and, therefore, sialic acids are de-*O*-acetylated. Agglutination was also observed when conidial cells were incubated in the presence of M1/5 and M1/5 HS8 strains of influenza A virus, which are specific for α2,6- and α2,3 sialylgalactosyl sequences, respectively. Agglutinating activity was higher in the presence of M1/5 virus (Table 1), confirming data obtained by cytofluorimetry of the binding of FITC-MAA and FITC-SNA to conidia in which the α2,6-linked structure predominated. No agglutination of *F. pedrosoi* was observed when the fungal cells

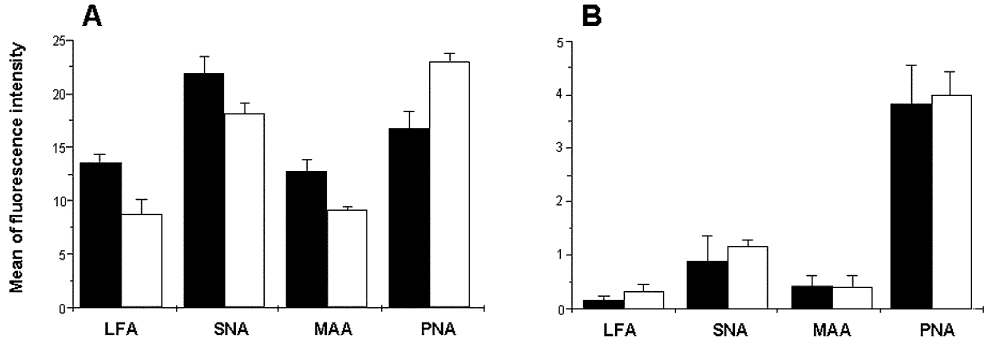

Fig. 2 Surface expression of sialic acids in conidial (**A**) or sclerotic (**B**) forms of *F. pedrosoi*. Non-treated (*black bars*) or sialidase-treated (*white bars*) fungal cells were incubated with FITC-LFA, -SNA, -MAA, or -PNA and analyzed by flow cytometry. Sclerotic forms reacted very poorly with all the sialic acid-specific lectins, while conidia were recognized by all of them. Sialidase-treated conidia were less reactive with FITC-labeled lectins, except for those incubated with PNA

Table 1 Agglutination of *Fonsecaea pedrosoi* conidia with different strains of influenza virus[a]

Virus	Agglutination titer
Influenza C	1:32
Influenza A, strain M1/5	1:32
Influenza A, strain M1/5 HS8	1:8

Influenza virus: C, specific for Neu5,9Ac_2; A, M1/5, specific for α2,6 sialylgalactosyl linkages; and A, M1/5 HS8, specific for α2,6 and α2,3 sialylgalactosyl sequences, respectively. Equal volumes of the conidia and virus suspensions were incubated at 4 °C for 2 h. Cell agglutination was scored by observation under a phase-contrast microscope. The agglutination titers were determined by using serially diluted influenza virus suspensions and were identical in two independent experiments

were incubated with allantoic fluids purified from uninfected, embryonated chicken eggs.

The nature of the sialic-acid-carrying glycoproteins in *F. pedrosoi* was analyzed by Western blotting. Crude protein extracts from *F. pedrosoi* conidia, mycelia, and sclerotic cells are shown in Fig. 3. After transfer to nitrocellulose membranes and incubation with peroxidase-labeled LFA, polypeptides with molecular masses corresponding to 56 and 40 kDa from mycelial and conidial extracts were shown to be sialylated. An additional band of 77 kDa was specifically detected by incubation of LFA with conidial extracts. Treatment of blots with periodate or incubation in the presence of sialylactose resulted in the abolishment of lectin binding. Proteins from sclerotic cells were not recognized by LFA, in agreement with the chemical analysis of sialic acids in these cells.

F. pedrosoi expresses surface and secretory sialidase activities

No detectable cleavage of 4-MU-Neu5Ac was observed when sclerotic cells or their culture fluids were assayed (Table 2), even when these cells were cultivated in the presence of sialic external sources (data not shown). In contrast, conidial and mycelial cells, as well as their culture fluids, demonstrated sialidase activity. The highest indices of 4-MU-Neu5Ac hydrolysis were observed in mycelial intact cells and their culture supernatants.

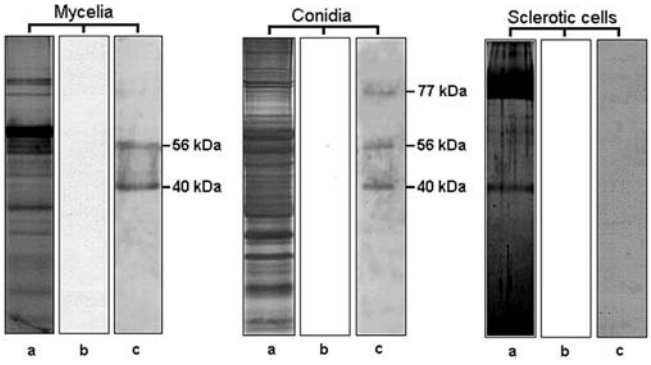

Fig. 3 Sialoglycoproteins from *F. pedrosoi*. Protein extracts were separated on SDS-PAGE and silver-stained (**a**) or transferred to nitrocellulose membranes (**b, c**). Blotted proteins were then incubated with peroxidase-labeled LFA in the presence of sialylactose (**b**) or with the lectin alone (**c**). Pre-treatment of membranes with sodium periodate also abolished lectin binding in a fashion similar to that demonstrated in **b** (not shown). Molecular masses (kDa) of sialylated glycoproteins are indicated on the *right*

Table 2 Surface and secretory sialidase activity in *F. pedrosoi* conidia, mycelium, and sclerotic cells. Sialidase activity was determined as the capability of 4-MU-Neu5Ac hydrolysis by cell suspensions or supernatant fluids. One unit (U) of enzymatic activity was expressed as 1 µmol Neu5Ac liberated per min of the assay. *BDL* Below detection limit

	Sialidase activity[a]		
	Mycelium	Conidia	Sclerotic cells
Fungal suspensions	10.2±1.6 mU mg^{-1}	1.66±0.2 mU mg^{-1}	BDL
Supernatant fluids	158.4±5.1 mU ml^{-1}	91.6±2.7 mU ml^{-1}	BDL

[a]The levels of 4-MU-Neu5Ac hydrolysis were expressed as miliunits (mU) of enzymatic activity per mass of fungal cells (mg; dry weight) in fungal suspensions or per volume (ml) of culture supernatants

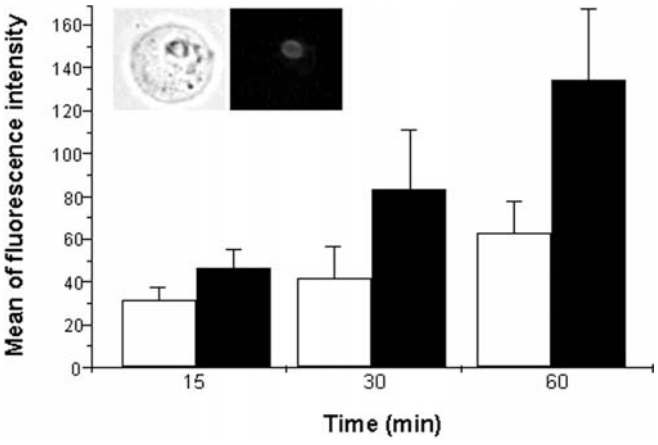

Fig. 4 Surface sialic acids protect *F. pedrosoi* conidia against phagocytosis by human neutrophils. Control (*white bars*) or sialidase-treated (*black bars*) conidial forms were stained with BCECF, incubated with human cells, and analyzed by flow cytometry. Levels of fungal attachment or internalization are expressed as the relative fluorescence intensity of neutrophils after incubation with conidia. *Inset* Representative sample of the interaction of BCECF-labeled conidia and human neutrophils under differential interferential contrast (*left*) or fluorescence (*right*) microscopy

Phagocytosis assay

To exclude an effect of activation of neutrophils due to isolation procedures, heparinized whole human blood was used to measure the phagocytosis of *F. pedrosoi* conidia. Control or sialidase-treated fungal cells were similarly stained with BCECF, which allows direct correlation between the fluorescence of host neutrophils after interaction with conidia and the association (attachment or internalization) between these cells. Binding of fungal cells to the neutrophils in fact resulted in an increase in the green fluorescence of human cells, as shown in Fig. 4. After 60 min, the fluorescence levels of neutrophils interacting with untreated conidia was around 50% lower than those observed when human cells were incubated with sialidase-treated fungal cells. This result indicated that conidia lacking surface sialic acids are associated to a greater extent with human neutrophils. The intracellular location of conidia after incubation with heparinized blood was confirmed by a combination of epifluorescence microscopy and simultaneous interference contrast microscopy in representative samples (Fig. 4, inset).

Discussion

In an early report (Souza et al. 1986), sialic acids were characterized as surface components of conidia and hyphae of *F. pedrosoi*, the major agent of chromoblastomycosis. In that study, sialidase-treated conidia had a reduced negative electrophoretic mobility and, compared to untreated cells, bound fewer particles of colloidal iron hydroxide and of cationized ferritin. *N*-acetylneuraminic acid was the only derivative found in the mycelium whereas conidia contained both *N*-glycolyl- and *N*-acetylneuraminic acids. Neuraminic acid derivatives were apparently determinants of morphogenesis and cellular integrity in *F. pedrosoi*. This structural function was suggested by the observation of marked alterations in the morphology of *F. pedrosoi* hyphae after sialidase treatment (Souza et al. 1986). However, the nature of the sialic acid-containing glycoconjugates in mycelia and conidia, as well as whether parasitic forms of *F. pedrosoi* (sclerotic cells) also express sialic acids, remained undefined.

Under our experimental conditions, sialic acids and sialylated glycoproteins were not detected in sclerotic bodies. Biochemical features of these cells, which are extremely resistant against destruction by phagocytes (Esterre et al. 1993; Rosen and Overholt 1996; Hamza et al. 2003), are very poorly known. Recent results from our laboratory (Alviano et al. 2003) indicated that the cell surface constitution of sclerotic cells markedly differs from that of conidia and mycelia with respect to carbohydrate expression and phosphatase activity. In addition, microscopy studies indicated that sclerotic forms of *F. pedrosoi* also differ from mycelial and conidial cells in that the former present a multi-layered cell wall with a large deposition of dark pigments (McGinnis and Hilger 1987; Silva et al. 2002; Hamza et al. 2003). In the present work, we have demonstrated that sialic acid expression serves as a differential marker for the morphological states of *F. pedrosoi*.

Knowledge regarding sialic acid-carrying compounds in fungal cells is still very preliminary. In *C. albicans,* it has been suggested that sialic acids are constituents of the surface-located iC3b glycoprotein receptor (Alaei et al. 1993). In the same study, sialidase treatment of three isolated glycoproteins resulted in an apparent decrease in the molecular masses of two of them. Further studies demonstrated that a hyphal 60-kDa mannoprotein from *C. albicans* reacted with a sialic-acid-binding lectin, and that sialidase treatment reduced the reactivity of this glycoprotein with a purified antibody to *Candida* CR2 (Wadsworth et al. 1993). The 60-kDa mannoprotein was associated with C3d, fibrinogen, and laminin binding (Bouali et al. 1987; Bouchara et al. 1990) and appeared to promote attachment of germ tubes to plastic (Tronchin et al. 1988). Sialylated glycolipids are absent in *C. neoformans* (Rodrigues et al. 2003), but sialylated glycoproteins with molecular masses corresponding to 38 (Hamilton et al. 1992; Rodrigues et al. 2003) and 67 kDa (Rodrigues et al. 2003) were detected in this pathogen. The latter molecule was recognized by influenza C virus at 4 °C, confirming a previous study in which 9-*O*-acetylated *N*-acetylneuraminic acid was detected (Rodrigues et al.1997). In *C. neoformans*, sialylation of glycoproteins apparently occurs through the action of a CMP-Neu5Ac-dependent sialyltransferase (Rodrigues et al. 2003).

This and a previous report (Souza et al. 1986) demonstrate that sialic acids in *F. pedrosoi* are glycosidically bound to galactose, forming α2,6- and, to a lesser extent, α2,3-sialylgalactosyl sequences. The detection of sialyl-

galactosyl sequences in fungal cells cultivated in a chemically defined medium is suggestive of a sialyltransferase-dependent sialylation but, as reviewed by Angata and Varki (2002), it cannot be ruled out that fungal cells have alternative pathways to synthesize and express sialic acids. We have demonstrated that *F. pedrosoi* mycelia and conidia express sialoproteins. One of these glycoproteins, the 77-kDa component, was specifically detected in conidial extracts, confirming that sialic acid expression is associated with fungal morphogenesis (Souza et al.1986). In addition, agglutination of conidia in the presence of influenza C virus was suggestive of the occurrence of a 9-*O*-acetylated sialic derivative. In some types of infections, *O*-acetylated sialic acids can affect the immunogenicity and pathogenicity of the infectious agent. Examples are the antigenicity of bacterial capsules, the modulation of alternative pathway of complement activation, and the partial or complete blocking of sialidase action (Hulb et al. 2000; Orskov et al. 1979). In this context, the presence of 9-*O*-acetylated sialic acids in conidial cells may explain their higher resistance to sialidase treatment, as observed in Figs. 1 and 2. The possible role of Neu5,9Ac_2 in the pathogenicity of fungi is still unknown.

Sialidases are essential tools in the metabolism of sialic acids, acting by hydrolyzing the *O*-glycosidic linkages between terminal sialic acids and subterminal monosaccharides of glycoconjugates (Schauer and Kamerling 1997). Sialidases have a potential role in bacterial pathogenesis, removing sialic acid from glycolipids, glycoproteins, and poly- and oligosaccharides (Jost et al. 2001). In fungi, the expression of sialidase activity is controversial. Two reports described the occurrence of this enzyme in *S. schenckii* (Uchida et al. 1974) and *C. albicans* (Royal et al. 1984). The latter study was subsequently contradicted by Roggentin et al. (1999), who proposed that *C. albicans* and *C. glabrata* do not produce sialidases. Similar results were observed using dermatophytes, which apparently do not synthesize either sialidases (Krug et al. 1997) or sialic acids (Esquenazi et al. 2003). We have demonstrated that mycelia and conidia, but not sclerotic cells of *F. pedrosoi*, produce surface and secretory sialidase activity. Since sclerotic cells did not produce sialic acids, a correlation between their biosynthesis and the expression of sialidase may occur. The putative role of sialidases in *F. pedrosoi* infection is unknown, but removal of sialic acids from cell-surface glycoproteins and glycolipids by fungal infecting forms possibly damages host cell glycans. In addition, removal of sialic acid alters the profiles of glycosylation in host cells, which could result in the exposition of additional sites for interaction with infectious agents. This process may reveal surface receptors for possible interactionwith fungi, contributing to increased adhesion, as proposed for pneumococci (Jedrzejas 2001). Once established inside the host, *F. pedrosoi* differentiates into sclerotic cells, which do not express sialidase activity.

Prior studies demonstrated that sialic acids protect the fungal pathogens *S. schenckii* and *C. neoformans* against phagocytosis (Alviano et al.1999). In the latter, a protective role for sialic acids during the initial steps of colonization of the human host was proposed. Infection by *C. neoformans* occurs via inhalation of poorly encapsulated cells, which resist phagocytosis by alveolar macrophages by unknown mechanisms (Rodrigues et al. 1999). Capsular polysaccharide, the main antiphagocytic structure in *C. neoformans*, is only expressed 5 h after initial contact of the fungus with the human host. Since sialic acids were identified as cell wall antiphagocytic components in *C. neoformans*, it has been suggested that these acidic sugars were responsible for the early fungal defense against host phagocytic cells, before full expression of the capsule is accomplished. In the present work, we demonstrated that sialyl residues protect *F. pedrosoi* conidia against phagocytosis by human neutrophils. The role of surface sialoglycoconjugates in the infection by *F. pedrosoi* may be similar to that proposed for *C. neoformans*, in that sialic acid, expressed by conidia and mycelia, could protect infecting propagules against destruction by host cells until fungal differentiation into sclerotic bodies. These cells, which are the parasitic forms of *F. pedrosoi*, are fully melanized and resistant against the action of effector immune cells (Esterre et al. 1993; Rosen and Overholt 1996; Hamza et al. 2003), probably by mechanisms in which sialic acid expression is not necessary. In this context, melanin has been proposed as the main structure in sclerotic cells contributing to the persistence of *F. pedrosoi* inside the human host (Farbiaz et al. 1992).

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References

Alaei S, Larcher C, Ebenbichler C, Prodinger WN, Janatova J, Dierich MP (1993) Isolation and biochemical characterization of the iC3b receptor of *Candida albicans*. Infect Immun 61: 1395–1399

Alviano CS, Pereira MEA, De Souza W, Oda LM, Travassos LR (1982) Sialic acids are surface components of *Sporothrix schenckii* yeast forms. FEMS Microbiol Lett 15:223–227

Alviano CS, Farbiarz SR, De Souza W, Angluster J, Travassos LR (1991) Characterization of *Fonsecaea pedrosoi* melanin. J Gen Microbiol 137:837–844

Alviano CS, Travassos LR, Angluster J, de Souza W (1992) Effect of environmental factors on *Fonsecaea pedrosoi* morphogenesis with emphasis on sclerotic cells induced by propranolol. Mycopathologia 119:17–23

Alviano CS, Travassos LR, Schauer R (1999) Sialic acids in fungi: a minireview. Glycoconj J 16:545–554

Alviano DS, Kneipp LF, Lopes AH, Travassos LR, Meyer-Fernandes JR, Rodrigues ML, Alviano CS (2003) Differentiation of *Fonsecaea pedrosoi* mycelial forms into sclerotic cells is induced by platelet-activating factor. Res Microbiol 154:689–695

Angata T, Varki A (2002) Chemical diversity in the sialic acids and related α-keto acids: An evolutionary perspective. Chem Rev 102:439–469

Arduino RC, Jacques-Palaz K, Murray BE, Rakita RM (1994) Resistance of *Enterococcus faecium* to neutrophil-mediated phagocytosis. Infect Immun 62:5587–5594

Bouali A, Robert R, Tronchin G, Senet JM (1987) Characterization of binding of human fibrinogen to the surface of germ tubes and mycelium of *Candida albicans*. J Gen Microbiol 133:545–551

Bouchara JP, Tronchin G, Annaix V, Robert R, Senet JM (1990) Laminin receptors on *Candida albicans* germ tubes. Infect Immun 58:48–54

Butterfield W, Jong SC (1987) Effect of carbon source on conidiogenesis in *Fonsecaea dermatitidis*, agent of chromomycosis. Mycopathologia 58:59–62

Castro LGM, Pimentel ERA, Lacaz CS (2003) Treatment of chromoblastomycosis by cryosurgery with liquid nitrogen: 15 years' experience. Int J Dermatol 42:408–412

Couceiro JNSS, Paulson JC, Baum LG (1993) Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 29:155–165

Esquenazi D, Rozental S, Alviano CS, Travassos LR, Schauer R (2003) Sialic acids are absent from the dermatophytes *Trichophyton mentagrophytes* and *Trichophyton rubrum*. Mycoses 46:1–6

Esterre P, Peyrol S, Sainte-Marie D, Pradinaud R, Grimaud JA (1993) Granulomatous reaction and tissue remodeling in the cutaneous lesion of chromoblastomycosis. Virchows Archiv A Patho Anat 422:285–291

Farbiarz SR, De Carvalho TU, Alviano CS, De Souza W (1990) Fine structure and cytochemistry of the interaction between *Fonsecaea pedrosoi* and mouse resident macrophages. J Med Vet Mycol 28:373–383

Farbiarz SR, Carvalho TU, Alviano CS, De Souza W (1992) Inhibitory effect of melanin on the interaction of *Fonsecaea pedrosoi* with mammalian cells *in vitro*. J Med Vet Mycol 30: 265–273

Hamilton AJ, Jeavons L, Hobby P, Hay RJ (1992) A 34 to 38 kilodalton *Cryptococcus neoformans* glycoprotein produced as an exoantigen bearing a glycosylated species-specific epitope. Infect Immun 60:143–149

Hamza SH, Mercado PJ, Skelton HG, Smith KJ (2003) An unusual dematiaceous fungal infection of the skin caused by *Fonsecaea pedrosoi*: a case report and review of the literature. J Cutan Pathol 30:340–343

Hulb U, Ishida H, Kiso M, Hasegawa A, Schauer R (2000) Studies on the specificity and sensitivity of the Influenza C virus binding assay for 9-*O*-acetylated sialic acids and its application to human melanomas. J Biochem 127:1021–1031

Jedrzejas MJ (2001) Pneumococcal virulence factors: structure and function. Microbiol Mol Biol Rev 65:187–207

Jost BH, Songer JG, Billington SJ (2001) Cloning, expression, and characterization of a neuraminidase gene from *Arcanobacterium pyogenes*. Infect Immun 69:4430–4437

Krug G, Roggentin P, Schauer R, Brasch J (1997) Dermatophyten bilden *in vitro* keine sialidase. Mycoses 40:17–21

Kwon-Chung KJ, Bennet JE (1992) Chromoblastomycosis. In: Medical Mycology. Lea & Febiger, Philadelphia, pp 337–355

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Limongi CL, Alviano CS, De Souza W, Rozental S (2001) Isolation and partial characterization of an adhesin from *Fonsecaea pedrosoi*. Med Mycol 39:429–437

Lowry OH, Rosebrogh NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

McGinnis MR, Hilger EA (1987) Infections caused by black fungi. Arch Dermatol 123:1300–1302

Mendoza L, Karuppayil SM, Szaniszlo PJ (1993) Calcium regulates *in vitro* dimorphism in chromoblastomycotic fungi. Mycoses 36:157–164

Oda LM, Kubelka CF, Alviano CS, Travassos LR (1983) Ingestion of yeast forms of *Sporotrix schenckii* by mouse peritoneal macrophages. Infect Immun 39:497–504

Oliveira LG, Resende MA, Lopes CF, Cisalpino EO (1973) Isolamento e identificação dos agentes da cromomicose em Belo Horizonte. Rev Soc Bras Med Trop 7:1 (in Portuguese).

Orskov F, Orskov I, Sutton A, Schenckman R, Lin W, Egan W, Hoff EG, Robbins JB (1979) Form variation in *Escherichia coli* K1 determined by *O*-acetylation of the capsular polysaccharide. J Exp Med 152:1375–1392

Potier M, Mameli L, Bélisle M, Dallaire L, Melançon SB (1979) Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-*N*-acetylneuraminate) substrate. Anal Biochem 94:287–296

Reuter G, Schauer R (1994) Determination of sialic acids. Methods Enzymol 230:168–199

Rivitti EA, Aoki V (1999) Deep fungal infections in tropical countries. Clin Dermatol 17:171–190

Rodrigues ML, Rozental S, Couceiro JNSS, Angluster J, Alviano CS, Travassos LR (1997) Identification of *N*-acetylneuraminic acid and its 9-*O*-acetylated derivative on the cell surface of *Cryptococcus neoformans*: influence on fungal phagocytosis. Infect Immun 65:4937–4942

Rodrigues ML, Alviano CS, Travassos LR (1999) Pathogenicity of *Cryptococcus neoformans*: virulence factors and immunological mechanisms. Microbes Infect 1:293–301

Rodrigues ML, Dobroff ASS, Couceiro JNSS, Alviano CS, Schauer R, Travassos LR (2003) Sialylglycoconjugates and sialyltransferases activity in the fungus *Cryptococcus neoformans*. Glycoconjugate J 19:165–173

Rogers GN, Herrier G, Paulson JC, Klenk HD (1986) Influenza C virus uses 9-*O*-acetylneuraminic as a high affinity receptor determinant for attachment to cells. J Biol Chem 261:5947–5951

Roggentin P, Krug G, Schauer R, Brasch J (1999) Lack of sialidase activity in *Candida albicans* and *Candida glabrata*. Mycoses 42:33–36

Rosen T, Overholt M (1996) Persistent viability of the medlar body. Int J Dermatol 35:96–98

Royal GC Jr, Nandedkar AK, Sampson CC, Faggett T (1984) Neuraminidase production by *Candida albicans*. J Natl Med Assoc 76:143–145

Rozental S, Alviano CS, De Souza W (1996) Fine structure and cytochemical study of the interaction between *Fonsecaea pedrosoi* and rat polymorphonuclear leukocyte. J Med Vet Mycol 34:323–330

Schauer R, Kamerling JP (1997) Chemistry, biochemistry and biology of sialic acids. In: Montreuil J, Vliegenthart JFG, Schachter H (eds) Glycoproteins II, vol 29b. New Comprehensive Biochemistry, Elsevier Science, Amsterdam, pp 243–402

Schnitzler N, Peltroche-Llacsahuanga H, Bestier N, Zündorf J, Lütticken R, Haase G (1999) Effect of melanin and carotenoids of *Exophiala* (Wangiella) *dermatitidis* on phagocytosis, oxidative burst, and killing by human neutrophils. Infect Immun 67:94–101

Silva JP, de Souza W, Rozental S (1999) Chromoblastomycosis: a retrospective study of 325 cases on Amazonic region (Brazil). Mycopathologia 143:171–175

Silva JP, Alviano DS, Alviano CS, De Souza W, Travassos LR, Diniz JAP, Rozental S (2002) Comparison of *Fonsecaea pedrosoi* sclerotic cells obtained *in vivo* and *in vitro*: ultrastructure and antigenicity. FEMS Immunol Med Microbiol 33:63–69

Soares RMA, Angluster J, de Souza W, Alviano CS (1995) Carbohydrate and lipid components of hyphae and conidia of human pathogen *Fonsecaea pedrosoi*. Mycopathologia 132:71–77

Soares RMA, Costa e Silva-Filho F, Rozental S, Angluster J, de Souza W, Alviano CS, Travassos LR (1998) Anionogenic groups and surface sialoglycoconjugate structures of yeast forms of the human pathogen *Paracoccidioides brasiliensis*. Microbiology 144:309–314

Soares RMA, Soares RMA, Alviano DS, Angluster J, De Souza W, Alviano CS, Travassos lr (2000) Identification of sialic acids on the cell surface of *Candida albicans*. Biochim Biophys Acta 1474:262–268

Souza ET, Silva-Filho FC, De Souza W, Alviano CS, Angluster J, Travassos LR (1986) Identification of sialic acids on the cell surface of hyphae and conidia of the human pathogen *Fonsecaea pedrosoi*. J Med Vet Mycol 24:145–153

Tronchin G, Bouchara JP, Robert R, Senet JM (1988) Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. Infect Immun 56:1987–1993

Uchida Y, Tsukada Y, Sugimori T (1974) Production of microbial neuraminidases induced by colominic acid. Biochim Biophys Acta 350:425–431

Wadsworth E, Prasad SC, Calderone R (1993) Analysis of mannoproteins from blastoconidia and hyphae of *Candida albicans* with a common epitope recognized by anticomplement receptor type 2 antibodies. Infect Immun 61:4675–4681

Warren L (1959) The thiobarbituric assay of sialic acids. J Biol Chem 234:1971–1975

Wasylnka JA, Simmer MI, Moore MM (2001) Differences in sialic acid density in pathogenic and non-pathogenic *Aspergillus* species. Microbiology 147:869–877