Anionogenic groups and surface sialoglycoconjugate structures of yeast forms of the human pathogen *Paracoccidioides brasiliensis*

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The surface anionogenic groups and sialoglycoconjugate structures of *Paracoccidioides brasiliensis* yeast forms were analysed by cell microelectrophoresis, binding assays with lectins and viral particles, ultrastructural cytochemistry, enzymic digestion and flow cytofluorimetry. *P. brasiliensis* yeast forms, particularly the budding primordia, reacted strongly with cationized ferritin. Binding assays showed that the reaction with sialic-acid-specific *Limax flavus* lectin (LFA) was distributed over the entire *P. brasiliensis* cell wall. Treatment of yeast forms with neuraminidase significantly reduced their negative surface charge and LFA labelling, which suggests that sialic acid residues are major anionogenic groups exposed on the *P. brasiliensis* surface. Furthermore, after neuraminidase treatment, labelling with *Arachis hypogaea* (peanut) agglutinin increased due to unmasking of subterminal β -D-galactopyranosyl residues. The sialic acid linkages to galactose are α 2,6 and α 2,3 as assessed, respectively, by fungal attachment to M1/5 and M1/5 HS8 strains of influenza A virus and binding of *Sambucus niger* and *Maackia amurensis* agglutinins. The α 2,6 linkage clearly predominated in both experiments. Flow cytofluorimetry analysis revealed the heterogeneity of *P. brasiliensis* yeast cell populations, which comprised young and mature budding yeasts. Both express binding sites to LFA and *Limulus polyphemus* agglutinin.

Keywords: anionogenic groups, influenza virus, *Paracoccidioides brasiliensis*, sialic acids, yeast forms

INTRODUCTION

Paracoccidioides brasiliensis, a dimorphic human pathogenic fungus, is the agent of paracoccidioidomycosis, a systemic mycosis endemic in several countries of South America. The yeast phase is associated with the infection, which starts in the lung, and may progress to eventual metastasis in other tissues and organs. Yeast cells are transiently found inside macrophages and extracellularly.

Abbreviations: CF, cationized ferritin; EPM, electrophoretic mobility; LFA, *Limax flavus* agglutinin; LPA, *Limulus polyphemus* agglutinin; MAA, *Maackia amurensis* agglutinin; PNA, *Arachis hypogaea* (peanut) agglutinin; SNA, *Sambucus niger* agglutinin.

A basic characteristic of the eukaryotic cell surface is its electrostatic charge, which is determined by the nature and number of ionogenic groups exposed on the plasma membrane (van Oss *et al.*, 1984). Sialic acid residues are constituents of many glycoconjugates and are the major ionogenic components contributing to the negative charge of many cell types (Schauer, 1982). Electrostatic forces are involved in the attachment of micro-organisms to several types of surfaces (van Oss *et al.*, 1986), and may be relevant to the interaction between the micro-organism and the host-cell (Hesketh *et al.*, 1987). In addition, sialoglycoconjugates have been associated with important functions and biological phenomena including malignant transformation and metastasis, also acting as receptors for hormones, lectins, viruses

and antibodies (Schauer, 1982; Varki, 1997). Sialic acid oligomers in $\alpha 2,8$ linkages as well as sialic acids $\alpha 2,3$ - and $\alpha 2,6$ -linked to galactose are present in sialoglycoconjugates (Powell & Varki, 1996).

The few reports available on the occurrence of sialic acids in pathogenic fungi suggested their presence in *Sporothrix schenckii* (Benchimol *et al.*, 1979; Alviano *et al.*, 1982; Oda *et al.*, 1983), *Fonsecaea pedrosoi* (Souza *et al.*, 1986) and *Cryptococcus neoformans* (Hamilton *et al.*, 1992). We have previously shown that both yeast and mycelial forms of *P. brasiliensis* also expressed surface sialic acid units (Soares *et al.*, 1993). In the present work we extend these studies to establish a correlation between sialic acids and the exposed cell surface anionogenic groups, determining the nature of the sialylated structures expressed in the yeast phase of *P. brasiliensis* grown in a chemically defined medium.

METHODS

Micro-organism. *Paracoccidioides brasiliensis* strain 339, originally obtained from Dr Angela Restrepo, Medellin, Colombia, was maintained in Sabouraud medium at room temperature, transferred every 2 months to fresh medium. Yeast forms were obtained by growth in a chemically defined medium (Gilardi & Laffer, 1962), pH 7.2 at 37 °C with shaking, for 14 d.

Enzymic treatment. Fungal cells were washed twice in PBS (0.01 M phosphate buffer 0.15 M NaCl), pH 6.0, and incubated for 30 min at 37 °C in the presence of 0.4 U ml⁻¹ of neuraminidase from *Clostridium perfringens* (Sigma, type X). For cell electrophoretic mobility (EPM), the neuraminidase from *Vibrio cholerae* (Sigma, type II) was also used at 0.4 U ml⁻¹, for 30 min at 37 °C. After treatment, cells were washed twice in PBS, fixed in 2.5 % (v/v) glutaraldehyde, and used for EPM studies.

Cell microelectrophoresis. The EPM of cells was determined in a Zeiss Cytopherometer by timing the passage of cells through a calibrated graticule when a current of 6 mA and a gradient of 5.5 V cm⁻¹ was applied to the electrophoresis chamber. Cell mobility was timed in both directions to minimize electrode polarization. Instrument calibration was controlled by measuring the electrophoretic migration of normal glutaraldehyde-fixed human erythrocytes. The measurements were made, in alternate directions, on 60 individual cells which were suspended in NaCl solution (ionic strength 145 mmol l⁻¹ at pH 7.2) at 25 °C. Electrophoretic mobilities were determined using the following equation: $EPM = (d \cdot t^{-1}) \times (D \cdot V^{-1})$, where d is the distance (in μ m) covered by the cells during measurements (usually 16 μ m); t is the time (in s) required by a cell to cover the distance d ; D is the distance between the two electrodes (18 cm); and V is the potential (in V) applied to the electrodes.

Ultrastructural cytochemistry. Glutaraldehyde-fixed cells were exhaustively rinsed with PBS and incubated in the presence of 10 μ g cationized ferritin (CF) ml⁻¹ (Sigma), at pH 7.2, for 1 h at room temperature (Danon *et al.*, 1972). Cells were post-fixed for 30 min in 1 % (v/v) osmium tetroxide, dehydrated in acetone, and embedded in Epon. Ultrathin sections were observed in a Zeiss 900 electron microscope at 80 kV.

Lectin binding. Both neuraminidase-treated and untreated cells were fixed in 4 % (v/v) paraformaldehyde made in PBS,

pH 7.2, for 1 h, rinsed with PBS and pre-incubated sequentially for 30 min in the same buffer containing 150 mM NH₄Cl, and then in PBS with 1 % bovine serum albumin (PBS-BSA) for 1 h. The lectins LPA (*Limulus polyphemus* agglutinin), LFA (*Limax flavus* agglutinin), PNA (*Arachis hypogaea* agglutinin), all from Sigma, MAA (*Maackia amurensis* agglutinin) and SNA (*Sambucus nigra* agglutinin) both from E-Y Lab (San Mateo, CA, USA) were conjugated to fluorescein isothiocyanate (FITC) for assays with yeast forms. LFA and LPA recognize all sialic acid linkages; N-acetylneuraminic acid rather than N-glycolylneuraminic acid is preferentially recognized by LFA. With both, the presence of an underlying saccharide is not required. SNA and MAA recognize sialic acids in $\alpha 2,6$ and $\alpha 2,3$ linkages respectively, whereas for SNA binding, Gal or GalNAc is the required sugar unit. For MAA the necessary underlying sequence is Gal β 1-4GlcNAc. Cells (5×10^6 ml⁻¹) were suspended in PBS, pH 7.2, and incubated with each of the lectin-FITC conjugates at the initial concentration of 1.2 μ g ml⁻¹ in PBS, pH 7.2, for 30 min at 23 °C. After incubation, the cells were washed three times in PBS and observed in a Zeiss epifluorescence microscope (Axioplan) or screened by flow cytometry analysis using an Epics Elite flow cytometer (Coulter Electronics) equipped with a 15 mW argon laser emitting at 488 nm. The system measures the fluorescence and laser light scattered from cells passing through a laser beam. The forward light scatter correlates with the particle size, and side scatter correlates with the granularity. The FITC fluorescence was measured in the 510–540 nm range, and both forward light scatter and side scatter were measured at 488 nm. The data obtained were run using listmode, which makes further analysis possible. Control cells were first analysed to determine their auto-fluorescence and relative size and granularity.

Virus binding and agglutination. Influenza A/Memphis/102/72 (M1/5) virus strain (provided by Dr Robert Webster, Memphis, TN, USA), and its clonal isolate M1/5 HS8 selected by growth in MDCK cells, in the presence of horse serum (Rogers *et al.*, 1983), were grown in the allantoic cavity of 10-d-old embryonated chicken eggs as described by Carroll *et al.* (1981). Virus strains were prepared by differential centrifugation, with subsequent standardization to 2.560 HAU (haemagglutination units) by haemagglutination assay with 1 % (v/v) fresh human erythrocytes. The agglutination of yeast cells induced by the virus particles was carried out in glass tubes at 4 °C for 1 h with occasional shaking. In these experiments, equal volumes of the cell suspension in PBS, pH 7.2, containing 6×10^5 cells ml⁻¹ and each virus suspension were rapidly mixed. Cell agglutination was scored visually after gently resuspending settled cells, and by observation under a phase-contrast microscope. The control was the supernatant fluids from uninfected chicken eggs. The specificity of virus binding was shown by pre-treating the yeast cells with neuraminidase.

RESULTS

Cell microelectrophoresis

The EPM of neuraminidase-treated and untreated yeast forms is shown in Table 1. Control cells moved towards the cathode with a mean EPM of -0.98μ m s⁻¹ V⁻¹ cm. The EPM values of yeast forms were significantly reduced by treatment with neuraminidase isolated from *Clostridium perfringens*. Treatment with neuraminidase isolated from *Vibrio cholerae* reduced the EPM even further, by 54.2 %.

Table 1. EPM of *P. brasiliensis* yeast forms before and after treatment with *Vibrio cholerae* (VC) and *Clostridium perfringens* (CP) neuraminidase

Treatment	EPM ($\mu\text{m s}^{-1} \text{V}^{-1} \text{cm}$)*	Reduction in EPM (%)†
None	0.98 ± 0.06	—
Neuraminidase (VC)‡	-0.53 ± 0.01	54.2
Neuraminidase (CP)‡	-0.68 ± 0.02	30.5

* Mean values obtained from three independent determinations. The values for the control and neuraminidase-treated cells are significantly different ($P < 0.01$).

† $[(\text{EPM}_c - \text{EPM}_t)/\text{EPM}_c] \times 100$, where c and t indicate control and neuraminidase-treated cells, respectively.

‡ 0.4 U ml^{-1} for 30 min.

Ultrastructural cytochemistry

Binding of cationized ferritin (CF) particles was observed at the cell surface of *P. brasiliensis* yeast forms. Particles appeared distributed over the entire cell surface of the yeast form. A great concentration of anionic groups was observed on newly formed budding yeast-cell primordia (Fig. 1a).

Lectin binding

Positive fluorescent labelling with FITC-LFA was observed over the entire surface of *P. brasiliensis* yeast cell walls (Fig. 2a). Neuraminidase treatment largely reduced FITC-LFA binding to whole yeast cells (Fig. 2c). Flow cytofluorimetric analysis of FITC-LFA, FITC-LPA (recognizing sialic acids) and FITC-PNA (recognizing β -D-galactose) lectins with *P. brasiliensis* yeast forms which were treated or not with neuraminidase is shown in Fig. 3 and Table 2. The yeast cell population was heterogeneous. Young and mature budding cells, as distinguished by their size, reacted with FITC-LFA (Figs 3b1, 2) and FITC-LPA (not shown). However, labelling with FITC-LFA was stronger (Table 2). Mature budding yeasts expressed much more sialic acid at the cell surface than did young cells. Also neuraminidase treatment yielded much more terminal β -galactose in the mature as compared with young cells.

P. brasiliensis yeast forms were labelled when incubated in the presence of FITC-SNA (specific for α 2,6-sialylgalactose) and FITC-MAA (specific for α 2,3-sialylgalactose) lectins, as also assayed by flow cytofluorimetric analysis. Preferential labelling was observed with the FITC-SNA lectin. The specificity of the reaction was confirmed by pre-treating the yeast cells with neuraminidase, which markedly reduced binding of both FITC-labelled lectins to *P. brasiliensis* (Table 2).

Virus binding

Two human influenza A virus strains were also used to determine the type of linkage of sialic acid to galactose in the surface sialoglycoconjugates of neuraminidase-

treated and untreated *P. brasiliensis*. These assays were performed with virus strains M1/5 and M1/5 HS8, which preferentially bind to the α 2,6- and α 2,3-sialylgalactose sequences, respectively. Both virus strains agglutinated the yeast cells; however, the reactivity of *P. brasiliensis* with M1/5 was greater than that with M1/5 HS8. In 18 of 20 assays the virus agglutination titres were equivalent to 256 HAU with M1/5 and 64 HAU with M1/5 HS8. Pre-treatment of yeasts with neuraminidase markedly reduced binding of the virus strains, as shown by the decrease in the agglutination titres: 16 HAU with M1/5, and 4 HAU with M1/5 HS8. No spontaneous agglutination of *P. brasiliensis* yeast forms was observed when the yeast cells were incubated with uninfected chicken egg supernatants.

DISCUSSION

The occurrence at the cell surface of anionogenic groups in *P. brasiliensis* was inferred from binding of CF to the fungal cell wall. These anionic groups impart a negative cell surface charge of about $-0.98 \mu\text{m s}^{-1} \text{V}^{-1} \text{cm}$, as evaluated by cell electrophoresis. Similarly, other human pathogenic fungi such as *Sporothrix schenckii* (Alviano *et al.*, 1982) and *Fonsecaea pedrosoi* (Souza *et al.*, 1986) showed a negative surface charge. The EPM values determined for *P. brasiliensis* resembled those observed in bacteria (Figueiredo *et al.*, 1995), protozoa (Schauer *et al.*, 1983; Silva-Filho *et al.*, 1990), as well as in human erythrocytes (Eylar *et al.*, 1962), all of them possessing a highly negative surface charge.

Most of the anionic sites on the eukaryotic cell surface result from the presence of carboxyl groups of acidic amino acids of proteins and glycoproteins (Weiss, 1969; Mehereshi, 1972), phosphate groups, and also carboxyl and sulphate residues of mucopolysaccharides (Burry & Wood, 1979). The carboxyl groups of sialic acids present in glycoproteins and glycolipids also seem to contribute strongly to the negative surface charge (James, 1979; Schauer, 1982). In the current study, treatment of yeast forms of *P. brasiliensis* with neuraminidase resulted in a significant decrease in the negative surface charge, showing that the carboxyl groups of sialic acids, which amount to 3.6×10^6 residues per cell (Soares *et al.*, 1993), effectively contribute to the electronegative surface charge. The enzymic removal of sialic acid residues was more effective with neuraminidase from *Vibrio cholerae* (54.2% reduction) than with that from *Clostridium perfringens* (30.5% reduction), suggesting the occurrence of different sialylated structures.

The biological significance of anionic groups which contribute to the electronegativity of *P. brasiliensis* is still unclear. Removal by neuraminidase of anionic groups from the external cell wall layers of *S. schenckii* rendered yeast cells 7.7-fold more susceptible to phagocytosis (Oda *et al.*, 1983). In *Cryptococcus neoformans* a species-specific sialylated exoantigen was recognized by a monoclonal antibody (Hamilton *et al.*, 1992) but its role in pathogenicity is not known. In *P. brasiliensis* the marked decrease in the negative surface charge, and in

Fig. 1. Anionic groups at the surface of *P. brasiliensis*. (a) A mature yeast cell with a bud strongly reactive with CF (small arrowheads); the cell wall of the mother cell is unreactive (large arrowheads). (b) Control, unlabelled, yeast cell. Bars, 1 µm.

Fig. 2. Labelling with FITC-LFA agglutinin of untreated (a) and neuraminidase-treated (c) *P. brasiliensis* yeast forms. (b, d) Corresponding fields observed by phase-contrast microscopy. Binding of lectin to the cells was much reduced by neuraminidase treatment (c). Bars, 10 µm.

the binding of fluorescent LFA to whole cells treated with neuraminidase, involved removal of terminal non-reducing units of *N*-acetylneuraminic acid from yeast surface sialoglycoconjugates. Accordingly, *N*-acetylneuraminic acid was the only type of sialic acid characterized chemically and spectroscopically in yeast forms of *P. brasiliensis* (Soares *et al.*, 1993). In addition to strain 339 used in this previous work we also found sialic acids in two other strains of *P. brasiliensis* (265 and 18) with distinct virulence (unpublished results).

Reaction with CF particles showed that the exposed anionic groups were distributed all over the cell surface of *P. brasiliensis* yeast forms. In *F. pedrosoi* the CF particles covered the entire cell surface of both conidial and mycelial forms (Souza *et al.*, 1986). In yeast forms of

S. schenckii a neuraminidase-sensitive double layer of surface acidic components was observed, as compared to the single acidic layer on saprophytic hyphae (Benchimol *et al.*, 1979; Alviano *et al.*, 1982). In this species the distribution pattern of anionic sites was tentatively associated with the fungal pathogenicity of the infective yeast.

In *P. brasiliensis* the binding of fluorescent LFA essentially confirmed the results with CF labelling, indicating that sialic acid residues are major anionic groups distributed on the cell wall external layer of yeast forms. Sialic acids are linked to β -D-galactose in sialylglycoconjugates, as also confirmed in *P. brasiliensis*, which became more strongly agglutinated by PNA after neuraminidase treatment (Soares *et al.*, 1993). The sialic acid-galactose bonds involve α 2,6 and α 2,3 linkages as determined in the current study, using, respectively, influenza A M1/5 and M1/5 HS8 virus strains as probes. The α 2,6-sialylgalactose sequences seemed to be more abundant, possibly indicating that sialoglycoproteins are the chief sialylated molecules. The α 2,3-sialylgalactose linkages are more common in sialoglycolipids, which may be cryptic on the cell surface (Lampio, 1988). In *P. brasiliensis* the predominance of the α 2,6 linkage was also confirmed by binding of the SNA lectin.

In some micro-organisms, including *Trypanosoma cruzi* and *T. brucei*, the acquisition of sialic acid is catalyzed by a parasite trans-sialidase that transfers sialic acid residues from glycoconjugates available in the environment to protozoan acceptor molecules (Previateo *et al.*, 1985; Schenkman *et al.*, 1993). The presence of sialoglycoconjugates in *P. brasiliensis* grown in a chemically defined medium, free of sialic acid, suggests that these residues are synthesized *de novo* and transferred to terminal galactosyl residues by a regular CMP-sialic-acid-dependent sialyltransferase.

Flow cytofluorimetric analysis with fluorescently labelled lectins showed two populations of cells of different sizes, the young, recently separated cells and the mature multi-budding mother cells, which carried a

Fig. 3. Flow cytometry (FACS) of untreated (1, 2) and neuraminidase-treated (3, 4) young (1, 3) and mature budding yeasts (2, 4) of *P. brasiliensis* incubated with FITC-LFA lectin (b), and PNA (c). (a) Control, unlabelled, cells.

Table 2. Percentage of fluorescent cells determined by flow cytofluorimetry of untreated and neuraminidase-treated young and mature budding yeasts of *P. brasiliensis* incubated with FITC-labelled lectins

Yeast population	Neuraminidase treatment*	Percentage of fluorescent cells†				
		LFA	LPA	PNA	SNA‡	MAA‡
Young cells	—	4.4 ± 0.4	3.1 ± 0.3	0.5 ± 0.2	—	—
	+	3.5 ± 0.2	2.6 ± 0.3	1.7 ± 0.3	—	—
Mature budding cells	—	45.3 ± 2.4	33.6 ± 0.7	6.5 ± 0.5	60.9 ± 1.3	20.1 ± 0.9
	+	38.2 ± 2.1	27.1 ± 1.0	8.4 ± 0.3	27.9 ± 0.9	6.1 ± 0.2

* Values with neuraminidase treatment are significantly different from those with untreated cells (Student's *t* test, *P* < 0.01).

† Means of three experiments, ± SD.

‡ In the SNA and MAA systems no separation of young and mature cells was evident.

number of highly anionic primordial protuberances. The mature budding cells contained more sialic acid residues than the young yeasts as shown by specific lectin binding. Such differences in the expression of surface sialoglycoconjugates may be correlated with the growth phase of *P. brasiliensis*. Noteworthy was the marked enrichment, as in erythrocytes (Eylar *et al.*, 1962), of terminal β -galactosyl residues in aged yeast cells of *P. brasiliensis*, which could favour their phagocytosis.

The present evidence for the expression of sialic acids in *P. brasiliensis* based on their recognition by specific ligands confirms the chemical characterization reported previously (Soares *et al.*, 1993).

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