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Sialic acids in fungi: A minireview

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The increasing number of reports on the presence of sialic acids in fungi (*N*-acetyl-, *N*-glycolyl- and 5,9-*N,O*-diacetylneuraminic acids) based on direct and indirect evidence warrants the present review. Formerly suggested as sialidase-sensitive sources of anionic groups at the cell surface of fungal species grown in chemically defined media (e.g., *Fonsecaea pedrosoi)*, sialic acids have also been found in *Sporothrix schenckii, Paracoccidioides brasiliensis, Cryptococcus neoformans* and recently, in *Candida albicans.* Methods used involved adequate hydrolysis and extraction procedures, HPTLC, gas-chromatography, colorimetry, mass spectroscopy, lectin and influenza virus binding. Apart from protecting fungal cells against phagocytosis (*S. schenckii, C. neoformans*) and playing a cellular structural role (*F. pedrosoi*), other biological functions of sialic acids are still being investigated.

Keywords: **fungi, sialic acids, anionic groups, *N*-acetylneuraminic acid, *O*-acetylated sialic acids, fungal pathogenesis**

Abbreviations: **BSM, bovine submandibular gland mucin; ClH, colloidal iron hydroxide; CMP, cytidine monophosphate; EI, electron impact; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; Gal, Galactose; GalNAc, *N*-acetyl-D-galactosamine; GLC, Gas-liquid chromatography; HPTLC, High-performance thin-layer chromatography; LFA, *Limax flavus* agglutinin; LPA, *Limulus polyphemus* agglutinin; MAA, *Maackia amurensis* agglutinin; MoAb, Monoclonal antibody; MS, Mass-spectrometry; Neu5Ac, *N*-Acetylneuraminic acid; Neu5,9Ac$_2$, 5-*N*-Acetyl-9-*O*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PNA, peanut agglutinin; SNA, *Sambucus nigra* agglutinin; WGA, wheat germ agglutinin**

Introduction

Sialic acids are a family of monosaccharides comprising several derivatives of neuraminic acid including *N*-glycolyl (Neu5Gc) and *N*-acetyl (Neu5Ac) groups, as well as *O*-acetyl, and other *O*-substitutions [1]. For a survey of the manifold sialic acid species see [2]. They are important terminal or subterminal units in oligosaccharides from complex glycoconjugates expressed at the cell surface. Naturally occurring sialic acids are known to mediate many important biological phenomena involving cell-cell and cell-matrix interactions [3]. They have been reported to play a role in microbial pathogenicity [2], and to influence the tumor cell metastatic potential [4,5].

In the past decades, sialic acids have been characterized mostly in animal cells, some viruses, bacteria and protozoan species [2,6–8]. Remarkably, sialic acids can also be found in fungi. They were first demonstrated by our group in yeast-like cells of dimorphic *Sporothrix schenckii* [9,10]. In the subsequent years, we have identified sialic acids in several other pathogenic species such as *Cryptococcus neoformans* [11,12], *Fonsecaea pedrosoi* [13], *Paracoccidioides brasiliensis* [14,15], *Candida albicans* [16–18, 18a]. Additional studies by other investigators have shown that sialic acids are also present in other species [19–20] (Table 1). In 1987, Benhamou and Ouellette [19] studied the phytopathogenic fungus *Ascocalix abietina* showing that sialic acid is an abundant cell surface component. Sialic acid and galacturonic acid units, also present at the cell surface of this species, were thought to influence fungal resistance to unfavorable environmental conditions. Analysis of the sugar composition of *Pneumocystis carinii* cyst wall showed trace amounts of sialic acid [20]. A very weak fluorescent signal was detected when this microorganism was incubated with FITC-labelled *Limax flavus* agglutinin and analyzed by flow cytometry, which corroborated the previous evidence [21]. In *C. neoformans,* Hamilton et al. [11] identified a sialoglycoprotein exoantigen reactive with a MoAb. Sialidase treatment precluded its recognition by this antibody. The presence of sialic acids in *C. albicans* was suggested by indirect methods. Sialidase treatment of three isolated glycoproteins resulted in the apparent decrease of molecular mass in two of them [16]. Additional studies on mannoproteins from blastoconidia and hyphae of *C. albicans* showed that sialidase treatment reduced the reactivity

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Table 1. Sialic acid density and distribution in different forms of pathogenic fungi.

Fungal species	*Cell form*	*Sialic acid content*	*Number of residues per cell*	*Sialic acid location*	*Sialic acid derivative*	*Reference*
F. pedrosoi	Conidia	21.0 ± 0.6 μg/100mg dry cells	1.2×10^5	Surface	Neu5Ac Neu5Gc	[13]
	Hyphae	17.7 ± 0.1 μg/100 mg dry cells	ND[a]	Surface	Neu5Gc	[13]
P. brasiliensis	Yeast	28.0 ± 0.1 μg/100 mg dry cells	3.7×10^6	Surface	Neu5Ac	[14,15]
	Mycelium	*13.0* ± 0.1 μg/100 mg dry cells	ND	Surface	Neu5Ac	[14,15]
C. neoformans	Yeast	5 μg/10^{10} cells	3.0×10^6	Surface	Neu5Ac Neu5,9Ac_2	[12]
				Exogenous glycoproteins	ND	[11]
C. albicans	Yeast	13 μg/10^{10} cells	2.8×10^6	Surface	Neu5Ac	[16–18, Soares *et al.,* in prep.]
S. schenkii	Yeast	10 μg/10^{10} cells	$2.0–2.8 \times 10^6$	Surface	Neu5Ac Neu5Gc	[9,10]
P. carinii	Cyst	Trace	ND	Surface and cell wall	ND	[19,20]

[a]ND: not determined

of a 60-kilodalton mannoprotein with a polyclonal rabbit antiserum [17]. Recent results from our laboratory [18a] and by Jones and co-workers [18] indicate that sialic acids are cell surface components of *C. albicans*. The relationship of these residues to fungal pathogenicity is the focus of future study.

The present minireview discusses the presence of sialic acid-containing structures in fungi, a topic which deserves more attention in the research field of microbial sialoglycoconjugates.

Anionic groups at the fungal cell surface

Relevant physicochemical properties such as electrical surface potential and interfacial interactions modulate biological associations of cells and organisms. Anionogenic groups, usually comprising carboxyl, sulfate and phosphate residues, confer to eucaryotic cells a high net negative charge [22–25]. Since electrostatic forces could mediate cell adhesion [26], the surface electronegativity may play an important role in fungal attachment to the host cells [9,13,14].

Sialic acid residues, which are the major ionogenic components in many cell types [1,2,6,27], possess a strongly acidic carboxyl group with a pK of approximately 2. Sialic acid-rich cells at physiological pH can use their negative charge for dispersion by self-repulsion, a characteristic of erythrocytes. In contrast to this repulsive effect, cell adhesion may otherwise be facilitated if mediated by positively charged substances or through Ca^{2+} bridges [28].

For the *in situ* visualization of anionogenic groups including sialic acid residues, several techniques are available, such as binding of cationized proteins and more specifically of colloidal iron hydroxyde (CIH) at very low pH. Cytochemical studies [29] showed that the outer surface of *S. schenckii* cell wall has a bilayered structure strongly reactive with CIH at pH 1.8. The presence of sialic acid residues in this antigenic bilayer was confirmed by sialidase treatment, which abolished CIH binding [9] (Fig. 1, A–C). Comparison of infective yeasts and saprophytic hyphae of *S. schenckii* showed that the infective forms have a double acidic layer rich in sialic acids, whereas hyphae have a single acidic layer. In this species the distribution of anionic sites on the infective yeast forms and external cell wall layer sloughed off from these cells was tentatively associated with fungal virulence of the infective yeast. Re-

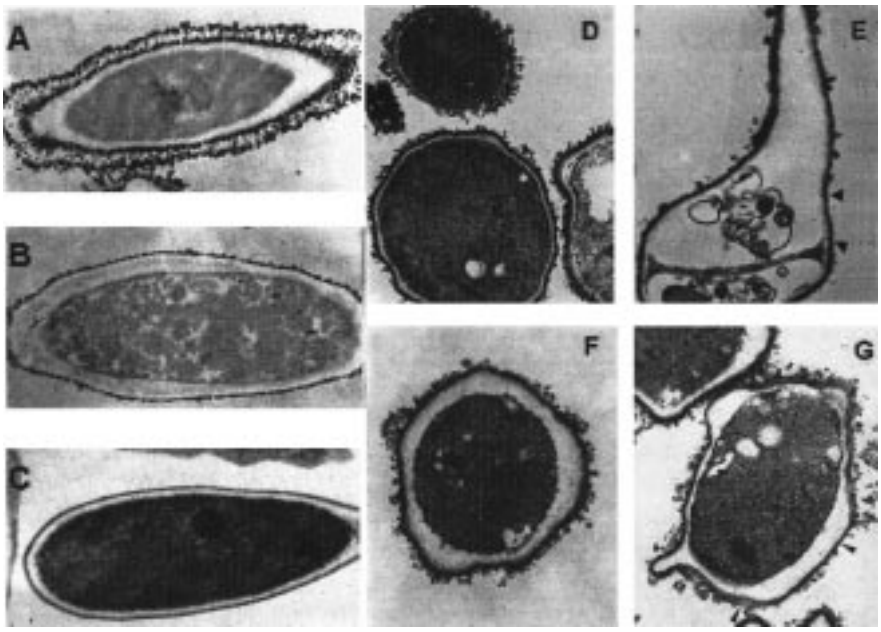

Figure 1. Staining with colloidal iron hydroxide of anionogenic groups at the cell surface of *S. schenckii* yeast forms and *F. pedrosoi* hyphae and conidia. **A,** Bilayer reactivity in untreated yeasts; **B and C,** partial and complete removal of anionic groups reacting with CIH after sialidase treatment, magnification × 16000; **D and E,** CIH staining of anionic groups on *F. pedrosoi* hyphae before (**D**) and after (**E**) sialidase treatment. Virtually no particles were seen on several sites of treated cells (arrow heads); **F and G,** similar staining and enzymatic treatment of *F. pedrosoi* conidia with few or no particles seen on treated (**G**) cells (arrow heads); magnification × 25000. Figure 1 is from Alviano CS, et al. (1982) *FEMS Microbiol Lett* **15**:223–227 and Souza ET, et al. (1986) *J Med Vet Mycol* **24**:145–153. Reproduced with permission from *FEMS Microbiology Letters* and *Journal of Medical and Veterinary Mycology.*

leased surface antigen in the form of aggregated membranous bodies could interact with antibodies and other elements of the immune system leaving the infective fungus unchallenged to proliferate and invade tissues [10]. The same methodology was used to evaluate the relevance of sialic acids as contributors to the negative charge of *F. pedrosoi* [13], and similar results were obtained (Fig. 1, D–G). However, the partial removal by sialidase treatment of sialic acid residues from conidial forms of *F. pedrosoi* suggests that some structures are inaccessible to the enzyme or are poor substrates for it.

Anionogenic groups in *P. brasiliensis* and *F. pedrosoi* impart a highly negative charge of about - 0,98 and - 1.17 $\mu m\ s^{-1}.V^{-1}$ cm respectively, as evaluated by cell electrophoresis [13,14] (Table 2). The enzymatic removal of sialic acid residues in *P. brasiliensis* was more effective when the fungus was treated with sialidase from *Vibrio cholerae* than with that of *Clostridium perfringens,* suggesting the occurrence of different sialylated structures or a different sterical conformation of the sialoglycoconjugates [15].

C. neoformans is a fungal pathogen that has a capsular polysaccharide, mainly composed of mannose, xylose and glucuronic acid. This fungus expresses a highly negative surface charge [30], which is mainly attributed to the uronic acid residues present at the capsular polysaccharide. Sialic

Table 2. Electrophoretic mobility (EPM) of *F. pedrosoi* and *P. brasiliensis* before and after treatment with sialidase, showing the contribution of sialic acids to the negative surface charge of these human pathogenic fungi.

Fungal species	*Sialidase treatment*	*EPM ± SD* ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)[a]	*Reduction in EPM (%)*
F. pedrosoi (conidial cells)	-	1.17 ± 0.06	—
	+[b]	0.75 + 0.01	35.9
P. brasiliensis (yeast cells)	-	0.98 ± 0.06	—
	+[b]	0.68 ± 0.02	30.5
	+[c]	0.53 ± 0.01	54.2

[a]Electrophoretic mobility is determined using the following equation: EPM = (*d*/*t*) × (*D*/*V*), where *d* is the distance (in μm) covered by cells during measurement (usually 16 μm); *t* is the time (in seconds) required by a cell to cover the distance *d; D* is the distance between the two electrodes (18 cm); and *V* is the potential applied to the electrodes.
[b]Treatment with 0.4 units/ml of sialidase from *Clostridium perfringens.*
[c]Treatment with 0.4 units/ml of sialidase from *Vibrio cholerae.*

acids, however, are also important contributors to the cryptococcal negative charge, since sialidase treatment of yeast cells results in reduced adhesion of *C. neoformans* to a poly-L-lysine coated surface [12].

Finally, Jones and co-workers [18] have compared the surface charge of untreated and sialidase-treated *Candida albicans,* by using the fluorescent probe 9-aminoacridine as an indicator of the electrostatic properties of the cell surface. They showed that sialidase-treated fungal cells possess a smaller electronegative surface potential, which indicates that sialic acids are components of the surface negative charge of this fungus.

Expression of sialic acids at the fungal cell surface

The ability of animal, plant, microbial, and fungal lectins to bind to specific carbohydrates has been used to determine the nature of the carbohydrate constituents expressed on a variety of microorganisms including protozoa and fungi [31–33]. Some sialic acid-binding lectins have been successfully used for in situ location of glycoconjugates. The most frequently used are the agglutinins from wheat germ (WGA), *Limax flavus* (LFA), *Limulus polyphemus* (LPA), *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA). With the latter two agglutinins, sialic acid α2,3- and α2,6-linkages can be distinguished [34]. Whereas some of these lectins recognize both Neu5Ac and Neu5Gc, others are specific for *N*-acetylneuraminic acid only. *O*-Acetylation may influence lectin binding, either in a positive or negative way.

Lectin binding (e.g., LFA and LPA) to whole cells was used to show the surface expression of sialic acids in fungi, which was abrogated by sialidase treatment (Fig. 2). Such treatment also rendered yeast cells increasingly more reactive with peanut agglutinin (PNA), suggesting that the underlying carbohydrate structures are similar to its preferential epitope Galβ1→3)GalNAc. In *C. neoformans, P. brasiliensis,* and *C. albicans* sialic acids are predominantly linked forming terminal α2,6-sialyl-galactosyl structures with much fewer α2,3-sialyl-galactosyl sequences [12,14,18]. In fact, fungal cells of these species were strongly agglutinated by influenza A H3N2 virus strain M1/5 (specific for α2,6-linkages) and poorly by its respective receptor variant M1/5 HS8 (specific for α2,3-linkages), which was selected by growth in the presence of horse serum [35]. These findings were essentially confirmed by using SNA and MAA lectins (Table 3), which preferentially bind Neu5Ac-α2,6-Gal/GalNAc and Neu5Ac-α2,3-Gal structures, respectively. The α2,6-sialyl-galactose sequence was more abundant, possibly indicating that sialoglycoproteins could be the chief sialy-

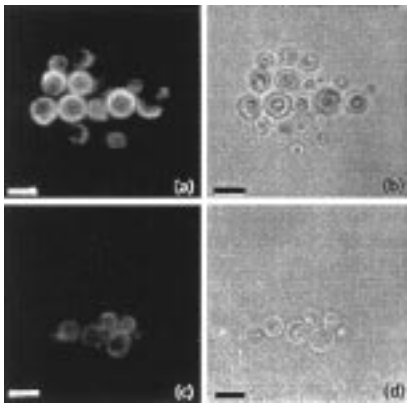

Figure 2. Labelling with FITC-LFA agglutinin of untreated (a) and sialidase-treated (c) *P. brasiliensis* yeast forms. Binding of lectin to the cells was much reduced by enzyme treatment (c). Corresponding fields observed by phase-contrast microscopy are shown in (b) and (d). Bars, 10 μm. Figure 2 is from Soares RMA, et al. (1998) *Microbiology* **144:** 309–14. Reproduced with permission from *Microbiology.*

Table 3. Percentages of fluorescent cells determined by flow cytofluorimetry of untreated and sialidase-treated yeasts of *P. brasiliensis* and *C. neoformans* incubated with FITC-labelled lectins.

			Fluorescent cells (means ± SD)				
Fungus	*Cell form*	*Sialidase treatment*	*LFA*	*LPA*	*PNA*	*SNA*[a]	*MAA*[a]
P. brasiliensis	*Yeast* (young cells)	−	4.4 ± 0.4	3.1 ± 0.3	0.5 ± 0.2	NE	NE
		+	3.5 ± 0.2	2.6 ± 0.3	1.7 ± 0.3	NE	NE
	Yeast (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
C. neoformans	Yeast	−	33.6 ± 5.7	ND[b]	ND	84.8 ± 3.9	13.1 ± 1.0
		+	15.1 ± 4.8	ND	ND	39.6 ± 6.1	11.1 ± 1.1

[a]In the SNA and MAA systems separation of young and mature cells of *P. brasiliensis* cells was not evident (NE).
[b]ND: not determined.

lated molecules. The α2,3-sialylgalactose linkage is more common in sialoglycolipids, which may be cryptic on the surface of adult cells [33,36].

Interestingly, in *C. neoformans,* the interaction with SNA was stronger than that with LFA [12]. This may reflect the presence of 9-*O*-acetyl groups in Neu5Ac, which preclude reactivity with LFA, but not with SNA [37]. The occurrence of $Neu5,9Ac_2$ in *C. neoformans* sialoglycoconjugates was further suggested by using influenza C virus which is a sensitive probe for 9-*O*-acetylated sialic acids [38–40]. Detectable agglutination of young, poorly encapsulated cryptococci mediated by influenza C virus showed that $Neu5,9Ac_2$ units are expressed at the surface of these cells (Fig. 3). This is the first report on the presence of $Neu5,9Ac_2$ in a pathogenic fungus. The 9-*O*-acetylated sialic acid derivative has also been demonstrated in the protozoan *Crithidia fasciculata* using gas chromatography-mass spectrometry [41]. In some infections, *O*-acetylated sialic acids can affect the immunogenicity and pathogenicity of the infectious agent [42], protect the host from attack by microbial agents [43], and also change some properties of sialic acid such as inhibition of the alternative pathway of complement [2,43,44].

To determine the expression of sialic acids in *C. neoformans* in relation to the capsule size, SNA binding to an encapsulated strain was compared with SNA binding to a poorly encapsulated form. The latter was markedly increased in comparison with the fully encapsulated strain as analyzed by flow cytometry (Fig. 4). These results suggested that the sialyl residues in *C. neoformans* are constituents of the cryptococcal cell wall and that capsule synthesis decreases their accessibility to external ligands [12]. The nature of the sialic acid-carrying component of the *C. neoformans* cell wall is still undefined.

In *P. brasiliensis,* it was observed that mature budding cells contained more sialic acid residues than young yeasts, as shown by specific lectin binding. Such difference in the expression of surface sialoglycoconjugates may be correlated with the growth phase of this fungus. Noteworthy was the marked enrichment, as in erythrocytes [24], of terminal β-galactosyl residues in aged yeast cells without budding, which, if also occurring in vivo, could favor their phagocytosis.

Our recent results [18a] on the presence of sialic acids in *C. albicans* agree with Alaei *et al.* [16], who showed that sialic acids are substituents of the surface-located iC3b glycoprotein receptor of *C. albicans,* and with Jones and coworkers [18], who demonstrated that sialic acid contributes to the surface negative charge of this fungus.

Isolation and characterization of sialic acids

The choice of the most suitable procedure for the isolation, purification and characterization of sialic acids is of crucial importance. This is particularly difficult when small amounts of sialic acid are present among other monosaccharides, es-

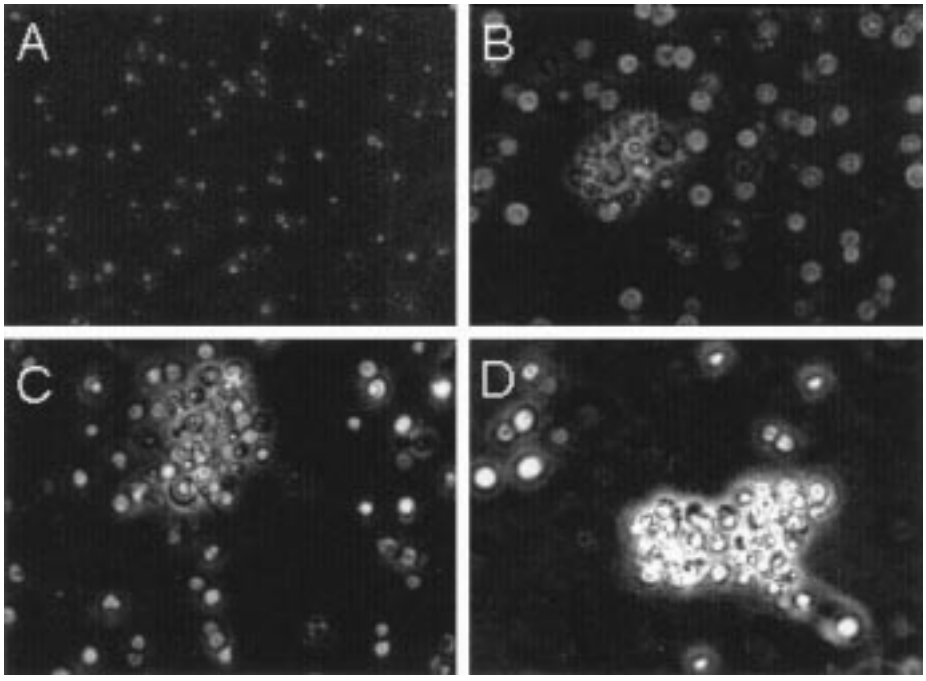

Figure 3. Phase-contrast micrographs of *C. neoformans* yeast cells agglutinated with influenza viruses (magnification, × 200 in A and × 400 in B, C, and D). **A,** Control; **B,** *C. neoformans* incubated with influenza virus A M 1/5 HS8 (specific for α2,3-sialyl-galactosyl sequences); **C,** Agglutination of yeast cells with influenza virus A M1/5 (specific for α2,6-sialyl-galactosyl sequences); **D,** Agglutination after incubation with influenza C virus (specific for $Neu5,9Ac_2$).

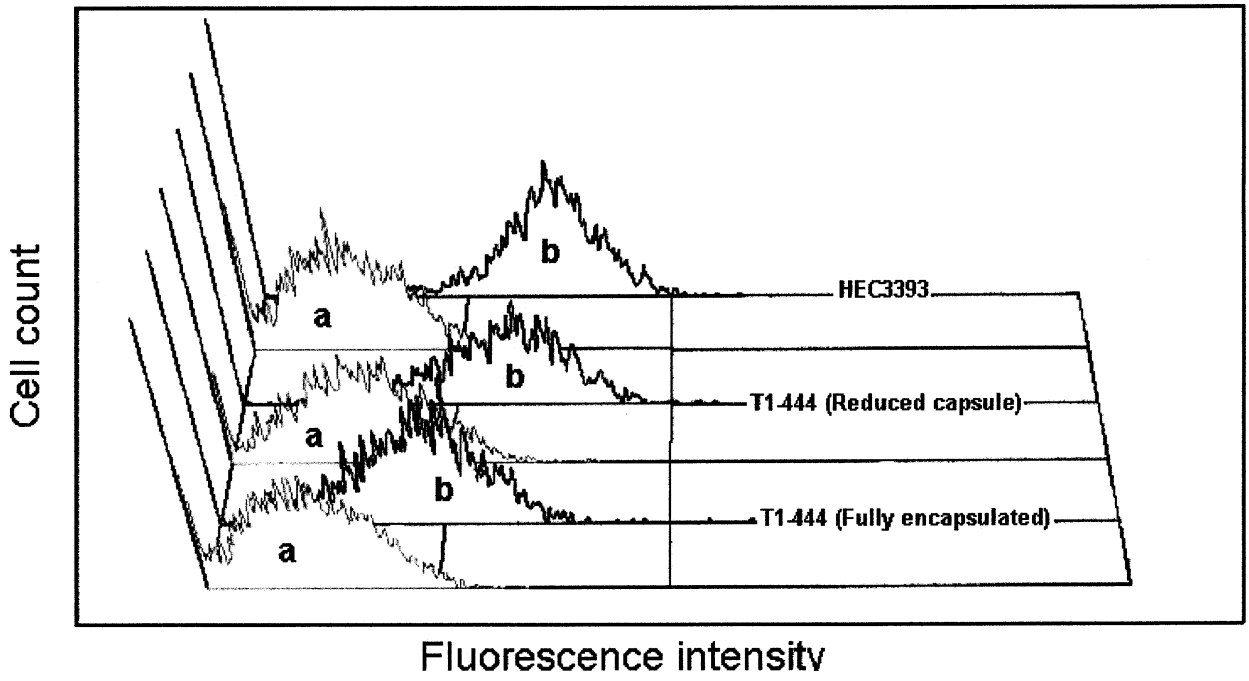

Figure 4. Flow cytometric analysis of binding of FITC-SNA to *C. neoformans* T_1-444 strain, T_1-444 with salt (CDM-NaCl)-induced capsule reduction, and HEC3393 strain, a sparsely encapsulated form. Analysis of 5,000 yeast cells is shown; (a) unstained cells and (b) cells incubated with FITC-lectin. This figure has been modified from Rodrigues, et al. *Infect Immun* **65:** 4937–42.

pecially pentoses, hexoses and uronic acids, which may interfere in the assays [1,2,45,46].

Most of the methods used for characterization of the various types of sialic acids require that these residues be previously released from their α-glycosidic bond. When mild acid hydrolysis and heat are used for this purpose, significant destruction of the *O*-substituents will occur even under the mildest possible conditions. Also, *O*-substitution renders the molecule relatively more resistant to acid hydrolysis than the nonsubstituted molecules [45,46].

In spite of this, the recommended method to release terminal sialic acids in high yield is the use of mild acid, which also liberates the majority of the *O*-acylated sialic acids. Based on this, acid hydrolysis for the extraction of sialic acids from fungi is performed as follows: fungal cells are suspended in water and the pH is adjusted to 2 with 50% (v/v) formic acid and the suspension incubated for 1 h at 70 °C [46]. The mixture is cooled in ice and then centrifuged. The supernatant containing released sialic acids is removed and the cell pellet suspended in water. The pH is adjusted to 1 with 3 M hydrochloric acid and the suspension incubated for 1 h at 80 °C. Under these conditions, which were used in other systems [46], de-*O*-acylation occurs to an extent of 30–50%. This fact observed with many glycoconjugates can be used for estimation of the amount of *O*-acetylated sialic acid originally present in the sample. It has to be noted that other acids, such as acetic acid or propionic acid, as summarized in Refs. 2, 6, 44 can also successfully be used for this purpose.

Enzymatic hydrolysis of sialoglycoconjugates is also not advantageous for quantitative sialic acid determination in many cases. Often lower amounts of sialic acids, especially from cell surfaces, are released rather by sialidases than by acids, which may be due to stereochemical hindrances and to the enzyme specificity. *N*-Glycolyl sialic acids are relatively poor substrates for several sialidases as demonstrated by comparing the enzymatic hydrolysis of e.g. Neu5Gc-α2,3-lactose and Neu5Ac-α2,3-lactose [1,2]. De-*O*-acetylated (alkali-treated) bovine submandibular gland mucin (BSM) is hydrolyzed faster than native BSM, indicating that *O*-acetylation of sialic acid strongly reduces the cleavage rate. The behaviour of *Trypanosoma brucei* sialidase and trans-sialidase towards 9-*O*-acetylated Neu5Ac of Neu5,9Ac_2-α2,3-lactose confirms this relationship and shows that both *O*-acetylation of the sialic acid side chain and *N*-glycolylation have about the same effect on enzyme activity [47]. The reactivity of *T. brucei* sialidase towards 4- and 9-*O*-acetylated sialic acids is in agreement with all sialidases studied so far. Due to these apparent sterical hindrances, the precise measurement of the degree and type of *O*-substitution of sialic acids in biological specimens is not possible [46].

Fungal sialic acid-containing hydrolyzates are ultracentrifuged (50,000 × *g*/30 min/4 °C) and the supernatants extracted twice with two volumes of diethyl ether [46]. The

water phase is concentrated in vacuum. The dry residue is suspended in 1 ml of water and separated from macromolecules in a Centricon 3 micropartition system (Amicon). The filtrate is then passed through a 2 ml-cation exchange column (Dowex 50WX8, 100 to 200 mesh, hydrogen form). After applying the sample, the resin is washed with 6 ml of water and the eluate collected together with the washings and lyophilized. After passage through the cation-exchange resin, the pool of sialic acids is adsorbed to an anion exchange column (Dowex 3X4A, 100 to 200 mesh, formate form). Elution from this resin is generally carried out with formic acid. The ion exchange chromatography should be carried out rapidly, because prolonged contact of *N,O*-acylneuraminic acids with the resin may result in degradation, *O*-acetyl-migration and/or de-*O*-acetylation. After evaporation in vacuum or lyophilization, the dry sample is dissolved in 0.3 ml of water and finally purified by gel filtration in a BioGel P-2 column (100 × 0.5 cm, minus 400 mesh). Elution is performed with 0.01 M acetic acid, keeping the flow rate at 1 ml/h. Fractions of 0.4 ml are collected and the presence of sialic acid is monitored by high performance thin-layer chromatography (HPTLC) on cellulose, with 1-propanol - 1-butanol - 0.1 M HCl (2:1:1, v/v/v) as solvent system [46], or silica plates, with n-propanol - 1M ammonia -water (6:2:1, v/v/v) as running solvent system [12]. HPTLC is one of the simplest and most reproducible method for the qualitative determination of sialic acids, being less affected by interfering substances when compared with other methods. This technique has been used for sialic acid analysis in *S. schenckii, C. neoformans, F. pedrosoi* and *P. brasiliensis* [9,12–14].

Quantitative determination of sialic acids in the samples from the purification procedure is carried out colorimetrically by the thiobarbituric acid method, according to Warren [48]. This method is negative for D-glucuronic acid (or D-glucuronolactone), an acidic sugar present at the cell surface of *C. neoformans* and *S. schenckii.* Using this assay, a density of 3×10^6 sialic acid residues per cell was found in *C. neoformans* and *P. brasiliensis,* a value similar to that found in human erythrocytes [28]. For comparison, *F. pedrosoi* contains 7.7×10^6 residues [13], whereas *S. schenckii* and *C. albicans* contain 2.0×10^6 residues [9, 18a]. The differential expression of sialic acid residues in dimorphic fungi may be related to the morphogenesis or may reflect a response to different growing conditions.

Volatile sialic acid derivatives are generated using mild derivatization procedures, such as esterification with diazomethane followed by trimethylsilylation or pertrimethylsilylation [46]. Subsequent gas-liquid chromatography (GLC) coupled with electron impact (El) mass spectrometry (MS), is the method of choice for the unequivocal identification of the presence and kind of sialic acids. In fungi, this method was used for identification of the neuraminic acid components of *P. brasiliensis* [14] and *C. albicans* [18a].

A combination of the procedures reviewed above allowed the identification of sialic acids in several pathogenic fungi (see also Table 1). In *F. pedrosoi,* Neu5Ac predominated in the mycelium, whereas the same monosaccharide was mainly found in conidia [13]. In *S. schenckii,* Neu5Gc was the main derivative encountered, which could be associated with the polar glycolipid fraction of yeast forms [9]. Neu5Ac was the only type of sialic acid characterized chemically and spectroscopically in the yeast forms of *P. brasiliensis* [14] and *C. albicans* [18a]. In *C. neoformans,* only Neu5Ac was detected by HPTLC [12], since the purification steps used for identification may have resulted in a partial loss of the labile *O*-acetyl groups, thus rendering difficult the detection of the small amounts of 9-*O*-acetylated sialic acid derivatives, which were identified by interaction with influenza C virus.

Biosynthesis of sialoglycoconjugates in fungal cells

The biosynthesis of glycoproteins in fungi, especially in yeast cells, has been studied in several species, including *Saccharomyces cerevisae, Pichia pastoris, Schizosaccharomyces pombe* and *Candida albicans.* Yeasts secrete and process glycoproteins in much the same way as mammalian cells [49]. They add *N*- and *O*-linked glycans to their glycoproteins, and much of the early processing of *N*-glycans is conserved in yeasts and mammals. Proteins when signaled to enter the ER, are glycosylated and transferred to the Golgi for further processing, then addressed to various organelles, to the plasma membrane and cell wall, or are secreted in the periplasm [50]. The addition of sialic acid residues to fungal glycoproteins is a completely unknown process. Some authors believed that yeast cells do not add peripheral sialic acids to their glycans [50], rather using phosphate, glucuronic acid [51] or pyruvate [52] to impart negative charges to their glycoproteins. However, the detection of sialic acids in *P. brasiliensis, C. neoformans* and *F. pedrosoi* grown in a chemically defined medium, free of sialic acid-containing substances, indicates that these acidic units are synthesized *de novo* and are transferred to terminal β-galactosyl residues probably by regular CMP-sialic acid-dependent sialyltransferases. Moreover, species such as *C. neoformans, C. albicans* and *S. schenkii,* also produced sialoglycoconjugates when grown in different complex media [9,11,16–18], which may suggest that sialic acid expression does not depend on the medium composition. No indication has been obtained so far for the presence of trans-sialidases in fungi as described in trypanosomal parasites [53]. Further studies on the biosynthesis of sialoglycoconjugates in fungal cells, including purification and cloning of glycosyltransferases, are needed to clarify this process.

Biological role of sialic acids

General functions of sialic acid residues at the cell surface are attributed to their physico-chemical properties such as

electronegativity, hydrophilicity and the relatively large size of the hydrated molecules. Their exposure on cells and their presence on secreted sialoglycolipids and sialoglycoproteins including mucins, determine and modulate cell interactions and carbohydrate-dependent physiological or pathophysiological responses. They can either mask recognition sites or serve as recognition determinants [1–3,6,28].

Surface molecules of fungal cells seemingly play an important role in their *in vivo* reactivity with the immune system, the protection against phagocytosis being one of the resistance mechanisms involved. Neutral and acidic heteropolysaccharides usually linked to proteins are thought to be the chief surface reactive components in fungi with less evidence for the role played by other fungal constituents, whose expression may vary with the growth stage and cell differentiation. Little information is thus available regarding the biological role of sialic acids as cell surface components in certain species. There is, however, some evidence supporting the hypothesis that sialic acids may contribute to the pathogenicity of fungi and that Neu5Ac is an effective inhibitor of phagocytosis [10,12].

Peritoneal macrophages and hepatocytes have receptors that recognize terminal β-galactose or *N*-acetylgalactosamine units in soluble or cell surface glycoproteins. Terminal β-galactosylated molecules or cells can thus be recognized by macrophages, with subsequent endocytosis. Sialic acids which are mostly linked to galactose, if not to other sialic acid units, prevent binding and degradation by phagocytic cells of a number of glycoconjugates as well as whole cells [2,3,6,28]. Removal of these acidic sugars, e.g. by sialidases or aging, uncovers the β-galactosyl residues which can now bind to the corresponding mammalian lectins [54,55].

Sialic acid residues expressed at the cell surface of *S. schenckii* and *C. neoformans* protect unopsonized fungal cells from phagocytosis by resident mouse peritoneal macrophages [10,12] (Table 4). Enzymatic removal of sialic acids from the external layers of *C. neoformans* and *S. schenckii* envelopes renders yeast cells more susceptible to phagocytosis. For sialidase-treated *S. schenckii* and *C. neoformans,* the phagocytic index was 7.7- and 2.0-fold that of untreated cells. Sialic acid removal generated newly exposed galactosyl units, which could mediate interactions of fungi with macrophages.

Table 4. Effect of sialidase treatment of *S. schenkii* and *C. neoformans* yeast cells on their ingestion by mouse peritoneal macrophages.

Fungal species	*Sialidase treatment*	*Enhancement of phagocytic index*
S. schenkii (yeast cells)	−	−
	+	7.7-fold
C. neoformans (yeast cells)	−	−
	+	2.0-fold

In the case of *C. neoformans,* the presence of surface sialic acids can be associated with pathogenesis. Due to size restrictions, poorly encapsulated *C. neoformans* yeast cells, and more likely basidiospores, are candidates for initiating an infection in the lung [56]. Once the infection is established, a capsule is synthesized which has anti-phagocytic properties. The increased expression of Neu5Ac and its 9-*O*-acetylated derivative at the cryptoccal cell wall of poorly encapsulated yeast forms could, at the initial stage of infection, play a role in protecting the fungus against phagocytosis by alveolar macrophages, until the full expression of capsular polysaccharides, the major virulence factor of *C. neoformans,* is accomplished.

In *F. pedrosoi,* a structural function was attributed to sialic acids. Marked alterations in the morphology of *F. pedrosoi* hyphae incubated in acid pH after sialidase treatment paralleled the increased deformability and decreased rigidity of certain mammalian cells after sialic acid loss or removal [1]. In fact, sialic acid units may increase the intrinsic viscosity of several glycoproteins [57] and this property presumably influences the structural role of these macromolecules.

Microbial pathogens such as viruses, mycoplasma, bacteria and protozoa use sialic acids to adhere to host cells. Very little is known about the role of fungal sialic acids in cell adherence. A hyphal 60-kilodalton mannoprotein from *C. albicans* reacted with WGA and sialidase treatment reduced its reactivity with a purified antibody to *Candida* CR2 [17]. Coincidently, this glycoprotein was associated with C3d, fibrinogen, and laminin binding [58,59] and appeared to promote attachment of germ tubes to plastic [60]. Complement receptors of *C. albicans* could then be adhesins for attachment to host cells and extracellular matrix constituents.

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References

1 Schauer R (1982) Chemistry, metabolism and biological functions of sialic acids. *Adv Carbohydr Chem Biochem* **40:** 131–234.

2 Schauer R, Kamerling JP (1997) Chemistry, biochemistry and biology of sialic acids. In *Glycoproteins II* (Montreuil J, Vliegenthart JFG, Schachter H, eds) pp 243–402. Amsterdam: Elsevier.

3 Kelm S, Schauer R (1997) Sialic acids in molecular and cellular interactions. *Int Rev Cytol* **175:** 137–240.

4 Harvey BE, Toth CA, Wagner HE, Stule GD Jr, Thomas P (1992) Sialyltransferase activity and hepatic tumor growth in a nude mouse model of colorectal cancer metastases. *Cancer Res* **52:** 1775–79.

5 Miyagi T, Sato K, Hata K, Tamiguchi S (1994) Metastatic potential of transformed rat 3Y1 cell lines is inversely correlated with lysosomal-type sialidase activity. *FEBS Lett* **349:** 255–59.

6 Varki A (1992) Diversity in the sialic acids. *Glycobiology* **2:** 25–40.

7 Corfield AP, Schauer R (1982) Occurrence of sialic acids. In *Sialic Acids—Chemistry, Metabolism and Function* (Schauer R, ed) pp 5–50. Wien: Springer.

8 Krauβ JH, Reuter G, Schauer R, Weckesser J, Mayer H (1988) Sialic acid-containing lipopolysaccharides of purple nonsulfur bacteria. *Arch Microbiol* **150:** 584–89.

9 Alviano CS, Pereira MEA, Souza W, Oda LM, Travassos LR (1982) Sialic acids are surface components of *Sporothrix schenckii* yeast forms. *FEMS Microbiol Lett* **15:** 223–27.

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

11 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:** 143–49.

12 Rodrigues ML, Rozental S, Coceiro JNSS, Angluster J, Alviano CS, Travassos LR (1997)Indentification of *N*-acetylneuraminic acid and its 9-*O*-acetylated derivative on the cell surface of *Cryptococcus neoformans*: Influence on fungal phagocytosis. *Infect Immun* **65:** 4937–42.

13 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:** 145–53.

14 Soares RMA, Alviano CS, Angluster J, Travassos LR (1993) Identification of sialic acids on the cell surface of hyphae and yeast forms of the human pathogen *Paracoccidioides brasiliensis. FEMS Microbiol Lett* **108:** 31–4.

15 Soares RMA, Silva-Filho FC, 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:** 309–14.

16 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:** 1395–99.

17 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:** 4675–81.

18 Jones L, Hobden C, O'Shea P (1995) Use of a real-time fluorescent probe to study the electrostatic properties of the cell surface of *Candida albicans. Mycol Res* **99:** 969–76.

18a Soares RMA, Soares RMA, Alviano DS, Angluster J, Alviano CS, Travassos LR (2000) Identification of sialic acids on the cell surface of *Candida albicans. Biochim Biophys Acta,* in press.

19 Benhamou N, Ouellette GB (1987) Ultrastructural characterization of an extracellular fibrillar sheath on cells of *Ascocalyx abietina,* the scleroderris canker agent of conifers. *Can J Bot* **65:** 154–67.

20 De Stefano JA, Cushion MT, Puvanesarajah V, Walzer PD (1990) Analysis of *Pneumocystis carinii* cyst wall. II. Sugar composition. *J Protozool* **37:** 436–41.

21 De Stefano JA, Trinkle LS, Walzer PD, Cushion MT (1992) Flow cytometric analyses of lectin binding to Pneumocystis carinii surface carbohydrates. *J Parasitol* **78:** 271–80.

22 Gasic GJ, Berwicz L, Sorrentino M (1968) Positive and negative colloidal iron as cell surface electron stains. *Lab Invest* **18:** 63–71.

23 Burry RW, Wood JG (1979) Contributions of lipids and proteins to the surface charge of membranes. An electron microscopy study with cationized and anionized ferritin. *J Cell Biol* **82:** 726–41.

24 Eylar EH, Maddoff MA, Brody OV, Oncley JL (1962) The contribution of sialic acid to the surface charge of the erythrocyte. *J Biol Chem* **237:** 1992–200.

25 Furchgott RF, Ponder E (1941) Electrophoretic studies on human red blood cells. *J Gen Physiol* **24:** 447–57.

26 Matta MAV, Alviano CS, Angluster J, De Souza W. Silva-Filho FC, Esteves MJG (1993) Surface charge and hydrophobicity of wild and mutant *Crithidia fasciculata. Cell Bioph* **20:** 69–79.

27 James AM (1979) Molecular aspects of biological surfaces. *Chem Soc Rev* **8:** 389–418.

28 Schauer R (1985) Sialic acids and their role as biological masks. *Trends Biochem Sci* **10:** 357–60.

29 Benchimol M, De Souza W, Travassos LR (1979) Distribution of anionic groups at the cell surface of different *Sporothrix schenckii* cell types. *Infect Immun* **24:** 912–19.

30 Nosanchuk JD, Casadevall A (1997) Cellular charge of *Cryptococcus neoformans:* contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. *Infect Immun* **65:** 1836–41.

31 Sharon N, Lis H (1990) Legume lectins—a large family of homologous proteins. *FASEB J* **4:** 3198–208.

32 Mandal C, Mandal C (1990) Sialic acid binding lectins. *Experientia* **46:** 433–41.

33 Goldstein IJ, Poretz RD (1986) In *The Lectins: Properties, Functions and Applications in Biology and Medicine* (Liener IE, Sharon N, Goldstein LI, eds) pp 35–244. New York: Academic Press Inc.

34 Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins. *J Biol Chem* **266:** 83–8.

35 Couceiro JN, 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:** 155–65.

36 Lampio A (1988) Exposure of major glycolipids in human P^k and p erythrocytes. *Glycoconj J* **5:** 513–20.

37 Varki A (1997) Sialic acids as ligands in recognition phenomena. *FASEB J.* **4:** 248–55.

38 Muchmore EA, Varki A (1987) Selective inactivation of influenza C esterase: a probe for detecting 9-*O*-acetylated sialic acids. *Science* **236:** 1293–95.

39 Rogers GN, Herrler G, Paulson JC, Klenk HD (1986) Influenza C virus uses 9-*O*-acetyl-*N*-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *J Biol Chem* **261:** 5947–51.

40 Zimmer G, Reuter G, Schauer R (1992) Use of influenza C virus

for detection of 9-*O*-acetylated sialic acids on immobilized glycoconjugates by esterase activity. *Eur J Biochem* **204:** 209–15.
41 Matta MAV, Aleksitch V, Angluster J, Alviano CS, De Souza W, Andrade AFB, Esteves MJG (1995) Occurrence of *N*-acetyl- and *N*-*O*-diacetyl-neuraminic acid derivatives in wild and mutant *Crithidia fasciculata. Parasitol Res* **81:** 426–33.
42 Ørskov F, Ørskov I, Sutton A, Schenckman R, Lin W, Egan W, Hoff GE, Robbins JB (1979) Form variation in *Escherichia coli* K1 determined by *O*-acetylation of the capsular polysaccharide. *J Exp Med* **152:** 1375–92.
43 Varki A, Kornfeld S (1980) An autosomal dominant gene regulates the extent of 9-*O*-acetylation of murine erythrocyte sialic acids. A probable explanation for the variation in capacity to activate the human alternate complement pathway. *J Exp Med* **152:** 532–44.
44 Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L (1995) Chemistry and biology of sialic acids. In *Biology of the Sialic Acids* (Rosenberg A, ed) pp 7–67. New York: Plenum Press.
45 Varki A, Diaz S (1984) The release and purification of sialic acids from glycoconjugates: methods to minimize the loss and migration of *O*-acetyl groups. *Anal Biochem* **137:** 236–47.
46 Reuter G, Schauer R (1994) Determination of sialic acids. *Methods Enzymol* **130:** 168–99.
47 Engstler M, Reuter G, Schauer R (1992) Purification and characterization of a novel sialidase in procyclic culture forms of *Trypanosoma brucei. Mol Biochem Parasitol* **54:** 21–30.
48 Warren, L (1959) The thiobarbituric acid assay of sialic acids. *J Biol Chem* **234:** 1971–75.
49 Kornfeld R, Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem.* **54:** 631–64.
50 Gemmill R, Trimble, RB (1999) Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. *Biochim Biophys Acta* **1426:** 227–37.
51 Fukazawa Y, Kagaya K, Shinoda T (1995) Cell wall polysaccharides of pathogenic yeasts. *Curr Top Med Mycol* **6:** 189–219.
52 Gemmill TR, Trimble RB (1996) *Schizosaccharomyces pombe* produces novel pyruvate-containing *N*-linked oligosaccharides. *J Biol Chem* **271:** 25945–49.
53 Schenkman S, Jiang MS, Hart GW, Nussenzweig V (1991) A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell* **65:** 1117–25.
54 Lee H, Kelm S, Michalski J-C, Schauer R (1990) Influence of sialic acids on the galactose-recognizing receptor of rat peritoneal macrophages. *Biol Chem Hoppe-Seyler* **371:** 307–16.
55 Bratosin D, Mazurier J, Debray H, Lecocq M, Boilly B, Alonso C, Moisei M, Motas C, Montreuil J (1995) Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins. Evidence that sialic acids control their life span. *Glycoconjugate J* **12:** 258–67.
56 Wickes BL, Mayorga ME, Edman U, Edman JC (1996) Dimorphism and haploid fruiting in *Cryptococcus neoformans:* association with the α-mating type. *Proc Natl Acad Sci USA* **93:** 7327–31.
57 Ahmad F, McPhie P (1980) The intrinsic viscosity of glycoproteins. *Int J Biochem* **11:** 91–6.
58 Bouali A, Robert R, Tronchim G, Senet JM (1987) Characterization of binding of hyman fibrinogen to the surface of germtubes and mycelium of *Candida albicans. J Gen Microbiol* **133:** 545–51.
59 Bouchara JP, Tronchin G, Annaix V, Robert R, Senet JM (1990) Laminin receptors on *Candida albicans* germ tubes. *Infect Immun* **58:** 48–54.
60 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.

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