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Identification of *N*-Acetylneuraminic Acid and Its 9-O-Acetylated Derivative on the Cell Surface of *Cryptococcus neoformans*: Influence on Fungal Phagocytosis

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Sialic acids from sialoglycoconjugates present at the cell surface of *Cryptococcus neoformans* yeast forms were analyzed by high-performance thin-layer chromatography, binding of influenza A and C virus strains, enzymatic treatment, and flow cytofluorimetry with fluorescein isothiocyanate-labeled lectins. *C. neoformans* yeast forms grown in a chemically defined medium contain *N*-acetylneuraminic acid and its 9-O-acetylated derivative. A density of 3×10^6 residues of sialic acid per cell was found in *C. neoformans*. Sialic acids in cryptococcal cells are glycosidically linked to galactopyranosyl units as inferred from the increased reactivity of neuraminidase-treated yeasts with peanut agglutinin. *N*-Acetylneuraminic acids are α-2,6 and α-2,3 linked, as indicated by using virus strains M1/5 and M1/5 HS8, respectively, as agglutination probes. The α-2,6 linkage markedly predominated. These findings were essentially confirmed by the interaction of cryptococcal cells with the lectins *Sambucus nigra* agglutinin and *Maackia amurensis* agglutinin. We also investigated whether the sialyl residues present in *C. neoformans* are involved in the fungal interaction with a cationic solid-phase substrate and with mouse resident macrophages. Adhesion of yeast cells to poly-L-lysine was mediated, in part, by sialic acid residues, since the number of adherent cells was markedly reduced after treatment with bacterial neuraminidase. The enzymatic removal of sialic acids also made *C. neoformans* yeast cells more susceptible to endocytosis by macrophages. The results show that sialic acids are components of the cryptococcal cell surface that contribute to its negative charge and protect yeast forms against phagocytosis.

The pathogenic fungus *Cryptococcus neoformans* is the agent of cryptococcosis, an increasingly important disease since *C. neoformans* has a worldwide distribution and is a frequent opportunistic pathogen in AIDS patients (9).

The cell envelope of *C. neoformans* is composed of a rigid cell wall, basically containing glucans, and a capsular polysaccharide, the glucuronoxylomannan (GXM), with mannose, glucuronic acid, xylose, and *O*-acetyl groups (2, 5), and at least two minor components, galactoxylomannan and mannoprotein (MP) (6, 40). Several properties have been attributed to the capsular components of *C. neoformans*. GXM, the major capsular polysaccharide of *C. neoformans*, is anti-inflammatory, antiphagocytic, and immunosuppressive (reviewed in reference 21) and activates the alternative complement pathway, leading to opsonization of encapsulated yeast cells (3), whereas MP is the immunodominant antigen inducing a cell-mediated immunity (6, 24). In addition, it has recently been demonstrated that the MP-induced proliferation of human peripheral blood mononuclear cells can enhance human immunodeficiency virus (HIV) replication (29).

The infectious process by *C. neoformans* is initiated after inhalation of sparsely encapsulated yeast cells or basidiospores (18). In the alveolar space, inhaled fungal cells are phagocytosed by alveolar resident macrophages (21); thus, the primary interaction of fungal cells and phagocytes is an important step determining the course of infection. Presumably, surface components of fungal cells play an important role in their protection against phagocytosis, but the nature of these constituents is still unclear. From the primary site in the lung, *C. neoformans* can disseminate to other tissues, particularly the brain, causing lethal meningitis.

As present in many cell surface acidic glycoconjugates, sialic acids belong to a family of 9-carbon carboxylated sugars (34). The most frequent is *N*-acetylneuraminic acid (Neu5Ac). A number of sialic acids O acetylated at the 4-, 7-, 8-, and 9-hydroxyl positions have also been identified in biological systems (34, 42). In pathogenic fungi, a few reports suggest the occurrence of sialic acids in *C. neoformans*, *Sporothrix schenckii*, *Fonsecaea pedrosoi*, and *Paracoccidioides brasiliensis* (1, 13, 37, 38), but there are no previous reports on the presence of O-acetylated derivatives in these species.

In the present report, we describe the identification of sialic acids in yeast forms of *C. neoformans*. They are surface components which contribute to the negative charge of yeast cells and have a protective effect against endocytosis by mouse resident macrophages. These results raise the possibility that sialic acids are important antiphagocytic components of *C. neoformans* in the initial phase of infection, before full expression of the capsular polysaccharide takes place.

MATERIALS AND METHODS

Chemicals. Culture components and standards of sialic acids, peanut agglutinin (PNA), and *Vibrio cholerae* neuraminidase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sabouraud dextrose medium was from Difco Laboratories (Detroit, Mich.). All fluorescein isothiocyanate (FITC)-conjugated lectins were from EY Laboratories (San Mateo, Calif.). Centricon 3 was from

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Amicon, and the chromatographic apparatus used was obtained from Bio-Rad (Richmond, Calif.).

C. neoformans. Encapsulated *C. neoformans* var. *neoformans* T_1-444, serotype A, obtained from the Federal University of São Paulo, Brazil, was kindly provided by Olga Fisherman; strain HEC3393, a poorly encapsulated form (serotype A), was obtained from Laboratorio de Micologia Medica, Hospital Evandro Chagas, FIOCRUZ, Rio de Janeiro, Brazil. Both strains were isolated from humans with meningoencephalitis and AIDS. Stock cultures were maintained on Sabouraud dextrose agar under mineral oil and kept at 4°C.

C. neoformans cells were grown in a chemically defined medium (CDM) containing (grams per liter) glycerol (6.25), a purine-pyrimidine mixture (adenine, thymine, cytosine, guanine and hypoxanthine) (0.001), calcium pantothenate (0.002), cysteine (0.5), $(NH_4)_2SO_4$ (1.5), K_2HPO_4 (1.8), and $MgSO_4 \cdot 7H_2O$ (0.1), along with 0.05 mg each of biotin and thiamine · HCl per liter. Cells were cultivated at room temperature for 5 days, collected by centrifugation, and washed twice in 0.01 M phosphate-buffered saline (PBS; pH 7.2). The presence of the capsular polysaccharide was confirmed by mixing 10 μl of the cell suspension with 10 μl of India ink and observation in a light microscope. To reduce the capsule size, strain T_1-444 was cultivated in the same culture medium but in the absence of thiamine and with the addition of 2.9% NaCl (CDM-NaCl) (10). To measure capsular sizes, India ink preparations were observed in a Zeiss LSM 410 Invert confocal laser scanning microscope by using the LSM software. Data were analyzed by using the independent Student *t* test ($n = 30$), and the results are expressed as means ± standard deviations (SD). Experiments in this work were performed by using strain T_1-444 cultivated in CDM, except for flow cytofluorimetric analysis, in which strain HEC3393 and strain T_1-444 cultivated in CDM-NaCl were also used.

Determination of sialic acid. Extraction and purification of the sialic acids from *C. neoformans* were carried out as described by Reuter and Schauer (31). Yeast forms of *C. neoformans* (10^{10} cells) were suspended in 2 ml of water. The pH was adjusted to 2 with 50% (vol/vol) formic acid, and the suspension was incubated for 1 h at 70°C. After incubation, the mixture was cooled in ice and then centrifuged for 10 min at 900 × *g*. The supernatant containing released sialic acids was removed, and the cell pellet was suspended in about 2 ml of water. The pH was adjusted to 1 with 3 M hydrochloric acid. After incubation for 1 h at 80°C, the suspension was cooled and centrifuged as described before. The combined supernatants were then ultracentrifuged (Beckman L7 Ultracentrifuge, rotor type 65; 50,000 × *g* for 30 min at 4°C). The supernatant was collected and lyophilized, and the dry residue was suspended in 1 ml of water and separated from macromolecules in a Centricon 3 micropartition system from Amicon. The lower layer was passed through a 2-ml cation column of Dowex 50WX8 (100 to 200 mesh, hydrogen form). After the sample was applied, the resin was washed with 6 ml of water and the eluate was collected together with the washings and lyophilized. The dry sample was dissolved in 0.3 ml of water and finally purified by gel filtration in a BioGel P-2 chromatography column (100 by 0.5 cm, minus 400 mesh). Elution was done with 0.01 M acetic acid with a flow rate of 1 ml/h. Fractions of 0.4 ml were collected, and the presence of sialic acid was monitored by high-performance thin-layer chromatography (HPTLC) as described below. Samples containing sialic acids were finally quantified colorimetrically by the thiobarbituric acid method, as described by Warren (43).

Identification of sialic acids by HPTLC. Sialic acids were analyzed by HPTLC on silica plates with the solvent mixture *n*-propanol–1 M ammonia–water (6:2:1, vol/vol/vol). Spots were visualized by reaction with resorcinol-HCl. Standards of Neu5Ac and *N*-glycolylneuraminic acid were used at 1 mg/ml.

Enzyme treatment. Yeast cells (10^7) were washed twice in 0.01 M PBS (pH 7.2) and incubated for 2 h at 37°C in the presence of *V. cholerae* neuraminidase (0.2 U/ml), pH 6.0, in 2 mM $CaCl_2$. After incubation, the cells were washed in PBS and used for experiments. To determine the influence of neuraminidase treatment on fungal viability, 10^7 yeast cells treated with neuraminidase or not treated were suspended in 50 μl of PBS and mixed with the same volume of 0.04% trypan blue in PBS. This mixture was incubated for 5 min at room temperature, and the viability of the yeast cells was determined by calculating the percentage of unstained cells in a total of 300 cryptococcal yeast cells. No difference in cell viability was observed when neuraminidase-treated and untreated yeast cells were compared.

Adhesion to poly-L-lysine. Neuraminidase-treated and untreated yeast forms of *C. neoformans* were washed twice with 0.01 M PBS (pH 7.2), counted, and adjusted to 1.0×10^6/ml, and 100 μl was pipetted on poly-L-lysine (0.1%)-coated or uncoated glass slides placed inside wells of a 24-well plate. After 1 h of incubation, nonadherent yeast cells were discarded, the slides were washed twice with PBS, and then 2 ml of PBS containing 2.5% (vol/vol) glutaraldehyde was added. Adhesion was assessed microscopically by counting the number of adherent cells on five random fields (×400 magnification) in triplicate sets for each experimental condition. The percentage of adherent cells per well was determined and statistically analyzed by using Student's *t* test.

Lectin binding. Neuraminidase-treated and untreated yeast cells (10^7) were titrated with *Arachis hypogaea* lectin (PNA), which has an affinity for the disaccharide Galβ-1,3GalNAc, at an initial concentration of 250 μg/ml for 1 h at room temperature. Results were evaluated by visualizing agglutination in a light microscope. For fluorocytometric analysis, neuraminidase-treated and untreated cells were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. Fixed yeast cells were washed twice in PBS and incubated sequentially for 30 min in PBS containing 150 mM NH_4Cl and then in 1% bovine serum albumin in PBS for 1 h. Cells (10^6/ml) were rinsed in PBS and then incubated in the presence of the FITC-labeled lectin *Limax flavus* agglutinin (LFA), *Sambucus nigra* agglutinin (SNA), or *Maackia amurensis* agglutinin (MAA), which specifically recognize Neu5Ac, α-2,6-sialylgalactosyl, and α-2,3-sialylgalactosyl residues, respectively, at 40 μg/ml for 1 h at room temperature. After incubation, the cells were washed three times in PBS and used for analysis ($n = 5{,}000$) in an EPICS ELITE flow cytometer (Coulter Electronics, Hialeah, Fla.) equipped with a 15-mW argon laser emitting at 488 nm. The data obtained were run by using listmode, which makes further analysis possible. Control cells were first analyzed to determine their autofluorescence.

TABLE 1. Sizes of *C. neoformans* T_1-444 and HEC3393 capsules

Culture conditions	Capsule size (μm)[a]	
	T_1-444	HEC3393
CDM	3.93 ± 0.76	1.48 ± 0.33
CDM-NaCl	1.77 ± 0.27	ND

[a] Capsule size was defined as the distance from the cell wall to the outer capsular border. Data were analyzed by using the independent Student *t* test ($n = 30$). Results are expressed as means ± SD. A significant difference in capsular size ($P < 0.0001$) was observed for strain T_1-444 grown in different media. The capsular sizes of T_1-444 and HEC3393, both cultivated in CDM, were also significantly different ($P < 0.0001$). ND, not done.

Virus samples. Paired clonal strains M1/5 and M1/5 HS8 of influenza A virus strain Memphis/102/72 and a standard sample of influenza C virus strain Taylor/1233/47 with known affinity for α-2,6-sialylgalactosyl, α-2,3-sialylgalactosyl, and 9-*O*-acetyl-Neu5Ac (Neu5,9Ac_2), respectively (7, 33), 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 for use.

Virus binding. Agglutination of cryptococcal cells by the virus particles was carried out in glass tubes at 4°C for 1 h. To determine the *O*-acetylesterase activity of the influenza C virus, the agglutination assay was carried out at 37°C for 1 h. Equal volumes of a yeast suspension in PBS (pH 7.2) containing 10^7 cells/ml and the virus suspension were rapidly mixed. Cell agglutination was scored visually, after gentle resuspension of settled cells, and by observation in a phase-contrast microscope. The control was the supernatant fluid from uninfected, embryonated chicken eggs.

Macrophage-*C. neoformans* interaction. Peritoneal macrophages were collected from Swiss mice. Animals were killed, and their peritoneal cavities were washed with Hanks solution. Cells were plated and maintained in a humidified 5% CO_2 atmosphere at 37°C. After 30 min, nonadherent cells were removed and macrophages were cultivated for 24 h in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Yeast forms of *C. neoformans* cultivated for 5 days (neuraminidase-treated or untreated cells) were washed twice in 0.01 M PBS at pH 7.2 and then suspended in Hanks solution to a ratio of 10 *C. neoformans* cells per macrophage on monolayers of host cells. The yeasts were maintained in contact with the macrophages for periods varying from 15 min to 1 h, and the cultures were rinsed twice with culture medium, at room temperature, to remove nonadherent fungi. Macrophages were plated on glass coverslips placed inside wells of a 24-well tissue culture plate. After the interaction, the macrophages were fixed with Bouin's solution, removed, and stained with Giemsa. The percentage of infected macrophages was evaluated under an optical microscope. The number of intracellular fungi per infected macrophage was also determined. Intracellular yeast cells were inside vacuoles, whereas adherent yeast cells were at the cell surface with no evidence of a surrounding vacuolar membrane. The phagocytic index was calculated by multiplying the percentage of infected macrophages by the number of intracellular fungal cells per infected macrophage. The Student *t* test was used for statistical analysis of data.

RESULTS

Capsule size and growth conditions. The capsule sizes of the different strains of *C. neoformans* are listed in Table 1. In accordance with Nosanchuk and Casadevall (27), the capsule size is the distance from the cell wall to the outer capsular border. The capsular size of strain T_1-444 cultivated in CDM was 2.65 times that of strain HEC3393. Capsule production by strain T_1-444 could be significantly reduced ($P < 0.0001$) by

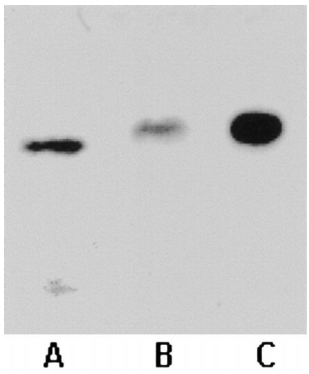

FIG. 1. HPTLC of sialic acids from *C. neoformans* T_1-444 yeast cells cultivated in CDM. (A and B) *N*-Glycolylneuraminic acid and Neu5Ac standards (Sigma Chemical Co.). (C) Sialic acid derivative from *C. neoformans*.

growing the yeast forms in a high-salt culture medium (CDM-NaCl). The CDM used, with glycerol replacing glucose or sucrose as the carbon source, was based on the medium of Butterfield and Jong (4), which was devised to enhance budding yeast-like single cells in *F. dermatitidis*, and was supplemented with growth-stimulating and dimorphism-regulating factors previously described in reports of studies with enteric yeasts and *Histoplasma capsulatum*, respectively (19, 39). The *C. neoformans* strains used in the present study grow rapidly in CDM, and the capsule size of strain T_1-444 after 5 days was as large as that of *C. neoformans* NIH 371 grown in a defined glucose-containing medium (27).

Determination of sialic acids. The purified acid hydrolysate of *C. neoformans* T_1-444 grown in CDM was analyzed by HPTLC, developed with *n*-propanol–1 M ammonia–water (6:2:1, vol/vol/vol). Only a blue spot with an R_f corresponding to that of Neu5Ac could be detected after reaction with the resorcinol-HCl reagent (Fig. 1). A density of 3×10^6 sialic acid residues per yeast cell was calculated by colorimetry (43). Similar results were obtained when *C. neoformans* was grown in brain heart infusion (data not shown).

Adhesion to poly-L-lysine. The influence of sialic acid on *C. neoformans* T_1-444 adhesion to the cationic substrate poly-L-lysine is shown in Fig. 2. Enzymatic removal of sialic acids from the fungus surface resulted in a decreased number of adherent yeast cells ($P < 0.01$) on the poly-L-lysine-coated glass slides. A high percentage (99.5%) of the cells of strain T_1-444 adhered to the cationic substrate, compared with 65.1% of neuraminidase-treated cells.

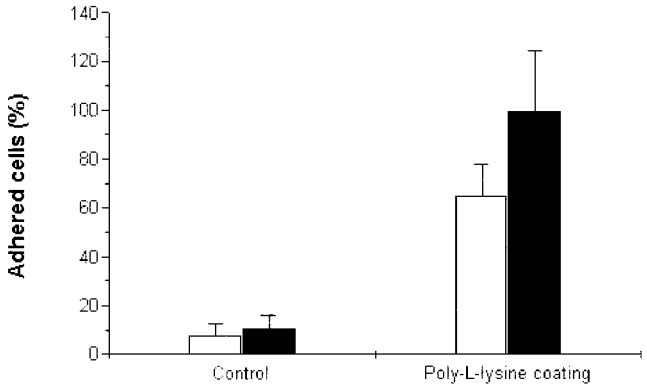

FIG. 2. Adhesion of neuraminidase-treated (white bars) or untreated (black bars) *C. neoformans* T_1-444 to uncoated (control) or poly-L-lysine-coated glass slides. Adhesion was assessed microscopically by counting the number of adherent cells on five random fields (×400 magnification) in triplicate sets for each experimental condition. The percentage of adherent cells per well was determined, and the Student *t* test was used for statistical analysis. Treatment with neuraminidase reduced cell adhesion to the cationic substrate ($P < 0.01$). Results of three experiments, expressed as means ± SD, are shown.

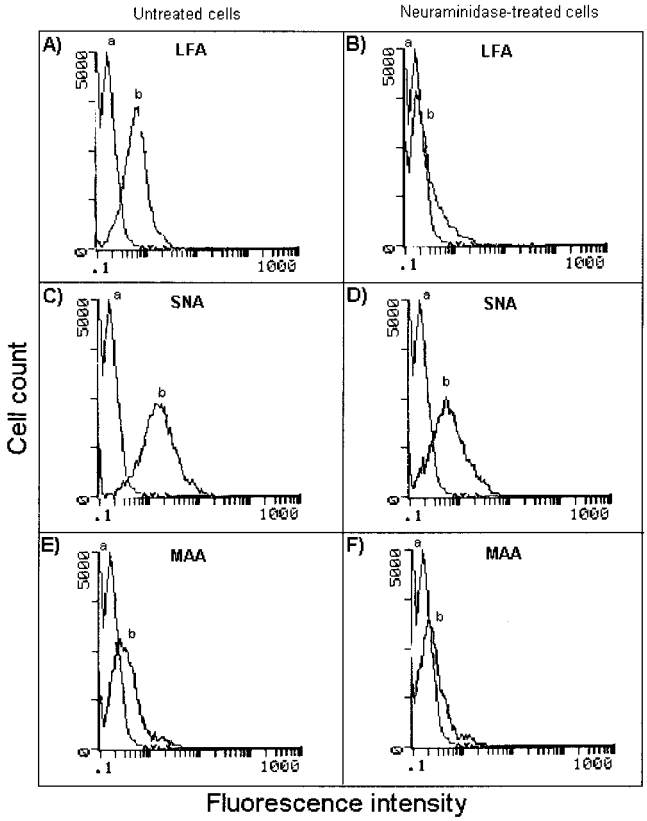

FIG. 3. Flow cytometric analysis of untreated (A, C, and E) and neuraminidase-treated (B, D, and F) *C. neoformans* T_1-444 yeast cells incubated with the lectins LFA, SNA, and MAA. (a) Control (autofluorescence inherent in unstained *C. neoformans* cells). (b) Incubation of untreated and neuraminidase-treated *C. neoformans* with FITC-labeled lectins. The greater reactivity with the lectin SNA suggests that sialyl residues are preferentially α-2,6 linked to galactose.

Lectin binding. Sialic acids in *C. neoformans* T_1-444 are glycosidically linked to β-galactosyl units, since cells are much more reactive with PNA after neuraminidase treatment, as assessed by the lower lectin concentration required for agglutination. Untreated yeast cells were agglutinated at a minimal PNA concentration of 250 μg/ml, whereas neuraminidase-treated cells required a minimum of 31.2 μg/ml for agglutination. Flow cytofluorimetric analysis with FITC-conjugated lectins reacting with neuraminidase-treated and untreated *C. neoformans* yeast forms is shown in Fig. 3. Detectable fluorescence was observed after incubation of the fungus with FITC-LFA (specific for Neu5Ac), confirming the surface expression of sialic acids. Neuraminidase treatment before interaction with FITC-LFA resulted in a decreased number of fluorescent cells. Binding of FITC-LFA was also abrogated by 100 mM Neu5Ac (data not shown). The nature of the sialylgalactosyl sequences was indicated by interaction of fungal cells with the FITC-MAA and FITC-SNA lectins, which recognize α-2,3- and α-2,6-sialylgalactosyl residues, respectively. Preferential binding was observed with the FITC-SNA lectin. The reaction between SNA and yeast cells was significantly reduced after

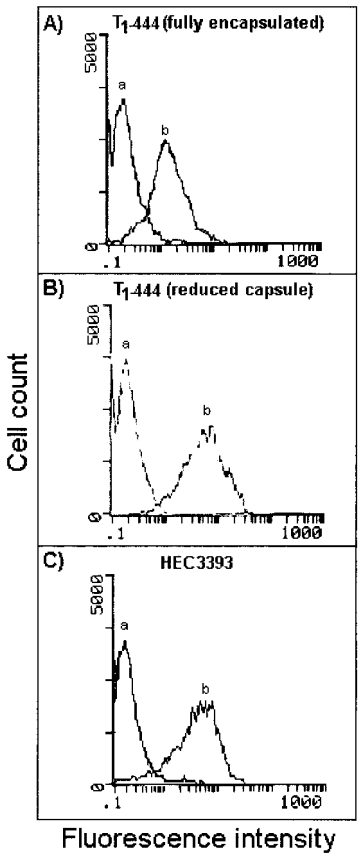

FIG. 4. Flow cytometric analysis of binding of FITC-SNA to *C. neoformans* T_1-444 (A), T_1-444 with salt (CDM-NaCl)-induced capsule reduction (B), and HEC3393, a sparsely encapsulated form (C). Analysis of 5,000 yeast cells is shown. (a) Unstained cells. (b) Cells incubated with FITC-lectin.

neuraminidase treatment. The determination of the sialic acid-reactive sites on *C. neoformans* was inferred from the binding of SNA, which showed the highest affinity for the yeast surface with poorly encapsulated cells (HEC3393) and with strain T_1-444 grown under special conditions for inhibition of capsule synthesis (Fig. 4 and Table 1). The reduction of capsular size in response to culture conditions (10) resulted in increased binding of FITC-SNA to *C. neoformans*. Binding of FITC-SNA to the surface of strain HEC3393 also led to strong staining of these cells.

Virus binding. Cells of yeast strain T_1-444 agglutinated in the presence of influenza C virus, which is specific for 9-*O*-acetyl-5-*N*-acetylneuraminic acid. They also interacted with influenza A virus strains M1/5 and M1/5 HS8, which are specific for α-2,6- and α-2,3-sialylgalactosyl sequences, respectively. Moreover, the agglutinating activity with the strain M1/5 virus was higher (Table 2), confirming data obtained by cytofluorimetry of the binding of FITC-MAA and FITC-SNA to cryptococcal cells in which the α-2,6-linked structure predominated. No agglutination of *C. neoformans* was observed when the fungal cells were incubated with allantoic fluids purified from uninfected, embryonated chicken eggs. Cell agglutination mediated by influenza C virus was inhibited when the cells were incubated at 37°C, due to its own *O*-acetyl esterase activity.

Interaction between mouse resident macrophages and fungal cells. The sialylated components of the *C. neoformans* cell surface had a protective effect against endocytosis by mouse resident macrophages. Treatment with bacterial neuraminidase made yeast cells approximately twofold more susceptible to phagocytosis ($P < 0.05$), as shown in Fig. 5.

TABLE 2. Agglutination of *C. neoformans* yeast cells with influenza virus[a]

Virus	Agglutination titer
Influenza C	1:30
Influenza A strain M1/5	1:150
Influenza A strain M1/5 HS8	1:24

[a] Influenza C virus is specific for $Neu5,9Ac_2$. Influenza A virus strain M1/5 is specific for α-2,6 sialylgalactosyl linkages. Influenza A virus strain M1/5 HS8 is specific for α-2,3-sialylgalactosyl sequences. Equal volumes of the yeast and virus suspensions were incubated at 4°C for 1 h. Cell agglutination was scored by observation in a phase-contrast microscope. Agglutination titers were determined by using serially diluted influenza virus suspensions and were identical in two independent experiments.

DISCUSSION

The occurrence of sialic acids in microorganisms and their biological roles have been suggested in several previous studies. Bacteria synthesize capsular polysaccharides (44) or lipopolysaccharides (22) containing sialic acid, which may help them to evade host defenses by inhibiting the direct activation of the alternative pathway of complement (11). Infectious protozoa, including *Plasmodium berghei* (36), *Trypanosoma cruzi* (35), and *Entamoeba invadens* (32), also possess sialic acids probably mediating cell-cell interaction and the cell invasion process. In fungi, Neu5Ac has been characterized in *P. brasiliensis* (37) and both *N*-acetyl and *N*-glycolyl derivatives were reported in *S. schenckii* and *F. pedrosoi* (1, 36). In *S. schenckii*, sialic acids protect yeast forms against phagocytosis (28), and in *F. pedrosoi*, neuraminic acid derivatives were associated with morphogenesis and cellular integrity (38).

Hamilton and coworkers have shown that sialic acid is a component of a *C. neoformans* glycoprotein produced as an exoantigen. Neuraminidase precluded its recognition by a monoclonal antibody (13). In the present work, we show that Neu5Ac and its 9-O-acetylated derivative ($Neu5,9Ac_2$) are found in acidic components of *C. neoformans* yeast forms. Neu5Ac could be detected by HPTLC, but this method and the purification steps used here for identification of neuraminic acid derivatives in *C. neoformans* usually result in partial loss of

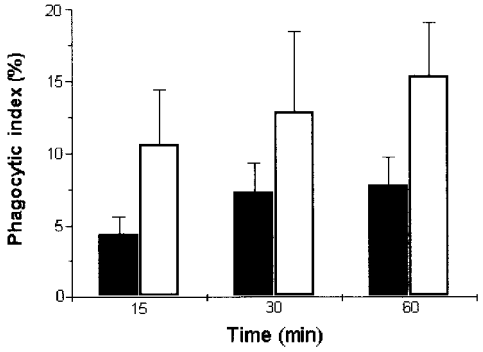

FIG. 5. Effect of neuraminidase treatment of *C. neoformans* T_1-444 yeast cells on their ingestion by mouse resident macrophages. Fungi, either untreated (black bars) or treated with neuraminidase (white bars) were incubated with macrophages at 37°C. The number of adherent or intracellular yeast cells in a total of 400 cells stained with Giemsa was determined. Treatment with neuraminidase rendered yeast cells approximately twofold more susceptible to endocytosis ($P < 0.05$). Results of three experiments are expressed as means ± SD.

the labile *O*-acetyl groups (31, 42). The presumably small amounts of 9-O-acetylated sialic acids in relation to total cellular sialic acids make difficult the detection of $Neu5,9Ac_2$ in sialoglycoconjugates (46). In the present work, we therefore used the interaction of influenza C virus with intact *Cryptococcus* yeast cells as a sensitive probe for 9-O-acetylated sialoglycoconjugates (23, 33, 46). The detectable agglutination of cryptococcal cells mediated by influenza C virus indicated the presence of $Neu5,9Ac_2$ and also showed that $Neu5,9Ac_2$ bearing sialoglycoconjugates are on the surface of the fungal cells. This is the first report on the presence of $Neu5,9Ac_2$ in a pathogenic fungus. The expression of the 9-O-acetylated sialic acid derivative in microorganisms has been demonstrated in the protozoan *Crithidia fasciculata* by gas chromatography-mass spectrometry (20). In some infections, O-acetylated sialic acids can affect the immunogenicity and pathogenicity of the infectious agent (30).

A density of 3×10^6 sialic acid residues per cell was found in *C. neoformans*. The periodic acid-thiobarbituric acid assay (43), used here for quantitative determination of sialic acids, has a 0.9% standard error for absorbance measurements. Considering that in this method the chromophore yield is drastically reduced in the presence of *O*-acetyl groups in the C-9 position (31) and that there is partial degradation of sialic acids in the purification steps, this number of sialic acid residues is probably underestimated. For comparison, *F. pedrosoi* contains 7.2×10^7 residues per cell (38), *S. schenckii* contains 2×10^6 (1), and *P. brasiliensis* contains 3×10^6 (37).

The surface expression of sialic acids in *C. neoformans* was confirmed by the binding of sialic acid-specific LFA to whole cryptococcal cells, which was abrogated by neuraminidase treatment. This treatment also made *C. neoformans* yeast cells markedly more reactive with PNA, suggesting that the sialic acid residues are glycosidically linked to a subterminal structure similar to Galβ(1→3)GalNac. The linkage involves mainly α-2,6-sialylgalactosyl sequences and very small amounts of α-2,3-sialylgalactosyl sequences. Indeed, cryptococcal cells were strongly agglutinated by virus strain M1/5 (specific for α-2,6 linkages) and poorly by strain M1/5 HS8 (specific for α-2,3 linkages). These findings were essentially confirmed by use of the SNA and MAA lectins. Interestingly, the fungal interaction with SNA was stronger than that with LFA. This may reflect the presence of 9-*O*-acetyl groups in Neu5Ac, which can block the interaction of Neu5Ac with LFA but not that with SNA (41).

To determine the expression of sialic acids at the *C. neoformans* cell surface in relation to capsule size, we compared SNA binding to encapsulated strain T_1-444 with SNA binding to strain HEC3393, a poorly encapsulated form. Binding of SNA to strain HEC3393 was markedly increased in comparison with SNA binding to strain T_1-444. The latter could, on the other hand, have its capsule production much reduced under certain culturing conditions (10). The reduction of capsular polysaccharide in this variant resulted in increased SNA binding to the fungal cell. These results strongly indicated that the sialyl residues in *C. neoformans* are constituents of the cryptococcal cell wall and that capsule synthesis decreases their accessibility to ligands such as SNA. The molecular organization of the cell wall of *C. neoformans* is poorly known, but composition analysis revealed that glucose (86%) and *N*-acetylglucosamine (7.3%) are the main sugar components (14). The nature of the sialic acid-carrying component of the *C. neoformans* cell wall is still undefined.

The present results also indicated that in *C. neoformans*, as in *S. schenckii* (28), sialic acids have a protective effect against phagocytosis. Removal of sialic acids from the external layers of *C. neoformans* by neuraminidase renders yeast cells markedly more susceptible to endocytosis. Sialic acid removal by neuraminidase generates new exposed galactosyl units, which could be involved in *C. neoformans* interaction with macrophages. In this regard, the inhibition by sialic acids of the interaction between galactose-recognizing receptors from rat peritoneal macrophages and galactose-exposing molecules has been shown (17).

The adhesion of yeast cells to the cationic substrate poly-L-lysine indicates that *C. neoformans* has a negative surface charge, which agrees with reports by Kozel et al. (15, 16) and Nosanchuk and Casadevall (27) regarding the negative cellular charge of cryptococcal cells, which is attributed mainly to the uronic residues of the polysaccharide capsule. We show here that the ionization of the carboxyl groups of sialic acids also contributes to the negative surface charge of *C. neoformans*, since treatment of cells with neuraminidase resulted in a significant decrease in the number of poly-L-lysine-adherent cells. Electrostatic forces are involved in cell adhesion (8) and may also be relevant in the interaction of phagocytic cells and microorganisms (25). Other human-pathogenic fungi, including *S. schenckii* (1) and *F. pedrosoi* (38), also have a negative surface charge, to which sialic acids are partial contributors.

Pulmonary cellular and exocellular elements play an important role in the initial defense against cryptococcal infection. Due to size restrictions, poorly encapsulated *C. neoformans* yeast cells, together with basidiospores, represent the most likely cryptococcal forms infecting the host (18, 45). These cells are those initially confronted by alveolar macrophages, since the encapsulated forms are detected at least 5 h after penetration of sparsely encapsulated yeast cells or basidiospores (12, 26). The increased expression of Neu5Ac and its 9-O-acetylated derivative could, at this stage, play an important role in protecting the fungus against phagocytosis by alveolar macrophages, until the expression of GXM, the major virulence factor of *C. neoformans*, is fully accomplished.

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