Differences in sialic acid density in pathogenic and non-pathogenic *Aspergillus* species

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***Aspergillus fumigatus* is a ubiquitous soil fungus that causes invasive lung disease in the immunocompromised host. The structure of the conidial wall has not been well characterized although it is thought that adhesins present on the surface are involved in attachment of the conidia to host lung cells and proteins, which is a prerequisite for the establishment of infection. Negatively charged carbohydrates on the conidial surface have been previously identified as the molecules responsible for attachment of conidia to extracellular matrix proteins. The aim of this research was to identify carbohydrates on the conidial surface that contribute to its negative charge. Direct chemical analysis and indirect binding assays have demonstrated that *A. fumigatus* possesses sialic acids on the conidial surface. Pre-treatment of *A. fumigatus* conidia with sialidase decreased binding of a sialic acid-specific lectin, *Limax flavus* agglutinin (LFA), to the conidial surface and decreased adhesion of conidia to the positively charged polymer poly L-lysine. Two other sialic acid-specific lectins, *Maackia amurensis* agglutinin and *Sambucus nigra* agglutinin, exhibited negligible binding to *A. fumigatus* conidia indicating that 2,3- α - and 2,6- α -linked sialic acids are not the major structures found on the conidial surface. Mild acid hydrolysis and purification of conidial wall carbohydrates yielded a product that had the same R_f as the Neu5Ac standard when analysed by high-performance thin-layer chromatography. A density of 6.7×10^5 sialic acid residues per conidium was estimated using a colorimetric assay. Conidia grown on a minimal medium lacking sialic acid also reacted with LFA, indicating that sialic acid biosynthesis occurs *de novo*. Sialic acid biosynthesis was shown to be regulated by nutrient composition: the density of sialic acids on the surface of conidia grown in minimal media was lower than that observed when conidia were grown on rich, complex media. It has previously been shown that pathogenic *Aspergillus* species adhere to basal lamina proteins to a greater extent than non-pathogenic *Aspergillus* species. To determine whether the expression of sialic acid on the conidial surface was correlated with adhesion to basal lamina, conidia from other non-pathogenic *Aspergillus* species were tested for their reactivity towards LFA. Flow cytometric analysis demonstrated that *A. fumigatus* had a significantly greater sialic acid density than three non-pathogenic *Aspergillus* species. Sialic acids on the conidial wall may be involved in adhesion to fibronectin, a component of the basal lamina, as binding of *A. fumigatus* conidia to fibronectin was strongly inhibited in the presence of a sialylated glycoprotein.**

Keywords: *Aspergillus fumigatus*, invasive aspergillosis, lectin, flow cytometry, adhesion

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Abbreviations: HPTLC, high-performance thin-layer chromatography; LFA, *Limax flavus* agglutinin; MAA, *Maackia amurensis* agglutinin; MALDI, matrix-assisted laser desorption ionization; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; SNA, *Sambucus nigra* agglutinin.

INTRODUCTION

Aspergillus species are saprophytic fungi found in soil, water and decaying organic matter. Of the 200 species in the genus only a few can cause disease including *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans* and *Aspergillus terreus*; however, over 90% of all cases are caused by *A. fumigatus* (Denning, 1998). In the normal host, *Aspergillus* species can cause a wide spectrum of diseases ranging from allergic manifestations and superficial infections (Bodey & Vartivarian, 1989), to a life-threatening invasive disease known as invasive aspergillosis. Initiation of infection begins with the inhalation of conidia, the infectious particles, which adhere to and germinate in the lung tissue (Latge, 1999). Hyphal invasion of the lung epithelium is followed by dissemination to other organs (Broderick *et al.*, 1996). Risk factors for invasive aspergillosis are prolonged neutropenia and/or long-term administration of high dose corticosteroids (Denning, 1998).

The frequency of invasive aspergillosis has increased 14-fold in the past decade (Groll *et al.*, 1996), mainly due to the greater numbers of bone marrow and organ transplant recipients (Wald *et al.*, 1997), more aggressive cytotoxic chemotherapy used to treat cancer patients (Bodey *et al.*, 1992) and the emergence of the human immunodeficiency syndrome (HIV-AIDS) (Minamoto *et al.*, 1992). There are currently only two antifungal agents used to treat the disease, amphotericin B and itraconazole, and their success rate is only 34%, even when used prophylactically (Denning, 1998). The increase in fungal infections worldwide and the lack of satisfactory antifungal therapy makes it critical to understand the mechanisms by which *Aspergillus* species adhere to and colonize the lung tissue.

Previous work in our laboratory has demonstrated that *A. fumigatus* conidia bind significantly better than those of other *Aspergillus* species to intact lung cell basal lamina and fibronectin (Wasylnka & Moore, 2000). Investigation of the mechanism of conidial binding determined that negatively charged carbohydrates occurring on the conidial surface may play a role in the adhesion of the conidia to host basal lamina (Wasylnka & Moore, 2000).

Sialic acids are members of a family of 9-carbon carboxylated monosaccharides that are the major ionogenic compounds in many cell types (Schauer, 1982). All compounds are thought to be derived from *N*-acetylneuraminic acid (Neu5Ac) with the most common modifications being the oxidation of the *N*-acetyl group to form *N*-glycolylneuraminic acid (Neu5Gc) and acetylation of the hydroxyl groups at the 4, 7, 8 or 9 position (Varki, 1992). In most glycoconjugates, the sialic acids are terminal or subterminal non-reducing sugars and are attached by 2,3- α or 2,6- α linkages to underlying galactose residues.

Sialic acids have been shown to be important in several aspects of microbial pathogenesis. Many viruses bind to their host via sialic acids on the epithelial cell surface.

For example, influenza A and B viruses recognize sialic acid whereas influenza C viruses bind exclusively to 9-O-acetylated sialic acids (Varki, 1997). Incorporation of sialic acids into the capsules of some pathogenic bacteria aids in evading host defences by inhibiting the direct activation of the alternative complement pathway (Wessels *et al.*, 1992). More recently, sialic acids have been identified in the cell walls of several pathogenic fungi including *Candida albicans* (Soares *et al.*, 2000), *Cryptococcus neoformans* (Rodrigues *et al.*, 1997), *Paracoccidioides brasiliensis* (Soares *et al.*, 1998) and, to a lesser degree, *Pneumocystis carinii* (De Stefano *et al.*, 1990). In pathogenic fungi, the biological function of these carbohydrates has not yet been determined but several studies have provided evidence that, as in other microbial pathogens, sialic acids may play a role in host recognition and in the evasion of host defences. For example, one report found that removal of sialic acids from *Cry. neoformans* yeast cells enhanced their susceptibility to phagocytosis by mouse macrophages (Rodrigues *et al.*, 1997). The authors postulated that these sialic acids might give the fungus a protective advantage in the host during the early stages of infection (Rodrigues *et al.*, 1997).

Preliminary analysis of the carbohydrate structures on the conidia of *A. fumigatus* has shown the presence of D-galacto-D-mannans (Barreto-Bergter *et al.*, 1981). Sialic acid substitution was not detected by these investigators because of the conditions used in the isolation procedures. Therefore, the aims of this study were: 1, to determine whether *A. fumigatus* conidia possess sialoglycoconjugates; 2, to identify the nature of the glycan linkages; and 3, to determine whether the *A. fumigatus* cell wall contains greater amounts of sialic acid than the cell walls of three non-pathogenic *Aspergillus* species.

METHODS

***Aspergillus* strains and growth conditions.** The following *Aspergillus* species were used in this study: *A. fumigatus* ATCC 13073, ATCC 42202 and CHUV (gift from M. Monod, Laboratoire de Mycologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland); *Aspergillus ornatus* ATCC 16921 and ATCC 66492; *Aspergillus wentii* ATCC 10584 and ATCC 1023; and *A. auricomus* ATCC 16890. All fungi were grown on YM agar (0.3%, w/v, yeast extract and malt extract; 0.5%, w/v, peptone; and 1%, w/v, glucose) for 5–14 d at 28 °C (except *A. ornatus* ATCC 66492, which was grown at 24 °C) until conidia were fully mature. For some experiments, *A. fumigatus* ATCC 13073 was grown on a sialic acid-free minimal medium [1%, w/v, glucose; 0.52 g KCl l⁻¹; 0.52 g MgSO₄·7H₂O l⁻¹; 1.52 g KH₂PO₄ l⁻¹; Hunters trace elements; 0.85 g NaNO₃ l⁻¹; vitamins (1 mg nicotinic acid l⁻¹, 2.5 mg riboflavin l⁻¹, 2 mg pantothenic acid l⁻¹, 0.5 mg pyridoxine l⁻¹, 0.01 mg biotin l⁻¹, 0.2 mg *p*-aminobenzoic acid l⁻¹) and 1.5%, w/v, agar]. Conidia were harvested by flooding the plate with PBS, pH 7.4/0.05% (v/v) Tween 20 and gently scraping with a sterile cotton swab. The suspension was then vortexed for 30 s to break up the chains of conidia and hyphal fragments were removed by filtration through sterile glass wool. Conidia were pelleted by centrifugation (1500 g, 2 min), resuspended in PBS and counted using a haemocytometer.

Sialidase treatment. Conidia were harvested from a mature plate of *A. fumigatus* ATCC 13073 and 2×10^7 conidia were resuspended in 50 mM sodium acetate buffer, pH 5.0 containing 0 or 1.1 U *Clostridium perfringens* 2,3- α -, 2,6- α -, 2,8- α -sialidase (Calbiochem) and incubated for 6 h at 37 °C.

Biotinylation of lectins. LFA (Calbiochem), *S. nigra* agglutinin (SNA; Sigma) or *M. amurensis* agglutinin (MAA; Sigma) (all 1 mg) were incubated with a 20 molar excess of n-hydroxy-succinimidobiotin long chain (Pierce) in 1 ml 0.1 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl, for 60 min at room temperature. The biotinylated lectins were then dialysed against PBS in a 4 ml Ultrafree membrane filter with an M_r 10000 cut-off membrane (Millipore).

Lectin labelling.

Sialidase-treated samples. Untreated or sialidase-treated *A. fumigatus* conidia were reacted with biotinylated LFA essentially as described by Rodrigues *et al.* (1997) with the following modifications. Cells were blocked for 60 min with PBS/10% goat serum and incubated with biotinylated LFA at 50 $\mu\text{g ml}^{-1}$ in PBS/10% goat serum for 60 min; bound lectin was detected by incubation for 60 min with Streptavidin–Oregon Green (Molecular Probes) diluted 1:150 in PBS/10% goat serum. Flow cytometry was performed on a Coulter EPICS Elite Esp flow cytometer using a 488 nm laser for excitation energy and a 550 nm dichroic filter capturing an emission band at 525 nm: 10000 events were recorded for each sample. Samples were also viewed with an Olympus VANOX AHBS3 microscope equipped with epifluorescence at 1000 $\times$ magnification. Bright field and fluorescent images for each sample were captured with a Sony 950 camera using Eclipse (Empix Imaging) image capturing software.

Determination of sialic acid linkage. To determine the sialic acid linkages in *A. fumigatus* conidia, an aliquot of 2×10^7 cells was lectin labelled as described above using 50 μg biotinylated LFA, SNA or MAA ml^{-1} and then analysed by flow cytometry or fluorescence microscopy as described above.

Competition of LFA binding by sialic acid. An aliquot of 3.6×10^7 *A. fumigatus* ATCC 13073 conidia was added to poly L-lysine coated coverslips for 60 min and reacted with biotinylated LFA in PBS/5% goat serum containing 0 or 100 mM Neu5Ac (Rose Scientific). The remainder of the protocol was as described above. Samples were mounted with ProLong mounting media (Molecular Probes) and analysed by fluorescence microscopy.

Lectin labelling of different *Aspergillus* species. Conidia from *A. fumigatus* ATCC 13073, ATCC 42202 and CHUV; *A. ornatus* ATCC 16921 and ATCC 66492; *A. wentii* ATCC 10584 and ATCC 1023; and *A. auricomus* ATCC 16890 were collected, and 1×10^7 conidia were labelled with biotinylated LFA and processed for flow cytometry analysis as described above.

Isolation of sialic acids from *A. fumigatus* conidia. Sialic acids from *A. fumigatus* conidia were isolated as described by Reuter & Schauer (1994) with minor modifications. Briefly, dry cells (5.5 g) were resuspended in 20–30 ml 88% formic acid for 60 min at 70 °C. The suspension, which should contain any O-acetylated sialic acids, was cooled on ice and centrifuged for 10 min at 1500 g. The supernatant was removed, and the pellet resuspended in 20–30 ml 3 M HCl and incubated at 80 °C for 60 min to remove all remaining sialic acids. This suspension was cooled and centrifuged as described above. The supernatants from both the formic and hydrochloric acid digests were then ultracentrifuged for 30 min at 50000 g and 4 °C, and the ultrasupernatants were lyophilized overnight. The dried residues were resuspended in 4 ml cold, deionized, distilled H_2O (dd H_2O) and then dialysed against

dd H_2O in a 5000 Da (HCl digest) or 10000 Da (HCOOH digest) molecular mass cut-off Ultrafree-4 centrifugal filter (Millipore). The diffusate was passed over a Dowex 50WX8 (100–200 mesh) cation exchange column (hydrogen ion form; Sigma) and washed with 3–4 bed vols cold dd H_2O . The eluate and washings were collected, lyophilized, resuspended in 5 ml cold dd H_2O and the pH adjusted to 4.0 with NH_4OH . This solution was then passed over a Dowex 2X8 (200–400 mesh) anion exchange column (formate form; Sigma), washed with 10 ml cold dd H_2O and then eluted with 20 ml 1 M formic acid. Fractions of 1.5 ml were collected and lyophilized. The dried residues were resuspended in 40 μl dd H_2O and 10 μl was spotted onto HPTLC (high-performance thin-layer chromatography) plates (Mandel Scientific) along with 5 μl Neu5Ac and Neu5Gc (Sigma) standards (at 1 mg ml^{-1}) and run in 1-propanol/1 M $\text{NH}_3/\text{H}_2\text{O}$ (6:2:1 by vol.). Plates were developed by spraying with orcinol/HCl spray reagent (81.4 ml 37% HCl, 0.2 g orcinol (Sigma), 2 ml 1% FeCl_3) diluted 3:1 with dd H_2O and then heated at 180 °C for 20 min.

Colorimetric quantitation of sialic acids. For colorimetric quantitation, 4×10^{10} conidia were hydrolysed with 0.1 M trichloroacetic acid for 3 h at 80 °C. The suspension was cooled and centrifuged at 9000 g for 10 min. The supernatant was then treated with 0.1 M HCl for 1 h at 80 °C and dialysed overnight in a 3500 Da dialysis cassette (Pierce) (4 °C, 750 ml dd H_2O , two changes). The next day the diffusate was concentrated by rotary vacuum until the final volume was 250 ml and this was lyophilized to dryness. Ion-exchange chromatography was performed as described above. To further purify the sialic acids from contaminants, anion exchange fractions were streaked onto HPTLC plates and the sialic acid containing bands scraped off and extracted with dd H_2O followed by 1:1 dd H_2O /methanol. These extracts were lyophilized by speed vacuum, resuspended in dd H_2O and analysed by colorimetric quantitation as described by Reuter & Schauer (1994) by measuring the absorbance at 572 nm of samples reacted with the orcinol/ Fe^{3+} /HCl reagent.

Matrix-assisted laser desorption ionization (MALDI) MS.

Mass spectra of purified sialic acids were obtained using a MALDI mass spectrometer from PerSeptive Biosystems with an accelerating voltage of 20 keV. An aliquot of purified sialic acids (2.5 μl) was mixed with 0.5 μl matrix (10 mg 2,5-dihydroxybenzoic acid ml^{-1}) and laser ionization focused around areas of good crystallization.

Adhesion to poly L-lysine. Sialidase-treated or untreated *A. fumigatus* conidia were added to poly L-lysine (Sigma) coated coverslips in 24 well plates at a concentration of 1×10^7 conidia ml^{-1} for 60 min at room temperature. Conidia were fixed with PBS/4% (w/v) paraformaldehyde, pH 7.4 for 60 min and then coverslips were mounted onto slides. Samples were viewed with an Olympus Vanox AHBS3 microscope at a 1000 $\times$ magnification. Sample enumeration was performed by collecting images from five random fields (Sony 950 video camera) and the number of conidia per field was calculated by the computer program Eclipse.

Adhesion assays. Experiments were performed as described previously (Wasylnka & Moore, 2000). Mucin (Type I-S, Sigma), asialomucin or sialic acid were co-incubated with peroxidase-labelled conidia at the indicated concentrations.

Miscellaneous chemicals and supplies. *Vibrio cholerae* sialidase was obtained from Calbiochem and *Arthrobacter ureafaciens* sialidase was from Sigma. All chemicals were reagent grade or better unless specifically noted. Asialomucin was a generous gift from Dr J. Esko (Division of Cellular and Molecular Medicine, University of California, San Diego, CA, USA).

Statistics. Differences in sialic acid densities between *Aspergillus* species were analysed by a one-way Kruskal–Wallis test for non-parametric data using JMP (SAS Institute). The Student's *t* test was used for statistical analysis of other data. Values shown are mean data from three independent experiments (unless otherwise stated). A *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

A. fumigatus conidia have sialic acids on their surface

We have previously shown that negatively charged carbohydrates present on the conidial surface mediate adhesion of *A. fumigatus* to basal lamina proteins. This finding, along with the recent identification of sialic

Fig. 1. LFA labelling of sialidase-treated or untreated *A. fumigatus* conidia. *A. fumigatus* conidia were untreated (a, c) or sialidase treated (b) and then incubated with biotinylated LFA and Streptavidin–Oregon Green (a, b) or Streptavidin–Oregon Green alone (c) and analysed by flow cytometry.

Fig. 2. Epifluorescence microscopy of LFA-labelled conidia demonstrates existence of sialic acids. Untreated (a) or sialidase-treated (c) conidia were incubated with biotinylated LFA and Streptavidin–Oregon Green. Untreated conidia were also incubated with biotinylated LFA in the absence (e) or presence (g) of 100 mM Neu5Ac followed by Streptavidin–Oregon Green. The corresponding differential interference contrast images are shown (b, d, f, h). Bars, 10 μ m. This figure is representative of two independent experiments.

acids in other pathogenic fungi prompted the investigation as to whether *A. fumigatus* conidia also possessed sialic acids. In earlier reports of *A. fumigatus* cell wall structure (Barreto-Bergter *et al.*, 1981; Hearn & Sietsma, 1994), sialic acids were not detected, which may have been a result of the carbohydrate purification scheme used. In addition, the study by Hearn & Sietsma (1994) investigated hyphal wall preparations, which may contain slightly different carbohydrate structures than conidia. For this reason we used sialic acid-specific lectins to detect sialic acids, since these proteins are very sensitive and specific for particular sialic acid derivatives (Varki, 1997).

A. fumigatus conidia were incubated with biotinylated LFA lectin, which reacts with any sialic acid linkage, (Schauer *et al.*, 1995) and then with a secondary fluorophore, Streptavidin–Oregon Green. *A. fumigatus* conidia were strongly fluorescent (compared to the sample which received Streptavidin–Oregon Green only), indicating the presence of sialic acids on the conidial surface (Fig. 1a). Analysis by flow cytometry (Fig. 1) and epifluorescence (Fig. 2a–d) of sialidase-treated and untreated conidia showed that pre-treatment

Fig. 3. Reactivity of *A. fumigatus* conidia with different sialic acid-specific lectins. *A. fumigatus* conidia were incubated with biotinylated LFA, SNA, MAA or no lectin, followed by Streptavidin–Oregon Green and analysed by flow cytometry. The histogram of flow cytometry data expresses the mean fluorescence (from three independent experiments) of MAA, SNA and no lectin as a percentage of the LFA fluorescence.

of conidia with *Clo. perfringens* sialidase significantly reduced the fluorescent signal. Three independent experiments using flow cytometry showed that sialidase-treated conidia had $48 \pm 18\%$ of the mean fluorescence when compared to the controls (set at 100%). We found that removal of sialic acids was only achieved at high concentrations of the *Clo. perfringens* sialidase (1.1 U). Lower concentrations of *Clo. perfringens* sialidase, *V. cholerae* sialidase or *A. ureafaciens* sialidase were ineffective at cleaving the sialic acids (data not shown).

Additional proof that the reaction of LFA was specific for sialic acid was obtained from lectin-competition experiments. Conidia were incubated with biotinylated LFA in the presence and absence of 100 mM sialic acid and then with Streptavidin–Oregon Green. The fluorescence of the conidia was almost eliminated when sialic acid was added with LFA, compared to the fluorescence observed with LFA alone (Fig. 2e–h). Therefore, the results from the sialidase treatment and the lectin-competition experiment both indicate that the LFA was specifically binding to sialic acids on the *A. fumigatus* conidial cell wall.

To determine the glycosidic linkage of the sialic acids, we reacted *A. fumigatus* conidia with lectins that bind specifically to different sialic acid linkages. Conidia were incubated with biotinylated LFA, MAA or SNA. MAA reacts with Neu5Ac2,3- α -Gal1,4- β -GlcNAc and SNA reacts with Neu5Ac2,6- α -Gal/GalNAc (Schauer *et al.*, 1995). The extent of MAA and SNA binding to *A. fumigatus* conidia was much lower than that observed with LFA: LFA-treated conidia were 13 times more fluorescent than SNA-treated conidia and 16 times more fluorescent than MAA-treated conidia as determined by flow cytometry (Fig. 3). To confirm that both SNA and MAA were functional and adequately biotinylated, the lectins were used as probes for binding to sialylated glycoproteins fetuin (MAA) and fibronectin (SNA) immobilized onto nitrocellulose. Both SNA and MAA reacted equally with these glycoproteins (data not

Fig. 4. HPTLC of sialic acids from purified *A. fumigatus* conidia hydrolysate. An HPTLC plate was spotted with purified hydrolysate of *A. fumigatus* conidia (1) or sialic acid standards (5 μ g) (2) and developed with the orcinol spray reagent.

shown), confirming that the lack of reactivity was due to the structure of the conidial carbohydrates. Since 2,6- α -sialic acid has been the predominant sialic acid structure identified in other pathogenic fungi (Rodrigues *et al.*, 1997; Soares *et al.*, 1998, 2000), the lack of reactivity with SNA was surprising. However, filamentous fungi such as *Aspergillus* may have novel sialic acids that are not found in yeast-like fungi such as *Candida* and *Cryptococcus*. In summary, the lack of reactivity of *A. fumigatus* conidia with SNA and MAA suggests that 2,3- α - or 2,6- α -linked sialic acids represent a minority of the sialic acid structures present on the *A. fumigatus* conidial cell walls. Alternatively, the sialic acid may be substituted in such a way that reduces SNA or MAA binding or that other sialic acid linkages, in particular 2,8- α or 2,9- α , may be present in *A. fumigatus* conidia. However, since there are no lectins that bind to these structures, this cannot be proven without a more detailed chemical analysis of purified sialic acids.

Identification of sialic acids in *A. fumigatus* by chemical purification

To provide direct chemical evidence for the presence of sialic acids on the surface of *A. fumigatus*, conidia were hydrolysed under mildly acidic conditions and then purified by ion-exchange chromatography using standard protocols (Reuter & Schauer, 1994; Rodrigues *et al.*, 1997). A spot that had the same R_F value as the Neu5Ac standard was identified by development with the orcinol reagent following HPTLC of the purified hydrolysate (Fig. 4). MALDI MS analysis identified a major peak at m/z 310, which is the $(M+H)^+$ peak characteristic of Neu5Ac (Fig. 5). These data provide direct chemical evidence for the existence of sialic acids in *A. fumigatus* and support the results of the lectin-binding experiments. A colorimetric quantitation assay of the purified fraction determined that the sialic acid

Fig. 5. MALDI MS analysis of purified sialic acid from *A. fumigatus* conidia. An aliquot of purified *A. fumigatus* sialic acid (2.5 μ l) was mixed with 0.5 μ l 2,5-dinitrobenzoic acid matrix and run on a PerSeptive Biosystems MALDI mass spectrometer. The 310 (M+H)⁺ peak is a major component of the sample. *m/z* fragments lower than 260 are due to the matrix. Higher molecular mass fragments are trace amounts of other unknown sugars.

content of our sample was 13.8 μ g in 4×10^{10} conidia, or 6.7×10^5 residues per cell. Using bovine fetuin as a control, our yield of sialic acid with this assay after hydrolysis and chromatography was approximately 50%. Therefore, the actual number of sialic acid residues per conidium may be greater than observed. This value is similar to sialic acid densities found in other fungal species: *Fonsecaea pedrosoi*, 1.2×10^5 cell⁻¹; *Par. brasiliensis*, 3.7×10^6 cell⁻¹; *Cry. neoformans*, 3.0×10^6 cell⁻¹; *Can. albicans*, 2.8×10^6 cell⁻¹ and *Sporothrix schenckii*, 2×10^6 cell⁻¹ (Alviano *et al.*, 1999).

Expression of sialic acids under different growth conditions

The protozoan parasite *Trypanosoma cruzi* can transfer sialic acids from sialic acid donors in the host onto mucin-like proteins on its surface using a parasite *trans*-sialidase enzyme (Burleigh & Andrews, 1995). In contrast, all fungi that have been studied synthesize sialic acids *de novo* (Alviano *et al.*, 1999). Therefore, we investigated whether *A. fumigatus* could synthesize sialic acids when grown on a chemically defined medium without sialic acid. Conidia grown on minimal medium or YM medium were incubated with biotinylated LFA and then with Streptavidin–Oregon Green. Observation of the samples by epifluorescence microscopy demonstrated that conidia grown on sialic acid-free minimal medium reacted with LFA (data not shown). This result confirms that *A. fumigatus* can synthesize sialic acids *de novo*. However, quantitation of sialic acids by flow cytometric analysis showed that the density of sialic acids from conidia grown on minimal medium was significantly less than that of conidia grown on YM

Fig. 6. Expression of sialic acid from conidia grown in minimal media (MM) or complex media (YM). Flow cytometric analysis of conidia grown on MM or YM after incubation with biotinylated LFA and Streptavidin–Oregon Green (+LFA) or with Streptavidin–Oregon Green alone (–LFA). The histogram of the flow cytometry data expresses the mean fluorescence of MM-grown conidia as a percentage of the YM-grown conidia fluorescence. The values from two independent experiments are shown (white bars, expt 1; black bars, expt 2).

medium (Fig. 6). Therefore, in environments of limiting nutrient availability *A. fumigatus* synthesizes less Neu5Ac, and/or less Neu5Ac is expressed on the conidia surface. Reactivity of conidia grown on minimal medium with SNA and MAA was negligible (data not shown). Therefore, the expression of sialic acids is regulated by the components in the growth medium. The observed difference in sialic acid expression may occur because complex media contain cofactors that stimulate *de novo* synthesis to a greater extent than minimal medium does. For example, sialyltransferase enzymes catalyse the transfer of CMP-Neu5Ac; thus the formation of the sugar nucleotide may have been limiting due to a low concentration of CTP in our minimal media (Gilbert *et al.*, 1998). Since our ultimate goal is to define the role of sialic acids in the virulence of *A. fumigatus*, it is more appropriate to grow *A. fumigatus* on a complex medium (such as YM) that is more representative of their natural environment. Thus, it is anticipated that the density of sialic acids expressed during growth in complex media is more comparable to the levels present in naturally occurring infectious conidia. Further experiments are required to determine whether there are any differences between the structures of sialic acids in conidia grown on complex medium versus minimal medium.

Adhesion to poly L-lysine

Sialic acids are the major anionogenic compounds in the cell surfaces of many species (Schauer, 1982). To determine whether sialic acids play such a role in *A. fumigatus*, conidia were treated with *Clo. perfringens* sialidase or buffer alone, added to poly L-lysine coated coverslips and bound spores were enumerated with an

automated counting program. In the first experiment ($n = 3$), untreated conidia adhered to poly L-lysine at a density of 121 ± 8 conidia per field. Pre-treatment of conidia with *Clo. perfringens* sialidase reduced the adhesion of conidia to poly L-lysine by 29% (87 ± 18 conidia per field) ($P < 0.05$). In a separate experiment, we observed a smaller decrease (15%) in adhesion following sialidase treatment of conidia; the value of the buffer-treated sample was 83 ± 5 conidia per field whereas the sialidase-treated sample was 71 ± 6 conidia per field ($P < 0.05$). These data suggest that the carboxylic acid groups of sialic acid are responsible for a significant portion of the electronegative charge on the conidia. The cell surface of a eukaryotic cell contains many other anionic groups including phosphate and sulfate groups, as well as carboxyl groups of acidic proteins and other polysaccharides, so that even with complete removal of sialic acids from the cell surface, other negatively charged groups remain that can adhere to the poly L-lysine polymer. Treatment of *A. fumigatus* conidia with *Clo. perfringens* sialidase removed approximately 50% of the sialic acids (Fig. 1), therefore the remaining sialic acid residues may be sufficient to allow binding to poly L-lysine. Other researchers have reported somewhat larger effects of sialidase on the adhesion of other fungi to poly L-lysine. For example, treatment of *Cry. neoformans* resulted in a 35% decrease in adhesion to poly L-lysine (Rodrigues *et al.*, 1997) and a 55% decrease was observed in *Can. albicans* (Soares *et al.*, 2000).

Conidia of pathogenic *Aspergillus* species possess greater amounts of sialic acid than non-pathogenic species

Previous work in our laboratory has shown that *A. fumigatus* conidia bind to purified fibronectin and intact basal lamina significantly better than other non-pathogenic *Aspergillus* species (Wasylnka & Moore, 2000). The biological function of sialic acids in pathogenic fungi is still unknown; however, they are important virulence factors in other micro-organisms such as *T. cruzi* (Burleigh & Andrews, 1995) and *Neisseria meningitidis* (Vogel *et al.*, 1996). To determine whether the density of sialic acids may be correlated with adhesion to basal lamina in *Aspergillus*, we measured the density of sialic acid on conidia from four species of *Aspergillus*. In addition to three strains of *A. fumigatus*, three non-pathogens were also studied: *A. wentii* (two strains), *A. ornatus* (two strains) and *A. auricomus* (one strain). *A. fumigatus* is the most common cause of invasive aspergillosis (Denning, 1998), while *A. wentii*, *A. ornatus* and *A. auricomus* have never been documented in human disease (Pitt, 1994). Adhesion to basal lamina has been previously measured in three of these four species (Wasylnka & Moore, 2000).

Conidia from all four *Aspergillus* species were collected from agar plates and incubated with biotinylated LFA followed by Streptavidin–Oregon Green. Analysis by fluorescence microscopy provided preliminary evidence that all three *A. fumigatus* strains contained greater

Table 1. Sialic acid density on conidia of pathogenic and non-pathogenic *Aspergillus* species

Conidia from all *Aspergillus* strains tested were collected and resuspended in PBS. An aliquot of 1×10^7 conidia were fixed with PBS/4% paraformaldehyde and incubated sequentially with PBS/150 mM NH_4Cl , PBS/10% goat serum, 100 μg biotinylated LFA ml^{-1} and then with a 1:100 dilution of Streptavidin–Oregon Green. Samples were adjusted to 5×10^6 conidia ml^{-1} and analysed on a Coulter EPICS Elite Esp flow cytometer using a 488 nm laser for excitation energy and a 550 nm dichroic filter capturing an emission band at 525 nm.

Strain	Fluorescence*
<i>A. fumigatus</i> CHUV	226.4
<i>A. fumigatus</i> ATCC 42202	213.8
<i>A. fumigatus</i> ATCC 13073	168.4
<i>A. auricomus</i> ATCC 16890	48.4†
<i>A. wentii</i> ATCC 1023	24.0†
<i>A. wentii</i> ATCC 10584	12.7†
<i>A. ornatus</i> ATCC 16921	25.2†
<i>A. ornatus</i> ATCC 66492	25.6†

* Sample/control of the mean log fluorescence channel (controls received Streptavidin–Oregon Green but no LFA).

† Statistically different from *A. fumigatus* strains as determined by the Kruskal–Wallis test. The data are representative of two independent experiments.

amounts of sialic acids than the other *Aspergillus* species tested (data not shown). Therefore, the experiment was repeated and the conidia analysed by flow cytometry to quantitatively measure sialic acid density in all four species. Because differences in the shape and size of conidia can affect the data spread in flow cytometry, each species was labelled with both LFA and Streptavidin–Oregon Green (sample fluorescence) and also with Streptavidin–Oregon Green alone as a control. By dividing the mean of the control fluorescence by the value of the sample fluorescence, a mean sample value was obtained which enabled a direct comparison between species. The mean fluorescence of *A. fumigatus* ATCC 42202 and CHUV was four times greater than the *A. auricomus* strain, 10 times greater than both *A. ornatus* strains and the *A. wentii* ATCC 1023 strain and 20 times greater than *A. wentii* ATCC 10584 (Table 1). The mean fluorescence of the other *A. fumigatus* strain, ATCC 13073, was three times greater than the *A. auricomus* strain, six times greater than both *A. ornatus* strains and the *A. wentii* ATCC 1023 strain and 13 times greater than *A. wentii* ATCC 10584 (Table 1). Statistical analysis of the mean fluorescence using the Kruskal–Wallis test demonstrated that the mean fluorescence of all the *A. fumigatus* strains was significantly greater than any of the non-*A. fumigatus* strains ($P < 0.05$). Therefore, these experiments showed that the expression of sialic acids on *A. fumigatus* conidial walls was 3–20 times greater than the conidia of the three non-pathogenic *Aspergillus* species. In addition, the flow cytometry data comparing sialic acid expression be-

Fig. 7. Binding of *A. fumigatus* conidia to fibronectin in the presence of sialylated carbohydrates. Microtitre plates were coated with fibronectin at 50 μg ml⁻¹ for 1 h at 37 °C and the next day a standard binding assay was performed. Peroxidase-labelled *A. fumigatus* conidia were added to the plates in the presence of mucin, asialomucin or Neu5Ac at the indicated concentrations for 1 h at 37 °C. Unbound conidia were washed off with PBS/Tween and the absorbance measured at 490 nm on a microtitre plate reader (Bio-Tek Instruments) after the addition of the peroxidase substrate. The values are the means ± standard deviations of triplicate wells and the figure is representative of two independent experiments.

tween *Aspergillus* species parallels our data on the adhesion of *Aspergillus* species to basal lamina: all *A. fumigatus* strains bound to basal lamina with 10–20 fold greater affinity than *A. ornatus* and *A. wentii* strains (*A. auricomus* was not tested) (Wasylnka & Moore, 2000). Taken together, these data indicate a correlation between sialic acid density and adhesion to basal lamina proteins.

This is the first report of differences in sialic acid expression between pathogenic and non-pathogenic species within a genus. The results indicate that non-pathogenic *Aspergillus* species synthesize sialic acids; however, either less sialic acid is transported to the cell wall or different glycosylation pathways occur in these species. Since sialic acids are known virulence factors in other micro-organisms, the fact that all *A. fumigatus* strains tested expressed higher levels of sialic acids on their surfaces than the non-pathogenic strains suggests that sialic acid density may contribute to the virulence of this fungus.

Role of conidial sialic acid in the binding of *A. fumigatus* to fibronectin and basal lamina

To confirm that the observed differences in sialic acid density between species correlated with adhesion to basal lamina, we tested the adhesion of *A. fumigatus* conidia to components of the basal lamina in the presence of sialylated carbohydrates. We used this method because, as stated earlier, sialidase treatment of conidia removes only 50% of the sialic acids from the

spore surface and requires large amounts of enzyme. We first investigated the adhesion of *A. fumigatus* conidia to purified fibronectin. Conidia were co-incubated with the heavily sialylated glycoprotein mucin (12% sialic acid by weight), asialomucin or sialic acid and allowed to adhere to fibronectin-coated microtitre plates. Binding of conidia to immobilized fibronectin was inhibited almost completely (by 85%) by 250 μg ml⁻¹ and only partially (62%) by asialomucin (Fig. 7). Furthermore, at 125 μg ml⁻¹, mucin still inhibited binding by 56%, whereas binding to fibronectin in the presence of asialomucin was essentially restored. Therefore, mucin had a dose-dependent inhibitory effect on conidia binding to fibronectin. The inability of asialomucin to strongly reduce binding suggests that it is the sialic acid residues on mucin which are directly involved in the inhibitory effect. Further evidence for the involvement of sialic acids in binding to fibronectin was demonstrated using sialic acid as an inhibitor in binding assays. Co-incubation of conidia with 20 mM sialic acid inhibited binding by 90% (data not shown), although part of the reduction in binding may have been due to the acid pH of the sialic acid solution (pH 3.5 at 20 mM).

These results are similar to those obtained by Bouchara *et al.* (1997) who demonstrated inhibition of binding of *A. fumigatus* conidia to fibronectin by mucin; however, their interpretations of the results led to a different conclusion on the mechanism of conidia binding. They concluded that the sialylated glycoprotein mucin was competing with the oligosaccharides on extracellular matrix proteins for a lectin on the conidial surface (Bouchara *et al.*, 1997). However, previous work in our laboratory showed that conidia bound to the heparin-binding domain of fibronectin and neither desialylation nor complete deglycosylation of fibronectin decreased conidial adhesion. Furthermore, negatively charged carbohydrates and high ionic strength buffers inhibited the adhesion of conidia binding to fibronectin (Wasylnka & Moore, 2000). In the present study we have provided evidence for the existence of sialic acids on the conidial surface of *A. fumigatus*. In addition, we have shown that a sialylated glycoprotein inhibited the adhesion of conidia to fibronectin. Taken together, our results indicate that *A. fumigatus* conidia bind to fibronectin in part via sialoglycoconjugates on the spore surface and that the mechanism of binding is an ionic interaction between the negatively charged sialic acids on the conidial surface and positively charged regions of the fibronectin protein.

We next investigated whether sialic acids on *A. fumigatus* conidia were involved in binding to immobilized basal lamina. Conidia were again co-incubated in the presence of mucin, asialomucin or sialic acid and allowed to adhere to basal-lamina-coated microtitre plates. In contrast to the results obtained with fibronectin, mucin (up to 500 μg ml⁻¹) had no effect on binding of conidia to basal lamina (data not shown). However, as seen with fibronectin, sialic acid was also able to strongly inhibit binding to basal lamina (69% at 20 mM) (data not shown). Therefore, in contrast to

fibronectin, the binding to intact basal lamina may be more complex; perhaps requiring the participation of surface sialic acids and/or other negatively charged carbohydrates on the spore surface. Nonetheless, it has been suggested that the basal lamina of diseased lungs have increased amounts of fibronectin and other extracellular matrix proteins (Roman & McDonald, 1997). Therefore, binding of *A. fumigatus* conidia to fibronectin *in vivo* may be important in the development of invasive aspergillosis.

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