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Humoral Immune Response in Aspergillosis: An Immunodominant Glycoprotein of 35 kDa from *Aspergillus flavus*

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Abstract. A glycoprotein preparation containing 70% carbohydrates and 30% proteins was isolated from the mycelium of two strains of *Aspergillus flavus* and fractionated by ConA-Sepharose affinity chromatography. An immunodominant 35-kDa antigen was detected in a ConA-bound fraction (B fraction). It contained mannose and galactose in a 1.4:1.0 ratio. This antigen seems to be able to elicit an antibody response in patients with aspergillosis and in rabbits immunized with *A. flavus* whole cells. The carbohydrate units of the BF fraction appeared to be responsible for the antigenicity, since treatment with periodate removed most of the antibody binding capacity.

Aspergillus species have been implicated as the etiological agents of various lung diseases including allergic asthma, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma (fungus ball), and invasive aspergillosis (IA). *A. fumigatus* is the most common agent, but other species such as *A. flavus, A. terreus*, and *A. niger* can also cause human infections. Allergic *Aspergillus* sinusites (AAS) is a comparatively new disease entity that occurs in atopic patients with chronic sinusitis and nasal polyps [5, 14]. *A. flavus* and *A. fumigatus* are the *Aspergillus* species commonly detected after analysis of surgical debris and mucus, and are also involved in invasive mold sinusitis [3, 18].

A few *A. flavus* antigens have been described in the literature. A 34-kDa (Asp fl 13) and a 33-kDa (Asp fl 1) alkaline serine proteases were the major allergens of *A. flavus* [6, 22]. Immunoblot inhibition studies indicated that IgE cross-reactivity occurs among the 34-kDa antigen of *A. flavus, A. fumigatus*, and *Penicillium citrinum* [6]. By using monoclonal antibodies (Mabs), a 97-kDa antigen was detected on the surface and in the supernatant fluids of *A. flavus*. This antigen, or a similar one, is present in *A. fumigatus* and other *Aspergillus* species [12]. In addition, a 58-kDa antigen [8] has been purified from *A. fumigatus* mycelial extracts. This antigen is stained by both Coomassie blue and the periodic acid Schiff stains, indicating that it may be a glycoprotein or a glucogalactomannan protein conjugate. It also reacts with antibodies present in sera from patients with IA due to *A. flavus*. It is apparently shared by both species of *Aspergillus*.

By using hydrophobic interaction and affinity chromatography, Kurup et al. [15] isolated a glycoprotein fraction from *A. fumigatus* with two components of 35 and 65 kDa. This fraction consistently showed binding to IgG and IgE antibodies in sera from patients with ABPA. The sera of patients with ABPA [11] recognized a 93-kDa glycoprotein from *A. fumigatus*, purified by single-step affinity chromatography with the mannose-specific snowdrop lectin. Its relationship with a previously described galactomannan antigen of 94 kDa [26], containing immunodominant β(1→5) galactofuranan side chains, needs to be established.

In this paper, we describe the characterization of the antigens present in a ConA-binding fraction of two *A. flavus* strains that were recognized by rabbit antiserum raised against whole cells of *A. flavus* and sera from patients with proven aspergillosis.

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Material and Methods

Microorganisms. *Aspergillus flavus* NCPF 2008 (N strain) was kindly supplied by C. K. Campbell of the Mycological Reference Laboratory, Bristol Public Healthy Laboratory, UK. The *A. flavus* SP065 (S strain) was isolated from a patient with sinusitis in Rio de Janeiro, Brazil.

Growth conditions. Fungal cells were grown on Sabouraud solid slants ($g.L^{-1}$): Difco peptone, 10; Difco yeast extract, 5; Difco agar, 20; Glucose, 40.Cells were inoculated into Erlenmeyer flasks (500 mL) containing Sabouraud liquid medium (200 mL), which was incubated with shaking for 7 days at 25°C. Cultures were then transferred to the same medium (1 L) and incubated for 7 more days at the same temperature with shaking. The mycelium then was filtered, washed with distilled water, and dried.

Extraction and fractionation of glycoproteins. Crude glycoproteins from both strains were obtained by extraction of the intact mycelium in a phosphate buffer 50 mM pH 7.2, at 100°C for 2 h [10].

Affinity chromatography. The crude glycoproteins were fractionated on a ConA-sepharose 4B column (0.5 × 20 cm) and equilibrated with 10 mM Tris-HCl, pH 7.2. The unbound material (UB) was eluted with 10 mM Tris-HCl, pH 7.2, and the bound material (B) was then eluted with 0.2 M α-methylmannoside. Three-milliliter fractions were collected and monitored at A_{280} for the presence of proteins.

Analytical procedures. The total contents of carbohydrates, proteins, phosphate, and hexosamines were determined by the phenol-sulfuric [7], Folin-phenol reagent [17], Ames [1], and Belcher [3] methods, respectively.

Component monosaccharides. Fractions from ConA-affinity chromatography (UB + B) were hydrolyzed with 3 *N* TFA for 3 h at 100°C. The resulting monosaccharides were converted to their alditol acetates, which were characterized and quantified by gas chromatography coupled to mass spectrometry (GC-MS) [20].

SDS-PAGE. Samples (crude glycoprotein, UB and B fractions) were separated by eletrophoresis in a 12% polyacrylamide gel and silver stained. For additional details see Haido et al. [10].

Human sera. Human sera were provided by Claudine Pinel from the Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Grenoble, Grenoble, France and Prof. Alfeu Franca, Hospital Universitario Prof. Clementino Fraga Filho, UFRJ, Rio de Janeiro, Brasil. Sixteen serum samples were from patients with or without asthma. For these patients, positive serology for *Aspergillus*, peripheral blood eosinophilia, and an increase in total immunoglobulin E (IgE) were detected. Serum antibody reactivity was monitored by both the Ouchterlony diffusion assay or ELISA by using somatic and metabolic extracts of *A. fumigatus*. Five (5) cases of proven invasive aspergillosis (IA) were diagnosed by histologic evidence of the presence of hyphae in tissue specimens and the in vitro growth of the *Aspergillus* species in culture. Two probable IA cases presented positive *Aspergillus* sp, in cultures from bronchoalveolar lavage fluid or bronchial aspirates. Two serum samples were from aspergilloma patients who had been confirmed by X-ray analysis, clinical examinations, serological reactions, and isolation of *A. fumigatus* and/or *A. flavus* from bronchial washings. Serum samples from patients with proven mycotic diseases (coccidioidomycosis, cryptococcosis, histoplasmosis, paracoccidioidomycosis), and serum samples from tuberculosis patients were also used. Seventy-five (75) control sera were obtained from healthy individuals.

Rabbit immune sera. Two white male rabbits were inoculated with freeze-dried mycelium of *A. flavus* NCPF 2008 (2 mg/mL dry weight), emulsified with an equal volume of complete Freund adjuvant, and aliquots of 1 mL of emulsion were injected intradermally over a period of several weeks. For further details, see Haido et al. [10].

Western blot analysis. Following eletrophoresis, the sample were electrotransferred onto PVDF membranes as previously described by Glass et al. [9]. Proteins were transferred for 60 min in 10% methanol, 12.2 mM Tris, 96 mM glycine, pH 8.3, at 400 mA in a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) onto PVDF. After transfer, the PVDF membranes were blocked with 3% skim milk plus 0.1% Tween 20 in PBS (10 mM phosphate buffer, 0.15 M NaCl) at room temperature and incubated for 1 h at 37°C with human sera diluted 1:200. The PVDF membranes were washed in PBS Tween plus 3% skim milk, incubated with goat anti-human IgG (Bio-Rad, USA) (1:800) in the same buffer, washed as above, and developed with diaminobenzidine (DAB) in 0.1 M PBS buffer and H_2O_2. For detection of ConA-binding antigens, the PVDFs were blocked with a 10% solution of fetal calf serum (FCS) in Tris buffer saline (TBS), rinsed with TBS containing 1% FCS, and incubated with a ConA-horseradish peroxidase conjugate (25–50 μg/mL) in blocking buffer for 30 min. After washing, the PVDFs were developed as described above.

Periodate oxidation. Four mg of the *A. flavus* bound fractions were treated with increasing concentrations (10–100 mM) of sodium metaperiodate according to Haido et al. [10]. These preparations were further tested in ELISA experiments.

ELISA. The method used for ELISA was essentially as described by Voller et al. [21]. The wells of the flat-bottomed microtiter plates (Hemobag) were coated with 100 μL of a 5 μg/mL solution of crude and ConA- separated fractions from each of the two strains of *A. flavus*. Human and rabbit sera were used at dilutions from 1:200 to 1:25,600 in the presence of BSA. The chromogen used was *O*-phenylenediamine added together with hydrogen peroxide [10]. Inhibition tests were performed with rabbit serum diluted 1:200 to 1:25,600 pre-incubated for 30 min with 5 μg/mL of the *A. flavus* BF fraction.

Results and Discussion

Fractionation of glycoproteins from *Aspergillus flavus*. Crude glycoproteins were extracted from the N and S strains of *A. flavus* with hot phosphate buffer and fractionated by ConA-Sepharose column chromatography. As shown in Fig. 1, the glycoprotein preparation was separated into ConA-unbound (UB) and bound fractions (B), after elution with 0.2 M α-methyl-mannopyranoside. The chemical composition of the crude glycoproteins and B and UB fractions from both strains of *A-flavus* is shown in Table 1. Analysis of the ConA-binding fraction showed that mannose, galactose, and glucose were the monosaccharide components of the carbohydrate portion. The proportion of mannose:galactose was 1.4:1.0, suggesting the presence of a galactomannan-protein conjugate. The ConA-binding fractions isolated in this study are visualized as a band of 35 kDa in Western blot with ConA-HRP (Fig. 2). The unbound fractions (Fig. 2, lanes 2 and 5) were not reactive against ConA, while B fractions strongly reacted with the lectin conjugate (Fig. 2, lanes 3 and 6).

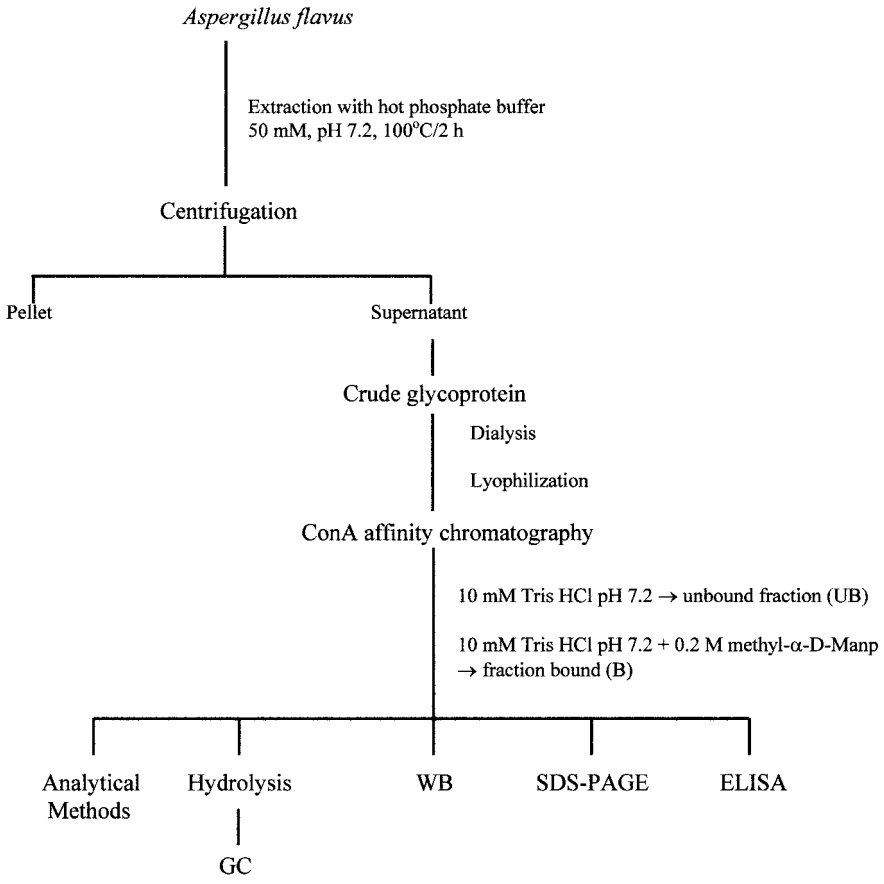

Fig. 1. Isolation and purification of *Aspergillus flavus* glycoprotein. For details of purification, see Materials and Methods.

Table 1. Chemical composition (%) of glycoprotein fractions of *Aspergillus flavus* N and S strains

Fractions	Sugar[a]	Protein[b]	Hexosamine[c]	Phosphate[d]	Monosaccharide Components (%)[e]		
					Man	Gal	Glc
N-crude GP	70	30	tr[f]	tr	62	32	6
N-UB	53	47	tr	tr	37	33	30
N-B	74	26	tr	tr	54	38	8
S-crude GP	65	35	tr	tr	43	46	11
S-UB	37	63	tr	tr	19	48	32
S-B	75	25	tr	tr	47	24	29

[a] Dubois *et al.* (1956).
[b] Lowry *et al.* (1951).
[c] Belcher *et al.* [3].
[d] Ames [1].
[e] *Determined by GC as alditol acetate derivatives.
[f] tr, traces.

Effect of chemical oxidation of carbohydrates on the antigenicity of the B fraction. The immunological relevance of the carbohydrate moiety of the B fraction was demonstrated in this study. The B fraction was treated with sodium metaperiodate, and its reactivity with rabbit hyperimmune serum was evaluated by ELISA. This chemical treatment reduced almost completely the recognition of the B fraction by antibodies, suggesting that its carbohydrate portion significantly contributes to antigenicity (Fig. 3). Similar results were described for peptidogalactomannans isolated from the mycelial surface of *A. fumigatus* [10], a 58-kDa antigen

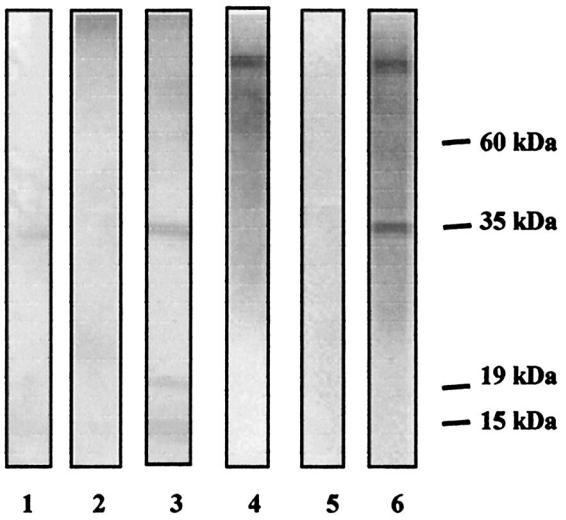

Fig. 2. Western blot analysis of N and S strain fractions of *Aspergillus flavus* probed with a ConA-HPR conjugate. Lanes (1,4) crude glycoprotein; (2,5) UB fraction; (3,6) B fraction.

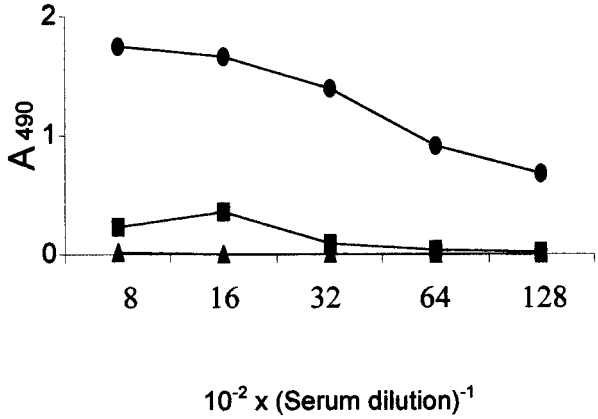

Fig. 3. Antigenicity of *Aspergillus flavus* untreated N-B (circles) and periodate-treated (squares) fractions probed with rabbit hyperimmune serum in ELISA experiments. Control of the reactivity of pre-immune rabbit serum is also shown (triangles).

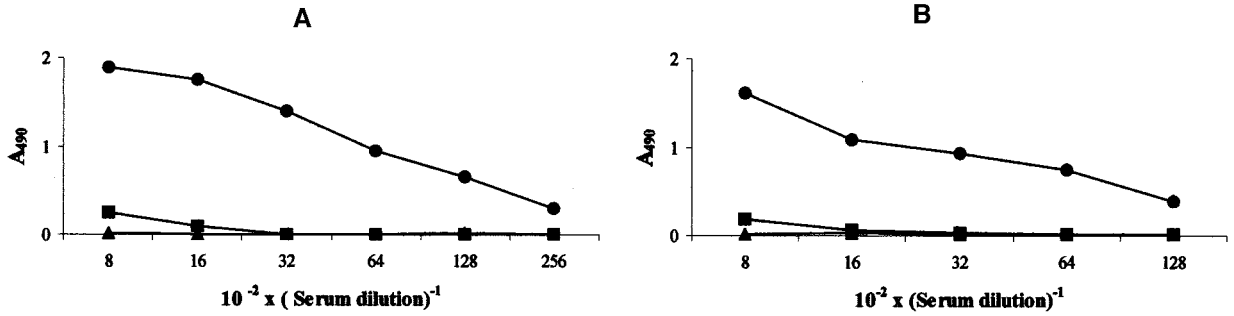

Fig. 4. ELISA experiments showing the antigenic reactivity of *Aspergillus flavus* N-strain fractions against rabbit hyperimmune serum. (a) UB fraction (squares), and B-fraction (circles). (b) Crude glycoprotein preparation (circles) or pre-incubation with B fraction (squares). Control systems in this experiment are represented by the reactivity of pre-immune rabbit serum (triangles).

from the mycelial extract of *A. fumigatus* [8] and other fungal antigens [19, 24].

Immunological reactivity of crude glycoprotein, UB and B fractions with sera from patients with ABPA. The reactivity of the fractions, after ConA-chromatography with sera from infected individuals, was determined by ELISA. The B fraction strongly reacted with a rabbit hyperimune serum, while the UB fraction was weakly reactive (Fig. 4a). The antigenic reactivity of the N-strain crude glycoprotein was completely inhibited by pre-incubation of the serum with fraction B (Fig. 4b). This result showed that the B fraction was a major antigenic compound present in the crude glycoprotein. The specificity of the antigen-antibody reaction was tested by using sera from several mycoses and from a patient with tuberculosis (Fig. 5), which revealed the occurrence of a weak cross-reactivity against the B fraction. The wide distribution and similar chemical structures of the galactomannans shared by pathogenic fungi including *C. immitis, H. capsulatum*, and *P. brasiliensis* [2] could be responsible for these serological cross-reactions. These results indicate that, under controlled conditions, serology with the B antigen may be a tool for differentiating infections caused by *Aspergillus* spp. from those caused by other fungi.

Immunoblotting analysis of the ConA-binding fractions. Following SDS-PAGE, the B fractions from the *Aspergillus* N and S strains were electrotransferred to PVDF membranes and probed with sera from patients with various forms of aspergillosis. The immunoblot pattern in 25 patients with aspergillosis revealed that the 35-kDa band of *A. flavus* is recognized by sera from IA, ABPA, and aspergilloma patients and is the immunodominant antigen present in the ConA-binding fraction of *A. flavus* (Fig. 6 a, b, c, d). There were no reactions on

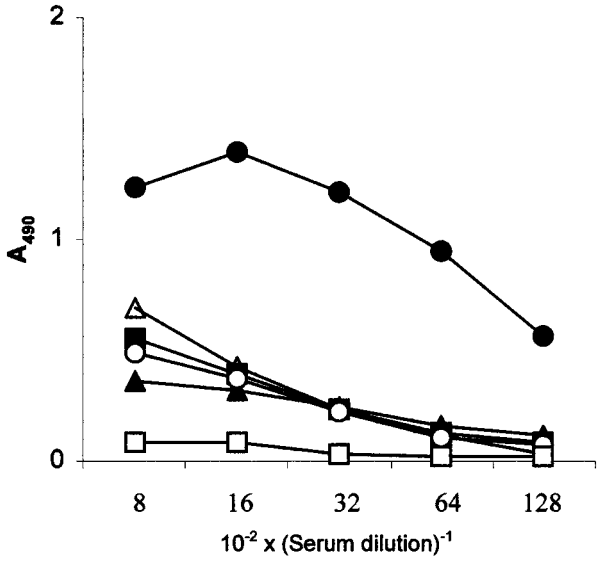

Fig. 5. Reactivity of *Aspergillus flavus* crude glycoprotein with sera from patients with aspergillosis (closed circles), cryptococcosis (open circles), coccidioidomycosis (closed squares), paracoccidioidomycosis (open circles), and tuberculosis (closed triangles). Sera from healthy individuals were also assayed (open squares).

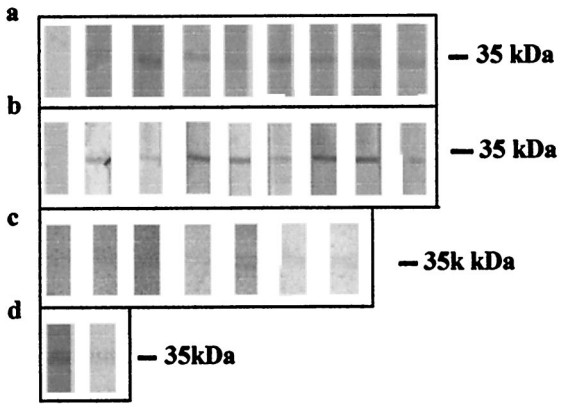

Fig. 6. Reactivity of GP35 isolated from N-strain of *Aspergillus flavus* with sera from patients with ABPA (a,b), invasive aspergillosis (c), and aspergilloma (d). Reactivity of sera from healthy individuals is shown in lane 1 (a, b).

the immunoblots incubated with a pool of normal sera (Fig. 6 a, b; lane 1). Recently, Yuen et al. [23] and Chan et al. [4] reported that patients with aspergilloma and invasive aspergillosis due to *A. fumigatus* developed specific antibodies against Afmp1p, an antigenic cell wall galactomannoprotein of *A. fumigatus*.

Knowledge of shared or unique components among different fungi is necessary for proper diagnosis and treatment of patients with respiratory mycoses. Little is known about the allergens of *A. flavus*. An alkaline serine proteinase was characterized as a major allergen of *A. flavus* that cross reacts with *A. fumigatus* and *P. citrinum* [6]. The ConA-binding antigen described in our study was expressed by both strains of *A. flavus;* it may be a useful tool for the diagnosis of some forms of aspergillosis.

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Literature Cited

1. Ames BN (1966). Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118
2. Azuma I, Kanetsuna F, Tanaka Y, Yamamura Y, Carbonell LM (1974) Chemical and immunological properties of galactomannans obtained from *Histoplasma duboisii, H. capsulatum, Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. Mycopathol Mycol Appl 54:111–125
3. Belcher RA, Nutten AJ, Sambrook CM (1954) The determination of glucosamine. Analyst 79:201–208
4. Chan CM, Woo PC, Leung AS, Lau SKP, Che XY, Cao L, Yuen KY (2002) Detection of antibodies specific to an antigenic cell wall galactomannoprotein for serodiagnosis of *Aspergillus fumigatus* aspergillosis. J Clin Microbiol 40:2041–2045
5. Chhabra A, Handa KK, Chakrabarti A, Mann SB, Panda N (1996) Allergic fungal sinusitis: clinicopathological characteristics. Mycoses 39:437–441
6. Chou H, Lin WL, Tam MF, Wang S, Han S, Shen H (1999) Alkaline serine proteinase is a major allergen of *Aspergillus flavus*, a prevalent airborne *Aspergillus* species in the Taipei area. Int Arch Allergy Immunol 119:282–290
7. Dubois M, Gillies KA, Hamilton JK, Rebers PA, Smith E (1956) Colorimetric method for determination of sugar and related substances. Anal Chem 28:350–356
8. Fratamico PM, Buckley HR (1991) Identification and characterization of an immunodominant 58-kilodalton antigen of *Aspergillus fumigatus* recognized by sera of patients with invasive aspergillosis. Infect Immun 59:309–315
9. Glass WF, Briggs RC, Hnilica LS (1981) Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Anal Biochem 115:219–224
10. Haido RMT, Silva MH, Ejzemberg R, Leitão EA, Hearn VM, Evans EGV, Barreto-Bergter E (1998) Analysis of peptidogalactomannans from the mycelial surface of *Aspergillus fumigatus*. Med Mycol 36:313–321
11. Haynes KA, Tuinstra P, Hughes TA, Wijnands LM, Rogers RR, Allens AK (1996) Purification and characterization of a 93 kDa *Aspergillus fumigatus* antigen with diagnostic potential. J Med Vet Mycol 34:421–426
12. Hetherington SV, Henwick S, Parham DM, Patrick CC (1994) Monoclonal antibodies against a 97-kilodalton from *Aspergillus flavus*. Clin Diagn Lab Immunol 1:63–67
13. Iwen PC, Rupp ME, Hinrichs SH (1997). Invasive mold sinusitis: 17 cases in immunocompromised patients and review of the literature. Clin Infect Dis 24:1178–1184
14. Katzenstein ALA, Sale SR, Greenberger PA. (1983) Allergic *Aspergillus* sinusitis: a newly recognized form of sinusitis. J Allergy Clin Immunol 72:89–93

15. Kurup VP, Ramasamy M, Greenberger PA, Fink JN (1988) Isolation and characterization of a relevant *Aspergillus fumigatus* antigen with IgG and IgE binding activity. Int Arch Allergy Appl Immunol 86:176–182
16. Latgé JP, Kobayashi H, Debeaupúis JP, Diaquin M, Sarfati J, Wieruszeski JM, Parra E, Bouchara JP, Fournet B (1994) Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. Infect Immun 62:5424–5437
17. Lowry OH, Rosebrough NJ, Fan AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem 193: 265–275
18. Morgan MA, Wilson WR, Neel HB, Roberts GD (1984) Fungal sinusitis in healthy and immunocompromised individuals. Am J Clin Pathol 82:597–601
19. Penha CVL, Lopes Bezerra LM (2000) Concanavalin A-binding cell wall antigens of *Sporothrix schenckii:* a serological study. Med Mycol 38:1–7
20. Sawardeker JS, Sloneker JH, Jeanes H (1965). Quantitative determination of monosaccharides as their acetates by gas-liquid chromatography. Anal Chem 37:1602–1604
21. Voller A, Bidwell D, Bartlett A (1976) Enzyme-linked immunosorbent assay. In: Rose NR, Freidman HM, eds. Manual of clinical immunology. Washington DC: American Society of Microbiology, pp. 506–512
22. Yu CJ, Chiou SH, Lai WY, Chiang BL, Chow LP (1999) Characterization of a novel allergen, a major IgE-binding protein from *Aspergillus flavus*, as an alkaline serine protease. Biochem Biophys Res. Commun 11:669–675
23. Yuen KY, Chan CM, Chan KM, Woo PC, Leung AS, Cao L (2001) Characterization of *AFMP1:* a novel target for serodiagnosis of aspergillosis. J Clin Microbiol 39:3830–3837
24. Zancopé-Oliveira RM, Bragg SL, Reiss E, Peralta JM (1994) Immunochemical analysis of the H and M glycoproteins from *Histoplasma capsulatum*. Clin Diagn Lab Immunol 1:563–568