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Identification and Characterization of an Immunodominant 58-Kilodalton Antigen of Aspergillus fumigatus Recognized by Sera of Patients with Invasive Aspergillosis

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Received 1 June 1990/Accepted 5 October 1990

Sera from 38 patients with invasive aspergillosis were tested by Western immunoblotting for the presence of antibodies to antigens of Aspergillus fumigatus present in a mycelial extract of the organism. All of the sera contained antibodies to an antigen of molecular weight 58,000, which was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was the only antigen recognized in approximately 90% of the sera tested. The 58-kDa antigen is an abundant component of mycelial extracts composing approximately 50% of the Coomassie blue-stained protein. The antigen also contains carbohydrate, since it is stained by the carboxyamide stain periodic acid-Schiff and it binds to the lectin concanavalin A. It was purified by immuno-affinity chromatography employing a monoclonal antibody directed against an epitope on the 58-kDa antigen. Analysis of the purified antigen by gas-liquid chromatography revealed the presence of mannose, galactose, and glucose residues in a 2:1:2 ratio. The ratio of protein to carbohydrate is 1.16:1. The protein is slightly acidic, containing relatively high quantities of glutamic and aspartic acids, glycine, alanine, serine, and threonine. The 58-kDa antigen also contains phosphate groups as part of its structure. Serological activity was totally destroyed after treatment with sodium metaperiodate and was partially destroyed after treatment with pronase. The 58-kDa antigen was not able to hydrolyze protein.

During recent years there have been marked increases in the incidence of invasive aspergillosis in immunocompromised patients, especially those with acute leukemia (12, 27, 42). Invasive infection is usually fatal in these patients (6, 27, 42), since antemortem diagnosis of aspergillosis remains problematic (41, 42). A definitive diagnosis of the disease requires histologic demonstration of hyphae in tissue. However, many immunocompromised patients cannot withstand invasive procedures such as open-lung biopsy. Blood cultures are virtually always negative (27, 42), and many patients have repeatedly negative sputum cultures (11, 27, 42). Nose cultures positive for Aspergillus species may be of some value in predicting a subsequent invasive aspergillosis infection (1). Early diagnosis of the disease would lead to more effective therapy and an increase in the survival rate.

Although the humoral antibody response to Aspergillus species in immunocompromised patients is weak, these assays have been developed for the detection of Aspergillus-specific antibodies. Immunodiffusion and counterimmunoelectrophoresis are the most widely used assays for measurement of antibody (5, 11, 23, 27, 31). Other methods that have been used include passive hemagglutination (15), radioimmunoassay (23), enzyme-linked immunosorbent assay (16), and immunoblotting (24). These tests are of value for diagnosis of other forms of aspergillosis, but they often fail in the immunocompromised patient with invasive disease even when more sensitive methods such as radioimmunooassay or enzyme-linked immunosorbent assay are used. Furthermore, it has been difficult to evaluate and compare these methods, since assay procedures and antigenic preparations have not been standardized and patient populations tested have differed. Immunoassays for antigen detection in serum appear to be more promising, since they do not depend upon normal host immune function. Most of these methods involve detection of an Aspergillus polysaccharide antigen (14, 30, 33, 34, 37, 39, 40), which appears to be cell wall galactomannan (7, 8, 19, 28). In some of these assays the antigen detected in vivo has not been well characterized. Although in the past 10 years much effort has been placed in the development of these methods, none has gained widespread acceptance. Improvements in sensitivity and practicality are needed before any of these assays becomes commercially feasible.

The use of highly purified, well-characterized preparations of serodiagnostically important Aspergillus antigens would aid in the development of a more sensitive and accurate immunoassay for invasive aspergillosis. The antigens could be utilized in both antibody and antigen detection assays. The immunoblot technique is a relatively simple and very useful method for identifying and characterizing by molecular weight the antigens to which an antigenic response is elicited (24). In this report we describe the identification by Western immunoblotting and the characterization of an immunodominant antigen of Aspergillus fumigatus which is recognized by antibodies in the sera of patients with invasive aspergillosis.

MATERIALS AND METHODS

Organism and culture conditions. A. fumigatus (strain F92) was obtained from the culture collection of the Department of Microbiology, The Thomas Jefferson University, Philadelphia, Pa. It was isolated from a sputum specimen from a patient with an aspergilloma. The organism was grown on Sabouraud plates for 5 to 10 days at 25°C. Conidia were harvested from mycelia by washing the plates with sterile 0.5% Tween 80. The suspension was inoculated into Czapek-Dox broth (Difco Laboratories, Detroit, Mich.) (approxi-
mately 8 x 10^6 conidia per ml of broth) and incubated on a gyratory shaker at 200 rpm for 4 days at 37°C.

Preparation of mycelial extract (ME). The mycelial portion was separated from the medium by filtering through 3MM (Whatman International, Ltd., Maidstone, England) chromatography paper. The mycelial fragments in phosphate-buffered saline (PBS; 0.05 M sodium phosphate [pH 7.4], containing 8% [w/vol] NaCl and 1 mM phenylmethylsulfonyl fluoride) were mechanically disrupted with glass beads (0.25-mm diameter) in a Braun homogenizer (B. Braun, San Francisco, Calif.). Hyphae were separated from these fragments by centrifugation at 17,000 × g for 20 min. The supernatant was again centrifuged (80,000 × g, 90 min, 4°C) and was filtered through Nalgene (Nalge Co., Rochester, N.Y.) 0.45-μm pore-size filter units and stored at −70°C. Protein was determined by the method of Lowry et al. (22).

ConA affinity chromatography. The ME was passed through concanavalin A (ConA)-Sepharose (Pharmacia, Uppsala, Sweden) affinity columns. The equilibration buffer was 0.05 M Tris hydrochloride (pH 7.4) containing 0.02% (wt/vol) NaN3, 0.001 M MgCl2, 6H₂O, and 0.001 M CaCl₂, 2H₂O. The unbound fraction (UBF) was collected, and the column was washed with equilibration buffer. To determine whether binding to ConA was specific or whether nonspecific electrostatic or hydrophobic interactions were involved, 4 M NaCl and 0.25% (vol/vol) Triton X-100 in equilibration buffer were passed through the column before elution with α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo.). The bound fraction (BF) was eluted from the column with 0.4 M α-methyl-D-mannoside in equilibration buffer. The fractions were dialyzed overnight against 0.01 M NH₄HCO₃ (pH 8.0), concentrated by using Centricon ultralfilter units (Poly-sciences, Inc., Warrington, Pa.) with a molecular weight cutoff of 10,000, and stored at −70°C. The BF and the UBF were tested for reactivity with rabbit and patient serum by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and Western blotting (36).

SDS-PAGE. The ME and ConA fractions were prepared for SDS-PAGE by the method of Laemmli (18). Briefly, samples were mixed with an equal volume of sample buffer and heated in a boiling water bath for 6 min. Electrophoresis was performed with 12.6% SDS gels. The running buffer was 0.025 M Tris hydrochloride (pH 8.3) containing 0.192 M glycine and 0.05% (wt/vol) SDS. Gels were either stained for protein with 0.1% Coomassie blue R-250 or for carbohydrate with periodic acid-Schiff stain (43) or were analyzed by immunoblotting.

Western blotting. Proteins were electrophoretically transferred from gels to nitrocellulose paper (0.45-μm pore size; Hoefer Scientific Instruments, San Francisco, Calif.) by the method of Towbin et al. (36). Gels (0.75 mm thick) were transferred for 35 min at 0.5 A in a Transphor transfer cell (model TE 52; Hoefer). The blotting buffer was 0.025 M Tris hydrochloride (pH 8.3) containing 0.192 M glycine and 20% (vol/vol) methanol. After transfer, the nitrocellulose paper was blocked in 5% (wt/vol) nonfat dry milk in PBS, rinsed in PBS containing 0.05% (wt/vol) Tween 20, and incubated overnight at room temperature with monoclonal antibody (MAb) or human or rabbit serum diluted 1:250 in PBS containing 2% normal goat serum. The nitrocellulose paper was washed in PBS–Tween 20 and incubated with goat anti-human, anti-rabbit, or anti-mouse immunoglobulin G, A, or M (H+L chains) conjugated to alkaline phosphatase (0.1 mg/ml; Kirkegaard and Perry, Gaithersburg, Md.) diluted 1:500 in PBS containing 2% normal goat serum. The immunoblot was developed with a 5-bromo-4-chloro-3-indolyl-phosphate–Nitro Blue Tetrazolium substrate.

Purification of 58-kDa antigen. The 58-kDa antigen was purified by immunoaffinity chromatography. 2.2-9, an immunoglobulin G1(x) MAb against the 58-kDa antigen, was purified from mouse ascites fluid by using the Affi-Gel protein A MAMS II kit (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions provided with the kit. Purified MAb was then coupled to Affi-Gel 10 (Bio-Rad) with 0.1 M NaHCO₃ buffer (pH 8.5), and a column was prepared. After equilibration with 0.05 M NaHCO₃ buffer (pH 8.0), the ConA BF was passed through the column. After the column was washed with equilibration buffer to remove unbound material, 0.1 M glycine (pH 2.5) was passed through the column to collect the BF. The pH was brought to 7.0 with 1 M Tris. The bound material was concentrated by using Centricon ultralfilter units (molecular weight cutoff, 10,000) and dialyzed against distilled water. A second column was prepared in the same manner but without coupling MAb to the Affi-Gel 10. This control was used to check for nonspecific binding of SDS–polyacrylamide gels and stained for carbohydrate with the periodic acid-Schiff stain by the method of Zacharius et al. (43). The fuchsin-sulfite stain was prepared by the method of Mcguckin and Mckenzie (25).

Characterization of the 58-kDa antigen. (i) Periodic acid-Schiff staining. Components in the ME, the ConA BF and samples of affinity-purified 58-kDa antigen were separated on 12.6% SDS–polyacrylamide gels and stained for carbohydrate with the periodic acid-Schiff stain by the method of Zacharius et al. (43). The fuchsin-sulfite stain was prepared by the method of Mcguckin and Mckenzie (25).

(ii) Gas-liquid chromatography. Carbohydrate analysis was performed with purified 58-kDa antigen on a Varian 3700 gas chromatograph (Varian, Sunnyvale, Calif.) with a 3% SE-30 column (Supelco, Inc., Bellefonte, Pa.) by the method of Clamp et al. (4) with some modifications. Methanolic HCl (500 μl; Supelco) was added to ampoules containing lyophilized samples and standards. The ampoules were sealed under vacuum, and the samples were hydrolyzed for 18 to 22 h at 100°C. The standards included D-glucose, D-mannose, and D-galactose, and all ampoules contained l-arabinitol as the internal standard. The samples were transferred to 1.5-ml polypropylene tubes and neutralized by the addition of silver carbonate. After a 10-min incubation, acetic anhydride was added, and the tubes were incubated for 3 h at room temperature with occasional vortexing. The samples were centrifuged, and the supernatants were placed in clean glass tubes and evaporated to dryness. The samples were washed twice with methanol, hydrolyzed with 500 μl of 0.5 N methanolic HCl, and heated at 65°C for 10 min. O-Tri-methylsilyl (TriSil-Z; Pierce Chemical Co., Rockford, Ill.) (100 μl) was added to each dried sample, and the tubes were heated at 65°C for 20 min to ensure complete derivatization. Chloroform (100 μl) and distilled water (500 μl) were added, and the tubes were centrifuged. The organic layer was washed twice with distilled water, and 5-μl samples were injected into the gas chromatograph.

(iii) Amino acid analysis. An amino acid analysis of the immunoaffinity-purified 58-kDa antigen was performed in the Biochemistry Department, Temple University, on an MM-70 amino acid analyzer (Glenco Scientific, Houston, Tex.). The sample was hydrolyzed in 6 N HCl for 21 h at 110°C.

(iv) Estimation of phosphate. The immunoaffinity-purified 58-kDa antigen was analyzed for the presence of phosphate groups by the method of Lowry and Lopez (21) with some modifications. Samples and NaH₂PO₄·H₂O standards (0 to 60 nM) were digested with a solution containing 30% (vol/vol) concentrated H₂SO₄ and 6.7% (vol/vol) HClO₄. The
samples were heated at 165°C for 2 to 3 h, and 1 ml of a solution containing 0.1 M sodium acetate, 0.25% (wt/vol) ammonium molybdate, and 0.06 M ascorbic acid was added. The tubes were then incubated in a water bath at 38°C for 2 h, and the A250 of the samples was measured.

(v) Pronase and sodium metaperiodate treatments. The ME (1 mg of protein) was incubated with 20 μg of pronase (Sigma) for 24 h at 37°C. Then 0.2 M sodium metaperiodate (NaIO₄; Sigma) was added to 1 mg of mycelial extract to give a final concentration of 0.05 M NaIO₄. Tubes were incubated at room temperature in the dark for 18 h, and the reaction was terminated by the addition of ethylene glycol (9). Samples from both treatments were applied to SDS-polyacrylamide gels, and the gels were silver stained (26) and analyzed by Western blotting.

Reagents. (i) Rabbit serum. Hyperimmune rabbit serum against a mycelial extract of *A. fumigatus* was raised by three subcutaneous injections of 0.7 to 1.0 mg of ME protein in incomplete Freund adjuvant.

(ii) Human sera. Human sera were provided by William Merz, Johns Hopkins University Hospital, Baltimore, Md.; Thomas Walsh, National Cancer Institute, Bethesda, Md.; and George Talbot, Hospital of the University of Pennsylvania, Philadelphia.

These sera were from patients diagnosed as having an invasive form of aspergillosis, with the majority having pulmonary involvement. Most of these patients were neutropenic and had an acute form of leukemia such as acute myelogenous or acute lymphocytic leukemia, or they had cancer. One patient had *Aspergillus* endocarditis, one had tuberculosis which led to acute necrotizing aspergillosis, two were transplant recipients, and one had *Aspergillus* sinusitis. The species isolated from patient specimens was either *A. fumigatus* or *Aspergillus flavus*. The normal human serum pool was obtained from healthy, nonhospitalized men and women.

(iii) Molecular weight standards. The low-molecular-weight standards were obtained from Pharmacia, Uppsala, Sweden. The preparation included phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa.

RESULTS

Fractionation of ME by ConA chromatography. The ME prepared by mechanical disruption of hyphae contained both cytoplasmic and cell wall components. The ME was fractionated by ConA affinity chromatography to separate components containing α-d-glucose and/or α-d-mannose residues. The components of the ConA column fractions were separated by SDS-PAGE. The UBF contained at least 29 Coomassie blue-stained bands ranging in molecular weight from >94,000 to 14,000 (Fig. 1). The BF contained components of approximately 80, 40, 38, 29, and 16 kDa and a major component of approximately 58 kDa (52 to 62 kDa). No major Coomassie blue-stained bands were detected in the fractions eluted with 4 M NaCl (lane 3) or 0.25% Triton X-100 (lane 4). Both fractions were concentrated, and almost the entire material was applied to the gel. Lane 6 is an immunoblot of the ConA BF incubated with MAb 2-2-9 directed against the 58-kDa antigen (13). The antibody reacted with a broad band at 58 kDa.

Immunoblot analysis of *A. fumigatus* antigens. Sera from 38 patients with invasive aspergillosis (a total of 172 serum samples) were tested by Western blotting for the presence of antibodies to antigens in the ME and in the ConA UBF and BF. Representative immunoblots are shown in Fig. 2. All of the sera studied had antibodies to an antigen with a molecular weight of 58,000. The 58-kDa antigen was also detected in the ConA BF (lane 2). In some of the immunoblots it appeared as a very broad band (52 to 62 kDa) (lanes 5 and 7), and occasionally there was diffuse background staining (lane 5). In approximately 90% of the sera tested, the 58-kDa antigen was the only antigen recognized. Blots 1 to 7 were incubated with sera from patients with invasive aspergillosis, all of whom were immunosuppressed. Blots 8 and 9 were incubated with serum from two patients who had an invasive form of aspergillosis but who were not immunosuppressed. They produced antibodies to the 58-kDa antigen, but their sera reacted also with at least eight other components in the ME. Hyperimmune rabbit serum (lane 10) also reacted with numerous *Aspergillus* antigens as well as the 58-kDa antigen. There was no reaction on immunoblots incubated with normal human serum (lane 11).

Purification and staining of the 58-kDa antigen. The highly
immunogenic 58-kDa antigen was purified by immunoaffinity chromatography with MAb 2-2-9, and it was partially characterized. Lanes 3 and 7 of Fig. 3 show the purified 58-kDa antigen stained with Coomassie blue and periodic acid-Schiff, respectively. There was a diffuse Coomassie blue-stained band at approximately 37 to 40 kDa which may be degradation products of the 58-kDa antigen (lane 3). A band of similar molecular weight (37,000 to 40,000) present in the ConA BF (lane 6) was stained by periodic acid-Schiff. The Coomassie blue-stained 58-kDa band in the ME and ConA BF (lanes 1 and 2) also was stained with periodic acid-Schiff (lanes 5 and 6). No protein was stained with Coomassie blue, and carbohydrate was not detected with periodic acid-Schiff in the fraction eluted from the column to which MAb was not bound (lanes 4 and 8, respectively). A silver-stained gel of the immunoaffinity-purified material showed only one major band at 58 kDa and a faint, diffuse 37- to 40-kDa band (data not shown).

Characterization of the 58-kDa antigen. Gas-liquid chromatography was employed for the analysis of the monosaccharide components of the 58-kDa antigen. The gas-liquid chromatogram of the immunoaffinity-purified antigen showed peaks which were identified as l-arabininol (internal standard), mannose, galactose, and glucose based on the retention times of known standards (Fig. 4). Galactose and glucose gave rise to three peaks and two peaks, respectively. The amount in micromoles of each component sugar in the mixture was read from the standard curve. The ratio of mannose to galactose to glucose was 2:1:2. Protein was measured by the method of Bradford (3). The ratio of protein to total carbohydrate was 1.16:1.

Results of the analysis of the amino acid composition of the immunoaffinity-purified 58-kDa antigen are shown in Table 1. The protein contains relatively high amounts of the acidic amino acids aspartic and glutamic acids. Threonine, serine, glycine, and alanine are also present in relatively high quantities, whereas cysteine, methionine, and lysine are present in low quantities. Tryptophan was not measured, since hydrolysis under strongly acidic conditions brings about complete destruction of this amino acid.

There have been reports describing the presence of phospho in Aspergillus cell walls (29) and of phosphate linked to fungal glycoproteins (20, 32). The 58-kDa antigen contains phosphate as part of its structure. Total phosphate of the immunoaffinity-purified 58-kDa antigen was estimated to be 1.1 mol of phosphate per mol of 58-kDa antigen.

ME was treated with pronase and sodium metaperiodate and was then tested for loss of serological activity by Western blotting with serum from patients with invasive aspergillosis. Pronase has nonspecific proteolytic activity and cleaves at many sites on the peptide chain. Periodate oxidation results in cleavage of the bond between carbon atoms substituted with hydroxyl groups. The intensity of the immunoreactive 58-kDa band decreased approximately 50% after treatment with pronase, but serological activity was eliminated (no immunoreactive bands) after treatment with periodate (data not shown).

The 58-kDa antigen was tested for the ability to hydrolyze protein. Purified antigen was not able to cleave azocoll or azocasein substrates, indicating that it is probably not a protease.

**DISCUSSION**

Since A. fumigatus is present in the hyphal form in lesions during an invasive infection, a mycelial extract prepared from the organism cultured at 37°C was used in this study. In this report we describe the identification and characterization of an immunodominant antigen present in mycelial extracts of A. fumigatus. The molecular weight of this antigen, determined by SDS-PAGE, is 58,000. However, depending on the batch of ME used, the antigen may appear as a broad band of 52 to 62 kDa. Also, the antigen sometimes appeared as a broad band, frequently with diffuse staining above and below the 58-kDa band on immunoblots incubated with patient serum (Fig. 2, lanes 5 and 7). The 58-kDa antigen was able to elicit an antibody response in patients with invasive aspergillosis and in rabbits immunized with ME subcutaneously. All of the patient sera tested reacted with epitopes on the 58-kDa antigen. Several of the sera...
The 58-kDa antigen was dissociated by the saccharide α-methyl-D-mannoside. The antigen was not detected in the ConA UBF. When the ConA BF and UBF were analyzed by immunoblotting with patient serum, the 58-kDa antigen was detected only in the ConA BF (Fig. 2).

The 58-kDa antigen contains phosphate groups as part of its structure. In fungal polysaccharides, phosphodiester linkages serve to link short oligosaccharides together to form high-molecular-weight polymers (17). Bortolotto (35) found that phosphate was involved in linking single sugar side chains of mannose to the mannan main side chains. The peptido-phospho-galactomannan complex of Cladosporium werneckii studied by Lloyd (20) contained phosphodiester linkages in the main chain of the polymer; the complex was also involved in linking side chains to the galactomannan main chain. Further studies are needed to determine the position and function of the phosphate groups in the 58-kDa molecule.

Analysis of the immunoaffinity-purified 58-kDa antigen by gas-liquid chromatography showed that mannose, galactose, and glucose were the monosaccharide components of the carbohydrate portion (Fig. 4). Galactoglucomannans have been isolated from the cell walls of seeds and plants (35). Akiyama et al. (2) showed that extracellular galactomannans in a suspension of cultured tobacco cells were composed of alternating units of D-mannose and D-glucose residues, and about two-thirds of the mannose residues carried side chains of galactose. Since the ratio of mannose-galactose-glucose in the carbohydrate moiety of the 58-kDa antigen is 2:1:2, there may be alternating units of mannose and glucose and a portion of the mannose residues may be substituted with galactose. At present the structure of the 58-kDa antigen is not known.

On Coomassie blue-stained gels of the immunoaffinity-purified 58-kDa antigen, a faint, diffuse 37- to 40-kDa band was also detected. When phenylmethylsulfonyl fluoride, a serine protease inhibitor, was not added during the preparation of ME or the ConA column fractions, there was a gradual decrease in the intensity of staining of the 58-kDa band on gels with a concomitant increase in the appearance of the 37- to 40-kDa band. This occurred after several freezing and thawing cycles of the samples or after storage at room temperature for several hours. Therefore, it appears that the 37- to 40-kDa band is related to the 58-kDa antigen and may actually be a degradation product. Furthermore, MAb 2-2-9 does not recognize the 37- to 40-kDa band by immunoblotting after transfer of proteins from denaturing or nondenaturing gels. Therefore, it should not bind to the immunoaffinity column. It is possible that the 37- to 40-kDa band is a degradation product produced by proteolysis of the 58-kDa antigen and that the degraded form is no longer recognized by the MAb. No protein or carbohydrate was stained on gels containing the fraction eluted from the column to which MAb was not bound (Fig. 3, lanes 4 and 8). Therefore, the 37- to 40-kDa band does not bind nonspecifically to the Affi-Gel 10 beads.

The relationship of the 58-kDa antigen to the immunoreactive carbohydrate conjugate purified by Weiner and Coats-Stephen (38) is not clear. Both stain with Coomassie blue and periodic acid-Schiff, bind to ConA, and are destroyed by perioate oxidation. The antigen identified by Weiner and Coats-Stephen appeared to be in polymeric form, since the molecular weight ranged from 45,000 to 300,000. The rela-

### Table 1. Amino acid composition of the 58-kDa antigen

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>nmol</th>
<th>μg of amino acid</th>
<th>No. of residues</th>
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<tr>
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<tr>
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<td>Leu</td>
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<tr>
<td>Arg</td>
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* a Tryptophan was not determined.
* b Nanomoles of each amino acid in 100 μg of protein. Protein was measured by the method of Bradford (3).
tionship of the 58-kDa antigen to cell wall galactomannan antigens found to be present in patient serum is also unclear. The 58-kDa antigen contains glucose residues as well as galactose and mannose, and protein accounts for approximately one-half of the molecular weight. Whether the 58-kDa antigen is present and detectable in patient serum during an invasive infection is not yet known.

ACKNOWLEDGMENTS

We thank Gerald Shockman for his discussions and for the use of his laboratory equipment. We are grateful to Reiko Kariyama for her excellent technical assistance in the use of the gas-liquid chromatograph. We thank Medical Communications for their photographic assistance and Gregory Harvey for his editorial assistance.

This work was supported in part by NRSRA Institutional Award 5 T32 AI-07101 from the National Institutes of Health.

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