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Detection of Galactomannan Antigenemia by Enzyme Immunoassay in Experimental Invasive Aspergillosis

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A sensitive enzyme immunoassay (EIA) for galactomannan antigenemia that avoids the use of radioisotopes was devised. Three carbohydrate-rich antigenic fractions were purified from *Aspergillus fumigatus* 2085: a cold alkali extract (CA) from mycelium, an acetone-precipitated pyridine extract (APSK-66) from mycelium, and a methanol precipitate from culture filtrate. CA and APSK-66 were further purified by gel filtration and ion-exchange chromatography, respectively. An acid hydrolysate of CA contained only mannose and galactose, as determined by gas-liquid chromatography. Rabbit antisera were raised against conidia, mycelia, and cell walls of *A. fumigatus*. By indirect EIA, the best immunoglobulin G response (1/8,000) was obtained against CA in rabbits immunized intravenously with cell walls. Antigenemia was detected by indirect EIA inhibition in heat-dissociated sera of four immunosuppressed rabbits that were infected intravenously but was absent in two uninfected controls. The circulating antigen was resistant to pronase, was adsorbed onto concanavalin A, and had a molecular size of 50 to 100 kilodaltons.

Invasive aspergillosis is an increasingly important cause of morbidity and mortality in immunocompromised patients, especially those with acute leukemia (23). Rapid diagnosis is of great importance because early treatment may be successful in resolving this potentially fatal infection (2). Unfortunately, diagnosis of invasive aspergillosis remains difficult. *Aspergillus* species can be cultured from antemortem sputum specimens in only 8 to 34% of cases (18, 34), and cultures of blood (18, 33) and urine (18) are almost uniformly negative. Fungal surveillance cultures may help predict invasive aspergillosis (1). Severe thrombocytopenia may limit transtracheal aspiration or lung biopsy, which have led to antemortem diagnosis in some patients (2). Detection of antibodies to the *Aspergillus* species by a number of immunoassay formats (14) has been used for many years, but unfortunately, the predictive value of these methods is unsatisfactory in profoundly immunosuppressed patients who are at greatest risk of invasive aspergillosis.

A different approach currently under investigation involves the detection of a polysaccharide antigen of the *Aspergillus* species (3, 4, 10, 17, 19, 22, 24, 27, 28, 30–32) that appears to be cell wall galactomannan (17, 19). Although existing methods for detecting this antigen seem promising, they have several limitations, including the short shelf life of reagents used in a radioimmunoassay (31), the inability to detect antigen concentrations in the low nanogram per milliliter range (28, 31), or a lengthy 48-h dialysis step, which is unsuitable for clinical use (24). In addition, the antigen, which is detected in vivo, has not been characterized in several of these assays. In this report we describe a simple enzyme immunoassay (EIA) for the detection of galactomannan from serum which can be completed in 4 h.

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MATERIALS AND METHODS

Microorganisms and culture conditions. *Aspergillus fumigatus* 2085 was obtained from the culture collection of the Division of Mycotic Diseases, Centers for Disease Control, Atlanta, Ga., where it is maintained as strain B2570. The fungus was cultured in a 17-liter glass carboy (19) filled with 13 liters of Czapek-Dox medium (Difco Laboratories, Detroit, Mich.) supplemented with 10^{-5} M $MnCl_2 \cdot 4H_2O$, 10^{-5} M $FeSO_4 \cdot 7H_2O$, 10^{-5} M $ZnSO_4 \cdot 7H_2O$, 10^{-6} M $CuSO_4 \cdot 5H_2O$, and 10^{-4} M $CaCl_2 \cdot 2H_2O$. Two 1-liter flasks, each containing 300 ml of medium, were seeded with 4-day-old conidia and incubated at 22°C for 48 h with gentle magnetic stirring. The carboy was inoculated with this starter culture, and incubation was continued for 96 h at 22°C with submerged aeration and magnetic stirring. Then, 150 ml of 1% thimerosal (Sigma Chemical Co., St. Louis, Mo.) was added, and the culture was stirred for 24 h without aeration. The growth was filtered over five layers of cotton muslin in a Büchner funnel, and the culture filtrate was reserved at 4°C. The mycelium was washed with 4 liters of 0.85% NaCl and 8 liters of deionized water, squeezed dry, and stored at −70°C.

Preparation of antigens. Galactomannan polysaccharide antigen was prepared by three different methods.

A cold alkali extract of mycelium (CA) was prepared by the method described by Reiss and Lehmann (19) and purified by gel filtration chromatography on Sephacryl S-200 Superfine (Pharmacia, Uppsala, Sweden) to separate galactomannan from glucan (19).

Mycelium was also twice defatted for 2 h with ethyl alcohol-ethyl ether (1:1) and extracted with 50% pyridine at 37°C for 24 h, as described by Azuma et al. (5, 8). The extract was centrifuged at 23,300 × *g* for 50 min, the supernatant was passed through a filter (Seitz), and the filtrate was lyophilized. The residue was dissolved in 70 ml of distilled water and centrifuged at 23,300 × *g* for 20 min, and the supernatant was reserved. The pellet was again

dissolved in 40 ml of distilled water and centrifuged, and the supernatants were pooled. APSK-33 and APSK-66 fractions were obtained by the addition of acetone to a final concentration of 0 to 33% and 33 to 66%, respectively (5, 8). The APSK-66 fraction, containing galactomannan, was chromatographed (5, 8) on a column of ion-exchange resin (AG 50W-X4; Bio-Rad Laboratories, Richmond, Calif.) and eluted with 25 ml of water followed by 56 ml of 0.2 M Na_2HPO_4.

Culture filtrate (7.5 liters) was evaporated under reduced pressure to 650 ml, and methanol was added at 4°C to a final concentration of 80%, as described by Sakaguchi et al. (25, 26) and Suzuki and Hayashi (29). The precipitate was centrifuged for 20 min at 10,400 × *g*, and the pellet was dissolved in 200 ml of water and dialyzed against distilled water for 48 h. The material was centrifuged at 25,000 × *g* for 30 min, and the supernatant (designated MET-S) was lyophilized.

Mannan of *Candida albicans* 20A (serotype A), was kindly supplied by Errol Reiss, Division of Mycotic Diseases, Centers for Disease Control.

Chemical analysis. Fractions were analyzed for total protein content by the Coomassie blue method (Bio-Rad) with respect to a standard of bovine serum albumin (Sigma). Total carbohydrate was estimated by the phenol-sulfuric acid method described by Dubois et al. (15), using glucose as a standard.

The monosaccharide composition of the antigenic fractions was determined as follows. Two milligrams of CA, APSK-66, or MET-S were dissolved in 1 ml of 1 N HCl and kept under N_2 for 6 h at 100°C with gentle magnetic stirring. The solution was neutralized with an equal volume of 1 N NaOH and dried under nitrogen at 37°C. Per-*O*-acetylated aldononitrile derivatives were prepared as described previously (12) and separated on a 30-m fused silica column with an internal diameter of 0.32 mm. The column was wall coated with 0.20 mm SPB-1 (Supelco, Inc., Bellefonte, Pa.). Analysis was performed on a gas chromatograph (5880; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector. Helium was used as the carrier gas and nitrogen was used as the makeup gas. The split ratio was 17:1. The injection port temperature was 315°C and the detector was 320°C. The column temperature was held at 150°C for 5 min and, in a stepwise fashion, reached a plateau of 290°C. Peaks were identified by comparing their retention times with those of authentic standards of D-glucose, D-mannose, and D-galactose (Pfanstiehl Laboratories Inc., Waukegan, Ill.). The area under each peak was calculated with a computing integrator and the molar ratios of monosaccharides determined for each antigenic preparation.

Antisera. New Zealand White rabbits (weight, 2 kg) were immunized by using three different protocols.

Cell walls of *A. fumigatus* 2085 were a gift from Errol Reiss and were prepared as described previously (21). Rabbits were injected once intramuscularly with 2.5 mg of cell walls in 2.5 ml of incomplete Freund adjuvant (Difco). Beginning 7 days later, 500 μg of cell walls, suspended in 0.1 ml of saline, was injected intravenously at weekly intervals for 6 weeks.

Mycelial homogenate was prepared by a modification of the method described by Kim and Chaparas (16). Two grams of dry mycelium was suspended in 25 ml of distilled water and briefly homogenized with a blender. The suspension, further homogenized for 10 min with a mortar and pestle, was lyophilized and reconstituted in sterile saline to contain 40 mg/ml. The suspension was emulsified with an equal volume of incomplete Freund adjuvant, and rabbits were given 4 weekly intramuscular injections of 10 mg of homogenate in 0.5 ml of emulsion.

A suspension of conidia (10^4 conidia per ml of saline) was prepared from *A. fumigatus* 2085 grown for 4 days on Sabouraud dextrose agar. Rabbits were immunized (6) by the subcutaneous injection of 1 ml of conidia suspension, followed by two intravenous injections of 0.5 ml of conidia suspension at 7-day intervals.

Blood was drawn weekly from the central ear artery of the rabbits, beginning at the midpoint of each immunization, and sera were stored at −20°C.

Production of antibodies against CA, APSK-66, and MET-S antigens was quantitated by indirect EIA. Microtiter plates (Immulon 1; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 0.2 ml of a 1-μg/ml solution of antigen in 0.06 M carbonate buffer (pH 9.6). Plates were incubated at 37°C for 3 h and then washed four times with double-strength phosphate-buffered saline (PBS-T; 0.02 M sodium phosphate buffer [pH 7.2], 0.28 M NaCl, containing 0.5 ml of Tween 20 [Fisher Scientific Co., Fair Lawn, N.J.] per liter). Nonspecific adsorption was blocked by incubating the plates with 1% bovine serum albumin (Sigma) and 1% polyvinylpyrrolidone (Sigma) in PBS-T for 30 min at 37°C. The plate was washed once with PBS-T and incubated with serial twofold dilutions of rabbit antisera for 1 h at 22°C. After washing four times with PBS-T, 200 μl of peroxidase-conjugated heavy chain-specific goat anti-rabbit immunoglobulin G (IgG) or IgM (Cappel Worthington, Malvern, Pa.) diluted 1/800 in PBS-T was added to each well. The plate was incubated for 1 h at 22°C and washed four times with PBS-T. *O*-Phenylenediamine (0.5 ml of a stock solution of 10 mg of methanol per ml) was diluted 1:100 with distilled water containing 0.1 ml of 3% hydrogen peroxide, and 200 μl was added to each well. The color was developed in the dark for 40 min at 22°C, and the reaction was stopped with 25 μl of 4 M H_2SO_4. The endpoint was defined as the highest dilution of serum that had an A_{490} of 0.200, as measured in a spectrophotometer (MR-600; Dynatech).

Rabbit model of invasive aspergillosis. Six female New Zealand White rabbits (weight, 2 to 3 kg) were immunosuppressed with daily subcutaneous injections of cortisone acetate (10 mg/kg per day), beginning 2 days before and ending 7 days after infection. After two base-line bleedings from the central ear artery, four of the animals were infected intravenously with 10^6 conidia of *A. fumigatus* 2085. This inoculum was prepared by suspending a 96-h slant culture on Sabouraud glucose medium with 3 ml of 0.9% NaCl, counting the suspension in a hemacytometer, and diluting to yield 10^6 conidia per ml. Blood samples were then drawn every 2 days until death. Autopsies were done on all animals, and both kidneys and a sample of liver were immediately removed for histopathology. A sample of each organ was placed in 10% phosphate-buffered Formalin (pH 7.0) and stained by the Gomori methenamine silver procedure. Two control rabbits received cortisone acetate but were not infected. Histopathology of both kidneys and liver in these control animals was also carried out.

Serum samples from all infected and control rabbits were assayed for galactomannan antigen.

Disseminated candidiasis in the immunosuppressed rabbits. Two female New Zealand White rabbits (weight 2 to 3 kg) were immunosuppressed with cortisone acetate, and after a base-line bleeding they were infected intravenously with 10^7 blastoconidia of *C. albicans* 3181 A, as described previously (11). The animals were sacrificed by exsanguination 4 days

after infection, when peak concentrations of mannan antigen in serum are detected (11). The kidneys, containing multiple microabscesses, were cultured quantitatively by homogenizing the tissue in 5 ml of phosphate-buffered saline (pH 7.2) and serially diluting and plating out the homogenate on Sabouraud glucose medium (11). Serum samples from these rabbits were also assayed for galactomannan antigen.

Inhibition EIA for galactomannan antigen. An EIA inhibition technique was used to detect galactomannan antigenemia. Galactomannan-serum complexes were dissociated (20) by boiling for 3 min in an equal volume of 0.1 M disodium EDTA (pH 7.2). The tubes were chilled, and the gel was centrifuged at 27,000 × *g* for 30 min. The liquid phase was incubated in a glass tube with an equal volume of a 1:250 dilution of anti-*A. fumigatus* cell wall antiserum for 90 min at 22°C. The sera were added, 200 μl per well, to a microtiter plate (Immulon 1) coated with 0.2 ml of a 1-μg/ml solution of CA antigen in 0.06 M carbonate buffer (pH 9.6). Subsequent steps were identical to those of the indirect EIA for antibodies against CA. Each test serum sample and a normal rabbit serum control sample were assayed in triplicate. Additional positive and negative controls were obtained by adding anti-*A. fumigatus* cell wall antiserum or normal rabbit serum directly to the microtiter plate coated with CA antigen. The percent inhibition was determined by the following formula: [(mean absorbance of normal rabbit serum − mean absorbance of test serum)/mean absorbance of normal rabbit serum] × 100. The percent inhibition was compared with a standard curve prepared in triplicate with known concentrations of CA antigen diluted in rabbit serum to determine the concentration of galactomannan.

Affinity chromatography on concanavalin A-Sepharose 4B. A concanavalin A (ConA)-Sepharose 4B column (0.6 by 7.0 cm; Sigma) was equilibrated with 0.02 M Tris hydrochloride buffer (pH 7.4) containing 0.5 M NaCl. Antigenemic serum dissociated by the boiling EDTA procedure was mixed with an equal volume of buffer, and 2.5 ml of the mixture was applied to the column. Elution was carried out at a flow rate of 15 ml/h at 22°C, and 1-ml fractions were collected. Bound material was subsequently eluted with equilibrating buffer containing 0.2 M methyl-α-D-mannopyranoside (Sigma). Fractions were assayed for galactomannan antigen by inhibition EIA.

Treatment with protease. Insoluble protease (pronase) from *Streptomyces griseus* attached to beaded agarose (Sigma) was dispensed by hypodermic syringe fitted with a 13-mm disk filter holder (Millipore Corp., Bedford, Mass.) containing a filter (pore size, 3.0 μm). The filter, retaining 1.55 mg of protease-agarose, was placed in a glass tube containing 0.5 ml of dissociated normal or antigenemic serum and incubated at 37°C for 18 h with magnetic stirring. The mixture was filtered on the disk filter device, and the filtrate was assayed for galactomannan antigen by inhibition EIA. The same experiment was also repeated without protease. As a positive control for enzyme activity, 300 μg of bovine serum albumin (Sigma) per ml of 0.1 M disodium EDTA (pH 7.2) was incubated with protease under identical conditions, and the filtrate was assayed for protein by the Coomassie blue method.

Ultrafiltration. The molecular weight of the antigen present in the heat-dissociated serum of an immunosuppressed, infected rabbit was estimated by using membranes that differed in their nominal molecular weight limits. The membrane ultrafiltration devices used were a microconcentrator (exclusion limit 30,000; Centricon-30; Amicon Corp., Danvers, Mass.), and a stirred ultrafiltration cell (model 8050; Amicon) equipped with membranes XM 50 (50,000 limit) or YM 100 (100,000 limit). Heat-dissociated antigenemic or normal rabbit serum (2 ml) was placed in the microconcentrator and centrifuged, and the filtrates and retentates were diluted to 2 ml with dissociated normal rabbit serum. The same experiment was repeated in the stirred ultrafiltration cell with 5 ml of heat-dissociated or normal rabbit serum. Filtrates and retentates were tested for galactomannan antigen by inhibition EIA.

TABLE 1. Yield and chemical composition of antigens CA, MET-S, and APSK-66 prepared from *A. fumigatus* 2085

Antigen	Starting material	Yield (mg)	% Protein	% Carbohydrate
CA	Mycelium, 177 g	120	50	50
MET-S	Culture filtrate, 7.5 liters	130	2	48
APSK-66	Mycelium, 38 g	52	23	42

RESULTS

Purification of antigens. In three experiments, the culture of *A. fumigatus* yielded 35, 38, and 177 g of compressed mycelium. The yield and chemical composition of antigens CA, MET-S, and APSK-66 are shown in Table 1. Purification of antigen APSK-66 by chromatography on the ion-exchange resin AG 50W-X4 produced a large early carbohydrate peak in the water eluate, and separation was obtained from a minor carbohydrate component that eluted with the disodium phosphate buffer. The monosaccharide composition of acid hydrolysates of the antigen preparations were CA, galactose and mannose (1:1.17); MET-S, galactose, mannose, and glucose (1:1.27:1.80); and APSK-66, galactose, mannose, and glucose (1.00:1.62:0.29).

Antisera. Antisera raised by immunizing rabbits with cell walls, mycelial homogenate, or conidia of *A. fumigatus* were tested by indirect EIA against antigens CA, APSK-66, and MET-S. The best IgG response (1/8,000) was obtained against antigen CA in rabbits immunized intravenously with cell walls, and titers of IgG against CA were 1/500 and 1/1,000 with antisera raised against conidia or mycelial homogenates, respectively. The antibody response against antigens MET-S and APSK-66 in rabbits immunized with cell walls, mycelial homogenates, or conidia was predominantly IgG (titers, 1/1,000 to 1/4,000), with a smaller IgM component. Antigen CA and the antiserum raised against cell walls were selected for further study.

Inhibition EIA for galactomannan antigen. A typical standard curve prepared in triplicate with known concentrations of CA antigen diluted in normal rabbit serum is shown in Fig. 1. The assay repeatedly detected as little as 10 ng of antigen per ml of serum. Mannan of *C. albicans* 20A could not be detected at concentrations of 10, 100, and 1,000 ng/ml.

Rabbit model of invasive aspergillosis. The four immunosuppressed rabbits died on day 4 after infection, and two uninfected control animals remained healthy and were sacrificed by exsanguination 16 days after the beginning of the immunosuppression. Areas of necrosis and hemorrhage were observed on the surface of the kidneys and livers of the infected rabbits. Invasive aspergillosis was demonstrated by histopathology of these organs in four infected rabbits but was absent in the uninfected controls. Numerous multifocal and confluent necrotizing pyogenic abscesses were observed that contained abundant neutrophils and typical septate hyphae with repeated dichotomous branching at an acute angle, with occasional invasion of blood vessels.

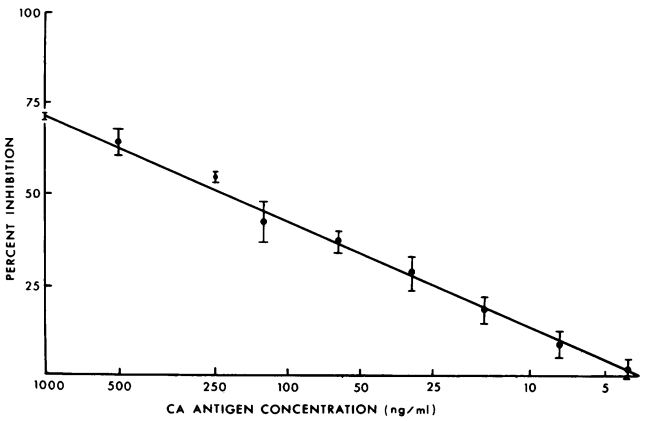

FIG. 1. A typical standard curve of the indirect EIA inhibition assay, prepared in triplicate with known concentrations of CA antigen diluted in normal rabbit serum. Each point represents the mean ± standard deviation.

Galactomannan in serum, which was negative before infection, was detected in the four infected animals on days 2 to 4. The peak concentrations of antigen were 95, 125, 170, and 350 ng/ml; this was determined by comparing the percentage inhibition with a standard curve prepared in triplicate with known concentrations of CA antigen diluted in normal rabbit serum. Galactomannan was undetectable in the serum of uninfected control rabbits.

Disseminated candidiasis in immunosuppressed rabbits. Cultural evidence of disseminated candidiasis was obtained at autopsy from the two infected rabbits. Infected kidneys were greatly enlarged with multiple microabscesses (11). The assay for galactomannan did not detect antigen in sera from these animals.

Affinity chromatography. Because galactomannan can bind to ConA-Sepharose 4B (19), the affinity of dissociated antigenemic serum to ConA was tested. The circulating antigen was almost entirely adsorbed onto ConA-Sepharose 4B and was eluted by methyl-α-D-mannopyranoside (Fig. 2).

Treatment with protease. In Table 2 it is shown that the circulating antigen was resistant to treatment with protease. A positive control of bovine serum albumin (300 μg/ml) incubated with the enzyme under identical conditions (18 h, 37°C) decreased to 60 μg/ml.

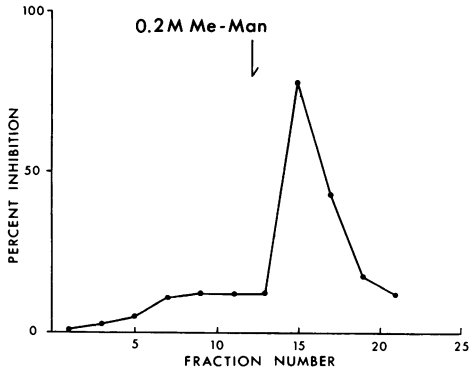

FIG. 2. ConA-Sepharose 4B affinity chromatography of dissociated antigenemic serum. Antigen was quantitated by inhibition EIA. Bound material was eluted by equilibrating buffer containing 0.2 M methyl-α-D-mannopyranoside (Me-Man).

Ultrafiltration. An estimate of molecular size was obtained by ultrafiltration of heat-dissociated antigenemic serum on membranes with different nominal molecular weight limits. The antigenic activity was retained by the XM 50 or Centricon-30 membranes, but not by the YM 100 membrane, suggesting a molecular weight between 50,000 and 100,000.

DISCUSSION

Clinical diagnosis of invasive aspergillosis is often difficult, and tests that detect antibody are often negative in profoundly immunosuppressed patients, who are most susceptible to invasion by this fungus (14). Methods have therefore been devised that directly detect circulating polysaccharide antigen of the *Aspergillus* species (3, 4, 10, 17, 19, 22, 24, 27, 28, 30–32). Existing methods have several disadvantages, however, including the use of radioactive material, insufficient sensitivity for the detection of antigen in the low nanogram per milliliter range, or a lengthy dialysis step that limits the clinical applicability of the method. We therefore devised a rapid and sensitive EIA for the detection of *A. fumigatus* galactomannan cell wall polysaccharide. The chemical structures of the galactomannans of the *Aspergillus* species have been elucidated (7, 9, 19), and this substance thus provides a well-characterized marker for antigen detection.

CA contained only mannose and galactose and was selected as the reference antigen. This preparation has been used previously by Reiss and Lehmann (19) and by Shaffer et al. (28) in studies on *Aspergillus* antigenemia. Rabbits immunized intravenously with cell walls of *A. fumigatus* produced the best IgG response to this antigen, and in this respect the method of immunization is similar to that used to raise antisera against *C. albicans* mannan (14).

The inhibition EIA that was devised can be completed in 4 h and obviates the need for radioisotopes. The method detected as little as 10 ng of antigen in serum per ml, and antigenemia was uniformly detected in four immunosuppressed, infected rabbits. The magnitude of antigenemia agreed with the data of Weiner and Coats-Stephen (31), who found antigen concentrations in the nanogram per milliliter range in a majority of rabbits 4 days after experimental infection. Because fungal antigenemia can occur at concentrations below the limit of sensitivity of this assay (13), further refinement will be possible by adapting the method to the inherently more sensitive double antibody sandwich format.

The circulating antigen had properties compatible with those of galactomannan. It was resistant to boiling for 3 min and to treatment with protease, and it was adsorbed onto

TABLE 2. Resistance of the circulating antigen to treatment with protease

Rabbit no.	Day[a]	% Inhibition[b]:	
		Without protease	With protease
1	−2	0	8
	+4	70	62
2	−2	0	2
	+4	62	71
3	−2	0	5
	+4	82	85

[a] −2, 2 days before infection; +4, 4 days after infection.
[b] Antigen in serum was quantitated by inhibition EIA.

ConA, a lectin with specificity for D-glucose and D-mannose residues. Furthermore, the molecular size of 50 to 100 kilodaltons is in agreement with the estimate of the molecular size of galactomannan (25 to 75 kilodaltons) obtained by Reiss and Lehmann (19).

A clinical evaluation of the EIA for galactomannan in serum is in progress in our laboratory.

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