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Rat Monoclonal Antibodies against *Aspergillus* Galactomannan

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Monoclonal antibodies (MAbs) against *Aspergillus fumigatus* galactomannan were produced in rats. Seven of them, EB-A1 through EB-A7, were characterized in more detail. They were all immunoglobulin M antibodies, reacting in an indirect enzyme-linked immunosorbent assay with purified *A. fumigatus* galactomannan, with avidity constants of between 2×10^9 and 5×10^9/M. Enzyme-linked immunosorbent assay inhibition experiments with modified galactomannan and synthetic oligomers of β(1→5)galactofuranose demonstrated that the MAbs bound to an epitope located on the β(1→5)galactofuranose-containing side chains of the galactomannan molecule. An identical or similar epitope also seemed to be present in other fungi. Immunofluorescence and immunoelectron microscopy experiments with EB-A2 revealed the presence of the antigen in the fungal wall and inside the cell. Immunoblotting experiments demonstrated that the epitope recognized by the MAbs was a common oligosaccharide moiety of a wide range of intracellular and extracellular glycoproteins in *A. fumigatus*. The characteristics of the MAbs justify their use in the diagnosis of invasive aspergillosis by antigen detection.

Aspergillus fumigatus and, to a lesser extent, other *Aspergillus* species as well can cause a variety of diseases, including allergic reactions ranging from mild asthma to allergic bronchopulmonary aspergillosis, superficial infections, and often lethal invasive infections (reviewed in reference 5). The latter have become an increasingly important threat to severely immunocompromised patients. The prognosis of the patients is very poor, so early diagnosis is of utmost importance.

The diagnosis of invasive aspergillosis is difficult. Clinical symptoms and roentgenographic pictures are not distinctive, especially in the early stages of the disease (39). Histochemical investigations are often impossible because of the lack of suitable samples from biopsies (39). Antibody tests are useful in the diagnosis of allergic bronchopulmonary aspergillosis and aspergilloma but have yielded conflicting results in the diagnosis of invasive aspergillosis in immunocompromised patients (14). Finally, the organism is rarely recovered by hemoculturing (14). The most promising diagnostic approach is the detection of *Aspergillus* antigens in the serum or urine of the patient. In several studies, the presence of antigens appeared to be a very specific indicator of invasive disease (reviewed in reference 12).

Polysaccharide and (glyco)protein antigens have been identified in patient serum and urine (19, 25, 41, 42, 53, 54). Most research in the last 10 years has been focused on galactomannan antigens (13, 17, 38).

Galactomannan is a major cell wall component in *Aspergillus* species and can also be secreted into the medium as a component of the exoantigen (ExA). In *A. fumigatus*, secreted galactomannan is composed of a branched core containing α(1→2)- and α(1→6)-linked mannose, with β(1→5)galactofuranose and/or β(1→4)galactopyranose moieties linked linearly in side chains terminated by galactofuranose nonreducing end units (31). However, the final structure of the galactomannan of *A. fumigatus* has not yet been established. Variations in the galactose/mannose ratio, the presence of galactopyranose or glucopyranose residues, branching of the galactan side chains and the mannan core, and the length of the side chains have been reported (2, 4, 13, 38).

The galactomannan is immunogenic (4, 35), and polyclonal antibodies against it have been used in antigen detection for the diagnosis of invasive aspergillosis (13, 17, 38) and in the detection of fungal contamination in foodstuffs (26).

In this paper, we describe the production and characteristics of seven rat monoclonal antibodies (MAbs) against *A. fumigatus* galactomannan and discuss their application in the diagnosis of invasive aspergillosis.

MATERIALS AND METHODS

Strains. *A. fumigatus* EV 601 and *A. flavus* EV 701 were from the Sanofi Diagnostics Pasteur collection and were originally isolated and kindly donated by J. Vandepitte (Catholic University of Leuven, Leuven, Belgium). Other fungal strains were obtained from the Centraalbureau voor Schimmelcultures (CBS; Baarn, The Netherlands), the National Institute of Public Health and Environmental Protection (RIVM; Bilthoven, The Netherlands), or the American Type Culture Collection (ATCC; Rockville, Md). The strains were maintained by subculturing on Sabouraud dextrose agar slants or on malt extract agar slants at room temperature.

Eleven bacterial strains were ATCC reference strains: *Shigella sonnei* ATCC 25931, *Serratia marcescens* ATCC 8100, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Enterobacter cloacae* ATCC

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23355, *Neisseria meningitidis* ATCC 13090, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. Four other bacterial strains were clinical isolates from different sources: *Acinetobacter* sp., *Yersinia pseudotuberculosis*, *Haemophilus parainfluenzae*, and *Streptococcus pneumoniae*.

Rats. LOU/C rats, which have kappa allotype IgK-1a, and LOU/C.IgK-1b rats, which have kappa allotype IgK-1b, were obtained from H. Bazin (Catholic University of Louvain, Brussels, Belgium).

Chemical analysis. Protein concentrations were determined either by the Bio-Rad Laboratories technique based on the method of Bradford (7) or with bicinchoninic acid (44) (Pierce, Polylab, Antwerp, Belgium) in accordance with manufacturer instructions. Hexose concentrations were measured by the orcinol-sulfuric acid method (8) or the phenol-sulfuric acid method (16).

Preparation of CIAs and ExAs. Conidia were inoculated in 50 ml of sterilized liquid 2% (wt/vol) glucose–1% (wt/vol) peptone medium in 150-ml flasks. Cultures were incubated at 25°C on a rotary shaker at 100 rpm for 4 to 7 days, depending on the strain. One hundred milliliters of these cultures served as inocula for 1.2-liter cultures prepared in 2-liter Biolafitte fermentors run under the following conditions: 800 rpm, 25°C, 0.6 liters of air per min. The mycelium was recovered during the active growth phase (30 to 48 h, depending on the strain) and separated from the medium by paper filtration. The mycelial mat was extensively washed with water and stored at −20°C until use (31).

For the preparation of crude intracellular antigen (CIAs), *A. fumigatus* CBS 331.90 was grown in different media: M1, containing 30 g of glucose and 10 g of yeast extract per liter (Difco); M2, containing 20 g of glucose and 10 g of mycopeptone per liter (Biokar; Prolabo, Paris, France); M3, containing 10 g of glucose, 5 g of asparagine, 1 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, and 3 mg of $FeSO_4 \cdot 7H_2O$ per liter; and M4, Vogel's medium (52). Because of differences in growth rates in these different media, the mycelium was recovered after different incubation times: 2 days in M1 and M2 and 3 days in M3 and M4. The CIAs were obtained after disruption of the mycelial mat in phosphate-buffered saline (PBS) by use of a glass bead (1-mm-diameter beads) MSK Braun homogenizer for 2 min under CO_2 cooling. Wall material was discarded after centrifugation at 8,000 × *g* for 10 min. Membranes were removed by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. The resulting supernatants (CIAs) were stored at −20°C until use.

ExAs were prepared by precipitation of culture filtrates with at least 3 volumes of ethanol overnight at 4°C. The samples were centrifuged at 4°C and 3,000 × *g* for 10 min. The precipitates (ExAs) were washed twice with ethanol and freeze dried (10) or resuspended, and aliquots were kept frozen at −80°C.

Purification of galactomannan. Pure galactomannan was obtained as described previously (31). In brief, the ExA of a 42-h-old culture of *A. fumigatus* CBS 143.89 was freeze dried. The ExA was extracted twice sequentially with hydrazine for 18 h at 100°C and 1.5 N HNO_2 for 2 h at room temperature. After extensive dialysis against water, the solution obtained was separated from any insoluble material appearing during the dialysis by filtration with 0.45-μm-pore-size filters and freeze dried. This solution only contained pure galactomannan (31).

MAbs. The MAbs described in this paper resulted from four different fusions. Three-month-old LOU/C rats were immunized in accordance with the following immunization schemes.

(i) Fusion 1. Formalinized spores of *A. fumigatus* EV 601 (200 μl) were injected into the footpad on days 0, 3, 6, and 10. Spore concentrations were 10^7/ml in the first two injections and 10^8/ml in the last two injections. *Bordetella pertussis* vaccine was used as an adjuvant in the first immunization. The fusion of cells from the lymph nodes in the hollow of the knee with myeloma cells was carried out 3 days after the last injection.

(ii) Fusion 2. A suspension (200 μl) of formalinized *A. flavus* EV 701 spores (10^8/ml) was injected into the footpad on days 0, 3, 7, and 11. *B. pertussis* vaccine was used as an adjuvant in the first immunization. The cells from the lymph nodes in the hollow of the knee were fused with myeloma cells on day 14.

(iii) Fusions 3 and 4. A mycelial extract (500 μl; 1 mg of protein per ml) of *A. fumigatus* EV 601 (fusion 3) or *A. flavus* EV 701 (fusion 4) was mixed with 500 μl of Freund's complete adjuvant (Difco, Pasture, Brussels, Belgium) and injected alternate subcutaneously, intraperitoneally, or intramuscularly at weekly intervals. After the seventh immunization, in which Freund's incomplete adjuvant (GIBCO, Life Technologies, Ghent, Belgium) was used, the immunization schedule was interrupted for 10 weeks. Intravenous booster injections of mycelial extract without adjuvant were given 2 and 4 days prior to fusion.

Cells from the lymph nodes in the hollow of the knee (fusions 1 and 2) or spleen cells (fusions 3 and 4) of the immunized rats were fused with IR983F rat plasmacytoma cells. The clones used in this study were cloned three times by limiting dilution on peritoneal feeder cells (9).

The isotypes of the MAbs were determined by a double-sandwich enzyme-linked immunosorbent assay (ELISA) (32). MAbs were produced in vitro in stationary culture flasks by use of Opti-MEM I (GIBCO) with 0.5% (vol/vol) fetal calf serum and 0.5% (vol/vol) horse serum or in vivo in LOU/C.IgK-1b rats (6, 28, 46). They were purified from cell culture supernatants or ascitic fluids by kappa allotype immunoaffinity chromatography (3) on an affinity matrix consisting of MAb MARK-3 coupled to Sepharose-4B, obtained from H. Bazin.

ELISA. Coating of the plates was carried out overnight at 4°C with 100 μl of antigen suspensions or solutions. Unless stated otherwise, antigen concentrations were 10^7/ml (cells) or 10 μg of protein per ml (soluble antigen preparations) in 0.15 M PBS (pH 7.2). One hundred microliters of the primary antibody (culture supernatant or purified MAb) in PBS with 0.1% (vol/vol) Tween 20 (PBS-Tw) was incubated at 37°C for 2 h. After three washes with PBS-Tw, a peroxidase conjugate of anti-rat immunoglobulin was added to the wells and incubation was continued for 1 h at 37°C. Then, the plates were washed four times with PBS-Tw, and positive reactions were revealed by the addition of the chromogen 3,3′,5,5′,-tetramethylbenzidine and H_2O_2. Color development was stopped by the addition of 100 μl of sulfuric acid (1 M), and the A_{455} was read with an LP100 ELISA plate reader (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). This protocol was used for screening of the hybridoma cultures and for the determination of species specificity in the initial characterizations of the MAbs.

Determinations of avidities. The avidities of the selected MAbs were determined with an ELISA as described above by use of plates coated with 10 μg of purified galactomannan per ml. Twofold serial dilutions of purified MAbs in PBS-Tw were added to the wells, starting at a maximal concentration

of 80 μg/ml. Each experiment was carried out in triplicate. The avidity constant (1/M) was calculated as the reciprocal of the average MAb molar concentration in the three assays at half-maximal binding (50).

Reactivity of MAbs EB-A1 and EB-A2 with ExAs of different fungal species. The cross-reactivity of EB-A1 and EB-A2 was studied by determining the avidity constants of these MAbs for ExAs and purified cell wall polysaccharides of different fungal species. ExAs from culture supernatants of *Trichophyton rubrum* and *Trichophyton interdigitalis* (both gifts from A. Rurangirwa, Liège, Belgium) and *Wallemia sebi*, *Trichoderma viride*, *Fusarium solani*, and *Cladosporium cladosporioides* (all gifts from M. van der Horst and R. Samson, Baarn, The Netherlands) were prepared by ethanol precipitation as described above. Exocellular polysaccharides from *Penicillium digitatum* (strain M58, from RIVM) and *Botrytis tulipae* (strain M18, from RIVM) were kind gifts from S. Notermans (Bilthoven, The Netherlands). *Saccharomyces cerevisiae* mannan was obtained from Sigma. *Candida albicans* mannan was prepared by Cetavlon precipitation (37). *Cryptococcus neoformans* glucuronoxylomannan was prepared by precipitation with sodium acetate-glacial acetic acid and ethanol as described by Dromer et al. (15). The antigens were used to coat wells at 100 ng/ml.

ELISA inhibition experiments. Inhibition experiments were performed by preincubation (37°C, overnight) of 60 μl of MAb (0.1 μg/ml) in PBS-Tw–1% (wt/vol) bovine serum albumin (BSA) with 60 μl of two- or threefold serial dilutions of the antigen under investigation. One hundred microliters of the mixture was added to duplicate microtiter wells, previously coated overnight with *A. fumigatus* ExA (1 μg/ml in 50 mM carbonate buffer [pH 9.6]). After 1 h at 37°C, the plates were washed with PBS-Tw. Then, 100 μl of peroxidase-conjugated goat anti-rat immunoglobulin (heavy and light chains) (Sigma), diluted 1:2,000 in PBS-Tw–1% BSA, was added to the wells. After 1 h of incubation at 37°C, the plates were washed with PBS-Tw and the binding of the MAb to the coating ExA was measured spectrophotometrically at 490 nm after the addition of a peroxidase substrate and ortho-phenylenediamine dihydrochloride as the chromogen. The percentage of inhibition was calculated as follows: [(OD without inhibitory antigen − OD with inhibitory antigen)/OD without inhibitory antigen] × 100, where OD is optical density. The percentages of inhibition were then plotted against antigen concentration. The 50% inhibitory concentrations were determined.

Serial dilutions of various compounds were used in these competition experiments: *A. fumigatus* galactomannan at a maximal concentration of 1 μg/ml; *A. fumigatus* galactomannan, hydrolyzed overnight at 100°C in 0.01 N HCl, at a maximal concentration of 350 μg/ml; *A. fumigatus* ExA, hydrolyzed overnight at 100°C in 0.01 N HCl, at a maximal concentration of 350 μg/ml; synthetic β(1→5)galactofuranose oligosaccharides, from trimers to heptamers, at a maximal concentration of 500 μg/ml (oligosaccharides were synthesized by Veeneman et al. [51]); branched and linear α(1→5)arabinan polymers isolated from plants, at a maximal concentration of 35 μg/ml (provided by J. P. Josseleau CERMAV, Grenoble, France); *C. albicans* mannan, prepared by Cetavlon extraction (37), at a maximal concentration of 3.5 mg/ml; and malto-oligosaccharides (tetraose to heptaose), obtained from Boehringer Mannheim, at a maximal concentration of 3.5 mg/ml.

In every inhibition experiment, the seven anti-*A. fumigatus* MAbs were tested in triplicate, unless otherwise indicated. MAb EB-Y8, specific for *Yersinia enterocolitica* O:8 (46), was used as a control antibody.

Immunofluorescence. Germinating conidia of *A. fumigatus* were obtained by the incubation of conidia from strain CBS 143.89 overnight at room temperature in 10-μl drops of Sabouraud medium, deposited on wells of immunofluorescence slides (Sanofi Diagnostics Pasteur), and maintained in a humid chamber. The germinating conidia adhering to the immunofluorescence slides were extensively washed in PBS and then incubated with MAb EB-A2 (20 μg/ml in PBS with 1% [wt/vol] BSA) for 1 h at room temperature. After washes in PBS with 0.1% BSA, the material was incubated with rabbit anti-rat immunoglobulin coupled to fluorescein isothiocyanate (Sigma, Pasture, Brussels, Belgium) (1/30 in PBS with 1% BSA). MAb EB-Y8 (46) was used as a control antibody.

For nongerminating conidia, the same protocol was used, but incubation was done in Eppendorf tubes and conidia were recovered after incubation and washes by centrifugation.

Immunoelectron microscopy. The mycelium was prepared for immunoelectron microscopy as described by Latgé et al. (30). In brief, the mycelium was fixed for 30 min in 2% paraformaldehyde and 0.1% glutaraldehyde and then submitted to several washes in 0.1 M sodium cacodylate buffer (pH 7.2) and overnight quenching of possible residual aldehyde radicals in 10 mM NH_4Cl.

For transmission electron microscopy, the fungal material was dehydrated in increasing concentrations of ethanol and embedded in Lowicryl K4M at 4°C. Ultrathin sections were incubated for 1 h at room temperature with MAb EB-A2 at 0.3 μg/ml in PBS–1% BSA. After several washes in PBS–0.1% BSA, the sections were incubated for 1 h at room temperature with goat anti-rat immunoglobulin coupled to 5-nm colloidal gold (Janssen) (1/50 in PBS–1% BSA). After several washes in PBS–0.1% BSA, PBS, and water, the grids were observed at 80 kV in a Philips CM 12 transmission electron microscope. MAb EB-Y8 (46) was used in negative control procedures.

For scanning electron microscopy, the material was sedimented onto polylysine (200 kDa; 0.01%)-coated glass coverslips. After the nonadhering material was washed off, the coverslips were successively incubated for 1 to 2 h with MAb EB-A2 (6 μg/ml in PBS–1% BSA), rabbit anti-rat immunoglobulin (1/50 in PBS–1% BSA; Sigma), and goat anti-rabbit immunoglobulin coupled to 30-nm colloidal gold particles (1/25 in PBS–1% BSA; Janssen), with intermediate washes in PBS–0.1% BSA. After a final PBS wash, the coverslips were incubated overnight at 4°C in 2% glutaraldehyde, washed with PBS, and postfixed with 1% OsO_4. After dehydration in ethylene glycol monoethyl ether and acetone, the specimens were submitted to critical point drying before carbon coating and observation with a JEOL scanning electron microscope by use of a back-scattering electron-imaging device. MAb EB-Y8, directed against *Y. enterocolitica* lipopolysaccharides (46), was used in negative control procedures.

Immunoblotting. Intracellular mycelial preparations were electrophoretically separated on 5 to 15% gradient or 7.5% continuous SDS-polyacrylamide gels as described by Laemmli (29). Electrophoresis was performed at 60 mA per 16-cm-wide gel for 3 to 4 h. After the separation, the proteins were electrotransferred to nitrocellulose paper (0.45-μm-pore size; Schleicher & Schuell) overnight at 4°C (47). Transfer efficiency and molecular weight markers were checked by staining in 0.3% (wt/vol) Ponceau S in 0.3%

TABLE 1. Inhibition by different fungal polysaccharides of the binding of MAbs to ExAs of *A. fumigatus* in an ELISA inhibition experiment

Galactomannan	Polysaccharide concn (μg/ml) reducing the binding of the indicated MAb by 50%[a]:						
	EB-A1	EB-A2	EB-A3	EB-A4	EB-A5	EB-A6	EB-A7
A. fumigatus	0.054 ± 0.041	0.053 ± 0.025	0.010 ± 0.004	0.031 ± 0.019	0.021 ± 0.016	0.012 ± 0.004	0.032 ± 0.009
Hydrolyzed[b]	>35	>350	>35	>35	>35	>35	>35

[a] Results are expressed as the average ± standard deviation for a minimum of four experiments with duplicate samples.
[b] No inhibition was observed at the indicated concentrations, the highest concentrations tested.

(wt/vol) trichloroacetic acid. The membranes were destained in PBS, and free binding sites were blocked in PBS-Tw containing 5% nonfat milk (Regilait) for 2 to 3 h at room temperature. The strips were incubated directly in the MAb solution (2 to 5 μg/ml) overnight at 4°C. After three washes in PBS-Tw, the blots were incubated for 1 h at 4°C in peroxidase-conjugated goat anti-rat immunoglobulin (1/2,000 in PBS-Tw; Sigma). The immunoreactive bands were visualized by incubation with hydrogen peroxide and diaminobenzidine tetrahydrochloride.

RESULTS

Selection of clones. The first screenings of hybridoma supernatants were performed on the crude antigen preparations used for the immunization. From the four different fusions, a total of 60 clones were characterized. This initial characterization included determination of reactivity with purified *A. fumigatus* galactomannan, reactivity with mycelial homogenates of different *Aspergillus* species and either homogenates or whole cells of other fungal and bacterial species, and isotypes. Seven clones secreting antibodies against galactomannan were selected for further characterization. They all had immunoglobulin M isotypes and kappa light chains and reacted with the five *Aspergillus* species (*A. fumigatus* ATCC 1028, *A. flavus* ATCC 10124, *A. niger* ATCC 10549, *A. versicolor* ATCC 16845, and *A. terreus* CBS 106.25) tested but not with other medically important fungi, such as *Sporothrix schenckii* ATCC 14096 and *Candida* spp. (25 strains, including 10 ATCC strains, 2 CBS strains, and 13 clinical isolates, representing seven species, i.e., *C. albicans*, *C. tropicalis*, *C.* [*Torulopsis*] *glabrata*, *C. pseudotropicalis*, *C. parapsilosis*, *C. guilliermondii*, and *C. krusei*). They also failed to react with the 15 different bacterial species tested. The seven antibodies were called EB-A1 through EB-A7.

Although the rats had been immunized with different antigen preparations from different species and according to different immunization protocols, the four fusions yielded antigalactomannan MAbs. EB-A4 resulted from fusion 1; EB-A1 resulted from fusion 2; EB-A2, EB-A3, and EB-A5 resulted from fusion 3; and EB-A6 and EB-A7 resulted from fusion 4.

Reactivity of MAbs with galactomannan. Estimation of the avidities of the MAbs for *A. fumigatus* galactomannan in an indirect ELISA revealed only relatively small differences in avidity between the different MAbs. The avidity constants ranged from 2×10^9/M to 5×10^9/M: two antibodies (EB-A3 and EB-A6) had an avidity constant of 2×10^9/M, two (EB-A1 and EB-A7) had an avidity constant of 4×10^9/M, and the three remaining MAbs (EB-A2, EB-A4, and EB-A7) had an avidity constant of 5×10^9/M.

ELISA inhibition experiments showed that very low concentrations of galactomannan reduced the binding of the MAbs to the coating ExA (Table 1). The 50% inhibitory concentration of galactomannan varied somewhat from MAb to MAb. EB-A3 could be most easily inhibited by galactomannan (10 ng/ml). The highest galactomannan concentrations were required for EB-A1 and EB-A2 (50 to 55 ng/ml).

Identification of epitopes. Purified galactomannan submitted to 0.01 N HCl treatment (100°C overnight) to remove the galactose-containing side chains (31) had lost its binding activity (Table 1). The hydrolysates of both *A. fumigatus* ExA and purified galactomannan, at concentrations of up to 350 μg/ml, failed to inhibit the binding of the MAbs to the coating ExA.

Synthetic β(1→5)galactofuranose oligomers of increasing length were studied in ELISA inhibition experiments with EB-A2. At 500 μg/ml, the trimer was not inhibitory, but the other oligomers, from the tetramer to the heptamer, the longest chain tested, reduced EB-A2 binding to the ExA by 88% (Fig. 1). Malto-oligosaccharides (tetraose to heptaose) were negative in the inhibition assay.

Different concentrations of the tetramer were tested against the different MAbs to determine the 50% inhibitory concentrations and the percentages of inhibition at the maximal tetramer concentration tested (500 μg/ml) (Table 2). The 50% inhibitory concentrations could only be estimated for three antibodies. They were 80 μg/ml for EB-A1, 100 μg/ml EB-A4, and 300 μg/ml for EB-A3. For the other four MAbs, these concentrations were higher than the maximal concentrations tested. Inhibition could nevertheless be ob-

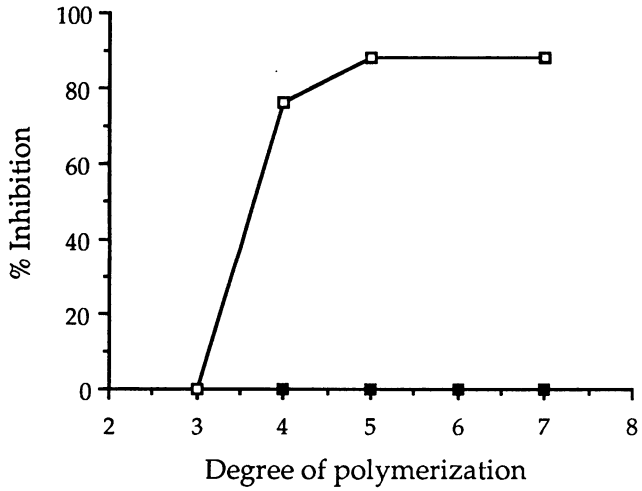

FIG. 1. Inhibition by synthetic oligosaccharides of different chain lengths of EB-A2 binding to coated *A. fumigatus* ExA in an ELISA inhibition experiment. The tetramer and longer oligomers of β(1→5)galactofuranose at 500 μg/ml were effective inhibitors (□). Malto-oligosaccharides at 3.5 mg/ml had no effect (■).

TABLE 2. Inhibitory effect in an ELISA inhibition experiment of the tetramer of β(1→5)galactofuranose on MAb binding to *A. fumigatus* ExA

MAb	50% Inhibitory concn (μg/ml)	% Inhibition at 500 μg/ml
EB-A1	80	97
EB-A2	>500	47
EB-A3	300	76
EB-A4	100	96
EB-A5	>500	39
EB-A6	>500	10
EB-A7	>500	39

served for most antibodies when the tetramer was used at the highest concentration (500 μg/ml). It ranged from virtually complete inhibition (EB-A1 and EB-A4) to about 40% inhibition (EB-A5 and EB-A7). The binding of only one MAb, EB-A6, was not significantly reduced by the tetramer.

Neither the branched α(1→5)arabinan with galactopyranose traces nor the linear α(1→5)arabinan, both at 35 μg/ml, showed binding activity in the inhibition assay. *C. albicans* mannan also yielded negative results, even at a concentration of 3.5 mg/ml.

Cross-reactivity of EB-A1 and EB-A2 with different fungal polysaccharides. To study the degree of cross-reactivity of EB-A1 and EB-A2, we determined the avidity constants for the reactions of these MAbs with different fungal ExA preparations (Table 3). The avidity constants for the binding of these antibodies to the exopolysaccharides from *P. digitatum*, *T. rubrum*, *T. interdigitalis* (with EB-A2), *B. tulipae*, and *W. sebi* were higher than 10^9/M, suggesting a reactivity comparable to that with the *A. fumigatus* galactomannan. Avidity constants on the order of 10^8/M were obtained with *T. interdigitalis* (with EB-A1) and *C. cladosporioides* exopolysaccharides. No reaction could be observed with ExAs from *F. solani* and *T. viride*, the mannans from *C. albicans* and *S. cerevisiae*, or the glucuronoxylomannan from *C. neoformans*.

Immunofluorescence. Indirect immunofluorescence revealed that EB-A2 reacted heterogeneously with different developmental structures from *A. fumigatus*. This result is clearly illustrated by the fluorescence pattern in germinating conidia (Fig. 2A and B). The germ tubes were intensely stained, while the conidia reacted, but did so weakly. On the other hand, among nongerminating conidia, young conidia, which could be identified by their poor refraction in phase-contrast microscopy, were strongly fluorescent (Fig. 2C). Controls tested with MAb EB-Y8 at the same concentration as MAb EB-A2 were negative.

TABLE 3. Reactivities of EB-A1 and EB-A2 with ExA preparations from different fungi, as determined by indirect ELISA experiments

Fungus	Avidity constant (1/M) for MAb:	
	EB-A1	EB-A2
Aspergillus fumigatus	2×10^9	5×10^9
Penicillium digitatum	3×10^9	5×10^9
Trichophyton rubrum	1×10^9	5×10^9
Trichophyton interdigitalis	4×10^8	2×10^9
Botrytis tulipae	1×10^9	4×10^9
Wallemia sebi	5×10^9	3×10^9
Cladosporium cladosporioides	8×10^8	2×10^8
Fusarium solani	$<10^7$[a]	$<10^7$
Trichoderma viride	$<10^7$	$<10^7$
Candida albicans[b]	$<10^7$	$<10^7$
Saccharomyces cerevisiae[b]	$<10^7$	$<10^7$
Cryptococcus neoformans[c]	$<10^7$	$<10^7$

[a] $<10^7$, the avidity constant was too low to be calculated.
[b] Purified mannan.
[c] Purified glucuronoxylomannan.

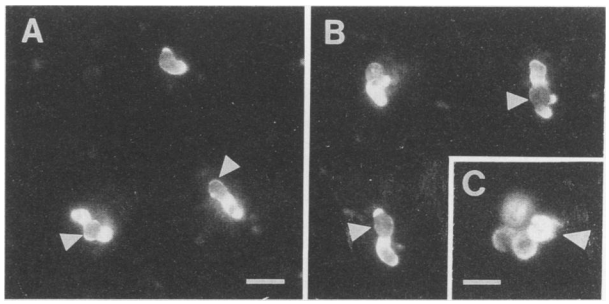

FIG. 2. (A and B) Indirect immunofluorescence of germinating conidia of *A. fumigatus* with EB-A2 (20 μg/ml). The conidia were only weakly stained (arrowheads), whereas the mycelial germ tubes were more reactive. Bar, 25 μm. (C) The arrowhead indicates a more brightly stained, young conidium. Bar, 5 μm.

Ultrastructural localization of EB-A2-reactive material. Ultrathin sections of *A. fumigatus* mycelium, incubated with EB-A2 and colloidal gold-labelled anti-rat immunoglobulin antibodies, showed that material secreted and released into the medium, the cell wall, and the intracellular compartment of the mycelium were labelled by this immunocytochemical procedure (Fig. 3A). The labelling inside the cell did not seem to be associated with any particular cell organelle. The presence of surface components reacting with EB-A2 was confirmed by scanning immunoelectron microscopy (Fig. 3B). Controls tested with MAb EB-Y8 at the same concentration as MAb EB-A2 were negative in transmission and scanning immunoelectron microscopy.

Immunoblotting. Immunoblotting experiments with ExA from *A. fumigatus* demonstrated that most glycoprotein antigens recognized by all the MAbs had molecular masses of between 41 and 90 kDa. No significant differences could be observed between the different MAbs (Fig. 4).

Culturing of *A. fumigatus* in the different media tested did not significantly modify the profiles of the intracellular immunoreactive bands (Fig. 5). However, the intensity of the labelled bands varied, indicating that the same antigens could be present at different concentrations. Striking variations were observed when the intracellular proteins of two strains, *A. fumigatus* CBS 331.90 and CBS 143.89, were compared (Fig. 5).

DISCUSSION

Several experiments confirmed that our MAbs reacted with galactomannan and more precisely with β(1→5)galactofuranose residues of galactomannan (Fig. 1 and Tables 1 and 2). The disappearance of the immunoreactivity of galactomannan after mild hydrolysis with 0.01 N HCl at 100°C demonstrated that the MAbs recognized the galactofuranoside-containing side chains (Table 1). Previous studies showed that this mild acid treatment only removed the galactoside side chains of *A. fumigatus* exopolysaccharides. The other linkages of the mannan core, α(1→2)- and α(1→6)-linked mannose and branched mannose residues substituted

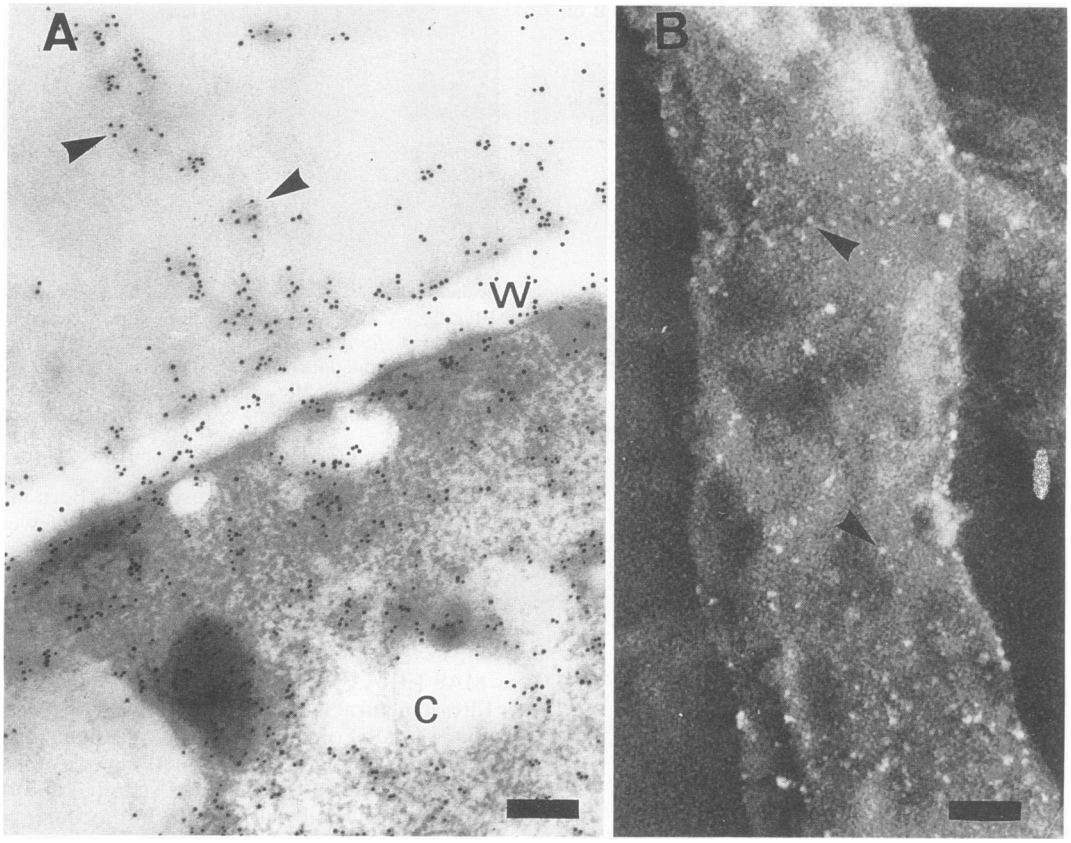

FIG. 3. (A) Reactivity of EB-A2 with an epitope located in the cell wall (W) and in the cytoplasm (C) of an *A. fumigatus* hypha. Material secreted exocellularly (arrowheads) also bound to the MAb. Bar, 0.13 μm. (B) Binding of EB-A2 to the cell surface of the *A. fumigatus* mycelium. Gold particles (arrowheads) were visualized by use of back-scattering electron imaging. Bar, 0.5 μm.

at C-2 and C-3 and substituted at C-2 and C-6, were not attacked (31). Further evidence for reactivity with the β(1→5)galactofuranose-bearing side chains was provided by the ELISA inhibition experiments with synthetic β(1→5)galactofuranose oligomers (Table 2 and Fig. 1).

Bennett et al. (4) already showed that galactofuranosyl groups are immunodominant in *Aspergillus* galactomannan. Notermans et al. (35) and Kamphuis et al. (26) used synthetic β(1→5)galactofuranose oligomers to confirm this fact in a study of *Penicillium* and *Aspergillus* galactomannans. These studies were done with polyclonal rabbit antisera. MAb 1, a murine MAb raised against a cold alkali extract of mycelium by Ste-Marie et al. (45), recognized a periodate-sensitive, pronase- and heat-resistant epitope. The glycoproteins labelled by MAb 1 in immunoblots reacted with concanavalin A and the BS-1 lectin from *Bandeiraea simplicifolia*, suggesting that the antigens contained mannose and α-D-galactose (and not β-D-galactose, present in galactomannan) or α-D-*N*-acetylgalactosamine (20), which is also secreted in vitro by *A. fumigatus* (31). MAb 1 also reacted strongly with *Fusarium* in an immunofluorescence assay, while our MAbs (36) (Table 3) as well as polyclonal antibodies that bind β(1→5)galactofuranosides (26, 27) failed to bind *Fusarium* ExAs. Analysis of the structures of the extracellular polysaccharides from *Fusarium moniliforme* and *F. solani* demonstrated the presence of D-galactose in the furanose form. However, unlike the galactofuranoside residues of the galactomannan of *A. fumigatus*, the galactofuranoside residues of the polysaccharide of *F. moniliforme* are 1→2- or 1→6-linked, with some branching at C-2 or C-6 (43). In *F. solani*, only 1,2,6-linked galactofuranoside residues could be demonstrated (34). In neither species were galactofuranosides located at terminal positions. Although the chemical nature of the epitope recognized by MAb 1 was not directly investigated (45), the arguments mentioned above suggest that the MAbs described in this paper are different from MAb 1.

In this study, there was some variation in the concentrations of β(1→5)galactofuranose needed to inhibit the MAbs. In the case of EB-A6, no clear inhibition could be demonstrated. This result suggests that the antigen-binding sites of the MAbs were not identical. The inhibitory concentrations of β(1→5)galactofuranose were relatively high, suggesting that the MAbs may recognize a more complex epitope, including, for example, a mannose residue of the core or galactopyranose residues of the side chains bearing β(1→5)galactofuranose. It might therefore be interesting to study the reactivity of hetero-oligomers in ELISA inhibition experiments. Such a study might help explain the cross-reactivity with *Trichophyton* ExA, in which no β(1→5)galactofuran has been reported until now.

Wall antigens of *A. fumigatus* are very immunogenic. Immunogold labelling of ultrathin sections has demonstrated that sera from aspergilloma patients or from rabbits immunized with *A. fumigatus* fractions bound almost exclusively to the mycelial wall (22). The anti-galactomannan MAb EB-A2, however, bound to intracellular and extracellular material and to wall components (Fig. 3). Its binding pattern

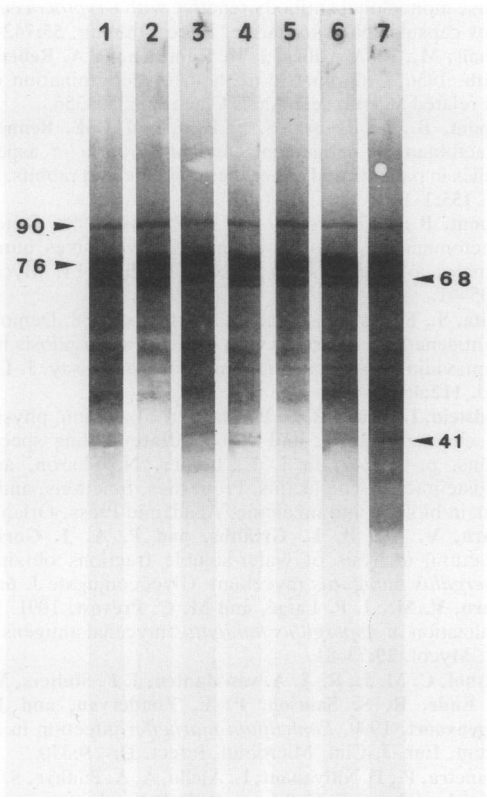

FIG. 4. Immunoblot of CIAs of *A. fumigatus* CBS 331.90 (25 μg of protein per lane) grown in Vogel's medium with the seven MAbs as probes. The recognized epitope was commonly present on a wide range of glycoproteins. The different MAbs revealed almost identical banding patterns. Lanes: 1, EB-A1; 2, EB-A2; 3, EB-A3; 4, EB-A4; 5, EB-A5; 6, EB-A6; 7, EB-A7. Numbers are in kilodaltons.

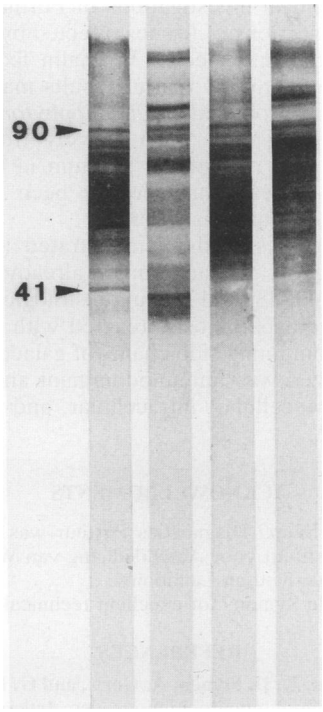

FIG. 5. Immunoblot of CIAs of *A. fumigatus* CBS 331.90 grown in three different media and of *A. fumigatus* CBS 143.89 grown in M3 with EB-A2 as a probe. Culturing in different media only caused quantitative variations in the intensities of some bands. However, the pattern of banding of *A. fumigatus* CBS 143.89 strikingly differed from that of *A. fumigatus* CBS 331.90. Lanes contained, from left to right, CBS 331.90 in M4 (25 μg of protein per lane), CBS 143.89 in M3 (25 μg of protein per lane), CBS 331.90 in M3 (10 μg of protein per lane), and CBS 331.90 in M1 (25 μg of protein per lane). Numbers are in kilodaltons.

resembled that seen when thin sections were labelled with a rabbit antiserum raised against the concanavalin A-binding fraction of a water-soluble preparation of a mycelial extract, which is known to be rich in galactomannan (21). Similarly, MAb 1 recognized an epitope located in the wall and intracellular compartment of hyphae and conidia (45).

In immunofluorescence experiments, EB-A2 intensely bound to germ tubes and to young conidia (Fig. 2). The weak fluorescence in older conidia suggested that the immunoreactive material was less accessible to the antibody because of the presence of a hydrophobic outer layer on the surface of mature conidia. The EB-A2 reaction patterns confirmed the differences existing in cell wall structure between different developmental phases of *Aspergillus* spp. (33). The positive reactions obtained with all stages of *A. fumigatus*, albeit of different intensities, were consistent with the fact that immunization with either conidia or mycelia yielded similar MAbs.

EB-A2 has been used to detect circulating antigen in the serum of patients with invasive aspergillosis by ELISA inhibition. The sensitivity (95%), specificity (100%), positive predictive value (100%), and negative predictive value (99%) of the ELISA for the diagnosis of invasive pulmonary aspergillosis, compared with the situation in a group of patients with other infections or no fungal infections, were very good (40). Antigenemia antedated clinical suspicion by a median of 24 days. However, in serial samples antigenemia was transient and galactomannan concentrations fluctuated significantly, confirming earlier findings (17, 19, 41) and suggesting that regular testing of patients is necessary to maximize the efficiency of antigen testing. Interestingly, in the same study (40) urine samples were positive more often than serum samples.

EB-A2 has also been used to coat latex beads. This latex agglutination test (Pastorex *Aspergillus*; Sanofi Diagnostics Pasteur) yielded sensitivities of 93.3% for a group of patients with proven invasive aspergillosis and 94.4% for a group of patients suspected of having this disease, while no false-positive results were observed (18).

Penicillium species have galactomannans immunologically similar or identical to those of *Aspergillus* species, so the cross-reactivity with *P. digitatum* is not a surprise. *Penicillium marneffei* occasionally causes disseminated infections (11, 23, 24). Patients with these infections have not yet been investigated with EB-A2-based ELISAs or latex agglutination tests, but guinea pigs with experimentally induced invasive *P. marneffei* infections yielded positive results with Pastorex *Aspergillus* (48, 49), although the antigen titers were lower than those in animals with experimentally induced invasive aspergillosis.

In the same studies, animals suffering from invasive *Trichophyton mentagrophytes* infections yielded negative results (48, 49). In a protocol developed by Arrese Estrada et al. (1), EB-A1, which reacted with *Trichophyton* antigens in the ELISA experiments presented here, was negative in

immunocytochemical investigations of Formalin-fixed, paraffin-embedded sections of tissues infected by *Trichophyton* spp. Although the influence of Formalin fixation must be taken into account, the two latter results may also suggest that the antigens produced by *Trichophyton* spp. in vivo differ qualitatively and/or quantitatively from those produced in vitro. The role of the medium in the amount of galactomannan secreted has already been shown for *A. fumigatus* (unpublished observation).

In conclusion, this study demonstrated that rat MAbs against *A. fumigatus* galactomannan, already applied to the serological (18, 40, 48) and immunohistological (1, 36) diagnosis of invasive aspergillosis, reacted with the β(1→5)galactofuranose-containing side chains of galactomannan. The epitope recognized was immunodominant and carried by a wide range of exocellular, intracellular, and wall glycoproteins.

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