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Chemical and Immunological Characterization of the Extracellular Galactomannan of *Aspergillus fumigatus*†

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The galactomannan (GM) produced extracellularly by *Aspergillus fumigatus* has been purified by a double sequential hydrazine-nitrous acid treatment of the ethanol precipitate of the culture filtrate. Nuclear magnetic resonance and gas-liquid chromatography–mass spectrometry analysis have been performed on intact GM, acid-hydrolyzed GM, and oligomers resulting from the acetolysis of the acid-hydrolyzed GM. Results show that *A. fumigatus* GM is composed of a linear mannan core with an α-(1-2)-linked mannotetraose repeating unit attached via α-(1-6) linkage. Side chains composed of an average of 4 to 5 β-(1-5)-galactofuranose units are linked to C-6 and C-3 positions of α-(1-2)-linked mannose units of the mannan. The immunoreactivity of GM and HCl-hydrolyzed GM was studied by use of human sera from aspergillosis patients and an antigalactofuran monoclonal antibody. The α-(1-2)(1-6)-mannan core is not antigenic. The immunogenic galactofuran is found amongst several exocellular glycoproteins. According to a direct enzyme-linked immunosorbent assay with GM as the detector antigen, only 26% of the serum samples from aspergilloma patients (all positive by immunodiffusion assays) give optical density values superior to a cutoff estimated as the mean ± 3 standard deviations of values obtained with control sera.

Galactomannan (GM) is a polysaccharide widely distributed amongst most *Aspergillus* and *Penicillium* species (3, 4, 6–8, 11, 16–18, 35, 39, 43). Because of their immunoreactivity, GM antigens are of special interest in medical mycology. In addition, several publications have mentioned that GM is present in the biological fluids of patients with invasive aspergillosis and its detection can be used for the immunodiagnosis of this life-threatening mycosis (3, 11, 12, 14, 35, 38). Several groups have reported on the chemical composition of GM produced in vitro. However, no definite structure has been established until now. The only consensus existing is the presence of a mannan core with side chains containing galactofuranosyl units. Even the structure of the galactofuran side chains, which have been studied extensively because of their immunoreactivity, remains controversial. These galactofurans have been characterized as linear chains of (1-4)-linked galactopyranose (Gal-p), (1-5)-linked galactofuranose (Gal-f), or branched chains with (1-5)- and (1-6)-linked Gal-f with 5,6-disubstituted Gal-f (4, 6, 30, 46). The discrepancy between the different studies could originate from the strain and culture conditions chosen and the methods used to extract, purify, and subsequently analyze the GM produced in vitro.

The study of the chemical characterization of the GM of *Aspergillus fumigatus* presented in this paper was directed by two concepts (27). (i) A simple analogy suggests that extracellular antigens represent a better picture of the in vivo circulating antigens than components extracted from the cell wall. This approach was used successfully to isolate from a culture filtrate of *A. fumigatus* an antigen of 18 kDa which also occurred in urine of patients with invasive aspergillosis (28). Preliminary fermentation kinetic studies have shown that during its active growth phase *A. fumigatus* secretes in the culture medium a complex mixture of proteins and polysaccharides rich in GM (27). (ii) It is possible to perform purification of exocellular GM according to mild protocols to retain the chemical integrity of the polysaccharidic structure. This study shows a structural analysis of *A. fumigatus* GM isolated from a culture filtrate by an original chemical purification procedure.

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MATERIALS AND METHODS

Production of an ethanol precipitate of the culture filtrate. Strain CBS 143-89 of *A. fumigatus* was cultivated in a 20-liter fermenter (Chemap) at 25°C, 500 rpm, 0.5 vol of air/vol of medium/min with a 2% glucose and 1% peptone (peptone Chapotaut; Touzart & Matignon, Paris, France) medium (initial pH, 6.3). The inoculum, used at an 8% (vol/vol) ratio, was a 60-h-old mycelial culture grown in the same conditions in a 2-liter fermenter; the preinoculum was 100 ml of medium inoculated with pieces of mycelial mat and grown in Erlenmeyer flasks at 150 rpm. The culture filtrate was precipitated with 4 volumes of ethanol. The ethanol precipitate (EP), washed three times with ethanol, was resuspended in water and stored freeze-dried.

General chemical analysis methods. Total hexoses were measured by the phenol-H_2SO_4 method (13). Total amino

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acids were estimated after 24 h of HCl (6 N) hydrolysis at 100°C by the ninhydrin method (47). Total hexosamines were determined with *p*-(dimethylamino)-benzaldehyde reagent after 2 h of HCl (8 N) hydrolysis at 100°C (42). Phosphate determination was done by the method of Ames and Dubin with NaH_2PO_4 as the standard (2).

Monosaccharide composition was determined after methanolysis with 0.5 M HCl in methanol for 24 h at 80°C. Methylglycosides were identified as trifluoroacetyl or trimethylsilylated derivatives by gas-liquid chromatography according to previously published procedures (21, 31, 48). Preliminary experiments have shown that the same monosaccharide molar ratio was observed after 4 N trifluoroacetic hydrolysis for 4 h at 100°C.

Purification of the GM. Preliminary studies have shown that proteases or hot NaOH treatments were not useful for purification of GM from EP. Hydrazine, which cleaves the peptide linkages and *N*-deacetylates the *N*-acetylhexosamines, was then used in conjunction with nitrous acid, which hydrolyzes hexosamines.

Hydrazinolysis was performed on freeze-dried EP with anhydrous hydrazine (Pierce) (1 ml per 15 mg of EP) overnight at 105°C (31). The resultant slurry was precipitated with 4 volumes of ethanol, and the precipitate was washed with ethanol and dialyzed overnight against running tap water. Nitrous acid hydrolysis of the EP submitted to hydrazinolysis was performed by addition of acetic acid to sodium nitrite to reach a 1.5 N nitrous acid solution (10). After overnight incubation at room temperature, air was bubbled through the solution. The solution was dialyzed against distilled water and precipitated with 4 volumes of ethanol. The precipitate was washed with ethanol and freeze-dried. The double treatment with hydrazine and nitrous acid was repeated once.

Final purification of the GM was obtained after recovery of the nonbound fraction to a MonoQ column (Pharmacia) eluted with 25 mM Tris-HCl (pH 7.5) buffer. Molecular weight of the polysaccharide was estimated by high-pressure liquid chromatography gel filtration on a Superose 12 HR (30/10) column (Pharmacia) eluted with NaOH (50 mM). Dextran T10, T40, T70, and T200 (Pharmacia) were used for molecular weight standards.

Purified GM samples hydrolyzed in 0.01 N HCl for 90 min and 24 h at 100°C were applied to a TSK HW40 (100/10) gel filtration column. Elution was performed with water at a 0.6-ml/min rate. Dextran T10, malto-oligosaccharides (M4 to M7) (Boehringer, Mannheim, Germany), raffinose, and sucrose were used as molecular weight standards.

Periodate treatment. Intact GM was submitted to 30 mM periodic acid oxidation for 15 min at 4°C. Longer periodate treatment resulted in the cleavage of mannan chains of GM. After destruction of the excess periodate with ethylene glycol (10 μl for 300 μg of GM in 250 μl of 30 mM Na periodate), the product was dialyzed and reduced with Na borohydride (2 mg) for 2 h at room temperature. After addition of acetic acid to a pH of 5 to 6 and dialysis against 0.5% acetic acid and then distilled water, the reduced-periodate-oxidized GM was freeze-dried. The arabinose and galactose content was estimated as described above.

Fragmentation of GM by acid hydrolysis and acetolysis. Hydrolysis of GM in HCl (0.01 N) was performed at 100°C in sealed tubes for 1 to 32 h. Hydrolyzed GM was recovered by ethanol precipitation or by the Fehling solution method (24). A hydrolyzed sample (18 h at 100°C in HCl [0.01 N]) of GM was submitted to acetolysis with a 10:10:1 (vol/vol/vol) mixture of acetic anhydride-acetic acid-sulfuric acid for 13 h at 40°C (25, 26). After de-*O*-acetylation, the oligosaccharides resulting from the preferential cleavage of α-(1-6) linkages were fractionated on a column (100/25) of Bio-Gel P-2 by elution with water at 0.25 ml/min. A series of α-(1-2)-linked manno-oligosaccharides isolated from *Candida albicans* were used as standards (22).

Methylation analysis. Intact and hydrolyzed polysaccharides were methylated by the lithium methyl sulfinyl carbanion method (34). After methanolysis with methanol-HCl (0.5 N), the products were acetylated with a 1:9 pyridine-acetic anhydride mixture overnight at room temperature. Identification of the 1-*O*-α- and 1-*O*-β-methyl-*O*-methyl-*O*-acetyl-glycosides was performed by gas-liquid chromatography–mass spectrometry with a DB1-coated glass capillary column (100 to 200°C, 2°C/min, nitrogen pressure of 8×10^4 Pa) and a Quadripolar Riber R 1010 mass spectrometer with an electron energy of 72 eV (15). The chemical designations of the methylglycosides have been abbreviated in the Results section. For example, after the methylation-acetylation protocols used in these experiments, 2-substituted mannose of a mannan chain produced 1-*O*-α- and 1-*O*-β-methyl-2-*O*-acetyl-3,4,6-tri-*O*-methylmannoside. This derivative will be designated below as 3,4,6-tri-*O*-methylmannoside.

Reductive cleavage experiments undertaken in order to simultaneously identify the linkage position and the ring size of the galactose moiety were performed as previously described (33). Basically, the reductive cleavage method was carried out in two steps with trimethyl trifluoromethane sulfonate as a catalyst. The reductive cleavage step lasted for 5 h under N_2. Identification of anhydro hexositols was assessed by comparison of their retention times on gas-liquid chromatography with authentic standards or values reported in the literature. The presence of specific fragments in mass spectra confirmed the identification of the *O*-acetyl-*O*-methyl-substituted hexoses.

NMR analysis. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded from 0 to 250 ppm with a Bruker AM 400-Wb spectrometer at 100 MHz from 20-mg samples in 0.5 ml of D_2O at 333 K. Chemical shifts are expressed in parts per million downfield from an external reference of a 0.5 M solution of sodium (trimethyl silane) 1-propane sulfonate (TSP) in D_2O. ^1H NMR spectra of manno-oligosaccharides obtained after acetolysis were recorded on a Jeol JNM-G8 × 400 spectrometer operating at 400 MHz at 70°C with 5-mg samples per ml of D_2O; acetone was an internal standard (23, 25). Assignment of H-1 proton signals was assessed by comparison with ^1H NMR analysis of α-(1,2)-linked mannopyranose units of *C. albicans* mannan (23, 25). Assignment of ^{13}C chemical shifts was done by comparison of resonance patterns previously published on GM samples from *Aspergillus* sp. and *Penicillium charlesii* and on reference monosaccharides substituted at selected positions (6, 7, 43, 44). Identification of the substituted carbon (C) of the internal sugar unit was indicated by a significant downfield shift of the signal obtained in the nonreducing hexose unit, whereas the signals of the neighboring carbons of the substituted position were shifted upfield (1, 9, 18, 19, 23, 25, 44).

Immunoreactivity of GM. Serum samples (n = 118) from 57 patients with aspergilloma were used through this study. One to six precipitin lines were detected by Ouchterlony diffusion assays with somatic and metabolic extracts from *A. fumigatus* (Diagnostic Pasteur, Marnes, France). Reactivity of sera was confirmed by indirect hemagglutination assay with the kit from Fumouze (Clichy, France). Serum samples (n = 31) from control patients were negative by these assays.

Sera from patients with aspergilloma were analyzed for the presence of antibody to *A. fumigatus* GM by a direct enzyme-linked immunosorbent assay (ELISA) method. Antigens tested

were intact purified GM, purified GM hydrolyzed for different time intervals in 0.01 N HCl at 100°C, and a soluble extract of an ultrasonicate of EP (SEP) in 50 mM ammonium acetate obtained after 5 min of centrifugation at 11,000 × *g*. EP was sonicated for 1 min with a 3-mm microtip probe in a Sonifer B30 cell disrupter. Wells of microdilution plates (F form; Greiner, Frickenhausen, Germany) were coated with 100 μl of a solution of purified GM (20 μg eq of glucose per ml) or SEP (10 μg eq of bovine serum albumin per ml) diluted in 50 mM carbonate buffer (pH 9.0) and incubated overnight at room temperature. Binding of antibodies to the ELISA plate was estimated with a 1:500 dilution of aspergilloma patient sera and peroxidase-conjugated anti-human immunoglobulin G as previously described (28).

SEP was electrophoresed at a concentration of 30 μg of protein per well in a 10% acrylamide gel. After electrophoresis, the gel contents were transferred onto nitrocellulose membranes, which were probed with positive and negative human sera (1:1,000 dilution) or rat anti-GM monoclonal antibody (MAb) (1:5,000 dilution) (41). Electrophoresis and immunoblotting conditions were as previously described (28). Anti-GM MAb was supplied by D. Stynen (Sanofi-Pasteur, Genk, Belgium).

RESULTS

Purification of the GM secreted in vitro by *A. fumigatus*. In the fermentation conditions used, maximal growth (around 8 mg of mycelial dry weight per ml of culture medium) was attained after 45 to 50 h of culture at a time when the pH reached 4.2. The end of growth was concomitant with an increase in the pH.

Material was released into the culture medium immediately after growth started; the release of extracellular material was a primary metabolic event and not a result of mycelial lysis. The ethanol-precipitable material (EP) was essentially composed of polysaccharides and proteins in an average proportion of 1:1. The polysaccharidic fraction was composed of GM and galactosamine (as a mixture of galactosamine and *N*-acetylgalactosamine) in a ratio of 1:2. Protein and hexosamine secretion was parallel to growth, whereas maximal polysaccharide recovery was obtained at the end of mycelial growth (data not shown). GM accounted for 14% of EP (dry weight) and reached a value of 125 μg eq of glucose per ml of culture filtrate after 48 h of growth (average on seven different fermentation runs). EP contained mannose and galactose in the proportion 1:1.5. However, depending on the batch studied, the molar ratio of galactose to mannose can vary between 0.9 and 2.8. Slight variations can also be observed over time during growth (data not shown).

A double hydrazine-nitrous acid treatment allowed the recovery of a pure GM. Maximal contamination of the hydrazine-nitrous acid-treated EP with hexosamine or amino acids accounted for up to 5% of the total amount of the recovered GM. After dialysis, the GM was further purified as the nonbound fraction in a MonoQ anionic exchange column to remove the chemical impurities remaining from the chemical extraction. On a Superose 12 column, the GM had an M_r of 20,000 on the basis of a standard curve obtained with the dextrans T10, T40, and T70 from Pharmacia. Mannose-to-galactose ratios varied from 1:0.9 to 1:1.7 depending on the GM batch analyzed (average on 10 different GM samples, 1:1.2). No phosphate could be detected by the phosphomolybdate reaction after mineralization of the sample with magnesium nitrate.

Structural analysis of the purified GM. (i) Intact GM. The permethylated GM was acetylated after standard HCl-methanolysis or reductive cleavage. The different methyl-*O*-methyl-*O*-acetyl-substituted hexoses were identified by gas chromatography followed by mass spectrometry (Table 1). The presence of large amounts of 2,3,5,6-tetra-*O*-methylgalactoside and only traces of 2,3,4,6-tetra-*O*-methylmannoside indicated that GM was composed of a mannan core with galactan side chains. Results of the methylation analysis showed that the mannan chain was composed of 2-, 6-, 2,6- and 2,3-di-*O*-linked mannopyranose. Similar concentrations of 2-*O*-linked and 6-*O*-linked mannopyranose were found. In addition, the amount of terminal nonreducing galactofuranosyl residues was close to the total amount of disubstituted mannopyranose residues, suggesting that all these mannosyl residues were linked to the galactose side chains.

TABLE 1. Relative retention times and molar ratios of the different *O*-methyl-*O*-acetyl-glycosides obtained from *A. fumigatus* GM

Compound	Sugar linkage(s)	Relative retention time(s) (min)[a]	Molar ratio	
			GM	HCl-GM[b]
2,3,5,6-Me_4-Man-p	t-Man-p	6.7	—[c]	0.1
3,4,6-Me_3-Man-p	2-Man-p	8.4	1.3	2.6
2,3,4-Me_3-Man-p	6-Man-p	12.1	1	1.0
3,4-Me_2-Man-p	2,6-Man-p	15.5	0.8	0.4
4,6-Me_2-Man-p	2,3-Man-p	15.7	0.7	0.4
2,3,5,6-Me_4-Gal-f	t-Gal-f	6.8	1.1 (1.1)[d]	0.6
2,3,6-Me_3-Gal-f	5-Gal-f	8.7, 10.4, 10.9, 11.2	5 (5.2)	—

[a] Gas-liquid chromatography conditions: 25 m DB1, 120 → 200°C at 2°C/min; major isomeric peaks are mentioned.
[b] Hydrolysis for 18 h at 100°C in HCl (0.01 N).
[c] —, not detected.
[d] Values in parentheses were obtained after reductive cleavage.

Terminal galactose residues occurred only in the furanose form, since only 2,3,5,6-tetra-*O*-methylgalactoside was found (characterized in mass spectrometry by an *m/e* of E1 ion of 161). The presence of terminal galactose in the furanosyl form was confirmed by the formation of arabinose after GM oxidation by periodate, which is responsible for the cleavage between C-5 and C-6 of the galactofuranosyl residue (data not shown). Four 2,3,6-tri-*O*-methylgalactosides were found: α- and β-methyl-4-*O*-acetyl-2,3,6-tri-*O*-methylgalactopyranoside and α- and β-methyl-5-*O*-acetyl-2,3,6-tri-*O*-methylgalactofuranoside. These compounds corresponded to either 4-*O*-linked galactopyranosyl or 5-*O*-linked galactofuranosyl residues which standard methylation analysis did not discriminate because of constriction of the galactose ring. The reductive cleavage technique, which excluded the rearrangement of the sugar ring, showed that only 5-*O*-acetyl-2,3,6-tri-*O*-methyl-1,4-anhydrogalactitol was found (Table 1). This result indicated that the galactan side chains of the GM were composed only of 5-*O*-linked galactofuranosyl sugars.

These results were confirmed by ^{13}C NMR spectroscopy of the intact GM (Table 2 and Fig. 1A). Nonreducing terminal β-galactofuranosyl units were characterized by ^{13}C chemical shifts at 110.4, 110.5 (C-1), 84.0 (C-2), 79.5 (C-3), 85.8 (C-4), 73.5 (C-5), and 65.6 (C-6) ppm. Chemical shifts at 109.6, 109.8 (C-1), 84.1 (C-2), 79.3 (C-3), 84.6 (C-4), 78.5 (C-5), and 63.9 (C-6) ppm were assigned to internal galactofuranosyl units. The C-5 signal of the internal galactofuranosyl unit at 78.5 ppm appeared in the lower magnetic field from the C-5 signal of the nonreducing terminal unit, which was assigned at 73.5 ppm (19, 43, 44). In addition, signals for the C-4 and C-6 carbons of the

TABLE 2. ^{13}C chemical shifts of GM and assignment of carbon signals of galactofuranosyl side chains of GM from *A. fumigatus* and *P. charlesii*

Carbon	^{13}C chemical shift(s) (ppm)					
	G β→5			β→5G β→		
	This study	BB[a]	UG[b]	This study	BB	UG
C-1	110.4, 110.5	110.4	(110.5)	109.8, 109.6	109.6	109.6 (109.5)
C-2	84.0	ND[c]	ND	84.1	84.1	84.1 (84.0)
C-3	79.5	ND	(79.1)	79.3	79.3	79.5 (79.1)
C-4	85.8	85.6	ND	84.6	84.6	84.7 (ND)
C-5	73.5	73.4	73.3 (72.8)	78.5	78.5	78.3 (78.2)
C-6	65.6	65.6	(65.6)	63.9	63.8	63.8 (63.7)

[a] BB, assignments made by Barreto-Bergter et al. (6). An increment of 1.1 ppm has been added to each chemical shift cited from this publication in order to convert to our chemical shift reference system.
[b] UG, assignments made by Unkefer and Gander (43, 44). Assignments from 1979 are in parentheses.
[c] ND, not detected.

internal residues were shifted in the higher magnetic field at 84.6 and 63.9 ppm, respectively. These results indicated that prominent ^{13}C signals of the intact GM were due to β-(1-5)-linked galactofuranosyl residues constituting the galactan side chain.

The average size of the galactofuran side chains was determined from convergent but slightly different results obtained after various chemical modifications of the GM. After periodate treatment and borohydride reduction, the galactose/arabinose ratio (corresponding to the internal Gal-f/terminal Gal-f ratio) was 3.2. ^1H NMR data showed that the ratio of internal Gal-f residues to nonreducing terminal Gal-f residues had a value of 3.3 (data not shown). A value of 4 was calculated for the ratio of the concentration of 2,3,5,6-tetra-*O*-methylga-

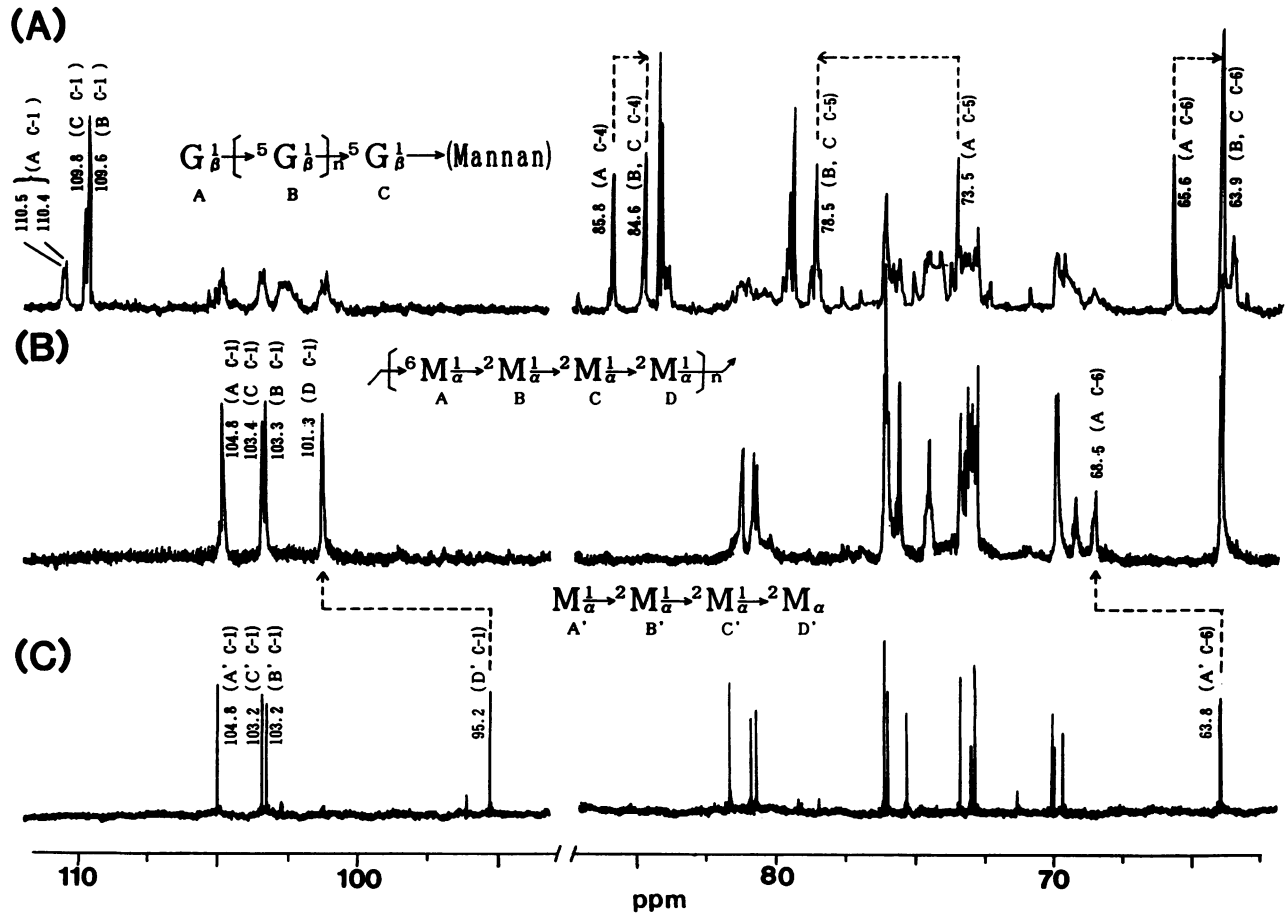

FIG. 1. ^{13}C NMR spectra of intact GM (A), GM hydrolyzed for 18 h at 100°C (pH 2) (B), and mannotetraose obtained by acetolysis from HCl-hydrolyzed GM (C). Chemical shifts from 62.5 to 111 ppm were recorded downfield from a 0.5 M TSP external reference.

lactofuranoside plus 2,3,6-tri-*O*-methylgalactofuranoside to 3,4-di-*O*-methylmannoside plus 4,6-di-*O*-methylmannoside. The calculation of the ratio of 2,3,6-tri-*O*-methylgalactofuranoside to 2,3,5,6-tetra-*O*-methylgalactofuranoside gave a value of 4.5 (Table 1). On the basis of these data, it can be estimated that the galactofuran side chain was composed of an average of 4 to 5 galactofuranose residues.

(ii) HCl-hydrolyzed GM. HCl (0.01 N) hydrolysis has been repeatedly described as an efficient treatment to remove β-(1,5)-galactofuranosyl residues from GM. Indeed, hydrolysis of the GM overnight at 100°C in HCl (0.01 N) removed the galactofuran side chains. Standard methylation analysis showed that HCl-hydrolyzed GM (overnight at pH 2.0 at 100°C) was mainly composed of 2,3,4-tri-*O*-methyl- and 3,4,6-tri-*O*-methylmannoside in a ratio of 1:2.6 (Table 2). Only traces of permethylmannoside were detected. The interpretation of these data is that the mannan core is a linear chain of (1-2)- and (1-6)-linked mannosyl residues. The increase in the amount of 2-*O*-linked mannopyranosyl residues following HCl hydrolysis (Table 2) indicated that (1-2)-linked mannose residues were the hexose residues involved in the branching of the galactan side chains to the mannan core. The presence of 3,4-di-*O*-methyl- and 4,6-di-*O*-methylmannosides in the intact GM indicated that the galactofuran side chains were bound on C-6 and C-3 of two different (1-2)-linked mannose residues.

The detection of 2,3,5,6-tetra-*O*-methylgalactoside and 4,6- and 3,4-di-*O*-methylmannoside in the HCl-hydrolyzed GM suggested that galactofuranosyl residues at the reducing end of the galactofuran side chains, i.e., the galactofuranosyl units involved in the branching to the mannan core, were more resistant to hydrolysis than all other galactofuranosyl residues of the side chains. Kinetic studies of the 0.01 N HCl hydrolysis showed that complete removal of the galactose without alteration of the mannan core was impossible. Hydrolysis of the galactofuranosyl subunits was always linked to some hydrolysis of the mannan, but to a lower extent (Fig. 2). The concomitant hydrolysis of mannan with galactan was confirmed by gel filtration studies of the hydrolyzed GM. The molecular mass of the hydrolyzed GM recovered after 24 h of HCl (0.01 N) hydrolysis was around 3 to 4 kDa (Fig. 3).

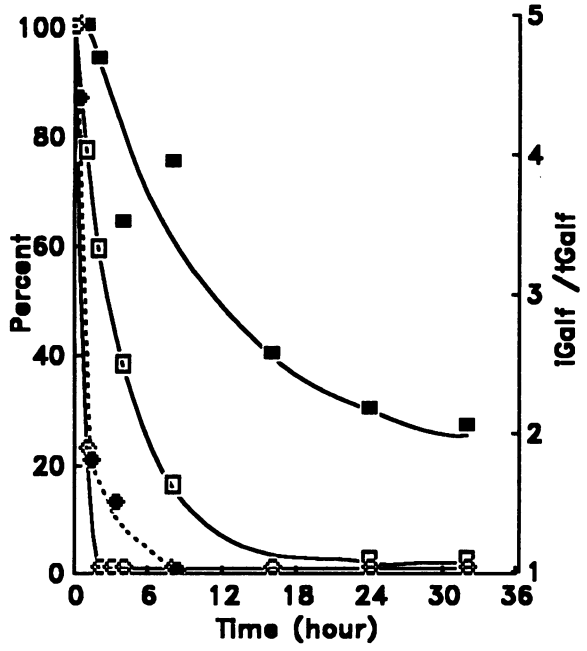

FIG. 2. Kinetic studies of hydrolysis of GM by HCl (0.01 N) at 100°C. Concentrations of mannose (■) and galactose (□) recovered in the ethanol precipitate of the different hydrolyzed samples are expressed as percentages of the initial amounts of each hexose in the nonhydrolyzed samples. Immunoreactivity of the hydrolyzed GM samples (○) is expressed as a percentage of the reactivity of the GM to a rat antigalactofuran MAb. The ratio of 2,3,6-tri-*O*-methylgalactofuranoside to 2,3,5,6-tetragalactofuranoside (iGalf/tGalf), which is an indication of the size of the galactofuran side chain (●), is also shown.

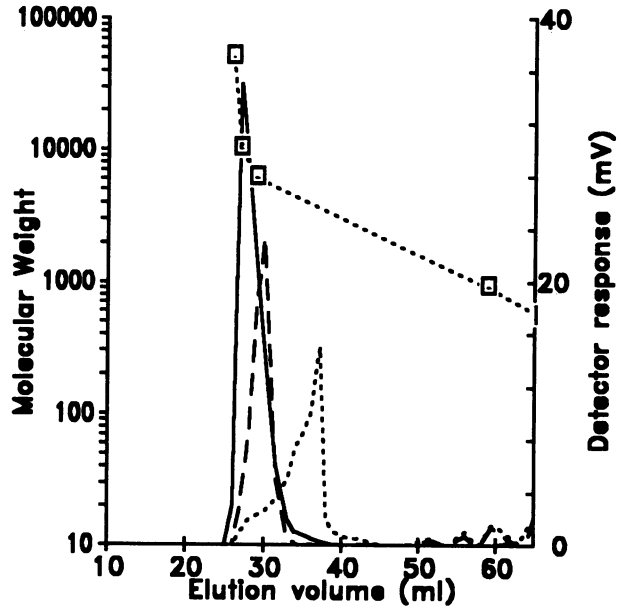

FIG. 3. Gel filtration on TSK HW40 of an intact GM (solid line) and of GM hydrolyzed at 100°C with HCl (0.01 N) for 90 min (dashed line) and 24 h (dotted line). Standard molecular weight markers (□) are shown on the left scale. Sugars were detected with a refractometer.

The ^{13}C spectrum of the HCl-hydrolyzed GM showed that the mannopyranosyl units are α linked (Table 3 and Fig. 1B). Comparison of the ^{13}C chemical shifts of the *A. fumigatus* HCl-hydrolyzed GM with those obtained by Unkefer and Gander after hydrofluoric acid treatment of the GM from *P. charlesii* (43) indicated the presence of a linear mannan with an α-(1-2)-linked tetraose repeating unit attached via α-(1-6) linkage. Characteristic chemical shifts of C-1 for this repeating unit are 104.8 ppm (-6M-2M), 103.3, 103.4 (-2M-2M), and 101.3 (-2M-6M) ppm. Assignments of signals of carbons C-2 to C-6 are in agreement with this repetitive tetraose structure.

The occurrence of α-(1-6)-linked mannopyranose units in the mannan core was assessed by acetolysis, which preferentially cleaves α-(1-6) linkages instead of α-(1-2) linkages. Results of the acetolysis of the GM hydrolyzed by HCl are presented in Fig. 4A. The acetolysate contained a large amount of tetramer in addition to low concentrations of trimer and dimer. ^{1}H NMR analysis showed that these oligosaccharides were composed of α-(1-2)-linked mannopyranosyl units (Fig. 4B) (23, 25). The major liberation of an α-(1-2)-linked mannotetraose during acetolysis was in agreement with the presence of a tetrasaccharide repeating unit of the form $(\text{-6M-2M-2M-2M})_n$ in the mannan core.

The ^{13}C chemical shifts assigned to the mannopyranosyl units of the α-(1-2)-linked mannotetraose are presented in Table 3 and Fig. 1C. Downfield shifts to 101.3 and 68.5 ppm

TABLE 3. Assignment of carbon signals of the core mannan of the GM of *A. fumigatus*

Carbon	^{13}C chemical shift(s) (ppm) of:											
	HCl-GM[a]									Acetolysis-HCl-GM (M4)[b]		
	$\xrightarrow{\alpha}$6M $\xrightarrow{\alpha}$2			$\xrightarrow{\alpha}$2M $\xrightarrow{\alpha}$2			$\xrightarrow{\alpha}$2M $\xrightarrow{\alpha}$6			M $\xrightarrow{\alpha}$2	$\xrightarrow{\alpha}$2M $\xrightarrow{\alpha}$2	$\xrightarrow{\alpha}$M2
	This study	BB[c]	UG[d]	This study	BB	UG	This study	BB	UG			
C-1	104.8	104.8	105.0	103.4, 103.3	103.4	103.6, 103.5	101.3	101.1	101.3	104.8[e]	103.2, 103.2[f]	95.2
C-2	73.4	73.2	73.8	80.7, 80.8	80.9	80.8, 80.9	81.2	81.1	81.3	73.1	81.2, 81.0	81.8
C-3	74.5	ND[g]	74.5	73.0, 73.1	ND	73.1, 73.2	72.8	ND	72.9	72.8	72.7, 72.7	72.7
C-4	69.2	ND	69.3	69.9, 70.0	69.8	70.0	69.7	ND	70.0	69.6	69.8, 69.8	69.8
C-5	75.6	ND	75.7	76.1, 76.1	76.0	76.2	76.0	ND	76.2	75.9	75.9, 75.9	75.2
C-6	68.5	ND	68.5	63.7, 63.7	63.8	64.0	64.0	ND	64.0	63.9	63.8, 63.8	63.8

[a] Mannan isolated by HCl hydrolysis from GM of *A. fumigatus* and *P. charlesii*.
[b] Mannotretaose (M4) isolated by acetolysis of the HCl-hydrolyzed GM of *A. fumigatus*.
[c] BB, from Barreto-Bergter et al. (6); an increment of 1.1 ppm has been added to each chemical shift cited from BB in order to convert to our chemical shift reference system.
[d] UG, from Unkefer and Gander (44).
[e] An increment of 1.8 ppm has been added to the chemical shift obtained with acetone as a reference in order to convert to our TSP reference system.
[f] Two peaks with a difference in chemical shift of <0.1 ppm are indicated with the same chemical shift values.
[g] ND, not detected.

from the C-1 signal of the reducing terminal unit (95.2 ppm) and the C-6 signal of the nonreducing terminal unit (63.8 ppm), respectively, indicated that the mannotetraose units were linked in a linear fashion via α-(1-6) linkage.

Immunoreactivity of purified GM. Direct ELISA data showed that GM reacted antigenically in a manner similar to that of an EP of the culture filtrate (Fig. 5). However, not all aspergilloma patients detected on the basis of positive immunodiffusion and hemagglutination tests were positive on the basis of ELISA with either GM or a soluble extract from EP. Only 36 and 26% of serum samples from aspergilloma patients (all positive in immunodiffusion assays) gave optical density (OD) values superior to the cutoff values for EP and GM, respectively. The cutoff value was calculated as the mean ± 3 standard deviations of OD values obtained with sera from control individuals who did not have any *Aspergillus* infection. In addition, no correlation between the number of precipitin lines and the ELISA values obtained with either GM or an ammonium acetate-soluble extract from the EP of the culture filtrate was found (Fig. 5). From these data, it can be concluded that (i) EP and GM are not suitable for detection of all aspergilloma patients by ELISA, and (ii) when present, high ELISA titers toward EP were due to antibodies directed against GM.

ELISA data were confirmed by Western blot (immunoblot) results. When EP was electrophoresed and blotted with an anti-gal-f MAb, several discrete glycoprotein bands with molecular masses between 35 and 94 kDa were identified. In addition, a very wide zone with molecular masses of >100 kDa was always detected (Fig. 6). Human sera positive in ELISA with both EP and purified GM reacted like the antigalactofuran MAb, i.e., with both the discrete low-M_r- and the wide high-M_r-positive bands. However, different Western blot patterns were seen according to the reactivities of the sera in ELISA. Sera highly reactive with GM were characterized by a strong reaction of the high-M_r wide band. In contrast, sera poorly reacting with GM reacted only with discrete low-molecular-mass protein bands and not at all with the >100-kDa wide band. The reactivities of these antigens to both rat MAb and human antibodies were destroyed by an overnight treatment of the blot with a 0.1 M periodate solution (data not shown).

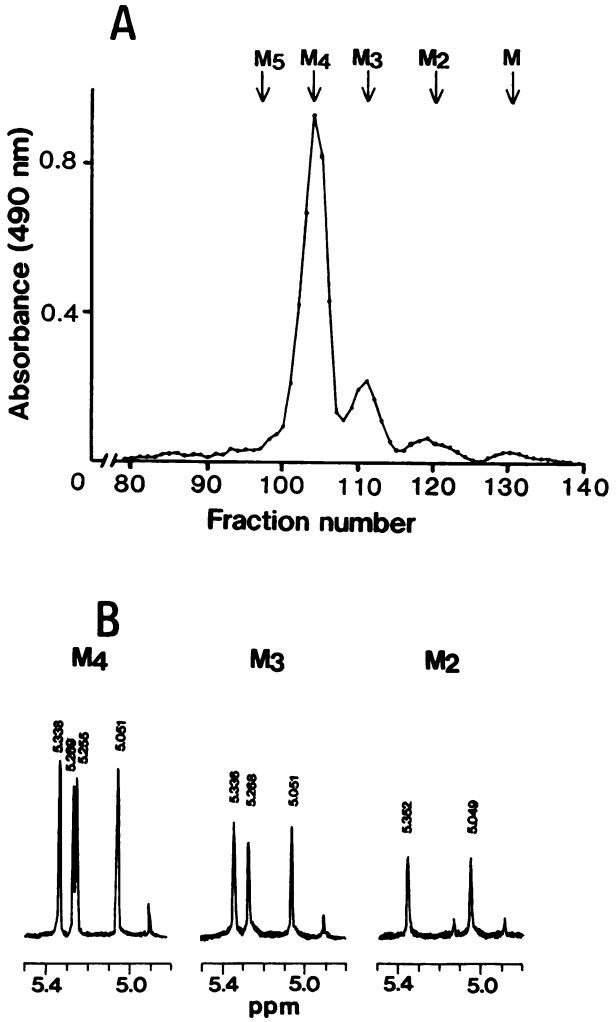

FIG. 4. Oligosaccharides obtained by acetolysis of an HCl-hydrolyzed GM. (A) Elution profile. Arrows indicate the elution positions of α-(1-2)-linked manno-oligosaccharides (pentaose to biose [M5, M4, M3, and M2] and mannose [M]). (B) ^{1}H NMR spectra of the oligosaccharides.

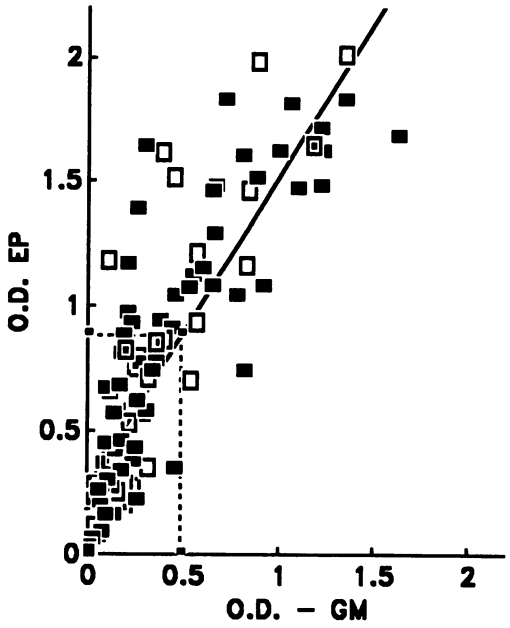

FIG. 5. OD values obtained with sera from aspergilloma patients in ELISA experiments with 1:500 dilutions of the sera. Each serum is characterized by two OD values (O.D.-EP and O.D.-GM), obtained with microtiter plates coated with either an ammonium acetate-soluble extract of an EP of the culture filtrate or a purified galactomannan (GM). The significance of the results can be estimated by comparison with the dotted lines parallel to the axes, which correspond to mean OD values ± 3 standard deviations obtained with control sera (□, serum samples showing at least four precipitin lines on Ouchterlony diffusion assays).

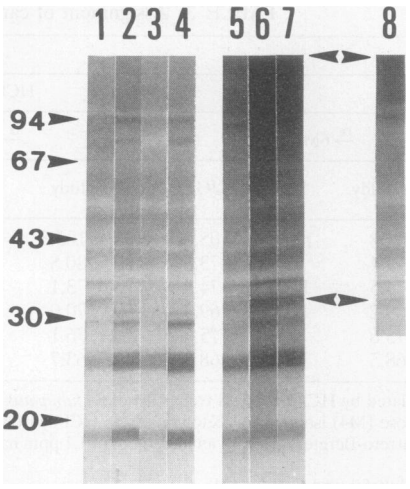

FIG. 6. Immunoblot analysis of EP with aspergilloma-positive human sera reacting differentially toward GM and EP in ELISA experiments. Lanes 1 to 4, sera reacting poorly with purified GM; lanes 5 to 7, sera reacting positively with GM. Lane 8, probed with an anti-GM rat MAb, shows that only components with molecular masses of >35 kDa contain the galactofuran epitope. Molecular masses (in kilodaltons) are shown at the left.

Galactofuran was the only antigenic part of these molecules, since the mannan core of the GM (recovered after acid hydrolysis) reacted only slightly with human sera from aspergilloma patients: ODs of 1:100 dilutions of the positive and control pool sera were respectively 0.75 and 0.62 in an ELISA experiment in which the microdilution plate was coated with hydrolyzed GM at a concentration of 10 μg eq of glucose per ml. Indeed, very limited hydrolysis of the galactofuran side chains resulted in the loss of the immune reactivity of the epitope (Fig. 2). When the side chain of the galactofuran had an average degree of polymerization of <2, no immunoreactivity with the anti-GM MAb was seen.

DISCUSSION

Several groups have reported on the chemical composition of *A. fumigatus* GM. However, no consensus structure has appeared from previous literature (Table 4). Discrepancy increases when structural data obtained on *A. fumigatus* GM are compared with data gathered from GMs of other species of *Aspergillus* and *Penicillium* which also are known to synthesize GM. Several reasons could explain the different results recorded during all structural analyses reported previously: (i) strains and culture conditions were different in each study, (ii) extraction methods vary from one study to another, (iii) several GM batches analyzed were insufficiently purified and contained contaminants such as, for example, proteins, and (iv) some of the analytical methods used were inadequate or insufficiently accurate.

It has been shown that changes, even slight, in the composition of the culture medium can be associated with important structural modifications of the secreted polysaccharide. For example, the use of a peptone from Touzart & Matignon results in the isolation of GM with a 1.5:1 ratio of galactose to mannose, whereas the use of Mycopeptone from Biokar is accompanied by the isolation of a glucogalactomannan with a Gal:Man:Glc ratio of 1:1:5. Glucose was also found associated with the GM isolated by Barreto-Bergter et al. (6), Mischnick and De Ruiter (30), and de Repentigny et al. (12). Similarly, the composition of the peptidogalactomannan isolated from *P. charlesii* is highly influenced by the pH of and the amount of phosphate in the culture medium (40). The strain chosen for GM studies may also be important. Although all strains tested were proven to produce GM, Gal:Man ratios found in the EPs of various strains of *A. fumigatus* can vary from 1:0.5 to 1:2. In addition, glucan present in EP can account for less than 10 to 50% of the total sugar content of EP (11).

The extraction methods and/or localization of the GM extracted by previous authors are different. With the exception of early studies by Sakaguchi et al. (39) and recent work by van Bruggen-van der Lugt et al. (46) and Mischnick and De Ruiter (30), all *A. fumigatus* GMs analyzed were extracted from disrupted mycelial cell walls by a harsh extractive procedure (most often, hot sodium hydroxide) and further purified by liquid chromatography (ion exchange, concanavalin A affinity, and size exclusion) (4, 6, 8, 12, 35). It is known that the monosaccharide composition of the polysaccharides of the cell wall and of the culture filtrate of *A. fumigatus* are very different (27). From results presented in Table 4, it seems that GM compositions could be clustered into two groups depending on the method of extraction used. GMs extracted from cell walls are characterized by the absence of 2,3-disubstituted mannopyranose (Man-p) and the presence of 4,6-disubstituted Man-p and high concentrations of nonreducing terminal Man-p. Bennett et al. (8) and Reiss and Lehmann (35) have even failed to detect (1-6) Man-p linkages. The presence of terminal Man-p residues and the identification of mannose, biose, triose, and tetraose after acetolysis of the mannan have led Barreto-Bergter et al. (6, 7) to suggest that the mannan core is a highly

TABLE 4. Comparison of relative molar ratios of components identified by various authors in different samples of *A. fumigatus* GM

Compound	Molar ratio[a] of indicated substance according to:						
	This study		Previous studies[b]				
	GM	HCl-GM[c]	GM-A[d]	GM-B[d,e]	HCl-GM-B[d,e,f]	GM-C[g]	GM-D
t-Man-p	—[h]	0.1	0.4	1.6	2.9	+	1.7
2-Man-p	1.2	4.2	1.1	1.9	5.5	+	1.0
3-Man-p	—	—	—	0.4	0.6	−	—
6-Man-p	0.9	1.6	0.2	1.4	3.6	−	—
2,3-Man-p	0.7	0.6	0.2	—	—	−	—
2,6-Man-p	0.6	0.6	0.7	1.6	2.0	−	0.7
4,6-Man-p	—	—	—	0.4	1.0	+	1.2
t-Gal-f	1.0 (1.0)[i]	1.0	1.0 (1.0)[i]	1.0	1.0	+	1.0
5-Gal-f	4.5 (4.7)	—	4.5 (4.4)	5.6[j]	tr[j,k]	+[j]	2.0[j]
6-Gal-f	— (—)	—	0.7 (1.3)	—	—	−	—
4-Gal-p	— (—)	—	— (0.5)	—	—	−	—
4,6-Gal-p	—	—	— (tr)	—	—	−	—
5,6-Gal-f	— (—)	—	tr (0.1)	—	—	−	—

[a] Calculated from permethylated alditol acetates obtained by standard methylation analysis and identified by comparison with standard alditol acetates.
[b] A, Mischnick and De Ruiter (30); B, Barreto-Bergter et al. (6); C, Bennett et al. (8); D, Reiss and Lehmann (35).
[c] Hydrolysis for 18 h in HCl (0.01 N) at 100°C.
[d] Glucitol acetates detected in the samples are not included in the table from which the values in this column are drawn.
[e] Averages calculated from data on GM from 5- and 10-day-old mycelia on OV 17 and OV 225 columns.
[f] Hydrolysis for 8 h at 100°C at pH 1.8.
[g] Molar ratios not given. +, present; −, absent.
[h] —, not detected.
[i] Values in parentheses were obtained after reductive cleavage.
[j] In the absence of reductive cleavage analysis, the presence of 5-Gal-f was not ascertained.
[k] tr, traces.

branched structure with mannosyl residues linked in a way similar to that of the yeast mannans. In contrast, extracellular GM from either *A. fumigatus* (Table 4) or *P. charlesii* (17) are characterized by the presence of 2,3-disubstituted Man-p, very low amounts of terminal Man-p, and the absence of 4,6-disubstituted Man-p. The methylation analysis, the NMR data, and the detection of tetraose mainly after acetolysis reported herein are all in favor of the presence of a linear (1-2)-(1-6) mannan in the exocellular GM of *A. fumigatus* similar to the mannan core of the GM produced by *P. charlesii*. Similar mannose repeating units have also been found in several *Hansenula* species (5).

GMs analyzed by previous authors were insufficiently purified, or their composition can be biased by the extraction procedure. For example, it is known that dilute alkalis degrade glycopeptides isolated from culture filtrates. In addition, concanavalin A affinity partition would select GM-containing compounds with numerous free nonreducing Man-p terminal groups, since these sugar residues have the highest affinity for concanavalin A. Moreover, the presence of contaminating proteins or hexosamine polymers in the GMs analyzed has not always been checked. When assessed, it was found that the GMs analyzed were contaminated by proteins. The amount of protein detected ranged from 10 to 50% of the material extracted (8, 12, 20). Similarly, the peptido-phosphogalactomannan isolated from *P. charlesii* contained 11% amino acids (36). The presence of contaminating proteins, if glycosylated, could account for an increase in the detection of nonreducing terminal Man-p, since N-glycosylated antigenic proteins which do not contain Gal-f have been detected in culture filtrates of *A. fumigatus* (22).

Nondestructive analysis such as NMR has advantages over the use of chemical methods, which always cleave, to some extent, specific sugar linkages. For example, acetolysis has always produced a mixture of monosaccharides and oligosaccharides with dp of 2, 3, 4, and even 5 (4, 6, 7, 17). These results are incompatible with the occurrence of a linear mannan with a (-2M-2M-2M-6M) repeating unit which should release a tetraose after acetolysis. However, when performed in mild conditions, acetolysis would result in the formation of a majority of mannotetraose (this study and references 4 and 17). In addition, the oligosaccharide that elutes in the position of the pentasaccharide can be a tetraose containing phosphorus and carbons from ethanolamine, as it occurs in *P. charlesii* glycopeptide (45).

Terminal galactofuranosyl residues have been identified in all GMs from *Aspergillus* and *Penicillium* species analyzed until now. Internal galactosyl residues have been assigned to 4-O-linked galactopyranosyl residues or 5-O-linked galactofuranosyl residues (4, 6, 8, 43, 44). Because of isomerization of the sugar ring, standard methylation analysis cannot correctly determine the configuration of the internal galactose sugar ring. Reductive cleavage analysis used in this study has confirmed nondestructive NMR data and indicated that galactan side chains are composed exclusively of β-(1-5)-galactofuranose. In contrast to previous studies (30, 46), neither β-(1-6)-linked galactofuranosyl residues nor 5,6-disubstituted galactofuranosyl residues were found in *A. fumigatus* GM. The reasons for these differences are unknown. It is not due to the analytical techniques used by De Ruiter and collaborators, since a GM extracted under our conditions did not show any (1-6)-linked galactofuranose in their experiments (12a). It may be due to a particular strain employed by these researchers, although several strains tested in our laboratory all produced exclusively β-(1-5)-linked galactofuranosyl side chains (data not shown). Another explanation is the difference in the methods of extraction of the GMs analyzed by both groups. The culture media are different, and these researchers have not controlled the purity of the GM. Alternatively, our hydrazine-nitrous acid treatment may have altered β-(1-6)-Gal-f residues.

The nature of the branching point of the galactofuran side chain to the mannan core was questionable. Azuma et al. (3) and Barreto-Bergter et al. (6) have indicated that Gal-f are

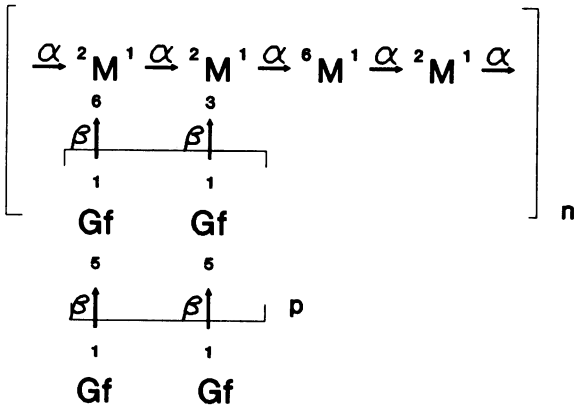

FIG. 7. Hypothetical structure of the repeating unit of the GM secreted by *A. fumigatus*. Average calculated values are 3 to 4 for p and 9 to 10 for n.

(1-6) linked to the mannan. Gander et al. (17, 44) demonstrated that in *P. charlesii*, galactofuran side chains are linked to (1-2)-mannopyranosyl units through (1-3) linkages, accounting for the 4,6-di-*O*-methyl mannoside. The other disubstituted 3,4-di-*O*-methyl mannoside would be derived from a mannopyranosyl residue in which a phosphoryl group is substituted at the C-6 or C-2 position. Our results showed that no phosphoryl residues are present in *A. fumigatus* GM. This would suggest that Gal-f are linked to (1-2)-mannose via (1-3) (similar to *P. charlesii*) and (1-6) linkages (as indicated in references 3 and 6). A recent report by Hearn et al. (20) has indicated the possibility that β-Gal-f is linked to α-Man-p with a variety of linkages including (1-2), (1-3), and (1-6) linkages. The final identification of the branching point will be possible only after extensive hydrolysis of the galactofuran by an exo-β-D-galactofuranosidase.

The structure of *A. fumigatus* GM presented in Fig. 7 can be deduced from our studies. Two matters remain undetermined: (i) the positions of the 2-*O*-linked mannosyl residues bound to the galactofuran side chains in the mannotetraosyl repeating unit were not assessed, and (ii) the definite size of each β(1-5)-galactofuran side chain has not been assessed accurately, since slightly different conclusions could be drawn from the results of periodate oxidation, methylation, and NMR analysis. In addition, it is still unknown whether all galactofuran side chains have the same dp or whether the distribution is uneven, with a single galactofuranosyl unit (like that in GMs isolated from several other human fungal pathogens [29]) and longer chains of dp ≥ 7. The presence of two signals at 110.4 and 110.5 ppm for the ^{13}C chemical shifts for the nonreducing galactofuranose unit could indicate the presence of two different types of galactofuran side chains. In addition, the occurrence of variable Gal:Man ratios in the different batches of GM analyzed could result from the presence of an exo-galactofuranosidase produced by *A. fumigatus* in the culture medium. In the case of *P. charlesii*, this enzyme is very active in the culture filtrate at acidic pH when the length of the polygalactofuranosyl chain in the glycopeptide increases (37). Slightly higher (5 to 6) dp of the galactofuranosyl units were obtained in *A. fumigatus* by Barreto-Bergter et al. (6) and Mischnick and de Ruiter (30). In the case of *Penicillium* species, the ratio of internal (1-5)-galactofuranose to terminal nonreducing galactofuranose was higher and reached values between 8 and 10 for *Penicillium digitatum* and *P. charlesii*. The presence of galactofuran side chains with an average dp of ≥4 in *A. fumigatus* is in agreement with immunochemical studies performed with antigalactofuran-specific rat MAbs. The epitope recognized by these MAbs is a β-(1-5)-oligogalactofuranosyltetraose (41).

This is the first study in which the antigenic potential of purified GM was assessed in the absence of contaminating proteins. It confirmed other findings, demonstrating that galactofuran side chains are immunodominant in *A. fumigatus* (8, 32). The fact that not all sera from aspergillosis-positive patients selected on the basis of positive immunodiffusion react with GM ELISA suggested that GM-containing antigens are not the precipitating diagnostic antigens. Two types of galactofuran antigens seem to be secreted by *A. fumigatus*. The first series is composed of glycoproteins with a short oligosaccharide moiety as shown by discrete bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis. The galactofuran can be O linked to these proteins, as suggested by Hearn et al. (20). The second antigenic entity can be a complex molecule similar to the lipo-peptidophosphogalactomannan isolated from *P. charlesii* (17, 36, 45). This polymer is composed of a peptide part containing 30 to 32 amino acids to which mono- and oligosaccharides were attached through O-glycosidic linkages of the terminal mannosyl residues to the hydroxyl groups of serine and threonine residues. In addition, it contained ethanolamine and choline linked through phosphodiester bonds to C-6 of the mannose residues. Because of its very low charge and the presence of lipids, this molecule could produce a wide smear after electrophoresis, in a way similar to the one observed for the wide >100-kDa band seen in Western blots with *A. fumigatus* extracts. The GM characterized in our study could be part of a similar molecule, since the purification methods would have cleaved such a molecule.

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