A peptidorhamnomannan from the mycelium of *Pseudallescheria boydii* is a potential diagnostic antigen of this emerging human pathogen

Marcia R. Pinto,¹ Barbara Mulloy,² Rosa M. T. Haido,³ Luiz R. Travassos⁴ and Eliana Barreto Bergter¹

Author for correspondence: Eliana Barreto Bergter. Tel: +55 21 590 3093. Fax: +55 21 5608344.
e-mail: immgbel@microbio.ufrj.br

¹ Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Ilha do Fundão, 21941-970, Rio de Janeiro, RJ, Brazil

² National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

³ Instituto Biomédico, Universidade do Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil

⁴ Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, Brazil

The ascomycete *Pseudallescheria boydii* is an emerging human pathogen frequently found in soil and polluted water. A peptidopolysaccharide antigen has been isolated from mycelial forms of *P. boydii*, and characterized using chemical and immunological methods. Monosaccharide composition, methylation analysis, and ¹H- and ¹³C-NMR spectra indicated the presence of a rhamnomannan with a structure distinct from those of similar components isolated from other fungi, containing Rhap(1 → 3)Rhap epitopes on side chains which may be linked (1 → 3) to (1 → 6)-linked mannose. The peptidorhamnomannan from *P. boydii* reacted poorly with an antiserum raised against whole cells of *Sporothrix schenckii* and strongly with one against *P. boydii* hyphae. These characteristics and immunological differences suggest that this major rhamnose-containing antigen of *P. boydii* may be useful for the specific diagnosis of infections attributable to this fungus.

Keywords: fungal pathogen, mycelium, peptidopolysaccharide, ¹H- and ¹³C-nuclear magnetic resonance

INTRODUCTION

The ascomycete *Pseudallescheria boydii* is frequently found in soil and polluted water. Several types of pathogenicity have been associated with *P. boydii*. It is known to cause human white-grain mycetoma (Rippon, 1998) and it has recently emerged as an agent of systemic and disseminated mycoses (Berenguer *et al.*, 1989). *P. boydii* resembles other fungal species in tissue specimens and is not easily distinguished from *Aspergillus* species. Antibody levels and skin tests may help diagnosis of these fungi, particularly if allergic disease is suspected (Lake *et al.*, 1990).

Very few studies on the antigenic composition and the immune response elicited during infection by *P. boydii* have been reported. Soluble antigens in culture filtrates were isolated and separated by anion-exchange

chromatography; they consisted of 70–96% carbohydrate and 4–16% protein (Lupan & Cazin, 1976). No detailed chemical composition of these antigens has been established. Here we report the characterization of a peptidorhamnomannan from mycelial forms of *P. boydii*, using GC, methylation analysis, ¹H- and ¹³C-NMR spectroscopy, ELISA and immunofluorescence techniques, and comparison with *Sporothrix schenckii*, another pathogenic fungus that also synthesizes rhamnose-containing antigenic structures (Travassos *et al.*, 1973; Mendonça *et al.*, 1976; Lopes-Alves *et al.*, 1994).

METHODS

Micro-organism and growth conditions. *Pseudallescheria boydii*, isolated from eumycotic mycetoma, was kindly supplied by Dr Bodo Wanke, Evandro Chagas Hospital, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. It was maintained in a medium containing (g l⁻¹): Difco peptone, 10; Difco yeast extract, 5; Difco agar, 20; glucose, 40. Cells were grown on Sabouraud solid medium and inoculated into

Abbreviations: COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; PAS, periodic acid/Schiff; TSP, trimethylsilylpropionic acid sodium salt.

Erlenmeyer flasks (500 ml) containing liquid Sabouraud medium (200 ml) which was incubated for 7 d at 25 °C with shaking. Cultures were then transferred to the same medium (3 l) and further incubated for 7 d at the same temperature with shaking; the mycelium was filtered with filter paper, washed with distilled water and dried.

Extraction and fractionation of peptidopolysaccharides. Crude extracts were obtained by extraction of the mycelium with hot phosphate buffer (50 mM, pH 7.2), at 100 °C for 2 h, and were then fractionated by Cetavlon precipitation, according to Lloyd (1970). The mother liquors from the first Cetavlon precipitation were adjusted to pH 8.8 with sodium borate and the resulting precipitate recovered by centrifugation to give the major fraction. This fraction, after uncoupling Cetavlon from the complex with acetic acid, was dialysed and lyophilized.

Fractionation of the peptidopolysaccharides (fraction B) on DE-52. The pH 8.8 Cetavlon-precipitated material was dissolved in 0.02 M sodium phosphate buffer, pH 6.1, and applied to a DE-52 column (Whatman, 9.0 × 2 cm). A discontinuous gradient of 0.1 M NaCl in 0.02 M sodium phosphate buffer to 1 M NaCl was used to fractionate the sample. Eluted fractions were monitored by A_{280} for protein, and colorimetrically (A_{490}) for carbohydrate (Dubois *et al.*, 1956).

SDS-PAGE. Peptidopolysaccharides were solubilized at 100 °C for 5 min in 0.06 M Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue under reducing conditions by adding 5% (w/v) 2-mercaptoethanol immediately before use (Laemmli, 1970). Electrophoresis was performed in a 12% polyacrylamide separating gel using a Bio Rad Mini-Protein II electrophoresis system. Gels were stained for carbohydrate with the periodic acid/Schiff (PAS) reagent and for protein with Coomassie blue.

Analytical procedures. Total carbohydrate was determined by the phenol/sulfuric acid method (Dubois *et al.*, 1956), protein by the Lowry method, phosphate by the procedure of Ames (1966), and hexosamine by the method of Belcher *et al.* (1954).

Component monosaccharides. Peptidopolysaccharides were hydrolysed with 3 M trifluoroacetic acid at 100 °C for 3 h; the resulting monosaccharides were analysed (a) by HPTLC in *n*-butanol/acetone/water (5:4:1, by vol.) (Ovodov *et al.*, 1967) and (b) as their alditol acetates, which were characterized and quantified by GC-MS (Sawardeker *et al.*, 1965).

Methylation analysis. Peptidopolysaccharides were methylated by the lithium methyl sulfinyl carbanion method (Parente *et al.*, 1985). The partially methylated derivatives were converted to the corresponding alditol acetates and were analysed by GC coupled to electron-impact mass spectrometry. The partially methylated alditol acetates were separated on a fused silica capillary column of OV-225 (30 × 0.25 mm, i.d.), programmed from 50 to 220 °C at a rate of 50 °C min⁻¹, then hold.

¹H- and ¹³C-NMR spectroscopy. ¹H- (500 MHz) and ¹³C- (125 MHz) NMR spectra were recorded using a Varian Unit 500 spectrometer. Samples (10 mg) were dissolved in 0.75 ml D₂O (99.96%) with trimethylsilylpropionic acid sodium salt (TSP) as internal reference. ¹³C chemical shifts were determined taking as reference the rhamnose methyl signal at 18.4 p.p.m. (Gorin, 1981). The DEPT (distortionless enhancement by polarization transfer) spectrum was recorded using the manufacturer's software. A double-quantum-filtered, gradient-assisted, magnitude-mode COSY spectrum was recorded using the pulse sequence GMQCOSY supplied by the spectrometer manufacturer.

Immunoreactivity of *P. boydii* peptidorhamnomannan. Rabbit sera against whole *P. boydii* hyphae and *Sporothrix schenckii* mycelium phase (strain 1099.18) were used in this study. They were analysed by a direct ELISA method (Voller *et al.*, 1976). Wells of flat-bottomed polyvinyl microtitre plates (Hemobag) were coated with 100 µl of a 10 µg ml⁻¹ solution of *P. boydii* peptidopolysaccharide. They were subsequently treated with PBS-Tween 20 (0.1%) containing 2% BSA as blocking agent; this and all subsequent incubations were at 37 °C for 1 h. The hyperimmune rabbit serum was used at a dilution of 1/200 to 1/1638400, and antibody binding was also measured using goat anti-rabbit IgG conjugated to horseradish peroxidase. The chromogen used was *o*-phenylenediamine, added with H₂O₂. Other steps were carried out as described by Haido *et al.* (1998).

Immunofluorescence. Indirect immunofluorescence analysis was carried out according to Haido *et al.* (1998). Fixed mycelia and conidia (2% formaldehyde in methanol) from *P. boydii* were treated with rabbit anti-*P. boydii* serum at a dilution of 1:50 and incubated for 60 min at 37 °C in a moist chamber. A pre-immune serum was used as control. After washing, fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:50 in PBS (10 mM, pH 7.2) was added. The slides were incubated for 60 min, washed and examined in a fluorescence microscope. For the inhibition tests, the rabbit anti-*P. boydii* serum diluted 1:50 was pre-incubated for 60 min at 37 °C with an equal volume of 50 and 100 µg ml⁻¹ peptidorhamnomannan from both *P. boydii* and *S. schenckii* (Lima & Lopes-Bezerra, 1997; Penha & Lopes-Bezerra, 2000) and with a peptidogalactomannan from *Aspergillus fumigatus* strain 2109 (Haido *et al.*, 1998).

RESULTS

Isolation and purification of *P. boydii* peptidorhamnomannan

The phosphate buffer extract of *P. boydii* mycelium (total carbohydrate 70%; protein 15%; yield 50 mg per g dry weight of mycelium) was fractionated by stepwise precipitation with Cetavlon at pH 7.0 and Cetavlon/borate at pH 8.8 (fraction B) as previously described (Haido *et al.*, 1998). The pH 8.8 Cetavlon-

Fig. 1. Elution profile of the peptidopolysaccharides from *P. boydii*, fractionated by DE-52 column chromatography. Fraction I is the unbound fraction. Fractions II, III, IV and V were subsequently eluted with a discontinuous gradient of NaCl as shown. The last component was eluted with 0.5 M NaOH. ○, Carbohydrate assayed colorimetrically (A_{490}); ●, protein determination by measuring A_{280} .

Fig. 2. SDS-PAGE of fraction B (FB) from Cetavlon pH 8.8 precipitation, and fractions I to V from DE-52 fractionation of *P. boydii* peptidopolysaccharides. The diffusely migrating bands in the 50–80 kDa range were stained by PAS (left panel). Protein staining by Coomassie blue of fractions I–V is shown in the right panel.

Table 1. Sugar component analysis of fractions I–V of peptidopolysaccharides (fraction B) obtained by DE-52 column chromatography

Monosaccharides were analysed as their alditol acetate derivatives using a fused silica capillary column of OV-225 (30 m × 0.25 mm i.d.) programmed from 50 °C to 220 °C (50 °C min⁻¹, then hold).

Fraction	Monosaccharide composition (%)			
	Rhamnose	Mannose	Galactose	Glucose
B	19.5	64.0	10.0	5.5
I	42.5	49.5	4.5	3.5
II	37.0	49.0	7.0	7.0
III	29.5	60.5	5.5	4.5
IV	36.0	54.0	5.0	5.0
V	40.5	47.0	6.5	6.0

Table 2. GC-MS analysis of *O*-methylalditol acetates derived from methylation analysis of rhamnomannans of *P. boydii*

Partially methylated alditol acetate	Relative retention time	Area (%)
1,5-Di- <i>O</i> -acetyl-6-deoxy-2,3,4-tri- <i>O</i> -methylrhamnose	0.84	12.3
1,3,5-Tri- <i>O</i> -acetyl-6-deoxy-2,4-di- <i>O</i> -methylrhamnose	0.99	12.6
1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylglucose	1.00	2.4
1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylmannose	1.04	13.8
1,2,5-Tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methylmannose	1.18	17.2
1,3,5-Tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methylmannose	1.21	10.5
1,4,5-Tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methylgalactose or glucose	1.25	7.5
1,5,6-Tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methylmannose	1.27	2.3
1,3,4,5-Tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methylmannose	1.41	4.7
1,2,5,6-Tetra- <i>O</i> -acetyl-3,4-di- <i>O</i> -methylmannose	1.66	2.4
1,3,5,6-Tetra- <i>O</i> -acetyl-2,4-di- <i>O</i> -methylmannose	1.68	14.2

precipitated material was further fractionated on a DE-52 column. The unbound fraction (fraction I) was eluted with 0.01 M phosphate buffer, pH 6.3, and fractions II, III, IV and V were subsequently eluted with a dis-

continuous NaCl gradient from 0.1 to 1 M (Fig. 1). Further analyses were carried out using chiefly fraction III, which contained very little protein and had a high carbohydrate content.

Table 3. Assignments of signals in the ^{13}C -NMR spectrum of rhamnomannans from *P. boydii*

Signal (p.p.m.)		Assignment
<i>P. boydii</i> *	<i>S. schenckii</i> and baker's yeast†	
103·6, 103·9	103·7	C-1 of α -D-Manp (non-reducing) end group and 3-O-substituted residue
101·7	102·0	C-1 of 2-O-substituted α -D-Manp residues
101·2	101·1	C-1 of 3,6-di-O-substituted α -D-Manp residues
99·6	100·3	C-1 of 2,6-di-O-substituted α -D-Manp residues
98·2	98·2	C-1 of α -L-Rhap (non-reducing) end group of O- α -L-Rhap-(1 $\rightarrow$ 3)-D-Manp
62·8	62·8	C-6 of unsubstituted α -D-Manp units
18·4	18·4	C of methyl group of α -L-Rhap units

* Relative to the rhamnose methyl signal at 18·4 p.p.m.

† Taken from Travassos *et al.* (1974). **Fig. 3.** (a) 125 MHz ^{13}C -NMR spectrum of fraction III peptidopolysaccharides from *P. boydii* recorded at 60 °C in D_2O ; (b) DEPT ^{13}C -NMR spectrum of the same molecules recorded at 45 °C in D_2O . Chemical shifts are relative to rhamnose C-6 at 18·4 p.p.m.

SDS-PAGE

Fraction B from Cetavlon pH 8·8 precipitation, and the unbound and bound fractions from DE-52 chromatography, were subjected to SDS-PAGE under reducing conditions in a 12% linear polyacrylamide gel as described in Methods. A broad band was observed in all

Fig. 4. 500 MHz ^1H -NMR of fraction III peptidopolysaccharide from *P. boydii* recorded at 60 °C in D_2O . Chemical shifts are relative to TSP at 0 p.p.m.

fractions, stained by PAS with apparent molecular mass in the 50–80 kDa range (Fig. 2).

Carbohydrate analysis of DE-52 column fractions

The content of neutral carbohydrate in the DE-52 fractions is shown in Table 1. Predominant components were rhamnose and mannose, suggesting the presence of a rhamnomannan.

Methylation analysis

A peptidorhamnomannan-enriched fraction (fraction III eluted from the DE-52 column with 0·25 M NaCl) was methylated, hydrolysed and the resulting units acetylated as described by Jansson *et al.* (1976). The O-methylalditol acetates are shown in Table 2.

Derivatives of 2,3,4-tri-O-methylrhamnose (12·3%) and 2,3,4,6-tetra-O-methylmannose (13·8%) originated from terminal rhamnose and mannose residues respectively.

Fig. 5. Double-quantum-filtered, gradient-assisted, magnitude-mode COSY spectrum of peptidopolysaccharide from *P. boydii* recorded at 45 °C in D₂O. Cross-peaks and groups of cross-peaks for the well-resolved H-1-H-2 and H-5-H-6 signals are indicated by dotted boxes. The suggested assignments are tentative. Chemical shifts are relative to TSP at 0 p.p.m.

Fig. 6. Reactivity of *P. boydii* fraction III peptidopolysaccharide (1 µg per well) with rabbit hyperimmune serum raised against whole mycelium of the fungus (○), with anti-*S. schenckii* (strain 1099.18, mycelial phase) serum (△), and with pre-immune rabbit serum (●). Serum dilutions were 1/200 to 1/819200.

Peaks corresponding to the derivatives of 2,4-di-O-methylmannose (14.2%) and 2,3,4-tri-O-methylmannose (2.3%) suggest α -(1 → 6)-mannopyranosyl residues substituted at the 3-positions by α -rhamnopyranosyl-containing side chains. In *Cerato-cystis ulmi* and *S. schenckii* (Travassos *et al.*, 1973; Gorin & Spencer, 1970) single rhamnosyl ramifications of the α -(1 → 6)-linked mannan were described. In *P. boydii* the presence of the 2,4-di-O-methylrhamnose derivative (12.6%) suggests that the rhamnose side chains could be disaccharides with the structure [α -Rhap-(1 → 3)- α -Rhap-(1 → 3)-] (Travassos *et al.*, 1973). Methylation data additionally show that (1 → 2)-linked mannose is also present (17.2%), with lesser amounts (10.5%) of (1 → 3)-substituted residues. Possibly the (1 → 2)-linked mannosyl units are also located in side chains that have α -mannosyl or α -rhamnosyl terminal non-reducing units.

NMR spectroscopy

Well-resolved signals in the ¹³C-NMR spectrum of *P. boydii* are listed in Table 3, with values for similar compounds taken from Travassos *et al.* (1974) for comparison.

Anomeric resonances in the ¹³C spectrum are consistent with the presence of mannose and rhamnose residues in

several different environments. Signals at 103.6 p.p.m. and 103.9 p.p.m. are consistent with the presence of terminal and 3-O-linked mannose (Gorin, 1981). Other intense anomeric signals are at 101.2 p.p.m., consistent with C-1 of 3,6-di-O-substituted mannose (Travassos & Lloyd, 1980) and at 98.2 p.p.m., consistent with non-reducing terminal rhamnose (Travassos & Lloyd, 1980). A signal at 101.7 p.p.m. has a chemical shift near that reported for C-1 of 2-O-substituted mannose (Gorin, 1980) but is of lower intensity than would be predicted by methylation analysis. The ¹³C-NMR spectrum (Fig. 3a) also contains other minor anomeric signals. No anomeric signal was assigned to 3-linked rhamnose, one of the major components by methylation analysis.

A DEPT spectrum (Fig. 3b) has signals from carbons having two protons (in this case C-6 of Gal/Glc and Man residues) inverted. Two inverted signals can be easily identified, at 62.8 p.p.m. (typical of unsubstituted C-6) and at 67.4 p.p.m. (typical of substituted C-6).

¹H-NMR spectroscopy of the peptidopolysaccharide (Fig. 4) reveals a strong spectrum originating from carbohydrate, with broad, weaker signals from the peptide. Distinctive signals arising from rhamnose H-6 methyl protons are seen at about 1.3 p.p.m.

A double-quantum-filtered, gradient-assisted, magnitude-mode COSY spectrum of the peptidopolysaccharide is shown in Fig. 5. Complete and definitive assignment of this spectrum is not possible due to severe signal overlapping, and broadening due to the high molecular mass of the peptidopolysaccharide. However, cross-peaks, and groups of cross-peaks for the well-resolved H-1-H-2 and rhamnose H-5-H-6 signals (identified by the distinctive high-field chemical shift of rhamnose H-6) are indicated by dotted boxes. A group of signals from anomeric protons (H-1) appears between 4.9 and 5.5 p.p.m., and the COSY spectrum (Fig. 5) indicates that several corresponding H-2 signals are found between 4.0 and 4.3 p.p.m., consistent with the presence of mannose and rhamnose. One H-1-H-2 cross-peak indicates an H-2 signal at about 3.56 p.p.m., consistent with α -glucose. The strong water (HOD) signal at 4.6 p.p.m. obscures the β -anomeric proton signal at about 4.5 p.p.m. with H-2 at 3.62 p.p.m., consistent with a β -galactosyl unit.

Fig. 7. Immunofluorescence of mycelium (B) and conidia (J) of *P. boydii* detected following incubation with rabbit anti-mycelium hyperimmune serum. Identical incubations were performed following pre-incubation of the serum with *P. boydii* fraction III peptidopolysaccharide (D, M), with *S. schenckii* peptidorhamnomannan (F, O) or with *A. fumigatus* peptidogalactomannan (H, Q) as described in Methods. Panels A, C, E, G, I, L, N and P show the same fields of *P. boydii* cells viewed by differential interference contrast microscopy. Bar, 10 µm.

These tentative assignments of H-1–H-2 cross-peaks agree with the results of monosaccharide analysis (Table 1).

Immunological reactivity of purified peptidorhamnomannan (fraction III)

Fraction III was tested in ELISA using hyperimmune rabbit antisera against mycelial forms of both *P. boydii* and *S. schenckii*. *P. boydii* fraction III reacted strongly with the homologous antiserum whereas a much lower reactivity was obtained with the anti-*S. schenckii* serum (Fig. 6).

Immunofluorescence

The reactivity of the rabbit anti-*P. boydii* serum with hyphae and conidia of *P. boydii*, detected by indirect immunofluorescence, is shown in Fig. 7. A strong

immunofluorescence reaction was observed with both cell types of this fungus, and the reaction was specifically inhibited by fraction III peptidorhamnomannan (Fig. 7). No reactivity was seen with the rabbit pre-immune serum (not shown). When the rabbit anti-*P. boydii* serum was incubated with a peptidorhamnomannan from *S. schenckii* or a peptidogalactomannan from *A. fumigatus*, no clear inhibition could be demonstrated (Fig. 7), suggesting the absence of cross-reactive determinants.

DISCUSSION

Rhamnomannans with traces of galactose or glucose, or both, have been identified in strains of *Ceratocystis*, *Graphium* and *Hyalodendron pyrium* (Travassos *et al.*, 1974; Spencer & Gorin, 1971). Among human-pathogenic fungi, however, rhamnomannans are seemingly restricted to *Sporothrix schenckii* (Travassos *et al.*,

1974). In the present work, the partial structure of a peptidorhamnomannan from *P. boydii*, an emergent human pathogen, was determined.

The family of peptidopolysaccharides from *P. boydii* which was fractionated by DE-52 column chromatography migrated in SDS-PAGE gels as a broad band in the 50–80 kDa range, stained by both PAS and Coomassie blue. No other protein band appeared in the gel, which suggested that a defined group of closely related peptidopolysaccharides had been isolated. The degree of heterogeneity among these glycoconjugates was determined by the carbohydrate content and carbohydrate/protein ratios of the different fractions analysed in this work. Methylation analysis suggests that α -L-Rhap(1 $\rightarrow$ 3)- α -L-Rhap disaccharides are present in side chains, possibly linked (1 $\rightarrow$ 3) to (1 $\rightarrow$ 6)-linked mannose residues. Mannose was also present as non-reducing end units, 2-O-, 3-O-, 6-O- and 2,4-di-O-substituted units and a few 2,6- and 3,4-di-O-substituted units. The relatively high proportion of 2-O-substituted mannosyl units could be due to their inclusion in side chains with and without rhamnose, or the presence of O-linked mannosyl oligosaccharides in the glycoconjugate.

Methylation analysis identified several structures for which prominent signals would be expected in the ^{13}C -NMR spectrum. Reasonable correspondence can be established between chemical shifts found in this study and literature values for several carbon signals in the anomeric and other well-resolved regions of the spectrum (Table 3). Unequivocal assignment of all NMR spectra awaits study of purified carbohydrate species from this rhamnomannan.

By comparison with the rhamnomannan extracted with hot dilute alkali from *S. schenckii*, *Ceratocystis stenoceras* and *C. ulmi* (Travassos *et al.*, 1973; Gorin & Spencer, 1970) it was evident that the rhamnomannan of *P. boydii* was significantly different. The dirhamnosyl side chains α -L-Rhap(1 $\rightarrow$ 2)- α -L-Rhap present in *S. schenckii* mycelial polysaccharides obtained at 25 °C (Mendonça *et al.*, 1976) were not found in the *P. boydii* rhamnomannan, which has α -(1 $\rightarrow$ 3)-linked rhamnosyl disaccharide.

The structural differences in the rhamnomannan of *P. boydii* as compared with a similar polysaccharide from *S. schenckii*, shown by methylation analysis, were confirmed by ^{13}C -NMR spectroscopy. A consistent difference was the absence of signals at 96.8 p.p.m. and 80.3 p.p.m. corresponding respectively to the C-1 of 2-O-substituted α -L-Rhap residues of α -L-Rhap(1 $\rightarrow$ 2)- α -Rhap and C-2 of 2-O-substituted α -L-Rhap units (Travassos *et al.*, 1974).

The ^1H -NMR spectrum (Fig. 4), and the COSY spectrum (Fig. 5), with a limited number of clearly defined cross-peaks, is indicative of regularity in the carbohydrate structure; the peptide signals are too broad and too weak to give visible cross-peaks. Technical difficulties arise in obtaining a complete structural

analysis by NMR of the whole peptidopolysaccharide, which has a high molecular mass and some degree of heterogeneity. However, studies of purified carbohydrate chains are currently being undertaken to determine their structures in detail.

Preliminary results on the nature of the antigenic determinants in *P. boydii* peptidorhamnomannan showed that fraction III contains antigenic determinants recognizable by anti-*P. boydii* antibodies, but that do not cross-react with anti-*S. schenckii* antibodies raised against whole cells of this fungus. This result can be explained on the basis of the immunodominance of the carbohydrate epitopes in the peptidorhamnomannans of both species. Lloyd & Travassos (1975) showed that anti-*S. schenckii* antibodies reacted with β -L-Rhap-(1 $\rightarrow$ 3)- α -D-Manp but Lopes-Alves *et al.* (1994) later showed that a much stronger reactivity was obtained with the O-linked chains containing 2-O- and 4-O-linked rhamnosyl units linked to α -D-glucuronic acid. The very weak cross-reactivity between *P. boydii* and *S. schenckii* depends therefore on the low-titre anti- α -L-Rhap(1 $\rightarrow$ 3)- α -D-Manp antibodies, since the α -L-Rhap-(1 $\rightarrow$ 3)- α -L-Rhap(1 $\rightarrow$ 3)-L-Rhap-(1 $\rightarrow$ 3)- α -D-Manp structure, with or without additional 2-O-linked mannopyranosyl units, appears to be unique to *P. boydii*.

By immunofluorescence we also showed that the rabbit antiserum against *P. boydii* mycelium strongly reacted with hyphae and conidia of this fungus, a reaction specifically inhibited by fraction III peptidorhamnomannan. A very weak inhibition was observed with the peptidorhamnomannan from *S. schenckii* (Penha & Lopes-Bezerra, 2000) or a peptidogalactomannan from *A. fumigatus* (Haido *et al.*, 1998).

To our knowledge, this is the first characterization of a chemically defined antigen from the mycelial forms of *P. boydii*. A major aim of this study was to determine whether this antigen could be useful for diagnostic purposes, mainly in mixed allergic bronchopulmonary fungal disease due to *P. boydii* and *Aspergillus* (Lake *et al.*, 1990). The chemical characterization of fungal antigens is important to allow a rational interpretation of the cross-reactivity between pathogenic species, thus helping to select immunodominant components that can be of diagnostic use. The present work provides evidence for the expression of antigens which are similar but do not cross-react with *S. schenckii* peptidopolysaccharides and differ from the major *Aspergillus* glycoconjugate. Further studies are currently under way to determine the fine structure of the *P. boydii* antigen.

ACKNOWLEDGEMENTS

We thank Maria de Fatima F. Soares for technical assistance and Dr P. A. J. Gorin for the GC-MS analysis. This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Conselho de Ensino e Pesquisa da UFRJ (CEPG), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Programa de Núcleos de Excelência (PRONEX).

REFERENCES

- Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8**, 115–118.
- Belcher, R. A., Nutton, A. J. & Sambrook, C. M. (1954). The determination of glucosamine. *Analyst* **79**, 201–208.
- Berenguer, J., Diaz-Mediavilla, J., Urrea, D. & Muñoz, P. (1989). Central nervous system infection caused by *P. boydii*: case report and review. *Rev Infect Dis* **11**, 890–896.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Swith, E. (1956). Colorimetric method for determination of sugar and related substances. *Anal Chem* **28**, 350–356.
- Gorin, P. A. J. (1981). Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. *Adv Carbohydr Chem Biochem* **38**, 13–104.
- Gorin, P. A. J. & Spencer, J. F. T. (1970). Structures of the L-rhamno D-mannan from *Ceratocystis ulmi* and the D-glucosyl-D-mannan from *Ceratocystis brunnea*. *Carbohydr Res* **13**, 339–349.
- Haido, R. M. T., Silva, M. H., Ejzemberg, R., Leitao, E. A., Hearn, V. M., Evans, E. G. V. & Barreto Bergter, E. (1998). Analysis of peptidogalactomannan from the mycelial surface of *Aspergillus fumigatus*. *J Med Mycol* **36**, 313–321.
- Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. & Longen, J. (1976). A practical guide to the methylation analysis of carbohydrates. *Chem Commun Univ Stockholm* **8**.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* **227**, 680–685.
- Lake, F. R., Tribe, A. E., McAleer, R., Frondist, J. & Thompson, P. J. (1990). Mixed allergic bronchopulmonary fungal disease due to *Pseudallescheria boydii* and *Aspergillus*. *Thorax* **45**, 489–492.
- Lima, O. C. & Lopes-Bezerra, L. M. (1997). Identification of a Concanavalin A-binding antigen of the cell surface of *Sporothrix schenckii*. *J Med Vet Mycol* **35**, 167–172.
- Lloyd, K. O. (1970). Isolation, characterization and partial structure of peptidogalactomannan from the yeast form of *Cladosporium werneckii*. *Biochemistry* **9**, 3446–3453.
- Lloyd, K. O. & Travassos, L. R. (1975). Immunochemical studies on L-rhamno-D-mannans of *Sporothrix schenckii* and related fungi by use of rabbit and human antisera. *Carbohydr Res* **40**, 89–97.
- Lopes-Alves, L., Travassos, L. R., Previato, J. O. & Mendonça-Previato, L. (1994). Novel antigenic determinants from peptidogalactomannans of *Sporothrix schenckii*. *Glycobiology* **4**, 281–288.
- Lupan, D. M. & Cazin, J., Jr (1976). Serological diagnosis of petriellidiosis (allescheriosis). I. Isolation and characterization of soluble antigens from *Allescheria boydii* and *Monosporium apiospermum*. *Mycopathologia* **58**, 31–38.
- Mendonça, L., Gorin, P. A. J., Lloyd, K. O. & Travassos, L. R. (1976). Polymorphism of *S. schenckii* surface polysaccharides as a function of morphological differentiation. *Biochemistry* **15**, 2423–2431.
- Ovodov, Y. S., Evtushenko, E. V., Vaskovsky, V. E., Ovodova, R. G. & Soloveva, T. F. (1967). Thin layer chromatography of carbohydrates. *J Chromatogr* **26**, 111–115.
- Parente, J. P., Cardon, P., Leroy, Y., Montreuil, J., Fournet, B. & Ricart, J. (1985). A convenient method for methylation of glycoprotein glycans in small amounts by using lithium methylsulfinyl-carbanion. *Carbohydr Res* **141**, 41–47.
- Penha, C. V. L. & Lopes-Bezerra, L. M. (2000). Concanavalin A-binding antigens of *Sporothrix schenckii*: a serological study. *Med Mycol* **38**, 1–7.
- Rippon, J. W. (1998). Pseudallescheriosis. In *Medical Mycology*, 3rd edn, pp. 651–680. Philadelphia: W. B. Saunders.
- Sawardeker, J. S., Sloneker, J. H. & Jeanes, H. (1965). Quantitative determination of monosaccharides as their acetates by gas-liquid chromatography. *Anal Chem* **37**, 1602–1604.
- Spencer, J. T. F. & Gorin, P. A. J. (1971). Systematics of the genera *Ceratocystis* and *Graphium*. Proton magnetic resonance spectra of the mannose-containing polysaccharides as an aid in classification. *Mycologia* **63**, 387–402.
- Travassos, L. R. & Lloyd, K. (1980). *Sporothrix schenckii* and related species of *Ceratocystis*. *Microbiol Rev* **44**, 683–721.
- Travassos, L. R., Gorin, P. A. J. & Lloyd, K. O. (1973). Comparison of the rhamnomannans from the human pathogen *Sporothrix schenckii* with those from the *Ceratocystis* species. *Infect Immun* **8**, 685–693.
- Travassos, L. R., Gorin, P. A. J. & Lloyd, K. O. (1974). Discrimination between *Sporothrix schenckii* and *Ceratocystis stenoceras* rhamnomannans by proton and carbon-13 magnetic resonance spectroscopy. *Infect Immun* **9**, 674–680.
- Voller, A., Bidwell, D. & Bartlett, A. (1976). Enzyme-linked immunosorbent assay. In *Manual of Clinical Immunology*, pp. 506–512. Edited by N. R. Rose & H. M. Freidman. Washington, DC: American Society for Microbiology.

Received 17 October 2000; accepted 21 February 2001.