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## NOTES

# Galactoxylomannan Does Not Exhibit Cross-Reactivity in the *Platelia Aspergillus* Enzyme Immunoassay<sup>∇</sup>

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**Given the recent report of a false-positive result in the *Platelia Aspergillus* enzyme immunoassay in a patient with cryptococcosis and in yeast extracts and purified galactoxylomannan of *Cryptococcus neoformans*, we evaluated culture extracts, purified polysaccharides, clinical specimens, and specimens from animals following experimental infection. Our results revealed no cross-reactions.**

Galactomannans (GalMs) are complex polysaccharides composed of D-galactose and D-mannose. Alkaline-extracted GalMs from *Aspergillus fumigatus* contain a core of (1→6)-α-D-mannopyranosyl residues, with β(1→5)-linked β-galactofuranosyl units. The pathogenic fungus *Cryptococcus neoformans* also contains complex capsular polysaccharides composed of glucuronoxylomannan (GXM) and galactoxylomannans (GalXM). GXM makes up about 90% of the capsular mass and is a polymer of up to six different repeating units that consist mostly of a linear α(1→3)-mannan trisaccharide with side groups consisting of β(1→2)-glucopyranosyluronic acid and β(1→2)- and β(1→4)-xylopyranosyl (2, 6). Additionally, the mannan backbone of GXM is modified by acetyl groups.

GalXM constitutes about 7% of the capsular mass (2) and has an α(1→6) galactan backbone containing four potential short oligosaccharide branch structures. The branches are 3-O-linked to the backbone and consist of an α(1→3)-Man, α(1→4)-Man, β-galactosidase trisaccharide with variable amounts of β(1→2)- or β(1→3)-xylose side groups (Fig. 1) (2). The GalXM backbone consists of galactopyranose and a small amount of galactofuranose (11), unlike GXM, which contains only mannopyranose (2). A recent study showed that GalXM was significantly smaller than GXM ( $1.7 \times 10^6$  Da), with an average mass of  $1 \times 10^5$  Da (6). Since GalXM has a smaller molecular mass, this implies that GalXM is the most numerous polysaccharide in the capsule on a molar basis, since there are 2 to 3.5 mol of GalXM for each mole of GXM. However, the often assumed conclusion that GXM was the most abundant molecule was based on the total amount of material that was recovered from the culture supernatant. These capsular components are shed into culture media, and GXM is found in

body fluids of patients, assisting in the diagnosis of cryptococcosis. However, whether GalXM is present in body fluids is unknown, as there is no diagnostic test available for its detection.

The cell wall GalM of *Aspergillus* spp. is likewise shed into culture media and body fluids of patients with aspergillosis, and it is the target of the *Platelia Aspergillus* enzyme immunoassay (EIA). β(1→5)-Galactofuranosyl side chains are the antigenic sites recognized by the monoclonal antibody used in the *Platelia Aspergillus* EIA (8).

Recently, the *Platelia Aspergillus* EIA was noted to be positive in a patient with cryptococcal meningitis (4). In that case, however, the patient was also receiving amoxicillin-clavulanate, a drug associated with false-positive results in the *Platelia Aspergillus* EIA. GalXM at concentrations of 100 ng/ml or higher also gave positive results in the *Platelia Aspergillus* EIA. GXM, however, the target of the cryptococcal latex agglutination (LA) test, was negative in the *Platelia Aspergillus* EIA. The investigators also demonstrated that *C. neoformans* yeast suspensions were positive in the *Platelia Aspergillus* EIA. Cross-reactivity with *C. neoformans* was not observed in earlier studies of the *Platelia Aspergillus* EIA (9) or the monoclonal antibodies used in that assay (8). In view of these conflicting findings, we undertook additional studies of cross-reactivity with *C. neoformans* in the *Platelia Aspergillus* EIA.

Cryptococcal GXM was prepared from *C. neoformans* strain 24067 and GalXM and GalXM/mannoprotein from strain cap67 according to published methods (3, 10, 11). Compositional analysis of GalXM was confirmed by gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The analysis revealed the moles percentages for GalXM's components were as follows: xylose, 17%; mannose, 28%; and galactose, 55% (10). These numbers are very similar to the moles percentages in Cherniak's studies: xylose, 22%; mannose, 29%; and galactose, 50% (11). Addi-

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TABLE 1. Sensitivity of detection of purified polysaccharide antigens

Antigen	Antigen concn (ng/ml)	GMI by <i>Platelia Aspergillus</i> EIA <sup>a</sup>	Result of Cn LA <sup>b</sup>
<i>A. fumigatus</i> GalM	1,000	8.28	0
	100	5.52	0
	10	0.72	0
	1	0.37	0
	None	0.06	0
<i>C. neoformans</i> GXM	1,000	0.07	3+
	100	0.08	2+
	10	0.09	1+
	1	0.05	0
	None	0.06	0
<i>C. neoformans</i> GalXM	1,000	0.17	3+
	100	0.07	1+
	10	0.06	0
	1	0.05	0
	None	0.06	0
<i>C. neoformans</i> GalXM-MP	1,000	0.11	0
	100	0.08	0
	10	0.09	0
	1	0.04	0
	None	0.06	0

<sup>a</sup> A result was considered to be positive if the GMI was greater than 0.5.

<sup>b</sup> Results are expressed semiquantitatively on a scale from 0 to 5+, with results from 1+ to 5+ considered to be positive. Cn, *C. neoformans*.

tionally, we ran a proton nuclear magnetic resonance spectrum for GalXM without further purification through an ion-exchange column as Cherniak had done earlier (11). The analysis showed that the anomeric region was between 5.4 and 4.3 ppm in a one-dimensional <sup>1</sup>H spectrum recorded at 600 MHz and 56°C and also similar to those reported previously (11). These antigens were tested in the cryptococcal LA test (Meridian Bioscience, Cincinnati, OH) and *Platelia Aspergillus* EIA (Bio-Rad Laboratories, Redmond, WA). GXM at ≥10 ng/ml and GalXM at ≥100 ng/ml were positive in the cryptococcal LA test but negative in the *Platelia Aspergillus* EIA (Table 1). This finding was verified by testing two different preparations of GXM from *C. neoformans* 24067 and three of GalXM, including preparations from *C. neoformans* cap67 grown in Sabouraud's medium and peptone supplemented with galactose. We also tested yeast colonies diluted in 1 ml of distilled water and agitated for 1 min, as described by Dalle et al. (4), and culture supernatants from *C. neoformans* (*n* = 4). All were positive in the cryptococcal LA test (3 to 4+ agglutination tested undiluted) but negative in the *Platelia Aspergillus* EIA.

The *Platelia Aspergillus* EIA positive kit control was positive in the *Platelia Aspergillus* EIA (3.54 index units) but negative in the cryptococcal LA test. A partially purified GalM was prepared from *A. fumigatus* by ethanol precipitation of culture supernatant (8), and a dilution containing about 1,000 ng/ml of GalM was prepared by comparison to the *Platelia Aspergillus* EIA cutoff control, which contained about 1 ng/ml of GalM. The *A. fumigatus* GalM was detected at a concentration of 1 ng/ml in the *Platelia Aspergillus* EIA but not in the cryptococcal LA test up to 1,000 ng/ml (Table 1). Furthermore, culture supernatants and mold colonies diluted in water from strains of *A. fumigatus* and *A. flavus* were positive in the *Platelia Aspergillus* EIA but negative in the cryptococcal LA test.

Additionally, we tested residual sera or cerebrospinal fluid from 25 patients that was positive in the cryptococcal LA test.

TABLE 2. Cross-reactivity in clinical specimens and experimental infection

Specimen source	Assay <sup>f</sup>	No. of specimens positive for antigen/no. tested	
		<i>Aspergillus</i>	<i>Cryptococcus</i>
Infected patients	Af GalM EIA	20/20 <sup>a</sup>	0/25 <sup>b</sup>
	Cn CPS LA	0/20 <sup>a</sup>	24/25 <sup>b</sup>
Mice experimentally infected with <i>C. neoformans</i>	Af GalM EIA	5/5 <sup>a</sup>	0/8 <sup>c</sup>
	Cn CPS LA	0/5 <sup>a</sup>	8/8 <sup>c</sup>
Rabbits experimentally infected with <i>A. fumigatus</i>	Af GalM EIA	7/7 <sup>d</sup>	0/8 <sup>e</sup>
	Cn CPS LA	0/7 <sup>d</sup>	8/8 <sup>e</sup>

<sup>a</sup> Serum.

<sup>b</sup> Serum or CSF.

<sup>c</sup> Spleen tissue.

<sup>d</sup> BAL fluid.

<sup>e</sup> Lung tissue.

<sup>f</sup> Af, *A. fumigatus*; Cn, *C. neoformans*.

The cryptococcal LA titer ranged from negative (one sample) to 1:65,336 (median, 1:1,024), but all were negative in the *Platelia Aspergillus* EIA. We also tested residual serum specimens from 20 patients that were positive in the *Platelia Aspergillus* EIA. The *Aspergillus* GalM galactomannan index units (GMI) ranged from 0.5 to 10.4 (median, 1.9), but all were negative in the cryptococcal LA test (Table 2).

Cross-reactivity was also studied in experimental models of invasive aspergillosis and cryptococcosis, according to institutional guidelines. BALB/c 6- to 8-week-old female mice (*n* = 8) from the National Cancer Institute (NCI) were infected intravenously with  $1 \times 10^4$  organisms of *C. neoformans* strain 24067 (1). The mice were sacrificed 7 days postinfection by asphyxiation with CO<sub>2</sub>, and sera and lungs were immediately harvested, placed in ice-cold phosphate-buffered saline, and frozen at -70°C. Tissues were placed in 2 ml of F-12 nutrient medium (Invitrogen Corporation, Carlsbad, CA) supplemented with glucose, glutamic acid, cystine, and HEPES and homogenized in sterile Ten Broeke (Vineland, NJ) grinders. The undiluted homogenates were positive in the cryptococcal LA test, with agglutination scores of 3+ or 4+ for five, 2+ for two, and 1+ for one animal. All were negative in the *Platelia Aspergillus* EIA.

Pulmonary aspergillosis in New Zealand White rabbits (*n* = 7) (Hazelton Research Products, Inc., Denver, PA) was established by inoculating them intratracheally with *A. fumigatus* (NIH isolate 4215; ATCC no. MYA-1163) (7). The studies were approved by the Animal Care and Use Committee of the NCI, and the rabbits were housed and monitored in facilities accredited by AAALAC International according to guidelines of the Public Health Service Policy for the Care and Use of Laboratory Animals. The rabbits were euthanized by intravenous administration of pentobarbital (65 mg/kg of body weight of pentobarbital sodium in the form of 0.5 ml of beuthanasia-D special euthanasia solution; Schering-Plough Animal Health Corp., Union, NJ). Undiluted plasma and bronchoalveolar lavage (BAL) fluid were positive in the *Platelia Aspergillus* EIA: the GMI ranged from 0.8 to 4.2 (median, 2.4) in plasma and 1.5 to 6.8 (median, 6.4) in BAL fluid. All specimens were negative in the cryptococcal LA test.

Cross-reactions between GXM, GalXM, and *Aspergillus*

GalM were not observed in this study. The reasons for the discrepant findings, compared to those of Dalle et al. (4), are unknown. The GalXM used in that study was prepared some years ago (5, 10) and may have changed during storage. Since the positive result in the *Platelia Aspergillus* EIA was noted only at high concentrations of cryptococcal GalXM (100 ng/ml), low-level contamination with *Aspergillus* GalM is also possible. The limit of detection of the *Platelia Aspergillus* EIA for *Aspergillus* GalM is about 0.5 ng/ml, permitting detection of low-level contamination. Also, subtle differences in methods for preparation of the GalXM may have contributed to the discrepant findings. Nevertheless, the biochemical and nuclear magnetic resonance characteristics of our GalXM were similar to that prepared by Cherniak et al. (5, 10), which was used in Dalle's study (4).

Previous studies had failed to observe cross-reactivity in culture supernatants from *C. neoformans* in the *Platelia Aspergillus* EIA (8, 9). Furthermore, positive results in the Pastorex *Aspergillus* LA test were not detected in plasma from guinea pigs with cryptococcosis (12).

A biochemical basis for cross-reactivity between cryptococcal GalXM and *Aspergillus* GalM is not obvious (2, 13). The structural dissimilarities among GalM, GalXM, and GXM suggest that the likelihood for shared antigenicity may be small (Fig. 1). Although  $\beta$ -galactofuranose, the epitope recognized by the monoclonal antibody used in the *Aspergillus* GalM antigen assay (8), is present in small amounts in GalXM (11), it is unknown if the two are similar antigenically. Thus, we were unable to confirm the cross-reactivity of *C. neoformans* GalXM or culture extracts or false-positive results in specimens from patients or animals with cryptococcosis in the *Platelia Aspergillus* EIA.

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