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Note

Glucuronoxylomannan of *Cryptococcus neoformans* obtained from patients with AIDS

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Cryptococcus neoformans has emerged as a major opportunistic pathogen in patients diagnosed with acquired immunodeficiency syndrome (AIDS)[1]. The yeast has a predilection for the central nervous system, where it causes cryptococcal meningoencephalitis[2]. In the pre-AIDS era both varieties of *C. neoformans* (*C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*) were isolated from clinical specimens[3], whereas today *C. neoformans* isolates from AIDS patients are almost invariably of the *neoformans* variety[4,5]. There appears to be a selective infection of AIDS patients with the *neoformans* variety and 99% of these are of the A serotype[6,7]. The results of our comprehensive investigation of the major capsular polysaccharide of *C. neoformans* serotype A revealed structural variability between isolates[8,9]. Of the eleven isolates studied, six (Group I[8]) conformed closely to the model originally proposed by Merrifield and Stephen[10] and Bhattacharjee et al.[11]. Two isolates (Group II[8]) are deficient in xylose to the extent that, based on molar ratios, they could easily be classified as serotype D[12–14] or A-D[15]. However, both ^{13}C NMR spectroscopy and methylation analysis clearly indicated that the two isolates of Group II differed from the typical D and A-D glucuronoxylomannans (GXM). The Group II GXMs have a significant quantity (10%) of Man*p* residues substituted with Glc*p*A at O-2 and Xyl*p* at O-4 whereas serotype D and A-D isolates do not. The Group II isolates were later confirmed to be serotype A[9] by using specific rabbit antisera (J.E. Bennett, National Institutes of Health). The remaining two groups, III and IV, contained substantial amounts of linkages thought to be distinctive of serotypes B[16–18] and C[19,20], i.e., Man*p* that are 4-*O*-glycosylated with β-D-Xyl*p*, while still possessing unsubstituted (1 → 3)-α-D-Man*p*.

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In this study we investigated the structure of GXM obtained from three clinical isolates (118, 150, and 194) of *C. neoformans* of the A serotype from patients with AIDS. The chemical data obtained in this study were acquired by using methods described in our previous investigations in which we group GXMs of *C. neoformans* serotypes A[8], serotype B[17], serotype C[20], and serotype D[13]. Isolates 118 and 194 corresponded in structure to Group I and the third isolate, 150, was a close match for Group III of serotype A as defined in ref 8.

RESULTS AND DISCUSSION

Purified, ultrasonically irradiated GXM (GXM-S).—The yield of GXM from the three isolates (~ 3.5 g for 150, ~ 2.5 g for 194, and ~ 0.5 g for 118) was generally equivalent to that observed in comparable studies of serotype A isolates[8,9]. However, this was true only if the initial 15-min treatment with ultrasonic irradiation was omitted from the general procedure. This variation in the general isolation procedure was necessary because the hexadecyltrimethylammonium bromide–GXM-S complex formed translucent gels that were difficult to recover.

Ion-exchange chromatography.—GXM-S from each isolate eluted as a single peak by ion-exchange chromatography on DEAE-Sepharose CL-6B. A representative elution for each isolate is shown in Fig. 1.

Carbohydrate composition.—The Xyl:Man:GlcA molar ratios of the substituent sugars and *O*-acetyl of GXM-S, determined by GLC and colorimetry, were calculated relative to mannose taken as 3.00 (Table I). The molar composition of GXM-S from 118 and 194 was similar to the Group I serotype A isolates recently described (see 201 in Table I)[8]. Whereas the molar composition of GXM-S from isolate 150 matched closely that of the Group III serotype A isolates described in the same study (see 371-3 in Table I). Of the eleven A isolates for which the molar

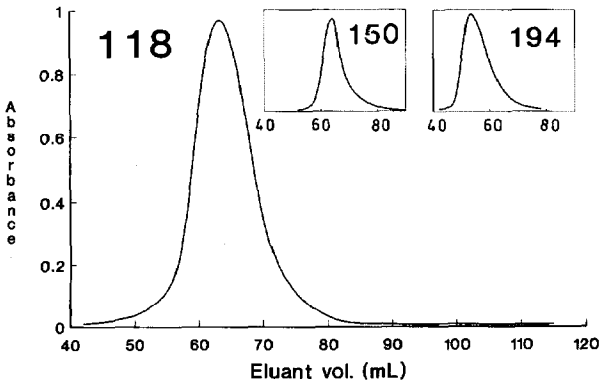


Fig. 1. Ion-exchange column chromatography of GXM-S on DEAE-Sepharose CL-6B using a linear gradient of 0.01 M Na_2HPO_4 to 0.01 M Na_2HPO_4–1.0 M NaCl. The eluant was assayed for neutral carbohydrate by the phenol–H_2SO_4 method, and absorbance measurements were obtained at 490 nm.

TABLE I

Molar ratios of GXM from AIDS isolates of *C. neoformans*, serotype A [a]

Isolate	D-Man	D-Xyl	D-GlcA [b]	*O*-Acetyl [b]
118	3.00	1.86	0.62	1.38 (6.7%)
194	3.00	2.18	0.70	2.07 (9.1%)
150	3.00	2.55	0.62	2.01 (8.5%)
201 [c]	3.00	1.97	0.58	1.88 (8.8%)
371-3 [c]	3.00	2.50	0.64	3.02 (12.3%)

[a] Composition data were obtained on sonicated GXM by GLC. [b] Uronic acid and *O*-acetyl were determined colorimetrically. [c] Data for non-AIDS serotype A isolates[8] for comparison.

ratios for the constituents are known[8], six are found in Group I and one is found in Group III. However, the major criteria for the group designations was the sequence of the sugar substituents on mannopyranan backbone as determined by ^{13}C NMR spectroscopy[14,18,21].

Methylation analysis.—The *O*-deacetylated GXM-S (GXM-D) of each isolate was methylated and analyzed by GLC–MS. The derivatives obtained from all isolates were: 2,3,4-tri-*O*-methylXyl, 2,4,6-tri-*O*-methylMan, 4,6-di-*O*-methylMan, and 6-*O*-methylMan. The molar ratios of the methylated derivatives of isolates 118 and 194 shown in Table II are similar to the data of Group I previously reported[8]. Data of a representative GXM-D of this group (201) is also shown in Table II. Isolate 150 has a distinctively higher molar ratio of 6-*O*-methylMan (Table II). These data conform to the Group III GXM-D represented by isolate 371-3 in Table II. The structural similarities between GXM-D from the AIDS isolates and the GXM-D representing Group I and Group III, defined in a previous study[8], are also observed by ^{13}C NMR spectroscopy (see below).

Nuclear magnetic resonance spectroscopy.—The ^{13}C NMR spectra of GXM-D (*O*-deacetylated GXM-S) samples for the three serotype A isolates obtained from the AIDS patients, and two spectra representative of Group I (isolate 201) and

TABLE II

GLC–MS methylation analysis of GXM from AIDS isolates of *C. neoformans*, Serotype A

Strain	Methylated PAAN derivatives (mol ratios)			
	Tri-*O*-Me		Di-*O*-Me	*O*-Me
	2,3,4-D-Xyl [a]	2,4,6-D-Man	4,6-D-Man	6-D-Man
118	2.14	0.24	2.76	n.d. [b]
194	1.75	0.63	2.29	0.08
150	2.38	0.29	2.42	0.29
201 [c]	2.14	0.39	2.50	0.11
371-3 [c]	2.43	0.26	2.43	0.32

[a] Identified by GLC–MS and molar ratios calculated based on the degree of substitution of mannose. [b] Not detected. [c] Data from non-AIDS serotype A isolates for comparison.

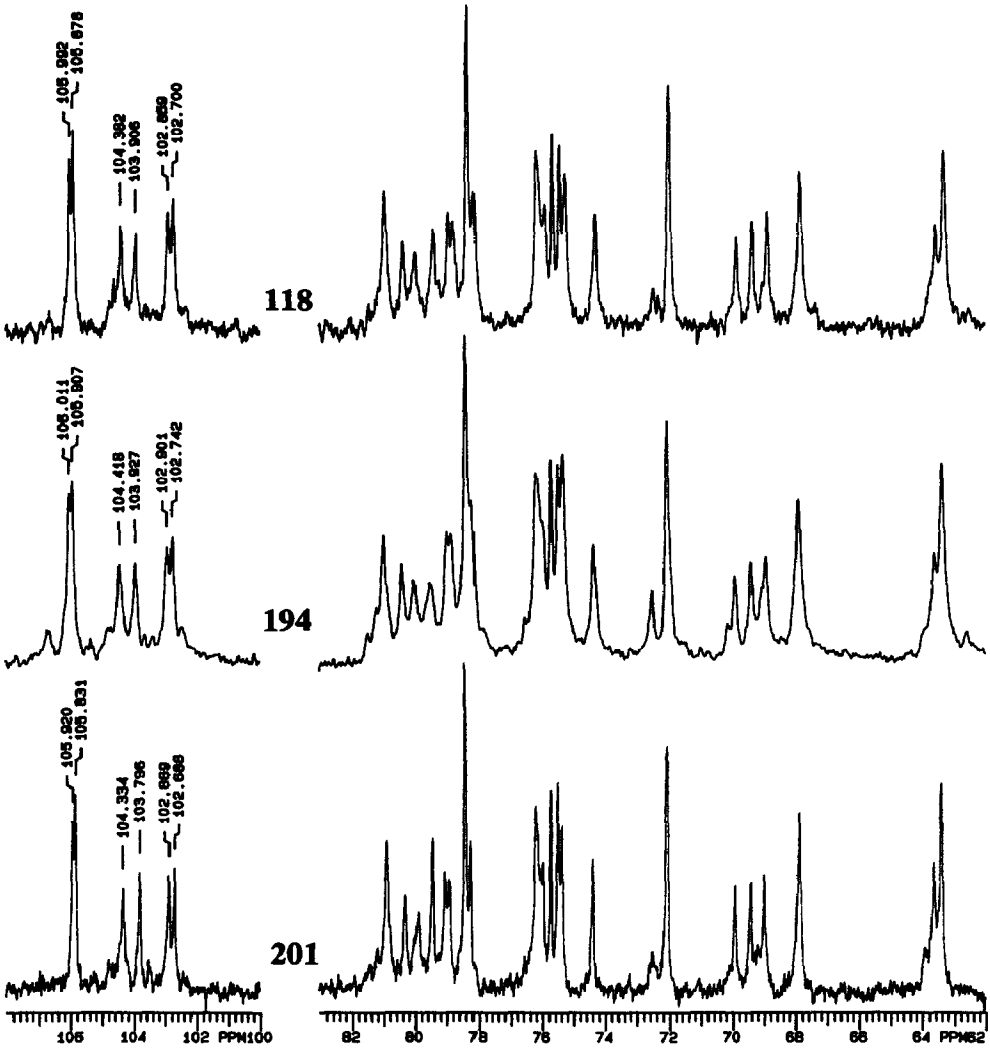


Fig. 2. Proton-decoupled ^{13}C NMR spectra (70°C, 100 MHz) of *O*-deacetylated GXM-S. The reference spectrum for serotype A isolate 201 is taken from ref 8.

Group II (isolate 371-3), respectively, are given in Figs. 2 and 3. Tabulation of the chemical shifts of the anomeric carbons for the GXM-D examined in this study and the two reference samples along with their putative linkage assignments are given in Table III. The two resonances at 105.88 and 105.99 ppm for isolates 118 are due to the anomeric carbons of β-D-Xyl*p* residues linked O-2 to Man*p* as shown in structure **1** and Table III; Man*b*Xyl2 (M*b*X2) and Man*c*Xyl2 (M*c*X2), respectively. The major resonance at 104.38 ppm results from the anomeric carbon atom of β-D-GlcA linked O-2 to Man*p* as shown in structure **1** and Table III: Man*a*GlcA2 (M*a*G). The three remaining major resonances for isolate 118 were

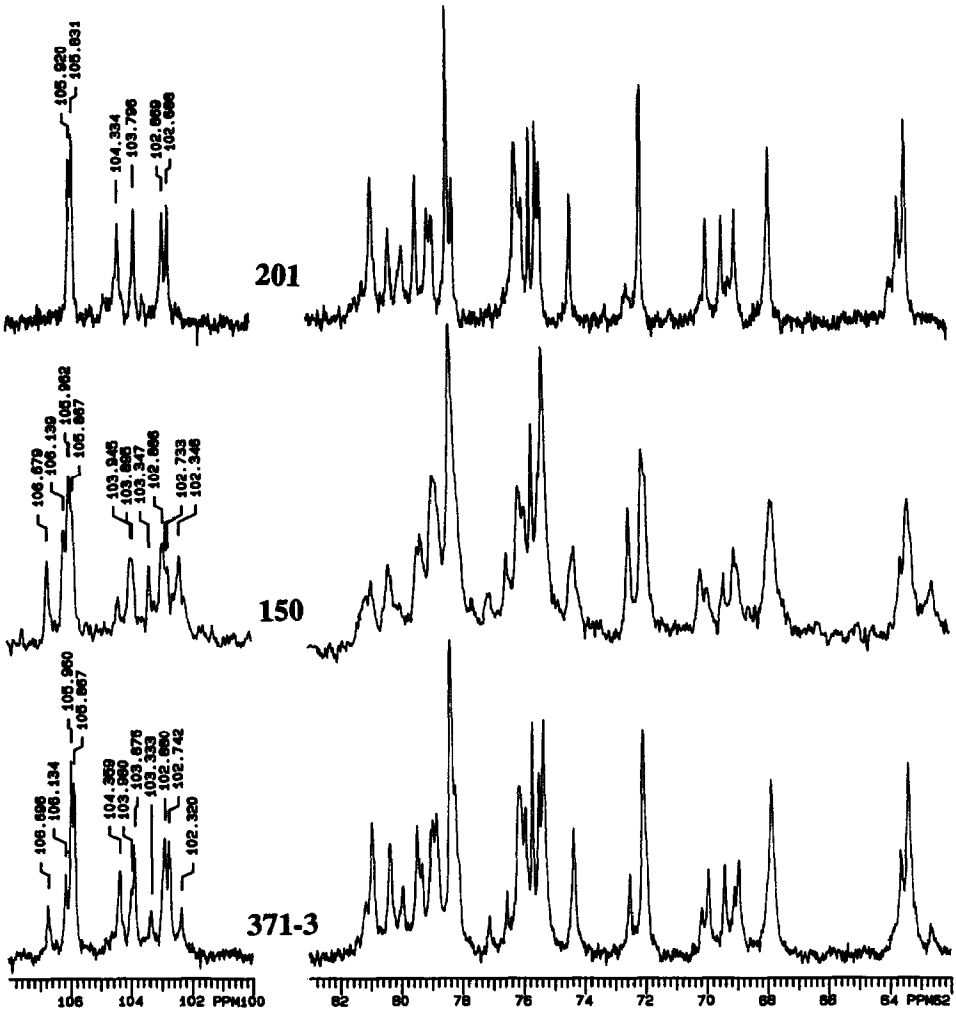


Fig. 3. Proton-decoupled ^{13}C NMR spectra (70°C, 100 MHz) of *O*-deacetylated GXM-S. The reference spectra for serotype A isolates 201 and 371-3 are taken from ref 8.

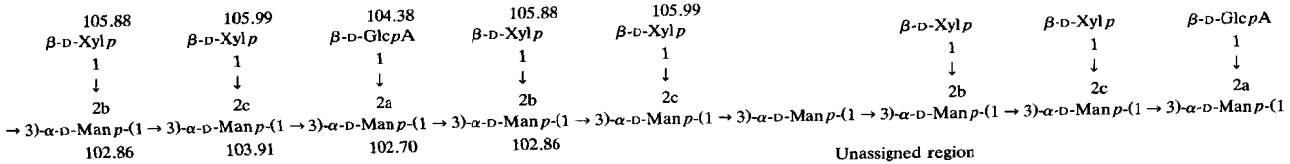
observed at 103.91, 102.86, and 102.70 ppm. These resonances are assigned to the anomeric carbon atoms of the three Man*p* residues substituted at O-2 with β-D-Xyl*p* and β-D-Glc*p*A as shown in structure **1** and Table III; Man*a*GlcA2 (M*a*G), Man*b*Xyl2 (M*b*X2), and Man*c*Xyl2 (M*c*X2), respectively. These data, for GXM-D from isolate 118, are in concordance with the ^{13}C NMR resonances observed for Group I as exemplified by the data for GXM-D of isolate 201 as shown in Table III and Fig. 2. Therefore, isolate 118 falls within the previously assigned Group I[8]. Almost identical chemical shift data for GXM-D from isolate 194 was recorded (structure **1** and Table III). It is assigned to Group I based on the same reasoning. GXM-D of isolate 150 presents a more complex spectrum and

TABLE III

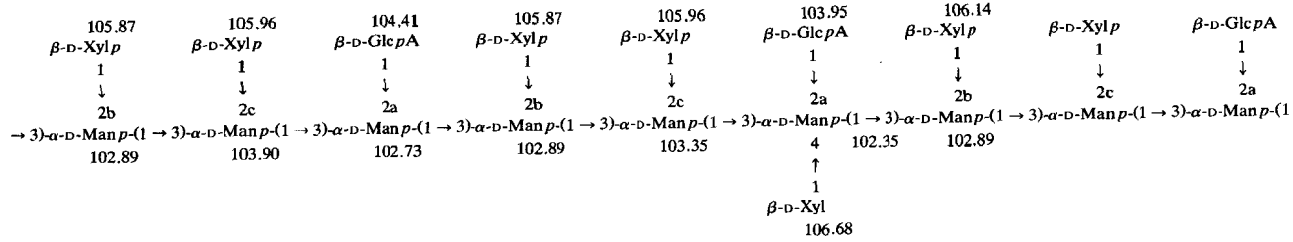
^{13}C NMR chemical shift [a] data for the anomeric carbons of GXM-D of *C. neoformans*, Serotype A

Isolate	M*a*GX4 [b]	M*a*GX4M*b*X2	M*b*X2	M*c*X2	M*b*	M*c*	M*a*G	M*a*GX4	M*a*G	M*a*GX4	M*b*X2	M*c*X2
Group I type isolates												
118			105.88	105.99			104.38		102.70		102.86	103.91
194			105.91	106.01			104.42		102.74		102.90	103.93
201 [d]			105.83	105.92			104.33		102.69		102.87	103.80
Group III type isolates												
150	106.68	106.14	105.87	105.96			104.41 [c]	103.95	102.73	102.35	102.89	103.90
												103.35
371-3 [d]	106.70	106.13	105.87	105.96			104.36	103.98 [c]	102.74	102.32 [c]	102.88	103.88
												103.33 [c]

[a] In ppm relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). [b] M*a*GX4 = Chemical shift of the underlined residue, (1 → 4)-β-D-Xyl*p*, in → 3)-Man*p* substituted O-2 and O-4 with Glc*p*A and Xyl*p*, respectively. See Structures **1** and **2**. Other entries follow the same nomenclature. [c] Underlined values are of low intensity. [d] From ref 8.



Structure **1**, Isolate 118



Structure **2**, Isolate 150

set of chemical shifts (Table III). The prominent anomeric carbon signals are those discussed above and found in Group I. However, there are four signals of low intensity at 106.68 (M*a*GX4), 106.14 (M*a*GX4M*b*X2), 103.95 (M*a*GX4), and 103.35 (M*c*X2) ppm (Table III) that are characteristic of serotype B[17,18]. A model for GXM-D from isolate 150 is given in structure **2**; regions containing unsubstituted Man*p* residues are not shown. These data, for GXM-D from isolate 150, are in concordance with the ^{13}C NMR resonances observed for Group III as exemplified by the data for GXM-D of isolate 371-3. (Table III and Fig. 3). Therefore, isolate 150 falls within the previously assigned Group III[8].

Signals corresponding to unsubstituted and disubstituted Man*p* of the mannan backbone were not observed in 1D ^{13}C NMR experiments performed in these studies. However, 2D NMR experiments for GXM-D from isolate 201[21] identified resonances of relatively low intensity for unsubstituted Man*p* that may occur in several different environments as shown in structure **1**. It is reasonable to assume that these minor resonances would also be observed for GXM-D of isolates 118 and 194 under the appropriate experimental conditions[21].

Thus, the GXM-D from three isolates of *G. neoformans* from patients with AIDS, confirmed as serotype A, were isolated, purified, and their structures determined by methylation analysis and ^{13}C NMR spectroscopy. Isolates 118 and 194 corresponded in structure to Group I, as typified by isolate 201, and the structure of the third isolate, 150, conformed to that of Group III, as typified by isolate 371-3 (Table III and Fig. 2) as defined in a comprehensive investigation of serotype A GXM-D[8].

EXPERIMENTAL

Culture and GXM Production.—One of the authors (T.G. Mitchell) provided clinical isolates of *C. neoformans* obtained from AIDS patients. Three cultures, 118, 194 and 150 were cultured for the preparative isolation of purified GXM. Each isolate was grown in 1.5 L of a chemically defined broth containing 2% glucose for 5 days at 35°C as previously described[9]. The A serotype of the three isolates was confirmed in the laboratory of S. Bragg and E. Reiss (Centers for Disease Control, Atlanta, GA). Exopolysaccharide was recovered from autoclaved culture supernatants by precipitation with EtOH. GXM was purified by selective precipitation with hexadecyltrimethylammonium bromide as previously described, except the initial 15 min treatment with ultrasonic irradiation (u.i.) was omitted[9]. The molecular weight of each purified GXM was reduced by u.i. (GXM-S)[17]. A portion of GXM-S (125 mg in 25 mL of H_2O) was adjusted to pH 11.25 with concentrated NH_4OH and incubated for 24 h at 23°C. The sample was dialyzed and lyophilized and gave the *O*-deacetylated derivative of GXM-S (GXM-D).

Analytical methods.—Neutral carbohydrate was detected by the phenol–H_2SO_4 method of Dubois et al.[22]. Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen[23]. *O*-Acetyl was estimated by the Hestrin procedure

with mannitol hexaacetate as the standard[24]. The constituent monosaccharides of the polysaccharides were identified and quantified as their per-*O*-acetylated aldononitrile (PAAN) derivatives by GLC as previously described[25,26]. Per-*O*-methylation of GXM-S was done by the method of Hakomori[27] as modified by Darvill et al.[28]. GXM-S, obtained from the three isolates, was analyzed by ion-exchange column chromatography using DEAE Sepharose CL-6B (Pharmacia) and a linear gradient of 0.01 M Na_2HPO_4 to 0.01 M Na_2HPO_4–1.0 M NaCl, pH 7.1, as previously described[9]. ^{13}C NMR spectra were recorded at 70°C with a Varian VXR 400 spectrometer, equipped with a 10-mm multinuclear probe, at 100.58 MHz. GXM-D (~ 100 mg) was dissolved in 3.1 mL of D_2O. Chemical shifts were measured relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) taken as 0.00 ppm. The deuterium resonance of the solvent, deuterium oxide, served as internal lock[17].

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