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Variation in the Structure of Glucuronoxylomannan in Isolates from Patients with Recurrent Cryptococcal Meningitis

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Capsular glucuronoxylomannans (GXM) of *Cryptococcus neoformans* var. *neoformans* isolates from patients with recurrent cryptococcal meningitis were analyzed by ^{1}H nuclear magnetic resonance spectroscopy and for reactivity with factor sera (Iatron, Tokyo, Japan). For each patient the initial and relapse isolates had previously been shown to be indistinguishable by DNA restriction fragment length polymorphism analysis. For patients J11 and J22 the GXM of the initial and relapse isolates were identical. For patients SB4 and SB6 the GXM of the initial and relapse isolates differed in structure and reactivity with factor sera. In patient SB4 the initial isolate had a serotype A/D structure, and the first relapse isolate had a serotype A structure. The second relapse isolate was a mixture of structures composed of serotype D components, glucuronomannan (GM), and a minor serotype A component. Analysis of the initial isolate from patient SB6 showed a structure composed mainly of serotype D, GM, and minor serotype A components and components not assigned to a particular serotype (N). The relapse isolate had the same composition as the initial isolate except for an increase in the serotype A component. This increase in the serotype A component of the relapse isolate resulted in a change in the serological specificity from serotype D to serotype A/D. The initial isolate from patient J9 had serotype D and GM structures. The first two relapse isolates had serotype D, N, and GM structures and a minor serotype A component. The third relapse isolate had mainly a serotype D structure. All the J9 isolates reacted only with serotype D-specific factor serum. These results indicate that some isolates obtained from patients with recurrent *C. neoformans* infections have undergone a change in GXM structure during the course of infection. The modification of GXM structure observed in some relapse isolates is reflected in changed serological properties. The results may have important implications for the design of vaccines and antibody-based therapeutic strategies against *C. neoformans*.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes life-threatening meningoencephalitis in 6 to 8% of patients with AIDS (13). *C. neoformans* has a polysaccharide capsule composed predominately of glucuronoxylomannan (GXM). The capsule is a primary virulence factor (7) because it functions as an antiphagocytic agent (24, 26). The capsular exopolysaccharides may also contribute to virulence by causing suppression of the immune response (25, 32, 43, 44), inhibiting leukocyte migration (17), and enhancing human immunodeficiency virus infection (35). In the absence of opsonins, phagocytosis of *C. neoformans* by host effector cells is inefficient (24, 37). Antibodies to the capsular polysaccharide are potent opsonins (37) that form the bases of two strategies being developed for the prevention and treatment of cryptococcal infections: vaccination with GXM-protein conjugates (15) and passive antibody therapy (16, 31).

Structural differences in GXM translate into antigenic differences which have been used to classify *C. neoformans* strains serologically. In 1935 Benham discovered antigenic differences in *C. neoformans* capsular polysaccharide by agglutination and precipitation reactions and used these differences to categorize strains (4). Later Evans introduced the serotype classification based on reactivity with defined absorbed polyclonal rabbit sera (19). Subsequent investigators have expanded on this classification by defining five serotypes, known as A, B, C, D, and A/D (21, 48). However, unlike other encapsulated pathogens, such as *Streptococcus pneumoniae*, for which serology proved a discriminating tool, the majority of *C. neoformans* clinical isolates have been grouped as serotype A (28). This is despite the evidence showing differences in capsular structure among strains grouped within a serotype (46, 47). For example, nuclear magnetic resonance (NMR) spectroscopy of serotype A capsular GXM has revealed significant differences in structure (11, 46, 47); polyclonal and monoclonal antibodies have revealed antigenic heterogeneity within strains assigned to a particular serotype (2, 40). The difficulties with serological classification may be due to the poor immunogenicity of cryptococcal polysaccharides and the reliance on rabbit sera (4).

MATERIALS AND METHODS

***C. neoformans* strains.** The J9, J11, J22, SB4, and SB6 strains were recovered from the cerebrospinal fluid of patients with meningitis (8, 41). Each of the five patients had a clinical recurrence of cryptococcal meningitis after initial treatment, and the absence of negative cultures demonstrated that none cleared the cerebrospinal fluid infection (8, 41). For each strain recovered from a patient, the "a" isolate is the initial isolate recovered when the diagnosis was first made and the "b," "c," and "d" isolates are sequential relapse isolates recovered during clinical recurrences of *C. neoformans* meningitis (8, 41). For patients J9, J11, SB4, and SB6 the initial and relapse isolates were not distinguished by URA5 and CNRE-1 restriction fragment length polymorphism (RFLP) analyses (41). For patients J11, SB4, and SB6, the initial and relapse isolates were not distinguishable by electrophoretic karyotyping (41). For patient J9, the electrophoretic karyotype of J9a differed from subsequent isolates at two chromosomal positions, and J9a was considered a variant of strain J9 (41). The J9b, J9c, and J9d isolates were not distinguishable by RFLP analysis or electrophoretic karyotyping (41). For patient J22, the initial and relapse isolates were not distinguishable by URA5 RFLPs (8). Each strain has been shown to be *C. neoformans* var. *neoformans* on

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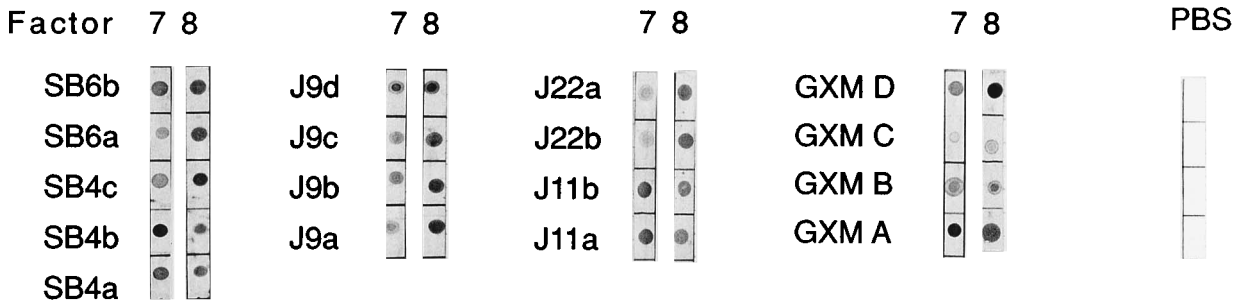

FIG. 1. Reactivities of serotype-specific factor sera 7 (serotype A) and 8 (serotype D) with purified GXM from patient isolates evaluated by DEA. Standard GXMs from serotypes A, B, C, and D are included for comparison. PBS, phosphate-buffered saline.

the basis of color reaction on glycine-canavanine-bromthymol blue agar (8, 41). Strains were maintained in Sabouraud dextrose agar at 4°C.

Antigen production. Each isolate was grown in 1 liter of chemically defined broth containing 2% glucose for 4 days at 37°C as described previously (11, 12). After growth the cultures were autoclaved and the cells were removed by centrifugation. Purified polysaccharide antigen (GXM) was isolated from the culture supernatant by selective precipitation with hexadecyltrimethylammonium bromide as previously described (11), except that the initial 15-min sonication step was omitted. A portion (50 mg) of the purified GXM was dissolved in 10 ml of H_2O and was O-deacetylated (GXM-D) by adjusting the pH to 11.25 with NH_4OH and stirring the resulting solution for 24 h at 23°C. Reference GXMs of each serotype ("reference" refers to isolates whose GXMs have molar ratios that approximate those suggested for serotypes A, B, C, and D by Bhattacharjee et al. [5]) and glucuronomannan (GM), prepared and characterized in previous studies, were available in the laboratory: isolate 6, serotype A (11); isolate 9375, serotype D (38); isolate CBS 132, serotype A/D (9); and GM (10).

Factor sera. Cryptococcal factor sera 5, 6, 7, and 8 (lot 910610), specific for serotypes B, C, A, and D, respectively, were provided by Iatron Laboratories, Inc., Tokyo, Japan. Sera were stored at −20°C except for short-term storage (4°C).

DEA. Dot enzyme (DEA) assays were done by the method of Tsang et al. (45) as modified by Belay et al. (3). Strips of nitrocellulose transfer membrane (0.5 by 5.0 cm) were spotted every centimeter with 1 μl of a freshly prepared solution of GXM (1 mg/ml of 0.06 M sodium carbonate buffer, pH 9.6) and processed as described (3). GXM solutions are used immediately after their preparation and then discarded since the carbonate buffer, pH 9.6, causes the slow saponification of the *O*-acetyl esters.

NMR. ^{1}H-NMR spectra were obtained with a Varian VXR-400 NMR spectrometer equipped with a 5-mm $^1H/^{19}F$ probe and operated at 399.952 MHz for ^{1}H observation. Spectra were recorded at 80°C, and chemical shifts were measured relative to HOD at 4.246 ppm. The HOD peak was referenced to the methyl groups of sodium 4,4-dimethyl-4-silapentane-1-sulfonate taken as 0.00 ppm. This procedure facilitated the direct recovery of NMR samples by lyophilization. All samples (from patient isolates and standards) consisted of approximately 10 mg of GXM-D dissolved in 0.5 ml of D_2O.

Spectra were processed by using the program FTNMR from Hare and Associates (now Biosym Technologies, San Diego, Calif.). Each spectrum was resolution enhanced by applying a sine bell window function over all real datum points. Spectra of GXMs from patient isolates were compared with spectra of reference GXMs whose chemical structures had previously been defined.

RESULTS

Purification. The yield of purified GXM from a 1-liter culture was generally 1 g, with a range of 0.5 to 2 g.

DEA. Dot blot results are shown in Fig. 1. For patients J11 and J22, the GXMs of the initial and relapse isolates showed no change in reaction with factor sera. J11 isolates react more strongly with factor serum 7 than with factor serum 8. J22 isolates react well with factor serum 8 but react weakly with factor serum 7. Neither J11 nor J22 reacted with factor sera 5 and 6 in DEA (data not given). The GXMs isolated from SB6a and SB6b gave equivalent, positive responses with factor serum 8, while the response with factor serum 7 was greater for the relapse isolate. SB6a and SB6b did not react with factor sera 5 and 6 in DEA (data not given). The three SB4 isolates gave varied responses in DEA with factor sera 7 and 8. SB4a gave strong positive responses with factor sera 7 and 8. SB4b gave a strong reaction with factor serum 7 and a weak reaction with factor serum 8. SB4c gave a strong response with factor serum 8 and a weak response with factor serum 7. Factor sera 5 and 6 did not react with the SB4 isolates (data not given). The J9 isolates (J9a through J9d) all gave strong responses with factor serum 8 but gave weak responses with factor serum 7. J9d GXM was the best responder to factor serum 7 of the J9 GXMs; however, that reaction was still weaker than the reaction observed with factor serum 8. The GXMs from J9b and J9c reacted very weakly with factor sera 5 and 6 (data not given). J9a and J9d did not react with factor sera 5 and 6 in DEA (data not given).

NMR. Figure 2 shows the resolution-enhanced mannose anomeric (C-1) region of the ^{1}H-NMR spectra of GXM-D standards used in the study. The anomeric protons due to the mannose residues are considered reporter groups for determining the presence of particular structural triads in GXM. The resonances in each spectrum have been labeled with a letter corresponding to a mannose in one of the structural triads depicted in Fig. 3. The reference serotype A GXM-D has three major anomeric resonances that correspond to the mannose residues of the polysaccharide. Each resonance represents a unique element of the structural triad for serotype A (Fig. 2 and Fig. 3A). The other minor resonances indicate the presence of a small amount of GM (Fig. 3GM). The major anomeric mannose resonances for GXM-D of the reference serotype D (Fig. 2) are contributed by the triad depicted in Fig. 3D. The low-intensity resonances are due to the presence of a minor quantity of GM (Fig. 3GM). The GXM-D of the reference serotype A/D isolate has structural elements representative of the four model structures depicted in Fig. 3. The major portion of the serotype A/D GXM-D is due to serotype D and GM triads (Fig. 3D and GM, respectively). There is proportionately much less serotype A structure (Fig. 3A). The corresponding NMR spectra are depicted in Fig. 2. Resonances assigned to the structural triad (N) not assigned to a particular serotype (Fig. 3N) are present at a very low intensity. At this time we do not know whether the various triads observed in each GXM-D are part of a single polymer or whether they are present as separate entities.

The structures of GXM-Ds from two sets of isolates (J11 and J22) did not change. The NMR spectra recorded for J11a and J11b show that these two isolates are essentially identical serotype A structures (Fig. 4). The spectra in Fig. 4 show the presence of a minor structural element that corresponds to the GM triad (Fig. 3GM). The J22 isolates also produced equivalent GXM-D structures (Fig. 5). Both isolates contain structural triads contributed by serotype D and GM. There is also evidence for the presence of a newly identified N triad (Fig.

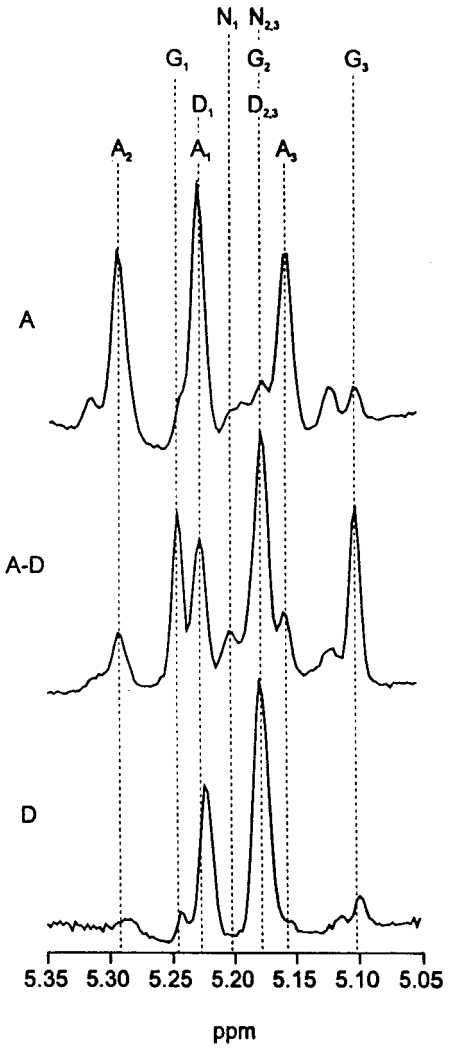

FIG. 2. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds of representative serotype structures for serotypes A, A/D, and D. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

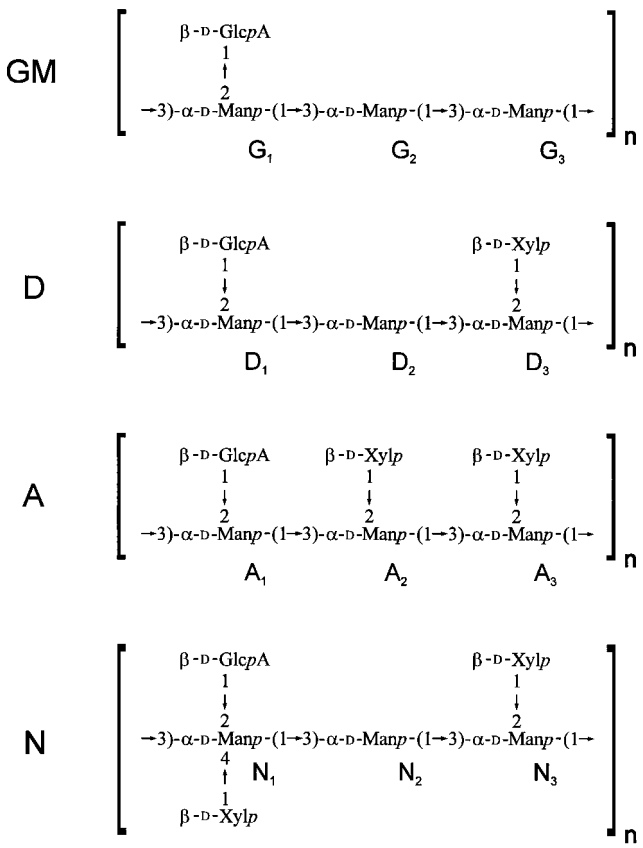

FIG. 3. Structures of the O-deacetylated GXMs of *C. neoformans*. Structures are shown for the GM repeating unit (9), the representative serotype D repeating unit (5), the representative serotype A repeating unit (5), and the newly identified repeating unit (N) (8). The mannose labels are used to identify structural elements in Fig. 2 and 4 through 8.

3N) as is indicated by the signals of low intensity in the NMR spectrum (Fig. 5, resonances N_1 and $N_{2,3}$).

GXM-Ds from the initial and relapse isolates of three patients (SB4, SB6, and J9) differed significantly in structure (Fig. 6 to 8). J9a produced GXM-D composed of triads that are characteristic of reference serotype D and GM structures (Fig. 3 and 8). J9b and J9c contain serotype D, N, and a small proportion of GM triads and a minor serotype A component (Fig. 8). GXM-D from J9d is composed almost completely of the reference serotype D structure with traces of serotype A and GM triads. The structures of the SB4 GXM-Ds varied the most. The GXM-D of SB4a contains serotype A, serotype D, and GM triads in about equal proportions (Fig. 6). SB4b contains only the reference serotype A triad in significant amounts. SB4c resembles the composition of the initial isolate except that there is less of the reference serotype A triad present. There may be a small amount of serotype A structure present. The SB6 isolates contain the main characteristic triads for serotypes A and D and GM (Fig. 7). SB6a also has a significant amount of the N triad. GXM-D from SB6b has a slightly greater proportion of the serotype A triad and only a minor amount of the N triad.

DISCUSSION

For patients J11 and J22, the GXM structures of the initial and relapse isolates were essentially identical. For patients J9, SB4, and SB6, the GXM structures of the relapse isolates differed from those of the initial isolates. The differences in GXM-D structure predicted by ^{1}H-NMR spectroscopy were confirmed with factor sera by DEA analysis of GXM for the SB4 and SB6 isolates but not for the J9 isolates. The fact that factor sera discriminated among sequential isolates for SB4 and SB6 indicates that the GXM structural differences determined by ^{1}H-NMR were recognized by antibodies and are therefore antigenically active. GXM is used instead of GXM-D in DEA because the integrity of the *O*-acetyl substituents is required for the observation of the serological activities of most factor sera. This observation indicates that serotype switching can occur during the course of human infection.

Serotype classification has been used to distinguish *C. neoformans* strains since the system was introduced by Evans (18, 19). Serotype determination is done with rabbit immune sera which have been absorbed with defined strains to remove antibodies to common determinants. However, serotype classification is not very discriminating; it groups the majority of strains isolated from patients and the environment as serotype

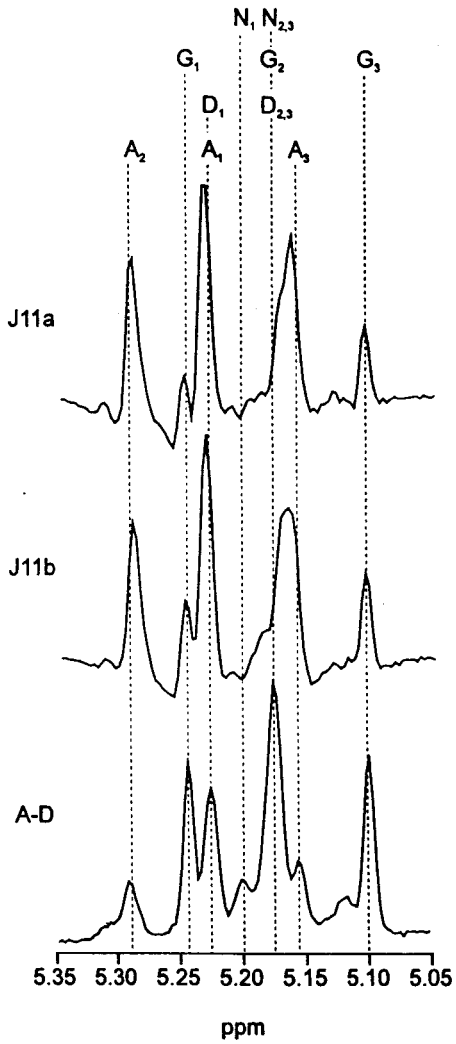

FIG. 4. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds from J11 isolates. The representative serotype A/D spectrum has been added for reference. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

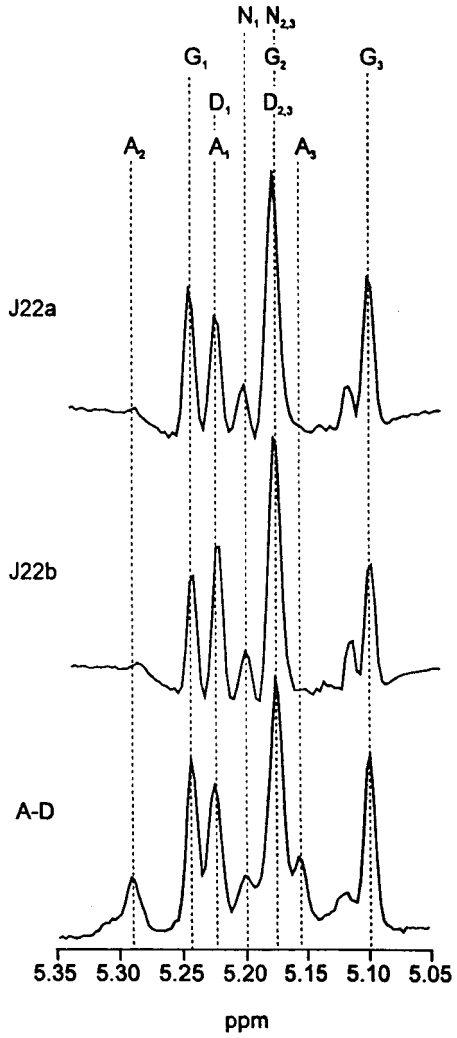

FIG. 5. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds from J22 isolates. The representative serotype A/D spectrum has been added for reference. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

A despite evidence for structural heterogeneity (11, 46) and antigenic variation (40) within serotypes. For the initial and relapse isolates of patients SB4 and SB6, changes in serotype were documented by NMR spectroscopy and serology. The SB4 and SB6 initial and relapse isolates were not distinguishable by the highly discriminatory DNA typing techniques of CNRE-1 RFLP analysis (14) and electrophoretic karyotyping (34) and were considered to belong to the same strain (41). The observations with SB4 and SB6 strongly suggest that serotype classification may not be a stable phenotypic marker for *C. neoformans*. In this regard it is noteworthy that some strains have been initially grouped within one serotype but subsequently reclassified as belonging to other serotypes (5). Strains assigned to serotypes A and D belong to the same variety and can mate (1, 27). Since mating implies genetic reassortment and recombination, it is not surprising that phenotypic antigenic characteristics could be variable and possibly interchangeable.

The mechanism(s) responsible for variation in GXM structure and serotype in initial and relapse isolates is not understood. For patients SB4 and SB6 the DNA typing results of the initial and relapse isolates were interpreted as consistent with persistence of the initial infection (41). For patient J9 the electrophoretic differences between J9a and subsequent isolates suggested infection with two related variants (41). However, J9b, J9c, and J9d were not distinguishable by RFLP analysis or electrophoretic karyotyping, and recurrence was also attributed to persistence of infection (41). Changes in strain serotype during persistence of infection imply GXM structural changes in vivo. GXM is a complex structure that is undoubtedly the result of a multienzyme synthetic process (22, 42, 48). Differences in enzyme regulation of capsule synthesis could translate into differences in GXM structure and consequently into antigenic differences. One possible mechanism for the generation of antigenic variants is immune selection. In bacterial systems immune mechanisms can select for antigenic variants (6). The capsule has epitopes which can elicit protective antibodies (16, 31, 36), and the generation of such antibodies could select for antigenic variants. Isolates of *C. neoformans* differ in their abilities to activate complement (49). Since complement provides important opsonins, it is conceiv-

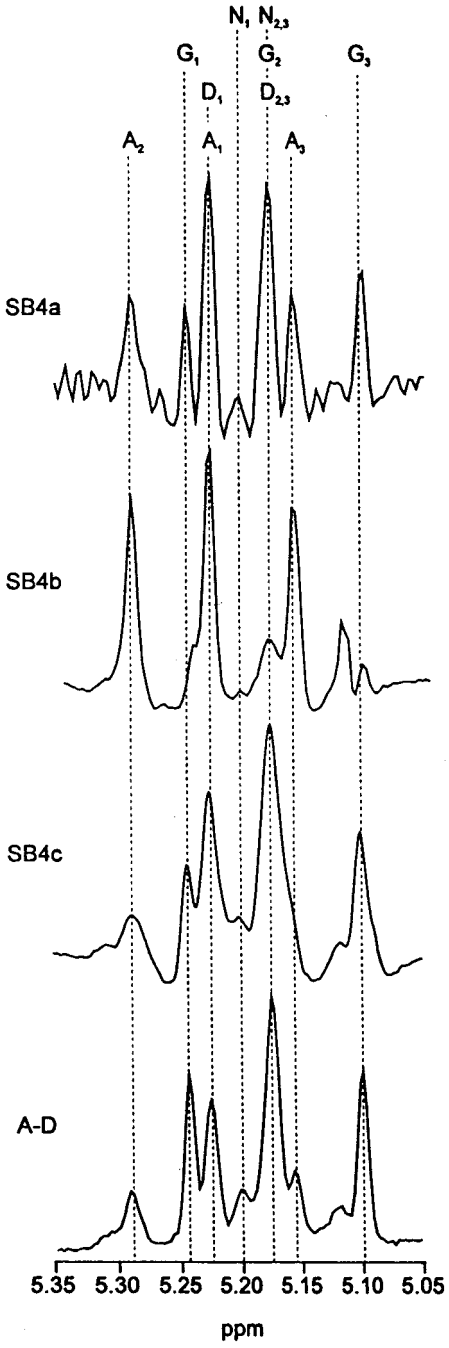

FIG. 6. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds from SB4 isolates. The representative serotype A/D spectrum has been added for reference. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

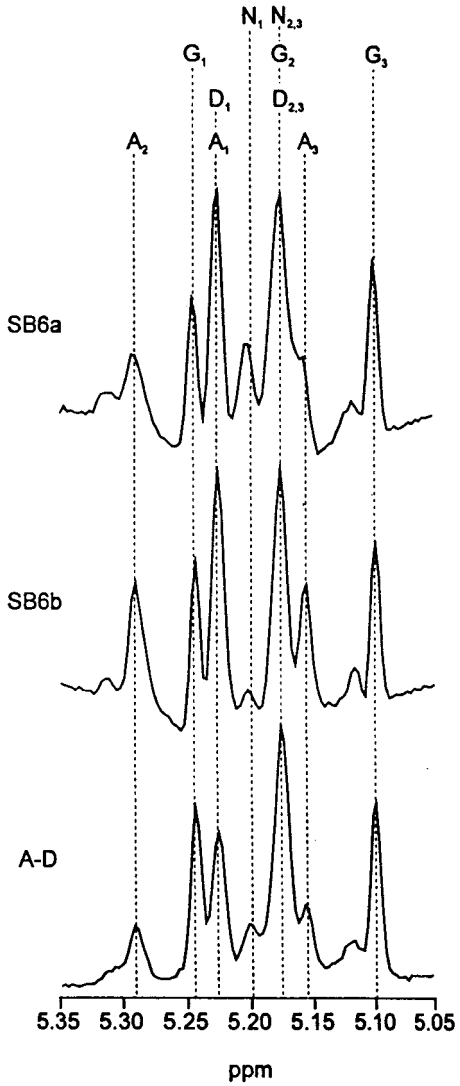

FIG. 7. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds from SB6 isolates. The representative serotype A/D spectrum has been added for reference. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

able that differences in complement activation could lead to the selection of structural variants. Similarly, lymphocytes have been shown to interact directly with *C. neoformans* (30, 33), and variation in capsular structure may be the result of selection by cell-mediated immune mechanisms. Whatever the mechanism, the recovery of new antigenic variants suggests genetic changes in vivo. Increased mutagenesis could conceivably occur in vivo as a result of exposure to antimicrobial free radicals generated by host effector cells (23). For some of the isolates in this study, the GXM changes are paralleled by changes in sterol composition, suggesting the selection of metabolically different variants (20). Alternatively, serotype variation within a strain of *C. neoformans* may represent a phenomenon analogous to phenotypic switching in *Candida albicans*, in which some strains can switch back and forth between phenotypes distinguishable by colony morphology (39). Nevertheless, it is important to note that the NMR spectra of the J9, SB4, and SB6 initial and relapse isolates are similar, but not identical, within the set of isolates for each patient. The magnitude of the inferred GXM structural changes is related to the relative areas of the resonances characteristic of polysaccharide structures (Fig. 2 to 8). Changes in GXM biosynthetic enzyme regulation may be responsible for the structural differences.

The finding of structural and serotype variations in sequential isolates of *C. neoformans* from some patients could have important implications for the development of vaccines and passive antibody therapies. If immune selection of antigenic variants occurs in vivo the result may be the recovery of new

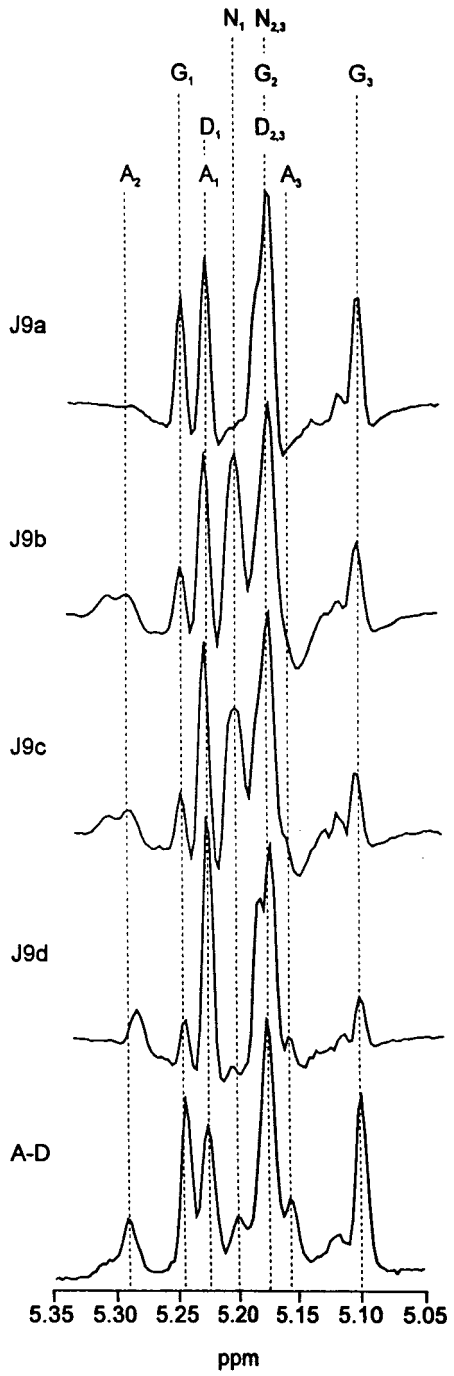

FIG. 8. Resolution-enhanced mannose anomeric region of the ^{1}H-NMR spectra of GXM-Ds from the J9 isolates. The representative serotype A/D spectrum has been added for reference. The mannose residues have been labeled with the corresponding structural elements (Fig. 3).

antigenic variants which differ significantly from the infecting strain. It is conceivable that some isolates from patients represent unique strains, having been selected in vivo, with no environmental counterpart. It is noteworthy that among *C. neoformans* var. *gattii* isolates, all environmental isolates are serotype B and serotype C isolates have been recovered only from patients (29). Our results raise the possibility that some serotype C strains are in vivo-selected variants of serotype B strains. Passage of strains in animals in the environment could contribute to antigenic heterogeneity of strains. In regard to vaccine development and antibody-based therapeutic strategies, the results strongly suggest the need for testing of reagents on both recent clinical isolates and environmental strains.

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