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Reactivity Patterns and Epitope Specificities of Anti-*Cryptococcus neoformans* Monoclonal Antibodies by Enzyme-Linked Immunosorbent Assay and Dot Enzyme Assay

TESFAYE BELAY,[1] ROBERT CHERNIAK,[1]* THOMAS R. KOZEL,[2] AND ARTURO CASADEVALL[3]

Department of Chemistry, LBCS, Georgia State University, Atlanta, Georgia 30303[1]; Department of Microbiology, University of Nevada, Reno, Nevada 89557[2]; and Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461[3]

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Cryptococcus neoformans **glucuronoxylomannans (GXM) are capsular polysaccharides important for virulence in cryptococcosis. This study used dot enzyme assays (DEA) and enzyme-linked immunosorbent assays (ELISA) to determine the reactivity patterns of 21 murine monoclonal antibodies (MAbs) with structurally defined GXMs from five serotypes. The MAbs were categorized into eight groups on the basis of DEA and five groups on the basis of ELISA. MAbs 302, 339, and 439 were studied extensively for their binding to various native and chemically modified GXMs. Quantitative variation in the inhibitory effects of GXMs on the binding of MAbs 302, 339, and 439 were observed by competitive ELISA. *O*-Deacetylation of serotype A, B, and D GXM resulted in the complete loss of their inhibitory properties. Carboxyl group reduction of GXMs from serotypes A and D resulted in a significant decrease of inhibitory activity for MAb. Xylomannans and methyl glycosides exhibited no detectable inhibitory activity on MAb binding to GXM. The results indicate (i) the existence of five to eight MAb-defined distinct epitopes in *C. neoformans* GXM that can elicit antibody responses, (ii) MAb detection of antigenic variation within GXMs assigned to a particular serotype, (iii) good correspondence between the patterns of MAb reactivities and polyclonal rabbit factor sera, (iv) good agreement between MAb molecular structure and serotype reactivity, and (v) a dependence of the serotype reactivity profile for a given MAb on the technique used to measure binding.**

Cryptococcus neoformans is a pathogenic yeast that causes a life-threatening meningoencephalitis in individuals with a weakened immune system (18, 32). The incidence of cryptococcosis has increased dramatically in recent years as a consequence of the AIDS epidemic, and it is a leading cause of death in patients with AIDS (5, 18, 34, 35, 44). *C. neoformans* is unusual among pathogenic fungi in that it has a polysaccharide capsule. The major capsular polysaccharide is glucuronoxylomannan (GXM), which is an important contributor to the virulence of *C. neoformans*. GXM is antiphagocytic and poorly immunogenic, and acapsular strains have significantly reduced virulence (6, 27). In vitro, GXM inhibits leukocyte migration (19), enhances human immunodeficiency virus infection in human lymphocytes (42), and promotes L-selectin shedding from neutrophils (20).

The typical GXM consists of a linear (1 → 3)-α-D-mannopyranan bearing β-D-xylopyranosyl (Xyl*p*), β-D-glucopyranosyluronic acid (Glc*pA*), and 6-*O*-acetyl substituents (4, 16, 50). Differences in GXM structure produce antigenic variability that has led to the classification of *C. neoformans* strains into five serotypes (A, B, C, D, and A-D) (26, 29, 31, 52). Despite considerable structural and antigenic diversity, a simple structural relationship exists between GXMs of reference isolates for the five serotypes. They are all comprised of a core repeating unit, illustrated in Fig. 1, to which (1 → 2)-linked and (1 → 4)-linked β-D-Xyl*p* units are added in increments of 1 to 4 residues. In this way, explicit typical molar ratios of Xyl/Man/GlcA in serotypes D, A, B, and C have been assigned as 1:3:1, 2:3:1, 3:3:1, and 4:3:1, respectively (4). GXM from serotypes A and D are mainly substituted with Xyl*p* at *O*-2, whereas GXM from serotypes B and C are substituted at *O*-2 and at *O*-4. Additional analytical data show that the precise molar ratios and typical substitution patterns, as proposed in the original models of GXM structure, are an oversimplification (12–16, 50) except for serotype B (49). In addition, substituent dispositions previously thought to be characteristic of one serotype have been identified in heterologous isolates (16, 50, 51). Chemical analyses of GXM documents the existence of antigenic multiplicity among the serotypes, particularly in serotypes A and C (15, 51).

Serotyping of *C. neoformans* isolates has been based on the reactivities of whole cells to polyclonal sera that have been selectively absorbed with whole yeast cells (2, 3, 29, 31). The presence of at least eight antigenic factors, distributed among the serotypes of *C. neoformans*, has been proposed based on the reactivities of the factor sera in yeast cell agglutination reactions (29, 31). Unfortunately, rabbit serotype-specific sera have low discriminatory power for determining structural heterogeneity, and greater than 90% of *C. neoformans* clinical strains have been classified as serotype A (35). Moreover, generation of rabbit serotype-specific sera is labor intensive, and the reagents are subject to lot variation and reactivity variation based on the *C. neoformans* strains used to absorb the antibody.

In recent years, monoclonal antibodies (MAbs) specific for GXM have been generated in various laboratories. The available MAbs have been studied by enzyme-linked immunosorbent assay (ELISA) (8, 11, 17, 24, 25, 46, 48), immunodiffusion (24, 25, 46), and immunofluorescence (23–25, 41, 48). Several MAbs are now used in diagnostic tests for capsular polysaccharide (28, 33, 47). MAbs have also been tested for their

* Corresponding author. Mailing address: Department of Chemistry, Georgia State University, University Plaza, Atlanta, GA 30303. Phone: (404) 651-3868. Fax: (404) 651-1416. E-mail (Internet): cherniak@gsu.edu.

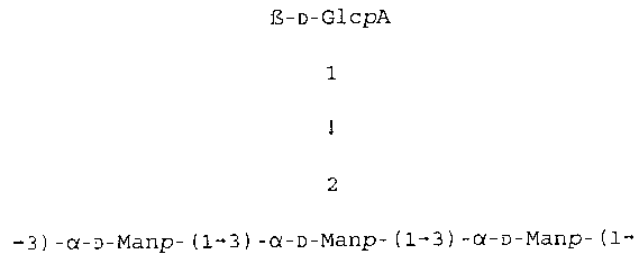

FIG. 1. Core repeating unit of GXMs of reference isolates for the five *C. neoformans* serotypes.

ability to protect mice against experimental cryptococcosis and were found to provide various levels of protection depending on the experimental model, the antibody isotype, and the fine specificity of the antibody (17, 21, 22, 37–40, 43, 53).

Previous studies indicate that the epitope specificity of an anti-GXM MAb can greatly influence selected biological activities of the antibody. For example, Mukherjee et al. (36) described two anti-GXM immunoglobulin M MAbs that were derived from the same B cell but recognized different epitopes on GXM. Passive immunization with one MAb prolonged survival, whereas the other MAb did not. In another study, the influences of various MAbs on serum activation and the levels of binding of C3 fragments to the yeast were compared (34a). Depending on the specificity of the antibody, anti-GXM MAbs could block, enhance, or have no effect on complement activation by encapsulated cryptococci. Since differences in biological activities can be associated with differences in apparent epitope specificity, it is important that the available anti-GXM MAbs be carefully characterized with regard to their reactivities with polysaccharides of differing serotypes.

Preliminary studies of serotype specificities of the anti-*C. neoformans* MAbs against GXMs have been done (8, 9, 11, 24, 25, 46, 48). However, a comprehensive comparison of the specificities of the various MAbs under standardized conditions has not been reported. In this study, we compare the reactivities of 21 different MAbs for *C. neoformans* GXM and define their serotype reactivities and epitope specificities.

MATERIALS AND METHODS

GXM and anti-GXM MAbs. *C. neoformans* isolates used in this study are listed in Table 1. Isolation and purification of GXMs was done as described previously (13). Structural derivatives of GXM (carboxyl reduced with retention of the *O*-acetyl *O*-deacetylated, and *O*-deacetylated-carboxyl reduced groups and xylomannan) were prepared as described previously (12–15). Methyl glycosides were purchased from Sigma Chemical Co., St. Louis, Mo. Reference GXMs of each serotype (the term reference GXMs refers to isolates of *C. neoformans* whose GXMs have molar ratios of mannose, xylose, and glucuronic acid that most closely fit the classical chemical delineation of the type-specific polysaccharides suggested for serotypes A, B, C, and D by Bhattacharjee et al. [4]) were bound to microtiter plates and nitrocellulose membrane strips for ELISA and dot enzyme assay (DEA) analysis, respectively. Reference isolates of the four serotypes of *C. neoformans* used in this study were as follows: isolate 6, serotype A (13); isolate 298 (DEA analysis) and isolate 34 (ELISA analysis), serotype C (15); isolate 409, serotype B (49); and isolate 9375, serotype D (45). The anti-*C. neoformans* MAbs examined in this study are described in Table 2.

DEA and ELISA. The preparation of antigen-coated nitrocellulose strips and their application in DEA were described previously (2, 3). Peroxidase-labeled goat anti-mouse immunoglobulin G (whole molecule) and metal-enhanced DAB substrate (Pierce, Rockford, Ill.) were used for antibody detection. A 1:40 or 1:80 dilution of the original concentration of each MAb (furnished by the provider laboratory) was assayed by DEA.

The optimal conditions for preparing the microtiter plates and performing the ELISA analyses were described previously (1–3, 11). The GXMs of different serotypes exhibit different efficiencies of binding to polystyrene microtiter plates. To circumvent this problem, all the GXMs were conjugated to the adipic acid dihydrazide derivative of bovine serum albumin (11). Binding of each MAb was evaluated by preparing serial dilutions of each antibody in 0.01 M Na_2PO_4–0.14 M NaCl (pH 7.4) with 0.05% Tween 20 (PBS-T). Alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (whole molecule) was used for antibody detection. Working concentrations for MAbs 302 (25 μg/ml), 339 (5 μg/ml), and 439 (7.7 μg/ml) for the inhibition studies were determined from the linear part of their respective titration curves. Competitive binding ELISA was done by preincubating 200 μl of GXM (0 to 100 μg/ml) in PBS-T with an equal volume of the predetermined dilution of each MAb in the same buffer for 90 min at 23°C. One-hundred-microliter portions of the incubated mixtures were then added in duplicate or triplicate to GXM-coated microtiter plates. A positive control experiment containing each MAb preincubated with buffer containing the coating antigen (0.0 to 100 μg per ml) was included in each ELISA. Negative controls consisted of the complete system without MAb or without the GXM coating antigen. Experimental protocols were repeated on two to three different occasions with plates prepared on different dates. The standard deviation of the measured optical density between replicate wells typically fell between 0.00 and 0.08 absorbance unit.

RESULTS

The DEA specificities of the MAbs available for study were determined with reference GXMs that correspond to the four defined serotypes of *C. neoformans* (Fig. 2). The specificities of the MAbs fell into eight categories as follows: group 1 was reactive (a high-intensity dot was observed) with GXM of all serotypes (MAbs 2H1, 383, 471, 21D2, 439, 3C2, and 17E12); group 2 was reactive with GXM of serotypes A, B, and C (MAbs 2D4 and 13F1); group 3 was reactive with GXM of serotypes A and B and showed some reactivity with GXM of serotypes D and C (MAbs 12A1, CD6, and BA4); group 4 was reactive with GXM of serotypes A, B, and D (MAbs 1255 and 339); group 5 was reactive with GXM of serotypes A and D (MAb 302); group 6 was reactive with GXM of serotype D and showed some reactivity (a low-intensity dot was observed) with GXM of the remaining serotypes (MAbs 4H3 and 7B13); group 7 was reactive with GXM of serotype D and showed some reactivity with serotype A GXM (MAbs BD1, 386, and CRND-8); and group 8 was reactive only with GXM of serotype A (MAb E1). The results are summarized in Table 3.

The binding specificities of the MAbs were also studied by ELISA using the same four reference GXMs (Fig. 3). The ELISA specificities of the MAbs fell into five categories as follows: group 1 was reactive with GXMs of all serotypes (MAbs 2H1, 383, 471, 21D2, 439, 3C2, 17E12, 2D4, 13F1, 386, 12A1, 4H3, and BA4); group 2 showed similar reactivity with

TABLE 1. *C. neoformans* isolates used in this study

Serotype	Isolate	Source and/or references[b]
A (I)[a]	6	T. G. Mitchell (Duke University) (14, 51)
A (II)	110	T. G. Mitchell (Duke University) (14, 51)
A (III)	150	T. G. Mitchell (Duke University) (14, 51)
A (IV)	271	K. J. Kwon-Chung (NIH) (51)
A (III)	371	K. J. Kwon-Chung (NIH) (51)
B	409	K. J. Kwon-Chung (NIH)
B	444	K. J. Kwon-Chung (NIH)
B	3172	E. Reiss (CDC)
B	3939	E. Reiss (CDC)
C	34	K. J. Kwon-Chung (NIH)
C	298	K. J. Kwon-Chung (NIH)
C	401	K. J. Kwon-Chung (NIH)
C	3183	E. Reiss (CDC)
D	9375	H. Jean Shadomy (Medical College of Virginia)
D	1254	E. Reiss (CDC)
D	125	E. Jacobson (Medical College of Virginia)
D	127	E. Jacobson (Medical College of Virginia)
A/D	132	T. Shinoda (Tokyo, Japan)

[a] Roman numbers in parentheses refer to the serotype A GXM chemical group (14, 51).

[b] NIH, National Institutes of Health; CDC, Centers for Disease Control and Prevention.

TABLE 2. Anti-*C. neoformans* MAbs

MAb	Immunogen: isolate (serotype)	Serotype specificity[a]	Assay	Source or reference[b]
2H1	GXM-TT[c]	A, B, C, D	ELISA	8
383	24064 (A) + RBC[d]	NR[e]	NR	T. Kozel
471	24064 (A) + RBC	A, B, C, D	ELISA, cell agglutination, immunodiffusion	46
21D2[f]	Infection by GH[g]	A, B, C, D	ELISA	10
439	24064 (A) + RBC	A, B, C, D	ELISA, cell agglutination, immunodiffusion	25
3C2	24066 (C) + RBC	A, B, C, D	ELISA, cell agglutination, immunodiffusion	46
17E12	GXM-TT	A, B, C, D	ELISA	8, 38
2D4	24066 (C) + RBC	NR	NR	T. Kozel
13F1	GXM-TT	A, B, C, D	ELISA	8, 36
12A1	GXM-TT	A, B, C, D	ELISA	8, 36
CD6	3183 (C) + BSA[h]	A, B, C, D	ELISA, DEA, cell agglutination, immunofluorescence	48
BA4	9759 (A) + BSA	A, B	ELISA, DEA, cell agglutination, immunofluorescence	48
1255	24064 (A) + RBC	A, B, D	ELISA, cell agglutination, immunodiffusion	25
339	24065 (B) + RBC	A, B, D	ELISA, cell agglutination, immunodiffusion	46
386	24067 (D) + RBC	A, D	Cell agglutination, immunodiffusion	46
302	24067 (D) + RBC	A, D	ELISA, cell agglutination, immunodiffusion	25
7B13	Infection by GH	D	ELISA	10
4H3	Infection by GH	D	ELISA	10
BD1	9759 (A) + BSA	A, D	ELISA, DEA, cell agglutination, immunofluorescence	48
CRND-8	NIH 512 (D) + keyhole limpet hemocyanin	D	ELISA, cell agglutination	30
E1	GXM (A)		ELISA, immunodiffusion, immunofluorescence	22

[a] Serotype specificity determined by the MAb provider laboratory.
[b] A cited reference number indicates where the MAb was first described.
[c] GXM-TT, a conjugate of GXM from isolate 371, serotype A, and tetanus toxoid (TT), for example.
[d] 24064 (A) + RBC, a conjugate of GXM from isolate ATTCC 24064 and erythrocytes (RBC), for example.
[e] NR, not reported.
[f] Originally reported as serotypes A and D specific (8) but later reported to be reactive to all serotypes (38).
[g] GH, a clinical isolate used to infect mice.
[h] 3183 (C) + BSA, a conjugate of GXM from isolate 3183 and bovine serum albumin (BSA), for example.

GXM of serotypes A, B, and D and reduced reactivity with serotype C GXM (MAbs CD6, 1255, and 339; 1255 is included in this group because of the low activity observed for most of the antigen concentrations used); group 3 was reactive with GXM of serotypes A and D (MAb 302); group 4 was reactive with GXM of serotype D (MAbs 7B13, BD1, and CRND-8); and group 5 was reactive with GXM of serotype A (MAb E1). The results are summarized in Table 3.

MAbs 302, 339, and 439 (molecular groups IV, III, and II, respectively) were selected for a detailed competitive binding analysis by ELISA based on their distinct serotype specificities, their respective specific activities, and their availability. In this competitive binding study, 19 structurally characterized native GXMs, representing all serotypes, GXM derivatives, and related methyl glycosides, were investigated. The data reported were obtained with MAb 439 (serotype A, B, C, and D specificity), MAb 339 (serotype A, B, and D specificity), and MAb 302 (serotype A and D specificity) by using competitive binding ELISA. The concentration of the inhibitor (GXM or its derivative) that reduced the control absorbance (no inhibitor present) by 50% (IC_{50}) was estimated by interpolation of the experimental curves. In each experiment, the inhibitory activity of a GXM or a derivative was compared with the inhibition observed with the homologous GXM without an inhibitor. The compounds studied represent a spectrum of molecular species found in the serotypes of *C. neoformans*. Residual binding of MAb was quantified following preincubation with increasing concentrations of the native GXM or GXM derivative. No significant differences in the results were observed when the experiments were repeated on different dates.

Inhibition of binding of MAb 302 to ELISA plates coated with serotype A GXM. Microtiter plates coated with the reference serotype A GXM (isolate 6) were used to determine the

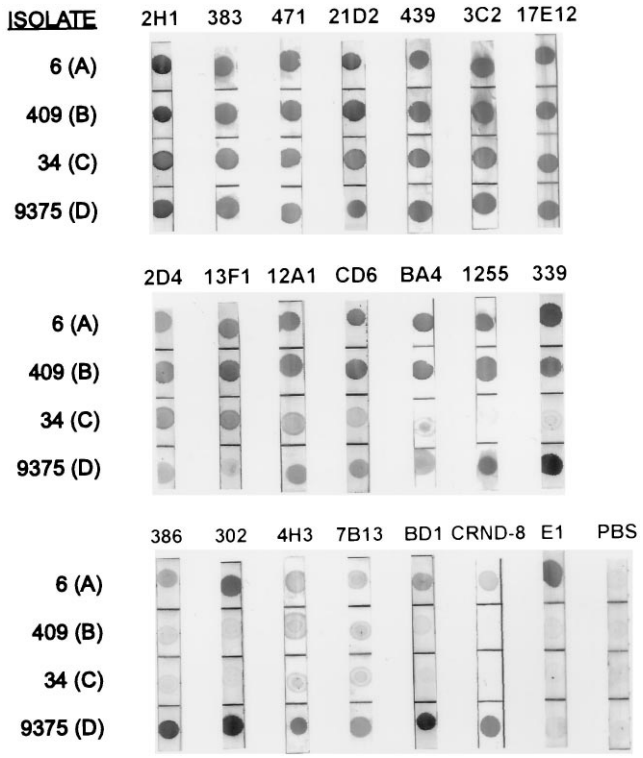

FIG. 2. Comparative specificities of MAbs reactive with *C. neoformans* GXM evaluated by DEA. Reference GXMs from serotypes A, B, C, and D were spotted onto nitrocellulose strips. Each assay was performed at least two times at an optimum dilution of each MAb. Serotypes are indicated in parentheses after the isolate designations.

TABLE 3. Characteristics of anti-GXM MAbs

MAb	Molecular group[a]	Serotype specificity		Antigen factor assignment	Reference or source[b]
		ELISA	DEA		
2H1	II	A ≈ B ≈ C ≈ D	A ≈ B ≈ C ≈ D	1	8
383	ND[c]	A ≈ B ≈ C ≈ D	A ≈ B ≈ C ≈ D	1	T. Kozel
471	II	A ≈ B ≈ C ≈ D	A ≈ B ≈ C ≈ D	1	46
21D2	II	A ≈ B ≈ C ≈ D	A ≈ B ≈ C ≈ D	1	10
439	II	A ≈ B ≈ D > C	A ≈ B ≈ C ≈ D	1	25
3C2	II	A ≈ B ≈ D > C	A ≈ B ≈ C ≈ D	1	46
17E12	II	A ≈ B ≈ D > C	A ≈ B ≈ C ≈ D	1	8, 38
2D4	ND	A ≈ B ≈ C ≈ D	A ≈ B ≈ C > D	Probable 1	T. Kozel
13F1	II	A ≈ B ≈ C ≈ D	A ≈ B ≈ C > D	Probable 1	8, 36
12A1	II	A ≈ B ≈ C ≈ D	A ≈ B > D > C	Intermediate: 1, 2, 3	8, 36
CD6	ND	A ≈ B ≈ D > > C	A ≈ B > D > C	Intermediate: 1, 2, 3	48
BA4	ND	A ≈ B ≈ D > C	A ≈ B > D > C	Intermediate: 1, 2, 3	46
1255	III	A ≈ B ≈ D > > C	A ≈ B ≈ D	2	25
339	III	A ≈ B ≈ D	A ≈ B ≈ D	2	46
386	IV	A ≈ B ≈ D > C	D > A	Intermediate: 2, 3	46
302	IV	A ≈ D	A ≈ D	3	25
4H3	V	D > B ≈ A > C	D > > A ≈ B ≈ C	Intermediate: 1, 8	10
7B13	V	D > > A > B ≈ C	D > > A ≈ B ≈ C	Intermediate: 1, 8	10
BD1	ND	D > > A ≈ B ≈ C	D > > A	8	48
CRND-8	ND	D > > A ≈ B ≈ C	D > > A	8	30
E1	I	A > > D > A ≈ C	A	7	22

[a] MAbs were assigned to molecular groups on the basis of variable region sequences (7).
[b] A cited reference number indicates where the MAb was first described.
[c] ND, not done.

residual activity of MAb 302 in the presence of competitive inhibitors. All serotype A GXMs were good inhibitors of the binding of MAb 302 to immobilized GXM of isolate 6 (Fig. 4). The range of IC_{50} values for the GXMs from serotype A isolates was 0.001 (isolate 110) to 1 (isolate 150) μg/ml. The differences in the IC_{50} values for isolates 150 and 110 likely reflect differences in the epitope distribution between the two. GXM from isolate 110 is comprised of structural elements assigned to serotypes A and D, and GXM from isolate 150 is comprised of structural elements assigned to serotypes A and B (45 and 55%, respectively) (14, 51). Therefore, GXM from isolate 110 would be expected to be a better competitive inhibitor of MAb 302 (A/D specificity). GXMs from serotype D were as effective as the GXMs from serotype A when compared. The inhibitory effects of the GXM from several isolates of serotype D were nearly equivalent to each other; therefore, only data for isolate 9375 are presented in Fig. 4. *O*-Deacetylation of the serotype A and serotype D GXMs resulted in an almost complete loss in their inhibitory effects when compared with that of the native polysaccharides (IC_{50}, >100 μg/ml) (Fig. 4).

Carboxyl group reduction of glucuronic acid residues, done under conditions in which most of the *O*-acetyl substituents are retained, resulted in a decrease of inhibitory activity when compared with that of native GXMs (IC_{50} shifted from 0.01 μg/ml to 0.1 to 1.0 μg/ml) (Fig. 4). Xylomannans prepared from serotype A, B, and C GXMs did not inhibit the binding of MAb 302 to microtiter plates coated with serotype A GXM (IC_{50}, >100 μg/ml) (data not shown).

Inhibition of binding of MAb 302 to ELISA plates coated with serotype D GXM. Microtiter plates coated with the reference serotype D GXM (isolate 9375) were used to determine the residual activity of MAb 302 in the presence of competitive inhibitors. Serotype D GXMs from several isolates were nearly equivalent in inhibiting the binding of MAb 302 (IC_{50}, ≈0.01 μg/ml). Thus, only the results obtained with the homologous GXM of isolate 9375 and serotype A isolates of different chemotypes are given in Fig. 5. Serotype A isolates 6 and 271 (IC_{50}, 0.1 μg/ml) and isolates 110 and 132 (IC_{50}, 1 μg/ml) were good inhibitors of MAb 302 binding to immobilized GXM of serotype D isolate 9375 (Fig. 5). Serotype A isolates of chemotype III (isolates 150 and 371) were the poorest inhibitors (IC_{50}, ≥100 μg/ml) (Fig. 5). *O*-Deacetylation of the serotype A and serotype D GXMs resulted in the complete loss of their inhibitory effects when compared with that of the native polysaccharides (IC_{50}, >100 μg/ml) (Fig. 5). Carboxyl group reduction done under conditions in which most *O*-acetyl substituents are retained resulted in a decrease of inhibitory activity when compared with that of native GXM (IC_{50}, >10 μg/ml) (Fig. 5).

Inhibition of binding of MAb 339 to ELISA plates coated with serotype A GXM. Microtiter plates coated with the reference serotype A GXM (isolate 6) were used to determine the residual activity of MAb 339 in the presence of competitive inhibitors. GXMs from serotype A isolate 6, serotype D isolate 9375, and serotype A/D isolate 132 were excellent competitive inhibitors of the binding of MAb 339 (IC_{50}, ≤0.1 μg/ml) (Fig. 6). The GXMs from other serotype A isolates 150, 371, and 271 (chemotypes III and IV) were less effective inhibitors (IC_{50}, 10 μg/ml) (Fig. 6). The inhibitory effectivenesses of all serotype D GXMs were essentially the same. The observed inhibition of binding of MAb 339 to serotype A (isolate 6) GXM-coated plates was equivalent to that observed for the homologous serotype A GXM (IC_{50}, ≤0.1 μg/ml); therefore, only exemplary data for serotype D isolate 9375 are presented (Fig. 6). *O*-Deacetylated GXMs generated from serotype A and serotype D did not inhibit the binding of MAb 339 (IC_{50}, ≥100 μg/ml) (data not shown). The inhibitory effect of the carboxyl-reduced GXM from serotype A/D (isolate 132) was slightly diminished when compared with that of native GXM. Carboxyl reduction of serotype A (isolate 6) GXM effected a substantial increase in the IC_{50} (IC_{50} shifted from 0.1 to 6 μg/ml). After carboxyl reduction, the weak inhibitory effects of several serotype A GXMs (strains 150 and 371) were eliminated (IC_{50},

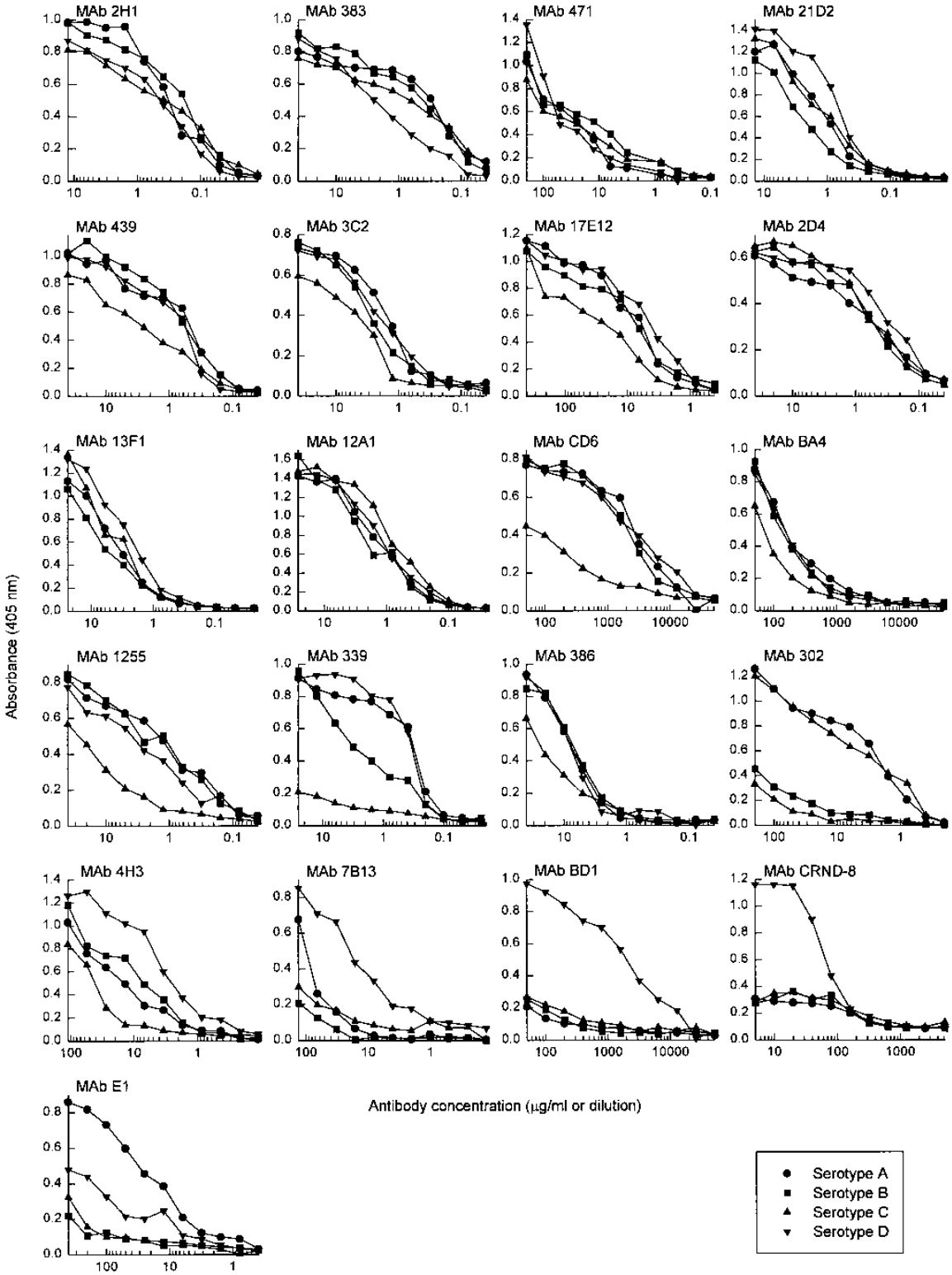

FIG. 3. ELISA titration of MAbs against reference GXMs of *C. neoformans* serotypes A, B, C, and D. Each point represents a background-corrected mean absorbance of four wells.

>100 μg/ml) (Fig. 6). The carboxyl-reduced GXMs from serotype D isolates were less effective inhibitors of MAb 339 binding to serotype A GXM-coated plates when compared with their native forms (IC_{50}, 10 and 0.01 μg/ml, respectively). Exemplary data for serotype D isolate 9375 are presented in Fig. 6. Serotype B GXMs were only slightly effective as inhibitors of the binding of MAb 339 to serotype A GXM-coated plates (data not shown). GXM from serotype B isolate 3172

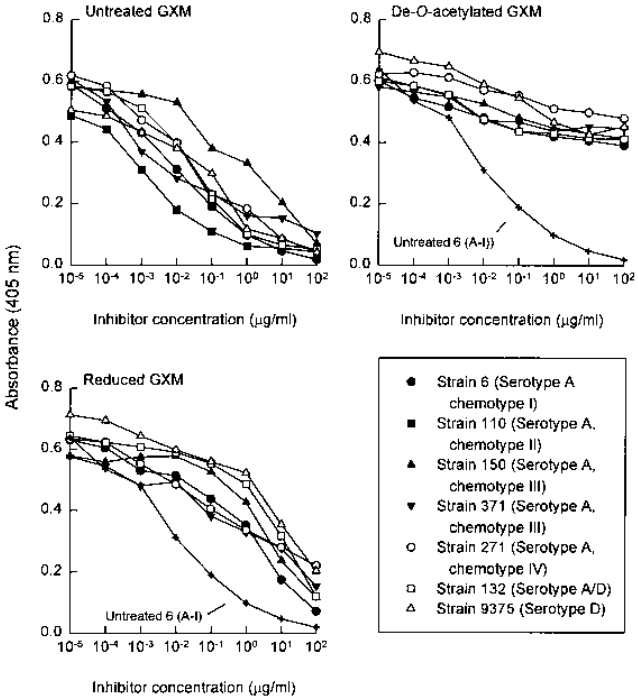

FIG. 4. Competitive inhibition by native GXM and GXM derivatives of the binding of MAb 302 to wells coated with serotype A (strain 6) GXM. MAb 302 (25 μg/ml) was preincubated with various amounts of native (upper left panel), *O*-deacetylated (upper right panel) or reduced (lower left panel) GXM, and the amount of residual binding of the MAb to wells coated with serotype A (strain 6) GXM was determined as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells. Native GXM from isolate 6 was included in each assay.

was the strongest, and serotype B isolates 444 and 409 were less effective, with observed IC_{50} values of 1 and 10 μg/ml, respectively. As observed with other serotypes, *O*-deacetylation of the serotype B GXMs resulted in the loss of all ability of the treated GXM to inhibit binding of MAb 339 to serotype B (isolate 409) GXM-coated plates (data not shown). Serotype C GXMs and xylomannan derived from serotype A were not inhibitory (IC_{50}, >100 μg/ml) (data not shown).

Inhibition of binding of MAb 339 to ELISA plates coated with serotype B GXM. Microtiter plates coated with the reference serotype B GXM (isolate 409) were used to determine the residual activity of MAb 339 in the presence of competitive inhibitors. GXMs from serotype A isolates 6 and 271, serotype D isolate 9375, and serotype A/D isolate 132 were good competitive inhibitors of the binding of MAb 339 to serotype B GXM-coated plates (IC_{50}, ≤0.1 μg/ml) (Fig. 7). GXM of serotype A chemotype III was markedly less effective (IC_{50}, 10 μg/ml for strain 371 and 100 μg/ml for strain 150) (Fig. 7). *O*-Deacetylated GXMs from serotype A isolates completely lost the ability to inhibit MAb 339 binding (IC_{50}, ≥100 μg/ml). Carboxyl reduction of serotype A GXMs had little effect on the ability of serotype A GXMs to inhibit binding of MAb 339 (data not shown). GXMs from serotype B isolates 409 and 444 were the strongest inhibitors of binding of MAb 339 to serotype B GXM-coated plates, while GXM from serotype B isolates 3172 and 3939 were weak inhibitors (IC_{50}, ≥10 μg/ml) (Fig. 7). *O*-Deacetylation of the serotype B GXMs greatly reduced their competitive binding to MAb 339 (IC_{50}, >100 μg/ml) (Fig. 7). Reduction of the carboxyl group of serotype B isolate 409 resulted in the loss of inhibitory effect (IC_{50}, >100 μg/ml) (data not shown). GXMs from serotype D isolates were strong inhibitors of the binding of MAb 339. A complete loss of inhibitory activity was observed after *O*-deacetylation of the serotype D GXMs (IC_{50}, ≥100 μg/ml) (data not shown). Xylomannans derived from serotypes A, B, and C and GXMs from serotype C did not inhibit the binding of MAb 339 (IC_{50}, >100 μg/ml) (data not shown).

Inhibition of binding of MAb 339 to ELISA plates coated with serotype D GXM. Microtiter plates coated with the reference serotype D GXM (isolate 9375) were used to determine the residual activity of MAb 339 in the presence of competitive inhibitors. GXMs from serotype A isolates 6 and 271 and serotype A/D isolate 132 were strong inhibitors of the binding of MAb 339 (IC_{50}, 0.1 μg/ml) (Fig. 8). GXM from serotype A chemotype III isolates 150 and 371 were less inhibitory, with respective IC_{50} values of 10 and >100 μg/ml (Fig. 8). *O*-Deacetylation of the serotype A GXMs resulted in a total loss in their inhibitory effects (IC_{50}, >100 μg/ml) (data not shown). Carboxyl-reduced GXMs of serotype A did not show reduced inhibitory activity when compared with untreated GXMs (data not shown).

GXMs from serotype D isolates were strong inhibitors of the binding of MAb 339 (IC_{50}, 0.1 μg/ml) (Fig. 8). The serotype D GXMs exhibited substantial loss of inhibitory properties after *O*-deacetylation (IC_{50}, ≥100 μg/ml). A 10-fold decrease in the inhibitory effect was observed for the carboxyl-reduced serotype D GXMs, with an increase in IC_{50} from 0.1 μg/ml for native GXM to 1 μg/ml for reduced GXM. Exemplary assays for *O*-deacetylated and carboxyl-reduced GXMs of serotype D are given in Fig. 8.

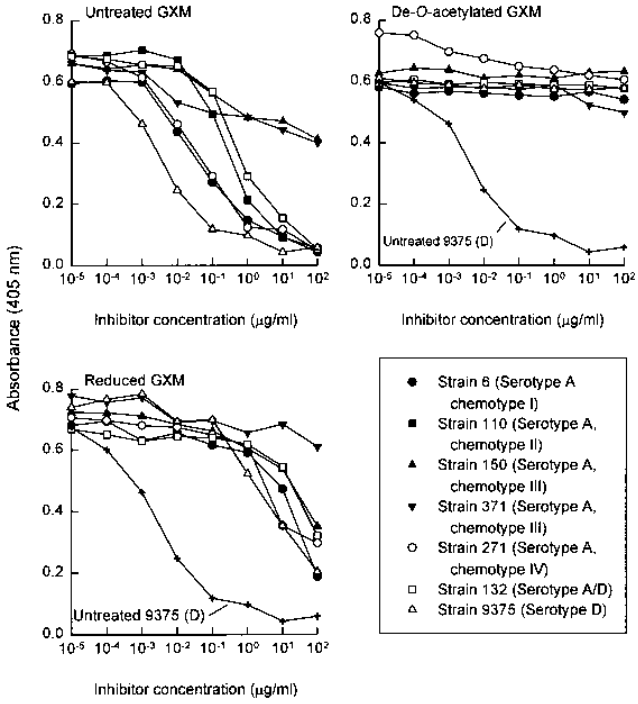

FIG. 5. Competitive inhibition by native GXM and GXM derivatives of the binding of MAb 302 to wells coated with serotype D (strain 9375) GXM. MAb 302 (25 μg/ml) was preincubated with various amounts of native (upper left panel), *O*-deacetylated (upper right panel), or reduced (lower left panel) GXM, and the amount of residual binding of the MAb to wells coated with serotype D (strain 9375) GXM was determined as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells. Native GXM from isolate 9375 was included in each assay.

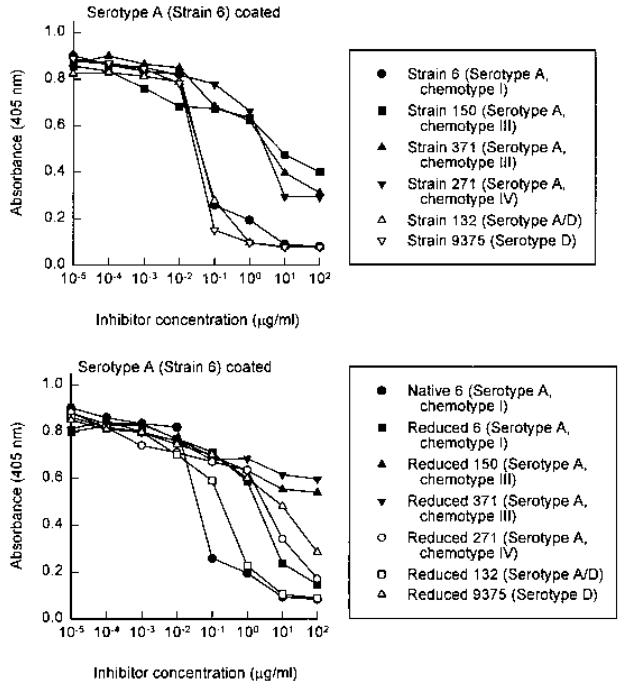

FIG. 6. Profile for competitive inhibition by native GXM and GXM derivatives of the binding of MAb 339 to wells coated with GXM of serotype A. MAb 339 (5 μg/ml) was preincubated with various amounts of each indicated preparation of GXM, and the amount of residual binding of the MAb to GXM-coated wells was determined as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells.

Native GXMs from serotype B isolates 409, 444, and 3939 were poor inhibitors of the binding of MAb 339 to serotype D GXM-coated plates (IC_{50}, >10 μg/ml), and any activity was lost on structural modification by *O*-deacetylation and carboxyl reduction (IC_{50}, >100 μg/ml) (data not shown). Xylomannans, methylglycosides, and native GXMs from serotype C isolates did not inhibit MAb 339 binding to microtiter plates coated with serotype D GXM (IC_{50}, >100 μg/ml).

Inhibition of binding of MAb 439 to ELISA plates coated with serotype A GXM. Microtiter plates coated with the reference serotype A GXM (isolate 6) were used to determine the residual activity of MAb 439 in the presence of competitive inhibitors. GXMs from serotype A isolates 6, 110, and 271 and serotype A/D isolate 132 were strong inhibitors of the binding of MAb 439 (IC_{50}, 0.03 μg/ml) (Fig. 9). The GXMs from serotype A isolates 150 and 371 (chemotype III) were weaker inhibitors (IC_{50}, >4 μg/ml) (Fig. 9). The serotype A GXMs lost their inhibitory effectiveness after *O*-deacetylation (Fig. 9). The reduction of the carboxyl group did not significantly alter the observed inhibitory effects of the serotype A GXMs (Fig. 9).

GXM from serotype B isolate 444 was the only serotype B GXM observed to effectively inhibit the binding of MAb 439 to microtiter plates coated with serotype A GXM (IC_{50}, 0.1 μg/ml). Other serotype B GXMs from isolates 3172 and 409 gave IC_{50} values that ranged from 10 to 100 μg/ml. The inhibitory effect observed for the GXM of isolate 444 was lost after *O*-deacetylation.

GXMs from serotype C isolates 401, 298, 3183, and 34 had IC_{50} values of 0.08, 1, 5, and 100, respectively (data not shown). This decrease in inhibitory activity generally paralleled the increase in mannosyl residues that are disubstituted with Xyl at *O*-2 and *O*-4. Once again, all inhibitory activity was lost after *O*-deacetylation of the serotype C GXMs (IC_{50}, >100 μg/ml).

The GXMs of the serotype D isolates 9375, 125, 127, and 1254 inhibited the binding of MAb 439, with observed IC_{50} values that ranged from 1 to 10 μg/ml (data not shown). The inhibitory activity was lost after *O*-deacetylation of the GXMs, but after carboxyl reduction, the inhibitory activity was not changed significantly from the original values observed for untreated GXMs.

Inhibition of binding of MAb 439 to ELISA plates coated with serotype B GXM. Microtiter plates coated with the reference serotype B GXM (isolate 409) were used to determine the residual activity of MAb 439 in the presence of competitive inhibitors. GXMs from serotype A isolates 6 and 271 and serotype A/D isolate 132 showed inhibitory activity, with IC_{50} values that ranged from 1 to 1.4 μg/ml (data not shown). GXM from serotype A isolate 150 was not inhibitory (IC_{50}, >100 μg/ml). The modest inhibitory effects of the GXMs from serotype A isolates 6 and 271 and serotype A/D isolate 132 were lost after *O*-deacetylation, but the inhibitory effects were not significantly altered by carboxyl reduction (data not shown). GXMs from serotype B isolates 409 and 444 were strong inhibitors of the binding of MAb 439 to microtiter plates coated with GXM from serotype B isolate 409 (IC_{50}, 0.005 μg/ml) (Fig. 10). GXM from serotype B isolate 3939 was a weak inhibitor (IC_{50}, 6 μg/ml), and GXM from serotype B isolate 3172 was not an inhibitor (IC_{50}, 100 μg/ml). The inhibitory effects of GXMs from isolates 409, 444, and 3939 were abrogated by *O*-deacetylation (Fig. 10). The competitive inhibitory effect of the homologous serotype B GXM from isolate 409 was significantly diminished by reduction of the carboxyl groups (IC_{50}, 1 μg/ml) (Fig. 10).

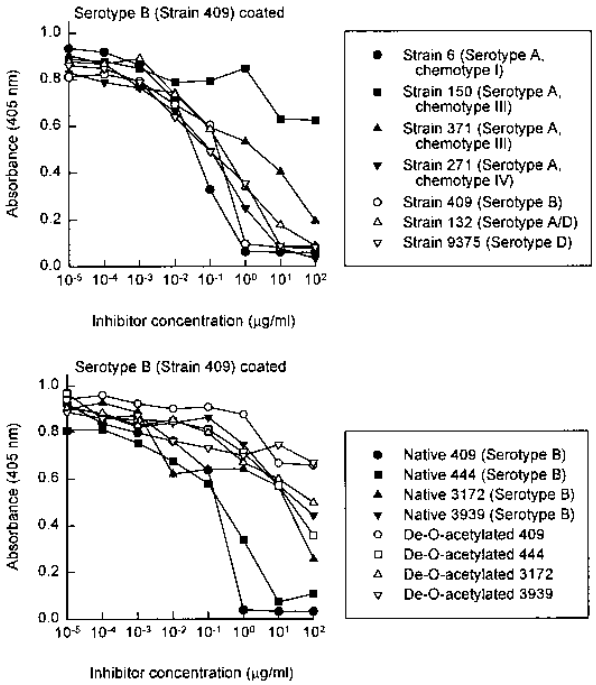

FIG. 7. Profile for competitive inhibition by native GXM and GXM derivatives of the binding of MAb 339 to wells coated with GXM of serotype B. MAb 339 (5 μg/ml) was preincubated with various amounts of each indicated preparation of GXM, and the amount of residual binding of the MAb to GXM-coated wells was determined as described in the Materials and Methods. Each datum point represents a mean absorbance value for two or more wells.

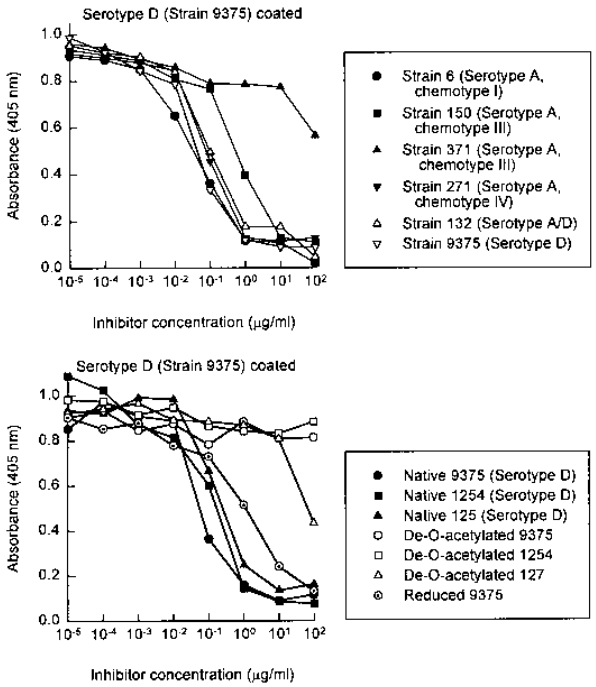

FIG. 8. Profile for competitive inhibition by native GXM and GXM derivatives of the binding of MAb 339 to wells coated with GXM of serotype D. MAb 339 (5 μg/ml) was preincubated with various amounts of each indicated preparation of GXM, and the amount of residual binding of the MAb to GXM-coated wells was determined as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells.

GXMs from serotype C isolates 401 and 3183 were moderately effective inhibitors (IC_{50}, 5 μg/ml) (data not shown), but the GXMs from serotype C isolates 298 and 34 were ineffective inhibitors of the binding of MAb 439 (IC_{50}, 100 μg/ml). The modest inhibitory activity observed for GXMs from isolates 3183 and 401 was lost after *O*-deacetylation (IC_{50}, 100 μg/ml).

GXMs from serotype D isolates 9375, 1254, 125, and 127 were weak inhibitors of the binding of MAb 439 to plates coated with serotype B GXM (IC_{50}, ≈10 μg/ml) (data not shown). The inhibitory activity of the serotype D GXMs was abrogated by *O*-deacetylation.

Inhibition of binding of MAb 439 to ELISA plates coated with serotype C GXM. Microtiter plates coated with the reference serotype C GXM (isolate 34) were used to determine the residual activity of MAb 439 in the presence of competitive inhibitors. The control absorbance obtained in this configuration was approximately half that observed in the other experiments reported above. Therefore, the confidence limit of IC_{50} values determined in these assays did not permit a reportable assessment of the competitive effects of GXM and GXM derivatives on binding of MAb 439 to plates coated with serotype C GXM.

Inhibition of binding of MAb 439 to ELISA plates coated with serotype D GXM. Microtiter plates coated with the reference serotype D GXM (isolate 9375) were used to determine the residual activity of MAb 439 in the presence of competitive inhibitors. GXMs from serotype A isolates 6 and 271 and serotype A/D isolate 132 were strong inhibitors (IC_{50}, ≈0.01 μg/ml) (data not shown), but GXMs from serotype A isolates 150 and 371 (chemotype III) were weaker inhibitors (IC_{50} ranged from ≈2 to 12 μg/ml). *O*-Deacetylation of the serotype A GXMs resulted in a total loss of the observed inhibitory effects (IC_{50}, >100 μg/ml) (Fig. 10). Carboxyl reduction of the serotype A GXMs resulted in a partial loss in the inhibitory effects (IC_{50} ranged from 1 to 10 μg/ml) (data not shown). GXMs from serotypes B and C were marginal inhibitors of MAb 439 binding to microtiter plates coated with serotype D GXM (IC_{50}, >10 μg/ml) (data not shown). GXMs from serotype C isolates 298 and 34, having the highest disubstitution with Xyl at *O*-2 and *O*-4 of the mannan backbone, were the least effective inhibitors of MAb 439 binding to immobilized GXM of serotype D (IC_{50}, >100 μg/ml). As shown in Fig. 10, slight variations in inhibitory effects for serotype D GXMs were observed. Although *O*-deacetylation of all the serotype D GXMs resulted in a complete loss of inhibitory activity, reduced GXM from the homologous serotype D GXM retained most of the inhibitory activity (Fig. 10).

DISCUSSION

This is the first comprehensive study to determine the specificity profiles for MAbs generated in five different laboratories by a variety of immunization conditions and hybridoma screening protocols. Two serological methods, indirect ELISA and DEA, were used to evaluate the binding activities and serotype specificities of 21 MAbs for purified and chemically defined GXM antigens. The ELISA and DEA results showed significant but not absolute correlation. A possible explanation for minor disparities in the activity patterns for the MAbs observed by ELISA and DEA is the difference in the mode of the physical binding of the GXM to the polystyrene microtiter plates and to the nitrocellulose strips. Microtiter polystyrene

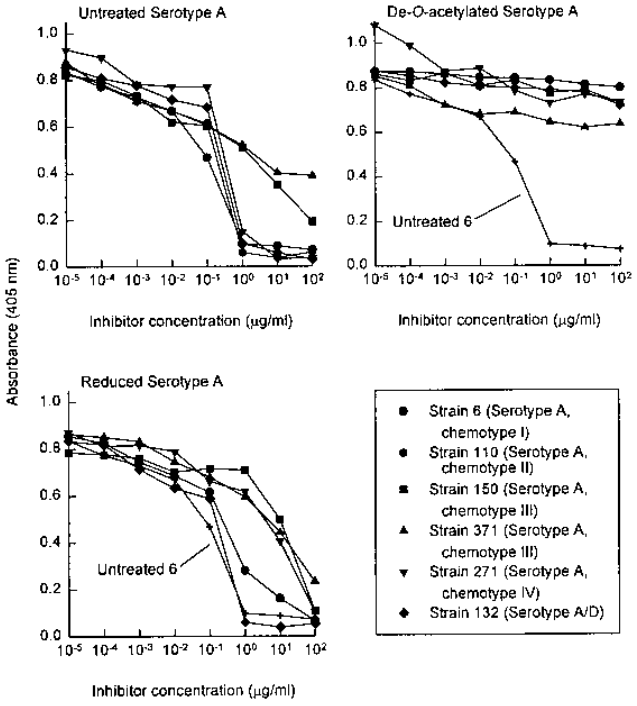

FIG. 9. Competitive inhibition by native GXM and GXM derivatives of the binding of MAb 439 to wells coated with serotype A (strain 9375) GXM. MAb 439 (7.7 μg/ml) was preincubated with various amounts of native (upper left panel), *O*-deacetylated (upper right panel), or reduced (lower left panel) GXM, and the amount of residual binding of the MAb to wells coated with serotype A (strain 6) GXM was determined as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells. Native GXM from isolate 6 was included in each assay.

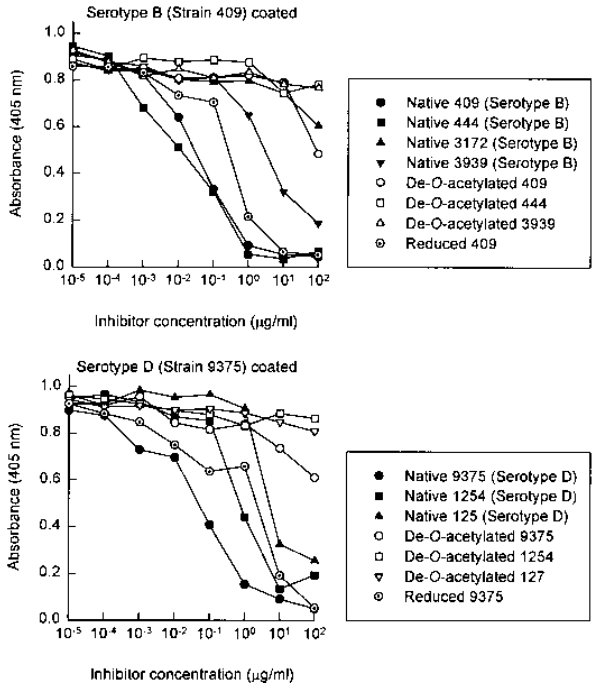

FIG. 10. Profile for competitive inhibition by native GXM or derivatives of GXM of the binding of MAb 439 to wells coated with GXM of serotype B (upper panel) or serotype D (lower panel). MAb 439 (7.7 μg/ml) was preincubated with various amounts of native or derivatized (*O*-deacetylated or reduced) GXM from serotype B (upper panel) or serotype D (lower panel) as described in Materials and Methods. Each datum point represents a mean absorbance value for two or more wells.

plates were primed with the adipic acid dihydrazide derivative of bovine serum albumin (11). Therefore, most of the bound GXM is not associated with the solid matrix, and it is probably free to bind to MAb. In DEA, the GXM is bound directly to the nitrocellulose surface and the polysaccharide may bind to the matrix over its entire length. Hence, only selective portions of the molecule may be exposed, and a particular epitope may be masked. Despite differences in the immunoassays, the ELISA and DEA results were consistent for most MAbs. The variation in serotype reactivity that was observed indicated that reactivity patterns for *C. neoformans* serotype are dependent on the method used. Hence, reactivity and specificity comparisons between MAbs should be made with data obtained under the same conditions.

Our results support and extend previous findings on all MAbs reported by using different immunoassays (8, 9, 24, 25, 46, 48). Most of the MAbs reacted by ELISA with GXMs from two or more serotypes. Many of the MAbs reacted with representative GXMs from all serotypes. The reactivities of the murine MAbs with serotype GXM observed in this study correlated well with the reactivities of the rabbit anticryptococcal polyclonal factor sera described by Ikeda et al. (31). Titration of the MAbs by ELISA and DEA showed that MAbs 2H1, 383, 471, 21D2, 439, 3C2, 17E12, 2D4, and 13F1 corresponded to factor serum 1. MAbs 339 and 1255 reacted like factor serum 2. The reactivity of MAb 302 was similar to that of factor serum 3. The reactivity of MAb E1 corresponds to that of factor serum 7, whereas the reactivities of MAbs BD1 and CRND-8 were similar to that of factor serum 8. The close correspondence in reactivity patterns between some MAbs and factor sera suggests that GXM antigenic determinants elicit antibodies with similar specificities in both rabbits and mice. Several MAbs showed reactivities that incorporated elements of the specificities of several factor sera. Such intermediate reactivities may reflect inherent differences between results obtained by ELISA and DEA. These intermediate reactivities also may be due to the finer specificities possible with MAbs relative to the specificities produced by cross-absorption of polyclonal antisera. Some of the intermediate reactivities suggest a continuum of epitope structures between the antigenic assignment made by use of factor sera.

In general, there was good correspondence between the reactivities of specific MAbs and their assignments to molecular groups on the basis of variable gene sequences (Table 2). For example, all of the group II MAbs reacted strongly with GXM from serotype A and B strains. Within a given MAb molecular group, there was variation in the reactivities of individual MAbs for the various serotype GXMs. This probably reflects the fact that none of the MAbs studied in this set have identical variable region amino acid sequences either as a result of somatic mutation in immunoglobulin genes or minor differences in variable gene family usage (7, 10).

Three MAbs, 439 (molecular group II), 339 (molecular group III), and 302 (molecular group IV), were studied extensively by ELISA competitive inhibition assays against various native and chemically modified GXM derivatives. Substantial variations in the IC_{50} ELISA values or intensities of reactivity by DEA showed that affinities of individual MAbs were isolate dependent. These differences in inhibitory effects imply that the epitopes required for binding of the MAbs are unequally shared among isolates. Alternatively, the variation in binding affinities may be due to the presence of partial epitope structures of lower affinity and/or steric hindrance.

The competitive binding data revealed significant differences in MAb binding to GXM from serotype A chemotypes (Fig. 4 to 9). This confirms previous reports that GXMs from strains assigned to a serotype can exhibit extensive variation in polysaccharide structure (15, 16, 50, 51) and that MAbs can discriminate serologically among strains assigned to a given serogroup (46). Previous studies have explained the differences between serotype A structures on the basis of differences in molar ratios of sugar residues (particularly *O*-2- and *O*-4-linked xylose) and the degree of *O*-acetylation (50, 51). Regardless of the type of GXM immobilized on ELISA plates (serotype A, B, or D), our results indicate that most GXMs of serotype A and D are strong inhibitors of MAb 339 while the serotype B GXMs are weak inhibitors. This reduced affinity may reflect the more complex structure found in serotype B GXMs (49) or possibly a distinctive distribution of *O*-acetyl esters.

For most MAbs, the *O*-acetyl is an important component of the epitope structure. The disposition of *O*-acetyl contributes to the antigenic multiplicity observed among GXMs obtained from all serotypes. This was true for most isolates, with the exception of the several highly substituted GXMs derived from serotype C which were serologically active in the absence of any detectable acetyl esters. The *O*-acetyl may be part of the epitope, it may serve to help fix the correct conformation, or it may serve in both capacities. Because the *O*-acetyl is such an overwhelming contributor to the epitope recognized by the MAbs, we were not able to define the role the polysaccharide portion of the GXM structures plays in specifying the epitopes involved in MAb binding. Thus, the specific structures responsible for serotype specificity remain undetermined.

In summary, our results indicate that despite some similarities in binding, many of the 21 MAbs studied are unique reagents. Depending on the serological method, the 21 MAbs were divided into five or eight groups based on their reactivi-

ties for GXM. The fact that MAbs (as a set) are more discriminatory than rabbit serotype-specific sera indicates that it may be possible to define *C. neoformans* strains on the basis of MAb reactivity. A MAb-based classification system would have several advantages, especially for defining strains on the basis of the existence of individual epitopes in the capsular GXM. MAbs also have an inherent advantage over rabbit sera because they are homogeneous, stable-invariant reagents. Important objectives that remain are to correlate MAb reactivities with actual GXM structures, define the molecular epitope structure for each MAb group, and determine the chemical disposition of the individual *O*-acetyl groups as they occur in the GXMs of the various serotypes. This information, in conjunction with the existing knowledge of GXM chemistry and structure, will provide a serological and structural database to interpret and classify the many biological effects associated with *C. neoformans* GXMs and the distinct biological activities associated with MAbs having differing epitope specificities.

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