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Molecular and Idiotypic Analysis of Antibodies to *Cryptococcus neoformans* Glucuronoxylomannan

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Antibodies to the *Cryptococcus neoformans* capsular glucuronoxylomannan (GXM) form the basis of two potential therapeutic intervention strategies, i.e., conjugate vaccines and passive antibody therapy. To better understand the molecular basis of the antibody response, the heavy- and light-chain immunoglobulin variable region (V_H and V_L, respectively) sequences of seven monoclonal antibodies (MAbs) to GXM were determined. Rabbit anti-idiotypic serum was made to the previously characterized murine MAb 2H1 and used to study MAb 2H1 idiotype expression in other GXM-binding MAbs and immune sera. MAb E1 originated from a C3H/HeJ mouse immunized with *C. neoformans* serotype A polysaccharide. MAbs 471, 1255, 339, 3C2, 386, and 302 originated from BALB/c mice immunized with polysaccharide of serotypes A, A, B, C, D, and D, respectively, conjugated to sheep erythrocytes. In the E1, V_H uses V_{11} from the T15 gene family and J_H3 and has a D segment of three amino acids, and the V_L uses a V_κSer-like gene family element and $J_\kappa5$. In MAbs 471 and 3C2, the V_H uses V_H7183-like gene family elements and J_H2 and has D segments of seven amino acids, and the V_L uses $V_\kappa5.1$ and $J_\kappa1$. In MAbs 1255 and 339, the V_H uses V_H10-like gene elements and J_H4 and has six codon D segments, and the V_L uses a $V_\kappa21$-like gene element and $J_\kappa5$. In MAbs 302 and 386, respectively, the V_H uses V_HGAM-like gene elements and J_H2 and J_H3 and has six and four codon D segments, and V_L uses $V_\kappa4/5$-like gene elements and $J_\kappa1$. V_H usage, MAb 2H1 idiotype expression, and fine specificity mapping define a minimum of three GXM epitopes which elicit protective antibodies. The results confirm that the antibody response is highly restricted, suggest a close relationship between molecular structure and serological properties, and provide insight into protein structural motifs important for GXM binding.

Cryptococcus neoformans is an encapsulated fungus that causes life-threatening meningoencephalitis in up to 9% of patients with AIDS (53). Treatment of cryptococcal meningitis in patients with AIDS is difficult because antifungal drugs often fail to eradicate the infection (49, 53). The high prevalence of *C. neoformans* infections in patients with AIDS and the difficulties associated with therapy have renewed interest in using antibodies for the prevention and treatment of cryptococcal disease (5, 12, 32, 33).

Immune rabbit serum modifies the course of murine cryptococcal infection (21, 26) and potentiates the antifungal effects of amphotericin B (25). Several monoclonal antibodies (MAbs) to the capsular glucuronoxylomannan (GXM) prolong survival and decrease fungal burden in murine cryptococcosis (14, 15, 17, 32, 38–42, 45) and potentiate amphotericin B efficacy in vitro and in vivo (14, 41). Passive antibodies most likely modify the course of infection by facilitating the interaction of effector cells (14, 15, 17, 31, 41, 46). Despite the potential protective action of antibodies, the host response to *C. neoformans* infection seldom elicits appreciable anti-GXM antibody responses in mice (6, 8, 34). GXM is a type 2 T-cell-independent antigen (19, 50) that is poorly immunogenic (2, 29). In contrast, GXM-protein conjugates elicit good antibody responses in mice (12) and are potential anti-*C. neoformans* vaccines. Although antibody quantity (15, 17), isotype (40, 45), and fine specificity (40) appear to be important for efficacy against murine *C. neoformans* infections, the molecular structural characteristics necessary for antibody binding and protective efficacy are unknown.

The majority of anti-GXM MAbs studied to date use an identical heavy- and light-chain variable region (V_H-V_L) combination: six MAbs from a mouse infected with a serotype A *C. neoformans* strain and 23 MAbs from a mouse immunized with a *C. neoformans* GXM-tetanus toxoid conjugate vaccine (GXM-TT) used the variable region combination V_H(7183 family)-J_H2–$V_\kappa5.1$-$J_\kappa1$ (38). The V_H structure of another MAb, 439, is V_H(7183 family)-J_H2 with a different light-chain combination (43). Earlier, a set of eight MAbs reactive with *C. neoformans* serotype D was found to use V_H441-J_H3 with λ light chains (8). Antibody variable region gene restriction has been found in various murine antibody responses, including those to several polysaccharides (3) and phosphorylcholine, nitrophenyl, and azophenlyarsonate haptens (10, 35, 47). The cellular and molecular mechanisms underlying antibody restriction are not understood. To further analyze the molecular genetics of the murine anti-*C. neoformans* capsular GXM antibody response, we determined V_H and V_L usage in seven additional MAbs generated by a variety of immunization schemes. Idiotype expression by these MAbs was studied with rabbit anti-idiotypic antiserum raised against a protective murine MAb, 2H1. Our results confirm that the antibody response to *C. neoformans* GXM is highly restricted and suggest relationships between idiotype expression, GXM binding, and molecular structure.

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TABLE 1. Origins, immunizing antigens, and references for antibodies in Table 2

MAb	Mouse strain	Immunogen	Reference
E1	C3H/HeJ	Serotype A ps[a]	18
439	BALB/c	Serotype A rbc[b]	20
471	BALB/c	Serotype A rbc	48
3C2	BALB/c	Serotype C rbc	48
2D10/2H1	BALB/c	GXM-TT	38
21D2	BALB/c	GH infection	8
13F1	BALB/c	GXM-TT	38
339	BALB/c	Serotype B rbc	48
1255	BALB/c	Serotype A rbc	20
302	BALB/c	Serotype A rbc	20
386	BALB/c	Serotype D rbc	48
4H3	BALB/c	GH infection	8
14A12	BALB/c	GH infection	8

[a] Serotype A ps, serotype A polysaccharide.
[b] Serotype A rbc, serotype A sheep erythrocyte conjugate.

MATERIALS AND METHODS

Hybridomas, MAbs, and polysaccharide antigens. Hybridomas 3C2, 471, 386, 339, 302, 1255, and E1 have been described (18, 20, 48). Hybridomas were maintained in 10% fetal calf serum in RPMI 1640 medium (GIBCO). Ascites was generated by injecting hybridoma cells into the peritoneal cavity of pristane-primed BALB/c mice. MAbs 471, 386, 339, 302, 1255, and 439 were purified by affinity chromatography on a cryptococcal GXM column as described previously (30). Serotype A GXM (GXM-A) from strain 371, GXM-TT, and *Citrobacter freundii* polysaccharide were kindly provided by J. Robbins and R. Schneerson (Bethesda, Md.). Table 1 summarizes the derivation of the MAbs with the appropriate references.

Southern blot hybridization. The V_H and V_L immunoglobulin (Ig) gene rearrangements in hybridoma cells were analyzed as described previously (8, 38). DNA was extracted from hybridoma, Sp2/0 myeloma (fusion partner for all hybridomas except E1), and BALB/c liver. For V_H analysis, hybridoma, Sp2/0 myeloma, and BALB/c liver DNAs were digested with the restriction enzyme *Eco*RI, and restriction fragments were separated in 0.8 to 1.0% agarose gels, blotted onto positively charged nylon membranes, and probed with the 2-kb *Bam*HI-*Eco*RI J_H3-J_H4 probe, J11. For V_L analysis, the hybridoma, Sp2/0 myeloma, and BALB/c liver DNAs were digested with the restriction enzyme *Hin*dIII, and the blots were probed with the 2.7-kb *Hin*dIII fragment $J_\kappa 1$-5 probe. Restriction fragments were transferred to the nylon membrane by using a solution of 10× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). Blots were hybridized at 65°C in a solution of 5× SSC, 7% sodium dodecyl sulfate (SDS), 10× Denhardt's reagent, and 10% dextran sulfate in 0.020 M sodium phosphate buffer (pH 7.2). Probes were labelled with [^{32}P]dCTP by using random primers. The restriction enzymes, random priming kit, and nylon membranes were purchased from Boeringer Mannheim (Indianapolis, Ind.).

mRNA sequence determination. mRNA was prepared by cell disruption in a guanidinium solution (4.23 M guanidine isothiocyanate [Fluka Biochemika, Buchs, Switzerland], 0.017 M *N*-lauroylsarcosine, 0.025 M sodium citrate [pH 7.0], 0.015 β-mercaptoethanol, and 0.011% antifoam A), CsCl gradient sedimentation, and oligo(dT) (Collaborative Research Inc., Bedford, Mass.) affinity chromatography. mRNA sequences were determined by reverse transcriptase sequencing with minor modifications of the method of Geliebter et al. (23). The sequencing primers were: TGGATGGTGGGAAGATG (V_κ), GACCCCAGAAAATCGGTT ($V_\kappa 5.1$), GGGGCCAGTGGA TAGAC (IgG1), TGTTCTTGGCATTGTCTCTG (MAb 7183 gene family), TGTTGATCTGCAAATAGGC (V_H of MAb 302), TGGTGAACCTGTCTTT (V_H of MAb 339), TCTC AGTGTGAAAGCAG and AGACTGGCCTGGCCTCTG (V_L of MAbs 339 and 1255), TAAGAGGTCCCAGACCC (V_L of MAbs 302 and 386), and GACTGAAATAAACTGCC (V_L of MAb E1). The primers were made at the Albert Einstein College of Medicine DNA synthesis facility.

For E1, 471, 3C2, 339, 386, and 1255, V_H sequences were also determined by amplification of VDJ variable region from mRNA cDNA by using the PCR, ligating amplified DNA to the pCR1000 of the TA cloning system (Invitrogen, San Diego, Calif.), and DNA sequencing by the dideoxynucleotide chain-terminating method with a Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio). For E1, 471, 3C2, and 339, sequencing of amplified cDNA was done to resolve RNA sequencing ambiguities. The V_H DNA of 471 and 3C2 was amplified by using the 5′ V_H 7183 primer CTTCACTTCGAC CACCTC and the IgG1-specific primer GGGGCCAGTG GATAGAC. The heavy-chain gene usage of V_H in MAbs 386 and 1255 was determined by sequencing PCR-amplified cDNA. The V_H DNA of MAbs 386 and 1255 was amplified by using the degenerate leader sequence primer ATG(GA)AAT G(GC)A(GC)CTGGGT(CG)(TA)T(TC)CTCT and constant region-specific primers. Two clones of MAbs 386 and 1255 were sequenced. Variable gene identification was done by sequence comparison with the GenBank database by using the Genetics Computer Group Sequence Analysis Software package (11) and the sequence compilation of Kabat et al. (28).

Rabbit antisera to MAb 2H1 (2H1αId). 2H1 IgG1(κ) of a protective murine MAb that binds GXM, has been described (6, 38–40). The anti-idiotypic reagent 2H1αId was prepared by immunization of a New Zealand White rabbit with MAb 2H1 in complete Freund's adjuvant. Rabbit antiserum was repeatedly absorbed with murine Ig coupled to Sepharose 4B (Pharmacia) until all reactivity with murine IgG1 was removed. The antiserum was then affinity chromatography purified on MAb 2H1 coupled to Sepharose 4B (Pharmacia LKB, Piscataway, N.J.). Preimmune serum from the same rabbit was also absorbed with the murine Ig column.

ELISAs. Several types of enzyme-linked immunosorbent assay (ELISA) were used, and details of specific ELISAs are given below. All ELISAs used polystyrene plates (Corning Glass Works, Corning, N.Y.) blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 25 mM Tris, 138 mM NaCl, 2.6 mM KCl [pH 7.2]). Unless otherwise stated, unlabelled and alkaline phosphatase (AP)-labelled goat anti-mouse reagents were obtained from Fisher Biotech (Fisher Scientific, Orangeburg, N.Y.). Antibody binding was detected with AP-labelled goat antisera and *p*-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, Mo.). AP-labelled goat anti-rabbit IgG (Fc) was obtained from Accurate Chemical and Scientific Corporation (Westbury, N.Y.). Optical densities were determined at 405 nm with a Ceres 900 reader (Bio-Tek Instruments, Inc., Winooski, Vt.).

Specificity of 2H1αId. Binding of the rabbit polyclonal 2H1 anti-idiotypic serum, 2H1αId, to 2H1 was determined by Western blot (immunoblot) and ELISA. For the Western blot, heavy and light chains of MAb 2H1 were separated by polyacrylamide electrophoresis in a 10% gel. Protein was electrotransferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences, Arlington Heights, Ill.) by using a trans-blot apparatus (MRA, Clearwater, Fla.). The membrane was blocked and incubated with 2H1αId and rabbit preimmune serum by the Amersham ECL protocol. Bound 2H1αId

TABLE 2. Serological and molecular characteristics of *C. neoformans* GXM-binding MAbs

Group	MAb	Serotype binding				Molecular structure					MAb 2H1 idiotype	Reference[a]
		A	B	C	D	V_H family[b]	J_H	D size	V_L	J_L		
I	E1	+	−	−	−	T15 (V_{11})	3	3	κ1	5	−	18
II	439	+	+	+	+	7183	2	7	κ1	1	++	20
	471	+	+	+	+	7183	2	7	κ5.1	1	++	48
	3C2	+	+	+	+	7183	2	7	κ5.1	1	+	48
	2D10 and 2H1[c]	+	+	+	+	7183	2	7	κ5.1	1	++++	38
	21D2	+	+	+	+	7183 (50.1)	2	7	κ5.1	1	+	8
	13F1	+	+	+	+	7183	2	7	κ5.1	1	+	38
III	339	+	+	+	+	10	4	6	κ21	5	−	48
	1255	+	+	+	+	10	4	6	κ21	5	−	20
IV	302	+	−	−	+	VGam	2	6	κ4/5	1	−	20
	386	+	±	±	+	VGam	3	4	κ4/5	1	−	48
V	4H3	−	−	−	+	X24 (441)	3	4	λ_1	λ_1	−	8
	14A12[d]	−	−	−	+	X24 (441)	3	4	λ_2	λ_2	−	8

[a] The reference indicates the article where the MAb was first described.
[b] The column lists the V_H family. The individual V_H family gene element is shown in parentheses if known.
[c] MAbs 2H1 and 2D10 were derived from one B cell in vivo and differ by a few amino acid substitutions resulting from somatic mutation. 2D10 and 2H1 are prototype members of a large set of 28 GXM-binding MAbs (38).
[d] MAb 14A12 is a representative of a set that includes another five IgM MAbs (8).

was detected with horseradish peroxidase-labelled goat anti-rabbit IgG as described in the Amersham ECL protocol. For ELISAs, polystyrene plates were coated with goat anti-mouse IgG1 and blocked with 1% BSA in TBS. The plates were then coated with MAb 2H1, incubated for 1 h at 37°C, and washed. Serial dilutions of rabbit antiserum were then added to the plate. Binding was detected by the A_{405} after incubation with AP-labelled goat anti-rabbit IgG (Fc) and *p*-nitrophenyl phosphate substrate. Reactivity of the antiserum to the IgG1 constant region was determined on myeloma MOPC 21 (Cappel, Organon Teknika Corp., West Chester, Pa.) captured in polystyrene plates by goat anti-mouse IgG1.

Inhibition of 2H1 binding to GXM by 2H1αId. Polystyrene ELISA plates were coated with 5 μg of GXM-A per ml as described previously (7). Plates were blocked with 1% BSA in TBS. A fixed concentration of 2H1 was added to GXM-coated ELISA plates. Rabbit antisera, preimmune or 2H1αId, was immediately added in decreasing concentrations. After incubation for 1 h at 37°C, the plates were washed, incubated with AP-labelled goat anti-mouse IgG1, and washed again, and binding was detected by A_{405} after the addition of *p*-nitrophenyl phosphate substrate. The 100% binding of 2H1 was determined by adding 1% BSA–TBS to the 2H1-coated wells. Soluble GXM-A, or *C. freundii* polysaccharide, in decreasing concentrations starting at 100 μg/ml, was also mixed with a fixed concentration of 2H1 and applied to ELISA plates coated with 2H1. After 1 h at 37°C, 2H1αId was applied to the plates. The plates were again incubated for 1 h at 37°C. Binding was detected by A_{405} by using AP-labelled goat anti-rabbit IgG (Fc) and *p*-nitrophenyl phosphate substrate.

Reactivity of 2H1αId with anticryptococcal MAbs. The reactivity of 2H1αId with a panel of murine anticryptococcal MAbs was studied by ELISA after binding the MAbs to GXM-A-coated plates. The MAbs studied included the seven MAbs described above and several that were described previously: 6 MAbs from a BALB/c mouse infected with a serotype A strain (6), 19 MAbs from a BALB/c mouse immunized with GXM-TT (6), 3 MAbs from a BALB/c mouse infected with strain GH (8), and MAb 439 (43). 2H1αId reactivity with the various MAbs was determined after MAb binding to plates coated with GXM-A. Briefly, MAbs were added to GXM-A-coated plates for 1 h at 37°C, the plates were washed, 2H1αId was added for 1 h at 37°C, the plates were washed again, and 2H1αId binding was detected by A_{405} after incubation with AP-labelled goat anti-rabbit IgG (Fc) and *p*-nitrophenyl phosphate substrate. To eliminate potential differences due to MAb affinity for GXM-A, the same experiments were repeated by using plates coated with goat anti-mouse kappa light chain and IgG1, which served to capture the MAbs for testing 2H1αId binding.

MAb fine-specificity analysis. 2H1 was applied to GXM-A-coated polystyrene plates at a concentration of 10 μg/ml and serially diluted. 2H1αId-negative MAbs (i.e., 302, 339, 386, and 1255) and 1% BSA were added immediately in duplicate at fixed concentrations of 1 μg/ml. After incubation for 1 h at 37°C, the plates were washed and 2H1αId was added. The plates were again incubated for 1 h at 37°C, and binding was detected with AP-labelled goat anti-rabbit IgG (Fc).

Serologic studies. Six BALB/c mice were immunized intraperitoneally with 25 μg of GXM-TT. Serum was obtained prior to immunization and on days 3, 5, 7, 10, 24, and 31 postimmunization. Sera were serially diluted on GXM-A-coated ELISA plates to determine the isotype profile of anti-GXM antibodies (7). Sera from the individual mice were studied by ELISA as described above for expression of the 2H1 idiotype. The sera of a single BALB/c mouse infected with the clinical isolate GH (8) was studied retrospectively for 2H1 idiotype expression.

RESULTS

Molecular analysis. Ig gene usage was determined by comparison of Ig variable gene sequences to the GenBank (Los Alamos, N.Mex.) database. On the basis of variable gene usage, the seven hybridomas studied here (i.e., E1, 3C2, 471, 339, 1255, 302, and 386) and 38 previously described (8, 38, 43) were classified into five groups (Table 2). For each MAb, the V_H and V_L sequences have been deposited in GenBank, and the accession numbers are listed in Table 3. Each group will be discussed separately.

MAb E1 was generated from a C3H/HeJ mouse immunized with serotype A polysaccharide and is primarily reactive with serotype A GXM (18). Since E1 was the first GXM-binding MAb described and shown to be protective in murine infection (15, 17), it is assigned to group I. In MAb E1, the V_H uses the

TABLE 3. GenBank accession numbers under which the MAb V_H and V_L sequences are deposited

Hybridoma	GenBank accession no.	
	V_H	V_L
E1	L30146	L30147
3C2	L30142	L30143
471	L30145	L30144
339	L30140	L30141
1255	L31512	L31513
302	L30138	L30139
386	L31895	L31516

V_{11} gene element from the T15 gene family (10), a short three-codon D segment, and J_H3. The E1 V_{11} gene differs from the V_{11} germ line gene from NZB/NZW and C57B mice by one base on the first codon. Since the C3H/HeJ germ line V_{11} is not available for comparison, this nucleotide change from the NZB/NZW germ line may be due to either somatic mutation or mouse strain polymorphism of the V_{11} gene. However, exact identity at other V_{11} codons and the germ line J_H3 indicates few somatic mutations in the generation of the E1 V_{11}. The D segment is very short and has four sequential G residues suggestive of generation by N sequence addition. E1 V_L uses a V_κ from the V_κSer group (24) and $J_\kappa5$.

MAbs 3C2 and 471 were generated from BALB/c mice immunized with polysaccharide of serotypes C and A, respectively, coupled to sheep erythrocytes (48). MAbs 3C2 and 471 bind to the GXM of the four *C. neoformans* serotypes (48). MAbs 3C2 and 471 have similar constructions, with the V_H using V_H7183-like gene family genes, a seven-codon D segment, and J_H2, and the V_L using $V_\kappa5.1$ and $J_\kappa1$ (Fig. 1; Table 2). This V_H-V_L configuration is very similar to that used for a set of 29 MAbs generated from BALB/c mice immunized with GXM-TT or infected with a serotype A strain (38), of which 2H1 and 2D10 are prototype members. Thus, group II includes MAbs with the V_H-V_L using 7183 gene family-J_H2-$V_\kappa5.1$-$J_\kappa2$, which binds to a GXM determinant found in all serotypes (Table 2). Southern blot analysis with the J_{11} probe was done to determine if hybridomas 3C2 and 471 shared V_H rearrangements (Fig. 2A). Hybridoma 2D10 (which has a V_H gene rearrangement identical to that of 2H1 [38]) was included in the Southern blot analysis to compare its V_H rearrangements with those of 3C2 and 471. For hybridomas 2D10 and 2H1, the productive rearrangement has been assigned to the band at 4 kb (38). For 3C2, two bands are apparent at 6.6 and 2.3 kb. The band at 6.6 kb is also found in liver DNA and is probably a

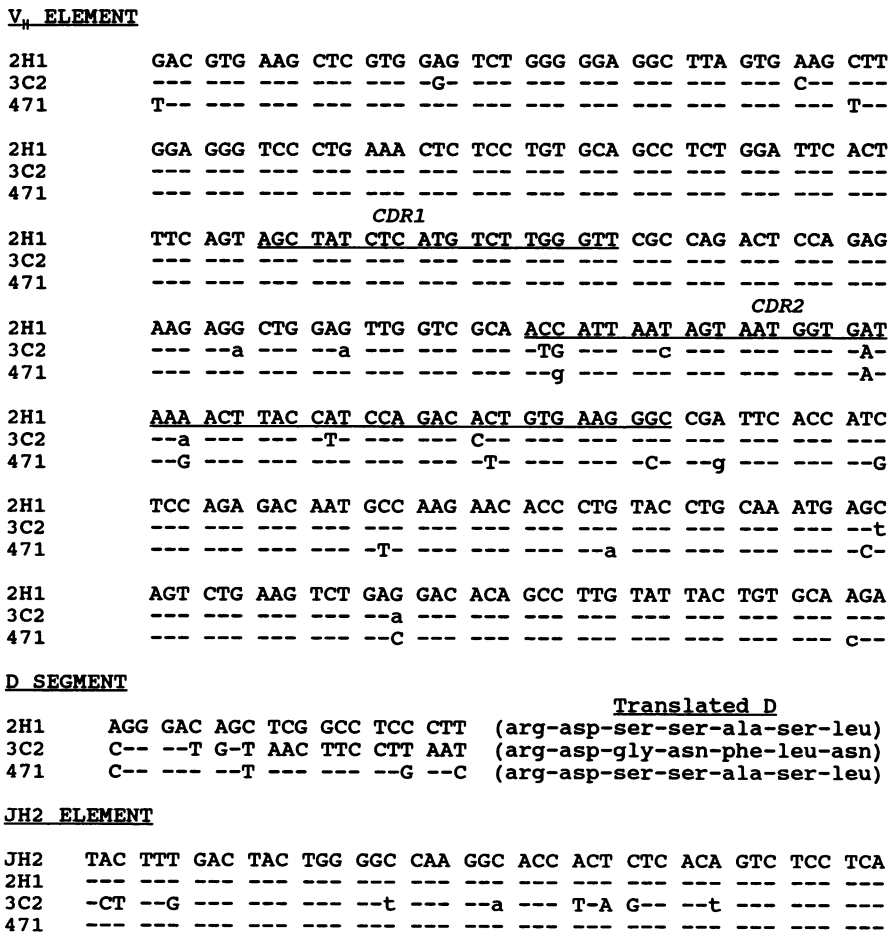

FIG. 1. Sequences of heavy-chain variable regions of MAbs 2H1, 3C2, and 471. The MAb 2H1 sequence was reported earlier (38) and is deposited in GenBank under accession number L05431. Nucleotide differences in the 3C2 and 471 sequences resulting in amino acid changes are shown in uppercase letters, whereas silent substitutions are shown in lowercase letters.

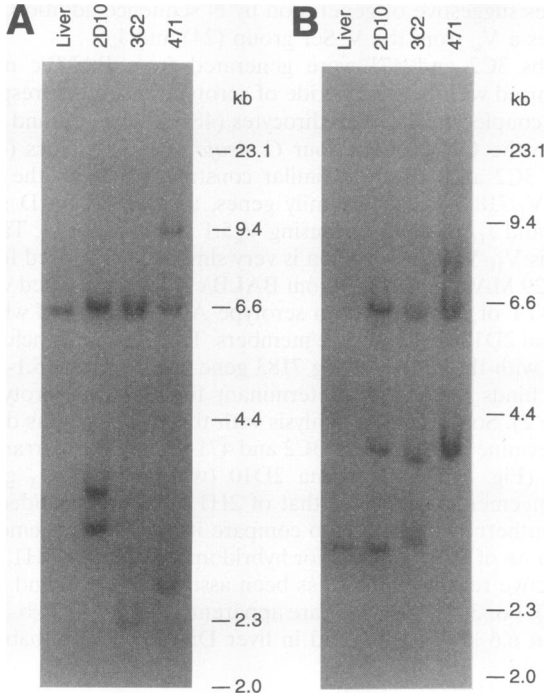

FIG. 2. V_H-V_L gene rearrangements in hybridomas 2D10, 471, and 3C2 as detected by Southern blot autoradiograms. (A) *Eco*RI digests of DNA from BALB/c liver and hybridomas 2D10, 3C2, and 471 probed with the J11; (B) *Hin*dIII digests of DNA from BALB/c liver and hybridomas 2D10, 3C2, and 471 probed with J_κ1-5. Hybridoma 2H1 gives a pattern like that of 2D10 (38).

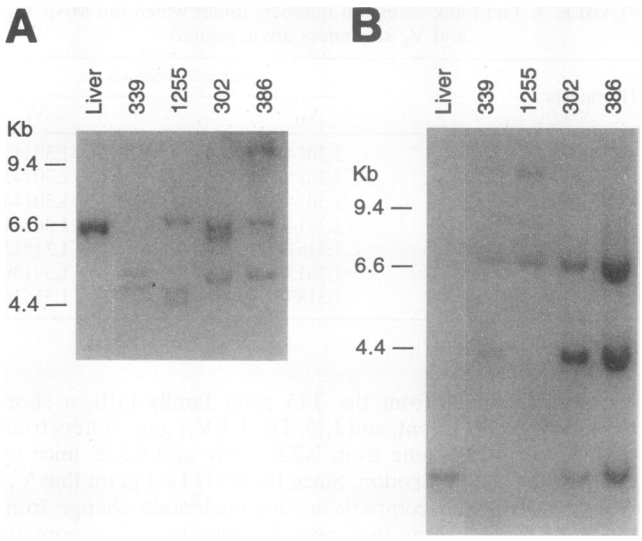

FIG. 3. V_H/V_L gene rearrangements in hybridomas 339, 1255, 302, and 386 as detected by Southern blot autoradiograms. (A) *Eco*RI digests of DNA from BALB/c liver and hybridomas 339, 1255, 302, and 386 probed with J11; (B) *Hin*dIII digests of DNA from hybridomas 339, 1255, 302, and 386 probed with J_κ1-5. Blots A and B are composites from the same gel where the liver DNA lane has been positioned next to the hybridoma 339 lane.

nonrearranged allele or a nonproductive rearrangement. *Eco*RI digests of Sp2/0 myeloma fusion partner DNA yielded a single band at ~6 kb, which was not found in either hybridoma 3C2 or 471 (not shown). The band at 2.3 kb is not found in either liver or Sp2/0 myeloma and is probably the productive rearrangement. For hybridoma 471, three bands are apparent at 2.5, 6.6, and 9.4 kb. The bands at 2.5 and 9.4 kb are Ig gene rearrangements not found in either liver or Sp2/0 myeloma and probably indicate productive and nonproductive Ig gene rearrangements. The V_H productive rearrangement in 2D10 or 2H1 at 4 kb is not present in either 3C2 or 471. These differences in V_H gene rearrangements suggest that despite close sequence identity, 3C2, 471, and 2H1 use different 7183 family gene elements. In contrast, Southern blot analysis of V_L with the J_κ1-5 probe (Fig. 2B) revealed that hybridomas 3C2 and 471 shared the band at approximately 4 kb which has been assigned to the productive rearrangement in 2H1 or 2D10 (38). The Southern analysis and the sequence data indicate that 3C2, 471, and 2D10 or 2H1 use the same V_L gene elements.

MAbs 339 and 1255 are generated from BALB/c mice immunized with polysaccharide of serotypes B and A, respectively, coupled to sheep erythrocytes (20, 48). MAbs 339 and 1255, like 3C2 and 471, bind to the GXM of the four cryptococcal serotypes (20, 48) but differ in molecular structure (Table 2). 339 and 1255 have similar constructions, with the V_H using a V_H10-like gene element, a six-codon D segment, and J_H4, and the V_L using a V_κ group 21-like gene element and J_κ1. Southern blot analysis of 339 and 1255 hybridoma DNA with J_{11} and J_κ1-5 probes revealed common bands at 4.7 and 6.6 kb, respectively, consistent with the sequence data, indicating the use of the same variable gene elements in V_H and V_L (Fig. 3). MAbs 339 and 1255 are distinct molecularly and idiotypically (see below) from the other MAbs and constitute group III.

MAbs 302 and 386 were generated from BALB/c mice immunized with polysaccharide of serotypes A and D, respectively, coupled to sheep erythrocytes (20, 48). MAbs 302 and 386 bind more strongly to GXM of A and D serotypes than to that of B and C serotypes. In MAb 302, the V_H uses a V_HGAM-like family gene element, a six-codon D segment, and J_H2. In MAb 386, the V_H also uses a V_HGAM-like family gene element, but unlike MAb 302, the MAb 386 V_H has a four-codon D segment and uses J_H3. MAbs 302 and 386 have similar V_Ls, which use V_κ4/5-like gene elements and J_κ5. Southern blot analysis of 302 and 386 hybridoma DNA with the J_{11} probe revealed common bands at 4.7 and 6.6 kb (Fig. 3). V_L Ig gene analysis with J_κ1-5 revealed identical band patterns for hybridomas 302 and 386 consistent with use of the same variable gene elements in V_L (Fig. 3). MAbs 302 and 386 are tentatively grouped together to form group IV since they use similar V_H and V_L gene elements (Table 2).

Specificity of 2H1αId. 2H1αId, the anti-2H1 rabbit antibody, binds to both the heavy and light chains of 2H1 in Western blots (data not shown). 2H1αId inhibits 2H1 binding to GXM. A 90% inhibition of MAb binding to GXM occurred at a 20-fold dilution of 2H1αId. Similarly, soluble GXM inhibits 50% of 2H1αId binding to 2H1 (data not shown). These results indicate that, in addition to private idiotopes, 2H1αId recognizes public idiotopes of both V_H and V_L in MAb 2H1, including determinants that are either in or near the 2H1 antigen binding site.

Reactivity of 2H1αId with anticryptococcal MAbs. 2H1αId reacted only with MAbs having a V_H composed of a 7183 family gene element, a seven-codon D segment, and J_H2 (Table 2). This included MAbs generated from infected and GXM-TT-immunized mice as well as MAbs 3C2, 439, and 471 generated from mice immunized with polysaccharide-erythro-

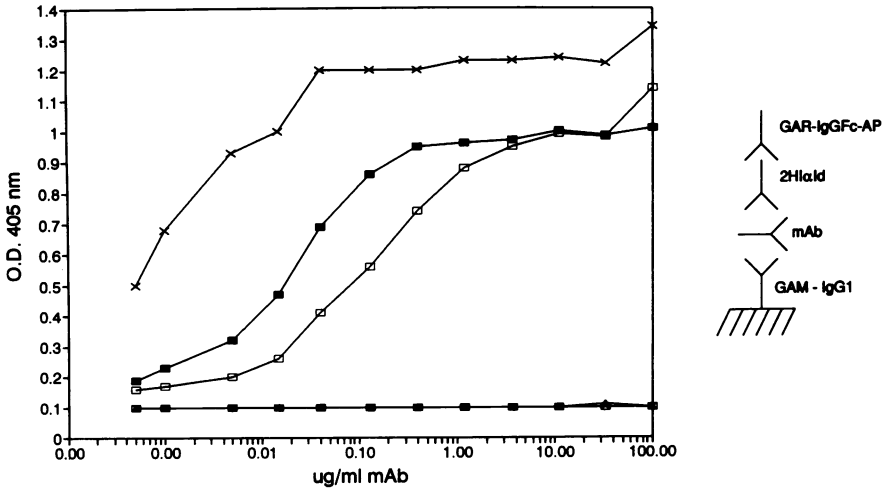

FIG. 4. 2H1 idiotype expression by MAbs 471 (closed square), 3C2 (open square), 339 (closed triangle), and 386 (open hourglass) compared with 2H1 (crosses). 302, 1255, and E1 had binding curves identical to those of 339 and 386 (not shown). 439 was idiotype positive and had a binding curve similar to that of 3C2 (not shown). The ELISA configuration is shown to the right of the figure. Abbreviations: O.D., optical density; GARIgGFc-AP, [alkaline phosphatase-conjugated goat anti-rabbit IgG(Fc)].

cyte conjugates. 2H1αId did not bind E1, 302, 339, 386, 1255, 14A12, or 4H3 and appears specific for group II MAbs (Fig. 4; Table 2). Among the group II MAbs, there were 2H1 idiotype expression differences which did not correlate with published affinity differences or structural motifs based on nucleic acid sequence analysis. The 2H1αId binding analysis was the same regardless of whether it was done on MAbs bound to GXM-A or captured by isotype- or light chain-specific goat antisera. Both 471 and 3C2 are recognized by 2H1αId. These MAbs have variable gene usage similar to 2H1, although they (like MAb 21D2 [8]), use different V_H7183 gene elements (Table 2; Fig. 3). MAb 439, a GXM-binding MAb that uses V_H7183-J_H2 but not J_κ5.1 (43), also expresses the 2H1 idiotype. Binding of 2H1αId to MAb 471 was roughly 10 times stronger than it was to MAb 3C2. The D segments of MAbs 471 and 2H1 are identical in protein sequence, whereas the 3C2 D segment differs by five amino acids (Fig. 2). Some differences in idiotype expression may be attributable to binding site idiotopes that could be obscured when idiotype expression is examined by antigen capture.

Fine specificities of idiotype-positive and idiotype-negative MAbs. MAbs 386, 302, 1255, and 339 are negative for 2H1 idiotype expression. These MAbs were used as competitive inhibitors of 2H1 binding to GXM to assess fine specificity. 2H1αId was used to detect binding since it will detect only 2H1. There was no competition between 2H1, 386, and 302 (Fig. 5A and B). There was minimal competition between 2H1, 339, and 1255 (Fig. 5C). This result could be due to steric hindrance if 339 and 1255 bind GXM near the 2H1 binding site or a shared binding site that is recognized more efficiently by 2H1. This result indicates that 2H1 and the idiotype-negative MAbs differ in fine specificity. E1 also failed to inhibit 2H1 binding to antigen (not shown).

Serologic studies. All six mice immunized with GXM-TT developed anti-GXM antibodies. As previously reported (6), IgM and IgG1 isotypes predominated (data not shown). All GXM-TT-immunized mice and the GH-infected mouse (8) had serum antibodies expressing the 2H1 idiotype. Idiotype expression was detected at the same time as anti-GXM IgG1, between days 7 and 10 postimmunization. 2H1αId titers of the six individual mice ranged from 1:150 to 1:4,000 and correlated with IgG anti-GXM titers. The GH mouse was infected with *C. neoformans* GH, and the spleen was fused on day 32 (8). Most of the MAbs generated from this mouse used the genetic elements shown for 14A12 (8), which is 2H1 idiotype negative (Table 2). However, one MAb, 21D2, recovered from this mouse used V_H50.1 of the V_H7183 family rearranged to V_κ5.1 (8) and is idiotype positive (Table 2). The serum of the GH-infected mouse was also 2H1 idiotype positive (data not shown).

DISCUSSION

The murine antibody response to *C. neoformans* GXM is a highly restricted, oligoclonal response. This conclusion is based upon antibody sequences from MAbs, reported here and elsewhere, derived from different BALB/c mice immunized with different immunogenic preparations of GXM (8, 38). Murine variable region antibody restriction has been reported for several haptens (10, 35, 47), including phosphorylcholine, a component of pathogenic bacteria (4, 10). Understanding gene usage in antibody responses is important because not all combinations may be equally protective. For example, some variable gene combinations are more protective in the mouse antibody response to phosphorylcholine (4), and protective human anti-*Haemophilus influenzae* antibodies express a limited number of variable region gene combinations (1, 36).

Including the 7 MAbs described here, a total of 45 MAbs to *C. neoformans* GXM have now been studied at the molecular level (8, 38, 43). Each of these MAbs can be classified into one of five groups on the basis of structure and serology (Table 2). The paucity of groups implies relatively little diversity in the antibody response to *C. neoformans* GXM. MAbs from groups I (E1), II (471, 2H1, 2D10, 18G9, 9F11, 17E12), and V (7B13) have been shown to modify the course of infection in murine cryptococcosis (15, 38–42, 45). However, membership in a group does not necessarily imply protective properties since certain group II (21D2) and group V (4H3) MAbs are nonprotective or minimally protective (40). Furthermore, MAbs within a group can differ in fine specificity (see below).

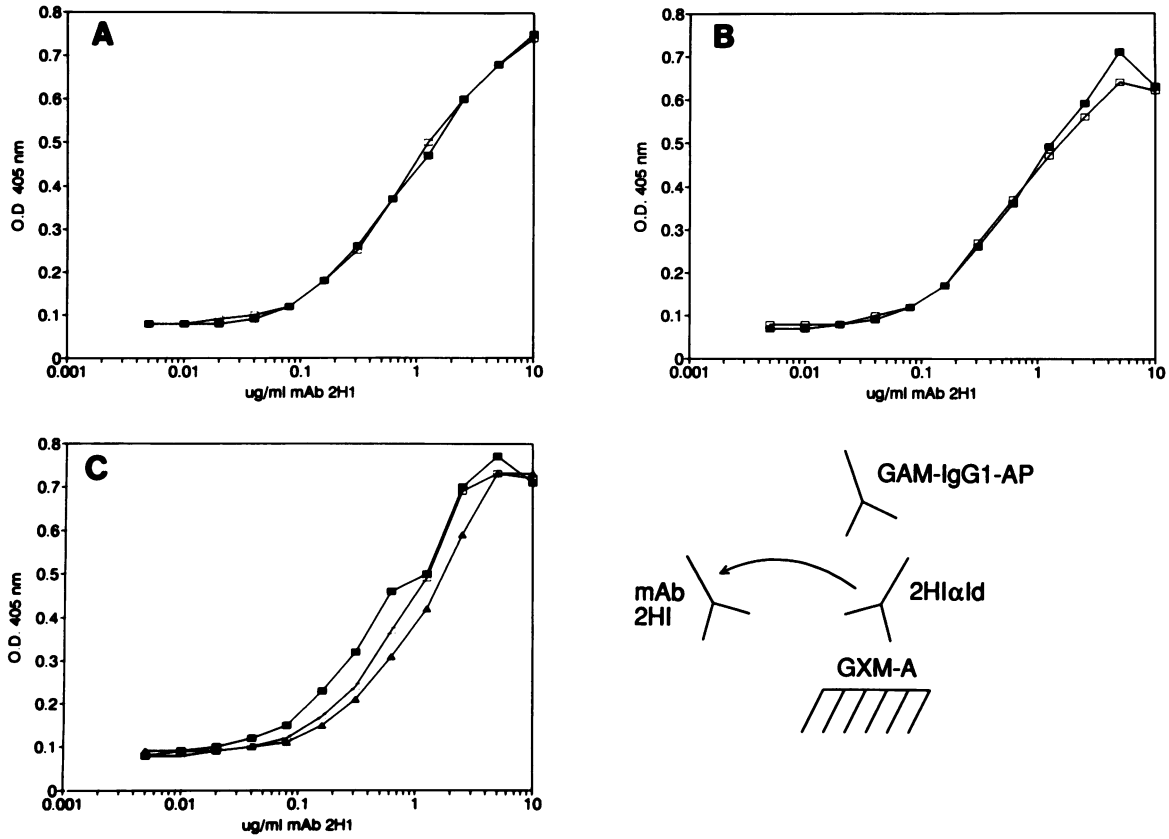

FIG. 5. Inhibition of 2H1 binding to GXM by 2H1 idiotype-negative antibodies. The ELISA configuration is as shown to the right of panel C. (A) 2H1 with 1% BSA in TBS (closed square) and 2H1 with 302 (open square); (B) 2H1 with 1% BSA in TBS (closed square) and 2H1 with 386 (closed square); (C) 2H1 with 1% BSA in TBS (closed square), 2H1 with 339 (open square), and 2H1 with 1255 (closed triangle). Idiotype-negative MAbs, used at a concentration of 2 μg/ml, were added immediately to titrations of 2H1. O.D., optical density.

Nevertheless, molecular and idiotypic analysis provides a framework for categorizing the various GXM-binding MAbs and for understanding differences in fine specificity, function, and GXM antigenic complexity.

E1 was the first MAb shown to modify the course of *C. neoformans* infection (15). E1 is 2H1αId negative and reacts primarily with serotype A GXM (16). A comparison of E1 with the protective 2H1αId-positive MAbs (i.e., 2H1, 471, and 2D10) reveals different variable gene usage and a short 3-amino-acid D segment. Differences in serotype specificity and variable gene usage by E1 and 2H1 idiotype-positive MAbs indicate different fine specificity. This implies that there are at least two epitopes in *C. neoformans* GXM that elicit protective antibodies.

MAbs 3C2 and 471 are similar in structure to MAb 2H1. These MAbs do not compete with 2D10, an IgM(κ) with the same structure and specificity as 2H1 (data not shown). In addition to gene elements from the V_H7183 family, 3C2, 471, and 2D10 or 2H1 all use identical J_H, V_κ, and J_κ gene elements and seven-codon D regions. 3C2 and 471 were generated in response to immunization with serotypes C (*C. neoformans* var. *gattii*) and A (*C. neoformans* var. *neoformans*) polysaccharide-erythrocyte conjugates, respectively. Therefore, there must exist a determinant in both varieties of *C. neoformans* which can elicit nearly identical antibody responses. The fact that 3C2 and 471 use the same or very similar variable gene combinations as that of MAb 2H1 provides a dramatic example of the restricted nature of the murine antibody response to *C. neoformans* GXM. In this regard, it is noteworthy that the translated CDR3 regions of 2H1 and 471 are identical despite significant nucleotide sequence divergence (Fig. 2). Southern blot analysis of Ig genes coding for 471 and 3C2 strongly suggest that these MAbs use different germ line V_H genes than 2H1 or 2D10. Although our data do not distinguish between 471 and 3C2 in terms of fine specificity, these antibodies do differ from 2H1 in fine specificity. MAb 13F1 is another example of a GXM-binding MAb with different fine specificity despite identical gene usage and 2H1 idiotype expression (6, 38) (Table 2). Fine-specificity differences between idiotype-positive MAbs may be attributable to somatic mutation or amino acid differences in the germ line V_H used. E1, 2H1, 2D10, and 471 all modify the course of experimental murine cryptococcosis and yet differ in fine specificity. Therefore, at least three *C. neoformans* GXM epitopes can elicit protective antibodies.

The D and J elements form the CDR3, part of the antigen binding site (28). Group II MAbs all have a D segment of seven amino acids (Table 2). The D regions of 2H1 and 3C2 differ significantly although both are idiotype positive. In phosphorylcholine-binding MAbs, the CDR3 length and not the sequence of the D segment appears to be important for determining the structure of the binding site and for V_H-V_L pairing (9). MAb 439 is an anticryptococcal MAb reported elsewhere (43) that uses the same set of V_H elements as 2H1

(Table 2). Despite using a different V_L, 439 expresses the 2H1 idiotype. Therefore, the 2H1 idiotype is encoded by multiple V_H7183 genes and may be expressed in the absence of V_κ5.1. The J_H2 segment is used in all of the idiotype-positive MAbs. This is analogous to restriction in the murine anti-azophenylarsonate and T15 antibodies in which J_H2 and J_H1, respectively, are used (9). The uniformity of structural components in group II antibodies expressing the 2H1 idiotype suggest that a specific protein structure is required to bind a determinant present in GXM. In all 32 group II MAbs studied, the first two amino acids in the D segment are arginine and aspartic acid, strongly suggesting that this amino acid sequence is an important structural motif for GXM binding. Molecular modelling of MAb 439 has suggested that the arginine is located at the surface of the antibody molecule within the putative binding site (43). The molecular model places the arginine in an ideal position to interact with the carboxyl group found in the glucuronosyl side chain on GXM (43). Thus, variable gene restriction in the anti-GXM antibody response may result from selection of those gene combinations which can form the protein structure necessary for binding GXM.

MAbs 339, 1255, 386, and 302 are 2H1 idiotype negative and have different molecular structures and fine specificities (Table 2) than MAb 2H1. MAbs 302 and 386 use identical V_κ genetic elements. MAbs 1255 and 339 use identical V_H and V_κ genetic elements. Therefore, the idiotype-negative as well as the idiotype-positive antibodies appear to use limited V_H-V_L combinations. Although several V_H-V_L combinations have been demonstrated among anti-GXM antibodies (Table 2), most mice that yielded multiple MAbs used only one variable region gene combination (8, 38). The V_H-V_L genes used in a given mouse may reflect priming by immunization with GXM or a cross-reactive antigen (8). If the anti-GXM antibody response is examined soon after immunization, the number of variable regions used in antipolysaccharide antibodies could be more numerous. The primary response to GXM could stimulate multiple B cells, but the secondary response may select only those B cells with the highest affinity for GXM, as has been reported for some antibody responses to haptens (52). The serologic studies suggest that 2H1 idiotype expression is temporally associated with isotype switching in BALB/c mice. A similar finding has been reported in the phosphorylcholine antibody response (22). Since isotype switching occurs in close association with somatic mutation, this could imply that a dominant idiotype emerges during the secondary response.

All anticryptococcal MAbs used J_H-proximal V_H gene family elements, including members of the T15, 7183, V_H10, V_HGam, and X-24 gene families (Table 2). Murine V_H gene families are arranged on one chromosome having arisen by duplication and sequence divergence of early genes (44). The neonatal antibody repertoire in mice demonstrates biased expression of J_H-proximal V_H gene family elements (37). J_H-proximal V_H gene family elements predominate in the response to GXM. This is striking given that V_H representation in adult murine B cells corresponds to the relative frequency of variable genes in the immunoglobulin repertoire (13). Preferential use of 3′ variable region gene elements by anticryptococcal GXM antibodies may be due to coincidental 3′ positioning of GXM-binding V_H genes or specific structural features of J_H-proximal V_H gene family elements that result in better affinity for *C. neoformans* GXM epitopes. Another characteristic of anti-GXM MAbs appears to be D segments which are shorter than average for murine MAbs (51). This would result in shorter V_H CDR3s, which may be another important structural requirement for binding GXM. For example, the length of the V_H CDR3 would be an important factor in positioning of the arginine into the putative binding site in class II antibodies.

Our observations have implications for ongoing efforts to develop antibodies for the therapy and prevention of *C. neoformans* infections. The finding that MAbs 2D10 or 2H1, 3C2, and 471 and the idiotype-negative MAbs 339 and 1255 react with different epitopes present in all four *C. neoformans* serotypes strongly suggests that the GXM antigenic structure is more complex than the eight-factor classification of Ikeda et al. (27). Group II antibodies that express the 2H1 idiotype include MAbs that modify *C. neoformans* infection. Therefore, the 2H1 idiotype may be a marker for the presence of protective antibodies in anticryptococcal sera elicited by either infection or GXM-TT immunization. The finding that at least three distinct epitopes elicited protective MAbs suggests that combinations of MAbs could be more effective and less likely to select for resistant antigenic variants than monoclonal preparations.

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