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Analysis of Human Monoclonal Antibodies Elicited by Vaccination with a *Cryptococcus neoformans* Glucuronoxylomannan Capsular Polysaccharide Vaccine

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The *Cryptococcus neoformans* capsular polysaccharide glucuronoxylomannan (GXM) has been conjugated to tetanus toxoid (GXM-TT) as an investigational vaccine. GXM-TT elicits antibodies that are protective in *C. neoformans*-infected mice. In an effort to characterize the fine specificity and molecular structure of human GXM-TT-elicited antibodies, we generated two GXM monoclonal antibodies (MAbs) from peripheral blood lymphocytes of a volunteer GXM-TT recipient and studied serum GXM antibody idiotype expression in 10 additional vaccinees. The MAbs, 2E9 and 3B6, are the immunoglobulin M(λ) isotype and bind capsular polysaccharides of *C. neoformans* serotypes other than the serotype A that was used for immunization. Neither antibody competes with murine GXM MAbs for antigen binding, suggesting that the human MAbs recognize a different epitope. The B-cell superantigen staphylococcal protein A binds both MAbs, and human immunodeficiency virus gp120 binds 2E9. MAb nucleic acid sequence analysis revealed that both antibodies use an identical V_λ1a-J_λ genetic element with different, somatically mutated, members of the V_H3 gene family and different D_H and J_H gene elements. The gene elements used by both MAbs occur in fetal B-lymphocyte repertoires, autoantibodies, and other polysaccharide antibodies. Post-GXM-TT vaccination GXM antibodies from 10 additional vaccinees expressed a shared idiotype defined by rabbit antiserum raised against MAb 2E9. Our data suggest that the human GXM antibody response is restricted and raise questions regarding the importance of specific variable-region elements and superantigens in the generation of human antibody responses to encapsulated pathogens.

Cryptococcus neoformans meningitis occurs in approximately 8% of human immunodeficiency virus (HIV)-infected individuals (18). In the setting of the profound immunosuppression of AIDS, cryptococcal infections are associated with high relapse and mortality rates (8). We and others have reported that antibodies against the glucuronoxylomannan (GXM) capsular polysaccharide (CPS) of *C. neoformans* are detectable in the serum of both HIV-positive and HIV-negative individuals (20, 23, 28). Naturally occurring antibodies reactive with CPSs of pathogenic bacteria have been detected in sera from children in the absence of exposure to or infection with such organisms (46, 47). Although a physiologic developmental impairment of antibody responses to polysaccharides prevents protective antibody responses to encapsulated bacteria in young children, naturally occurring antibodies against cross-reactive antigens are protective against given pathogens (46, 47). The existence of GXM antibodies in individuals with AIDS and cryptococcal meningitis (20) suggests that naturally occurring human GXM antibodies, like murine GXM antibodies (58), may be either protective or nonprotective.

C. neoformans shares pathogenic mechanisms with encapsulated bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* in that it has a predilection for the central nervous system and is surrounded by a polysaccharide capsule that is a virulence factor for infection (12). Antibody-mediated immunity is crucial for protection against encapsulated bacteria (10, 15, 46, 47, 53). Isotype and idiotype restriction of antibodies against pathogenic polysaccharide-encapsulated organisms has been documented for conjugate vaccine-elicited human antibodies against the CPS of *H. influenzae* type b, murine antibodies against GXM (10, 36), and infection-elicited human antibodies against *S. pneumoniae* and group A streptococcus (3, 4, 49, 50, 53). To determine whether human antibodies against the polysaccharide capsule of the pathogenic fungus *C. neoformans* are restricted similarly, we set out to characterize the molecular structural features of human antibodies elicited by GXM.

C. neoformans GXM has been conjugated to tetanus toxoid (TT) as an investigational vaccine (22). Murine antibodies that bind the *C. neoformans* GXM enhance the survival of mice with lethal cryptococcosis (40–42) and enhance phagocytosis of *C. neoformans* by macrophages in vitro (42). However, the protective efficacy of human GXM antibodies is unknown. Human GXM monoclonal antibodies (MAbs) rather than polyclonal antibodies are essential to address questions regarding the role of GXM antibodies in protection against cryptococcal infections. Therefore, we generated two human GXM MAbs from peripheral blood lymphocytes (PBLs) of a GXM-TT-immunized individual with the hope of beginning to decipher the nature of the human antibody response to GXM. This paper details the molecular structure and fine specificity of the two human GXM MAbs isolated, 2E9 and 3B6.

MATERIALS AND METHODS

Two lymphoblastoid cell lines were generated from the PBLs of a volunteer who had been immunized with GXM-TT at the National Institutes of Health, Bethesda, Md. PBLs and serum were kindly provided by J. Robbins, R. Schneerson, and J. Bennett (National Institutes of Health). Purified GXM from serotype A *C. neoformans* (strain 371) was provided by R. Schneerson. Informed consent was obtained for vaccination and venipuncture.

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Generation of Epstein-Barr virus-transformed cell lines. Mononuclear cells were isolated from whole blood by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.), washed in Hank's balanced salt solution (Sigma Chemical Co., St. Louis, Mo.) and resuspended in RPMI medium (Gibco, Grand Island, N.Y.), 20% fetal calf serum (HyClone Laboratories, Inc., Logan, Utah), 1% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 0.01 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) at 10^6 cells per ml. Two hundred microliters of the supernatant from the Epstein-Barr virus-infected marmoset cell line (B95-8; American Type Culture Collection, Rockville, Md.) and 50 ng of cyclosporine A were added to the cells as described in a published protocol (37). All wells yielded transformants which were tested for binding to GXM by an enzyme-linked immunosorbent assay (ELISA; see below). Cell lines were cloned within 3 weeks of transformation by limiting dilution. For cloning, supernatant from the J774.16 murine macrophage cell line was used to provide growth factors at a final concentration of 20%. B-cell growth factor (Cellular Products, Buffalo, N.Y.) was also used for cloning and then for growth of GXM-specific transformants in mass culture. Cell lines were grown in hybridoma serum-free medium (Gibco) in a Cellmax apparatus (Gibco).

Assay for GXM binding. Cell lines were tested for binding to GXM by an ELISA as described previously (20). Briefly, polystyrene ELISA plates (Corning Glass Works, Corning, N.Y.) were coated with 10 μg of GXM per ml diluted in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) in PBS. After washing in PBS–0.01% Tween 20, supernatants were applied to the ELISA plates in decreasing concentrations, and the plates were incubated at 37°C for 1 h. The plates were washed and incubated at 37°C for 1 h with a 1:1:1 mixture of alkaline phosphatase-labelled goat anti-human immunoglobulin G (IgG), IgA, and IgM (Fisher Biotech, Fisher Scientific). After washing, the plates were developed with *p*-nitrophenyl phosphate substrate (Sigma). The optical densities of the wells were determined at an A_{405} in a Ceres 900 ELISA reader (BioTek Instruments, Inc., Winooski, Vt.). Positive controls used were the following: (i) the murine GXM MAb 2H1 (33) (detected with goat anti-mouse IgG1; Fisher Biotech), kindly provided by A. Casadevall (Albert Einstein College of Medicine, Bronx, N.Y.), and (ii) serum from the GXM-TT-immunized donor. Negative controls used were the following: (i) no antibody; (ii) MAb 110.4 (43), an IgG1 murine MAb that does not bind GXM; and (iii) a human IgM MAb that does not bind GXM, kindly provided by A. Manheimer-Lory (Albert Einstein College of Medicine).

Assay for isotype of transformants. The isotype of secreted immunoglobulin was determined by an ELISA. ELISA plates (Corning) were coated with a 1:1:1 mixture of goat anti-human IgG, IgA, and IgM (Fisher Biotech) in PBS. After blocking with 1% BSA–PBS, 50-μl aliquots of supernatants from transformants were applied to 10 wells each. The plates were incubated at 37°C for 1 h and washed, and then two wells per supernatant were incubated with each of the following alkaline phosphatase-labelled reagents (Fisher Biotech) at 37°C for 1 h: (i) goat anti-human IgG, (ii) goat anti-human IgM, (iii) goat anti-human IgA, (iv) goat anti-human kappa light chain, and (v) goat anti-human lambda light chain. After incubation, the plates were washed and developed with *p*-nitrophenyl phosphate substrate (Sigma). A_{405} values were recorded with a BioTek Ceres 900 reader.

GXM binding of the transformants. Binding of secreted immunoglobulin from the cell transformants to purified capsular polysaccharide (CPS) of *C. neoformans* serotypes A, B, C, and D was determined by an ELISA. The CPSs were provided by A. Casadevall (Albert Einstein College of Medicine). The supernatants were adjusted to the same immunoglobulin concentration on the basis of concentrations determined by an ELISA: MAb supernatants or isotype-specific sera (Sigma) were added to goat anti-human isotype (Fisher Biotech)-coated ELISA plates (Corning) in serial threefold dilutions. After incubation and washing, alkaline phosphatase-labelled goat anti-human isotype-specific reagents were added (Fisher Biotech). After washing again, the plates were developed with *p*-nitrophenyl phosphate substrate (Sigma), and the A_{405} was determined. The concentration of each supernatant was derived from a standard curve of isotype-specific immunoglobulin (Sigma). For the GXM binding assays, a double-sandwich ELISA was used. ELISA plates were coated with goat anti-mouse IgG1 (Fisher Biotech). After blocking with 1% BSA–PBS, the plates were incubated at 37°C for 1 h with murine MAb 2H1 and washed. Each of the four GXMs was then added in duplicate at a concentration of 10 μg/ml. The plates were incubated at 37°C for 1 h and washed, and then supernatants of the GXM-binding cell lines were added in serially decreasing concentrations to the duplicate wells containing each GXM. After incubation at 37°C for 1 h, the plates were incubated with alkaline phosphatase-labelled goat anti-human isotype-specific reagents (Fisher Biotech), developed with *p*-nitrophenyl phosphate substrate (Sigma), and read as described above. Binding curves were created with Quattro Pro 4.0 software (Borland International, Inc., Scotts Valley, Calif.).

Immunofluorescence. The human MAb 2E9 and postimmunization serum from the PBL donor were tested by immunofluorescence for binding to *C. neoformans* serotypes D and A and an acapsular mutant (strains 24067, 371, and 52817, respectively [provided by A. Casadevall]). The organisms were grown for 72 to 96 h at 30°C in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.). Organisms (10^8) were mixed with serum at a dilution of 1:10 to 1:20 or with the MAb at a concentration of 100 μg/ml. After incubation at 37°C, the cells were washed extensively and labelled with fluorescein isothiocyanate-conjugated goat anti-human IgG (for serum antibody) or IgM (for MAbs; Fisher Biotech). The cells were incubated for an additional hour at 37°C, washed, and resuspended in mounting medium, and fluorescent and bright-field signals were collected with an MRC 600 confocal microscope (Bio-Rad, Hercules, Calif.) with a Kr-Ar laser, standard fluorescein isothiocyanate filter block provided by Bio-Rad, and a Nikon 60× planopo objective (numerical aperture, 1.4). Single optical sections from each field of cells were collected at 1.6-mm intervals. The equipment used was provided by the Image Analysis Facility of the Albert Einstein College of Medicine. Negative controls for immunofluorescent experiments were 1% BSA–Tris-buffered saline (TBS; 25 mm Tris, 126 mM NaCl, 2.6 mM KCl [pH 7.2]) (no antibody) and a human monoclonal IgG antibody that does not bind *C. neoformans* by an ELISA (provided by A. Manheimer-Lory).

Assays for polyreactivity of the transformants. Binding of the transformants to human insulin (Sigma), bovine thyroglobulin (Sigma), TT (Connaught, Inc., Swiftwater, Pa.), human IgG Fc (Calbiochem, San Francisco, Calif.), and double-stranded DNA (Sigma) was tested by an ELISA. ELISA plates were coated with insulin, thyroglobulin, and IgG Fc, each at a concentration of 10 μg/ml. After blocking, the MAbs were applied in duplicate, starting at a concentration of 10 μg/ml, and titrated serially. After incubation, the plates were washed and reincubated with goat anti-human IgM (Fisher Biotech). The plates were washed, and *p*-nitrophenyl phosphate substrate (Sigma) was added. The DNA binding ELISA was performed as described in a published protocol (19). Briefly, ELISA plates were coated with boiled, sonicated calf thymus DNA (Sigma) at a concentration of 10 μg/ml. The plates were then used as described above. The transformants were also tested for binding to *Crithidia lucidilae* by slide immunofluorescence (kits from ImmunoConcepts Diagnostic, Sacramento, Calif.). Positive controls for the assays were provided by Anne Davidson (human MAb with reactivity against insulin, thyroglobulin, and IgG Fc) and Betty Diamond (murine serum with reactivity against DNA) (Albert Einstein College of Medicine).

Assay for SPA and gp120 binding of the transformants. Binding of the transformants to staphylococcal protein A (SPA) and HIV type 1 gp120 was tested by an ELISA. For SPA binding, ELISA plates were coated with 1 μg of SPA (Sigma) per ml and blocked with 1% BSA–PBS as described above. Supernatants from the human MAbs, polyclonal human IgM used as a positive control (Sigma), and blocking buffer were applied to the plate and titrated 1:3 to a final volume of 50 μl per well. After incubation at 37°C for 1 h and washing, alkaline phosphatase-conjugated goat anti-human IgM (Fisher Biotech) was added. The plates were incubated, washed, developed with *p*-nitrophenyl phosphate substrate (Sigma), and read as described above. For binding to HIV type 1 gp120, each well of the ELISA plates was coated with 50 ng of HIV type 1 SF2 gp120 (catalog numbers 386 and 388; AIDS Research and Reference Reagent Program, Bethesda, Md.) diluted in carbonate buffer as described in a published protocol (5). After incubation and blocking with 0.1% Tween–PBS, MAbs, two murine anti-gp120 MAbs (43), and blocking buffer were added and titrated 1:3 to a final volume of 50 μg per well. The plates were incubated at 37°C for 1 h, washed, and reincubated with alkaline phosphatase-labelled goat anti-human or goat anti-mouse reagents (Fisher Biotech). After washing, the plates were developed with *p*-nitrophenyl phosphate substrate (Sigma) and read as described above.

Reactivity of the transformants with 2H1αld. 2H1αld is a rabbit antiserum that reacts with a group of highly protective murine GXM MAbs (13). Transformants were tested for binding to this reagent by an ELISA to determine if the human MAbs express the same idiotype as GXM-TT-elicited murine GXM MAbs. ELISA plates coated with purified 2H1αld and blocked with 1% BSA–PBS were incubated with serial dilutions of the human cell lines in decreasing concentrations. After incubation for 1 h at 37°C and washing, the plates were reincubated with alkaline phosphatase-labelled goat anti-human IgM (Fisher Biotech). After washing, the plates were developed with *p*-nitrophenyl phosphate substrate (Sigma) and read as described above. Murine MAb 2H1 detected with alkaline phosphatase-labelled goat anti-mouse IgG1 (Sigma) was used as a positive control.

Epitope mapping of the transformants. Competition experiments with murine GXM MAbs 2D10 [IgM(κ)] and 2H1 [IgG1(κ)] (38) were performed by an ELISA to determine if the human GXM MAbs recognize the same epitope as protective murine GXM MAbs do. ELISA plates were coated with 10 μg of the MAb per ml as detailed above. Serial dilutions of the murine MAbs (or 1% BSA–PBS) beginning at a concentration that saturated the plate were combined 1:1 with the human MAbs used at 50% saturation and added immediately to the GXM-coated ELISA plate in duplicate, and the plates were reincubated. Alkaline phosphatase-conjugated goat anti-human IgM was added to the plate, and the plate was processed further as detailed above. Inhibition of binding by the murine MAbs was calculated as follows: [(absorbance without inhibitor) - (absorbance with inhibitor)]/(absorbance without inhibitor).

Generation of polyclonal rabbit antiserum to MAb 2E9 (2E9 anti-idiotypic antibody). Rabbit anti-idiotypic serum was prepared against 2E9, one of the two IgM(λ) MAbs reported in this paper. The antiserum was prepared by subcutaneous immunization of a New Zealand White rabbit with purified 2E9 in complete Freund's adjuvant and chromatographic purification of serum. First, anti-isotype activity of the serum was removed by extensive absorption with human IgM Fc (Calbiochem) coupled to a Carbolink column (Pierce Life Sciences and Research Products, Rockford, Ill.). The pass-through was subsequently affinity

TABLE 1. Variable gene element utilization and GXM binding of GXM antibodies

Cell line	V_H family and gene element	D_H	J_H	V_L family and gene element	J_L	GXM binding[a]	Reference
2E9	V_H3 HHHG19G	NI[b]	J_H6	$V_\lambda1a$ Humlv117	$J_\lambda1$	A > B = D	35 (V_H); 55 (V_L)
3B6	V_H3 V_H26	DXP′1	J_H4b	$V_\lambda1a$ Humlv117	$J_\lambda1$	A = B = C = D	17 (V_H); 55 (V_L)

[a] Reactivity of GXM antibodies to *C. neoformans* serotypes A, B, C, and D.
[b] NI, not identified from available sequences.

purified with 2E9 coupled to Sepharose 4B (Pharmacia). The purified rabbit antiserum bound 2E9, 3B6 (another human MAb reported in this paper), and additional human MAbs that use human VH3 gene elements by an ELISA but not IgM Fc or MAbs that use VH4 and VH5 gene elements. The purified rabbit anti-2E9 reagent was used to detect 2E9 idiotype expression in pre- and postimmunization sera from the PBL donor and 10 additional volunteer GXM-TT vaccinees. Idiotype expression was determined by ELISA. Plates were coated with GXM and blocked as detailed above, incubated with pre- and postimmunization sera at a dilution of 1:10, and washed. Duplicate wells containing bound pre- and postimmune GXM antibodies were incubated with rabbit MAb 2E9 anti-idiotypic serum and preimmune rabbit serum as a control, washed, and reincubated with alkaline phosphatase-labelled goat anti-rabbit IgG Fc (Accurate Chemical and Scientific Corporation, Westbury, N.Y.). The plates were developed as detailed above. To determine the isotype of idiotype-positive GXM antibodies, the ELISA was repeated with sera diluted 1:1 with 0.15 M β-mercaptoethanol in TBS and incubated at 37°C for 1 h before use (20). Treatment of sera with β-mercaptoethanol reduces multimeric IgM, which because of its high avidity, competes with IgG antibodies and may mask their presence.

Sequence analysis of the transformants. Nucleic acid sequences of the GXM binding transformants were obtained by PCR amplification of RNA. Total RNA was isolated from 10^6 cells of each cell line with the Superscript kit (Gibco BRL) as described in the manufacturer's protocol. To obtain VDJ (heavy-chain [V_H]) and VJ (light-chain [V_L]) variable-region sequences, cDNA was generated by reverse transcription of RNA by use of constant-region primers for V_H, 5′-TGCTGCTGATGTCAGAGTTGT-3′, and for $V_{L(\lambda)}$, 5′-AGTGTGGCCTTGT TGGCTTG-3′, as described in the Superscript protocol. Six PCRs were performed with the same V_H constant-region primer and each of six (antisense) V_H primers representing six human V_H families (32). Six PCRs were also performed with the same V_L constant-region primer and each of six (antisense) V_L primers representing six human V_L families (32). Oligonucleotides were synthesized at the DNA Synthesis Facility of the Cancer Center of the Albert Einstein College of Medicine. After identification of the V_H and V_L used by each cell line, PCR products were cloned into the pCR1000 plasmid of the TA cloning system (Invitrogen, San Diego, Calif.). Two independent PCRs were done for all V_H and V_L, except one V_H from which there was insufficient RNA. Variable-region inserts were identified in plasmid DNA by restriction endonuclease analysis and hybridization with joining-region (J_H and J_L) primers. Plasmid DNA was isolated by use of the Maxi plasmid protocol (Qiagen, Inc., Chatsworth, Calif.). DNA sequencing was performed by the dideoxynucleotide chain-terminating method with the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio). Two clones of each V_H and V_L were sequenced. Variable-region genes were identified by sequence comparison with the GenBank database by use of the Genetics Computer Group (GCG; University of Wisconsin) sequence analysis software package (21) and the sequence compilation of Kabat et al. (31). Isoelectric points (pI) calculated for each V_H and V_L were determined with the GCG sequence analysis software package (version 7.3).

Nucleotide sequence accession numbers. The sequences identified in this study have been assigned GenBank numbers as follows: 2E9 heavy chain, U27187; 2E9 light chain, U27188; 3B6 heavy chain, U27189; and 3B6 light chain, U27190.

RESULTS

Cell lines and variable-region gene element utilization. Two GXM binding Epstein-Barr virus-transformed cell lines were isolated. As shown in Table 1, these cell lines are of the IgM(λ) isotype. The genetic elements used by the antibodies secreted by these cell lines are also detailed in Table 1. Both 2E9 and 3B6 use the identical $V_\lambda1a$-$J_\lambda1$ genetic element with different members of the human V_H3 immunoglobulin gene family, different D_H segments, and J_H6 and J_H4, respectively.

GXM binding of Epstein-Barr virus transformants. The binding curves of 2E9 and 3B6 are shown in Fig. 1. In the experiments illustrated by this figure, purified CPS (not GXM) was used for antigen capture. Both antibodies bind to the CPS from *C. neoformans* serotypes other than serotype A: 2E9 binds to CPS from serotypes A, B, and D (Fig. 1A), and 3B6 binds to CPS from all four serotypes (Fig. 1B). However, the binding of 2E9 is at least fivefold greater than the binding of 3B6 for each CPS (other than that from serotype C).

Immunofluorescence. Cell line 2E9 and postimmunization serum of the donor from whom 2E9 was generated both demonstrated similar patterns of capsular staining of *C. neoformans* cells by immunofluorescence (Fig. 2). The same patterns were observed with *C. neoformans* serotypes A and D. The hetero-

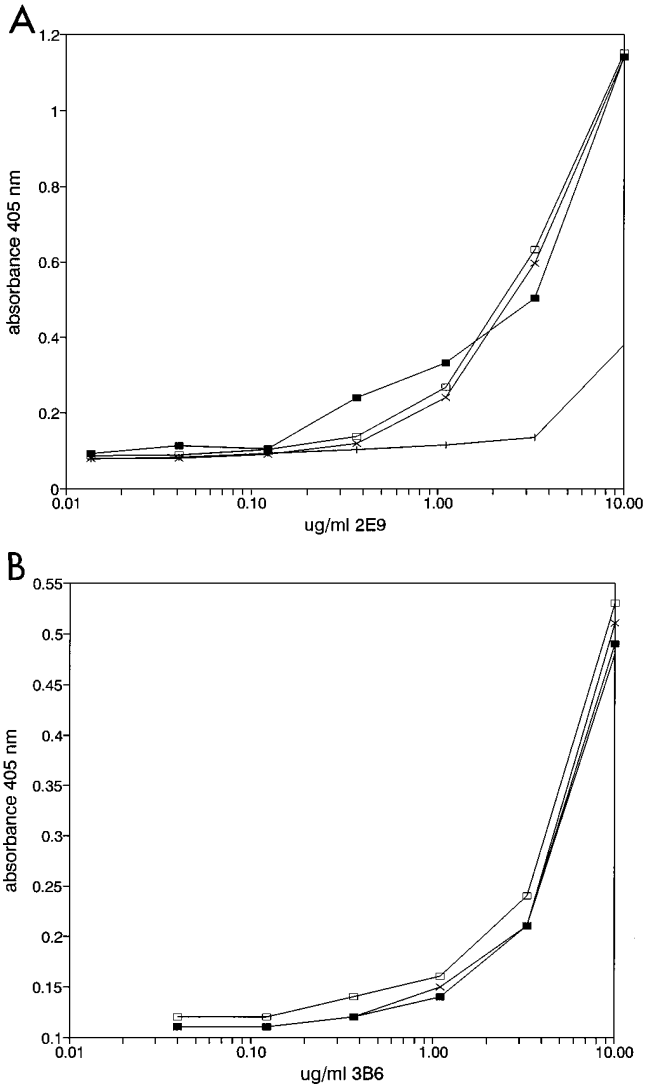

FIG. 1. Serotype specificity of GXM MAbs 2E9 and 3B6. Binding of 2E9 (A) and 3B6 (B) to purified CPS from *C. neoformans* serotypes A (■), B (×), C (+), and D (□) was tested by sandwich ELISA. Details of the protocol are described in the text. Binding, indicated by A_{405} on the *y* axes, is plotted for the concentrations of the antibodies indicated on the *x* axes.

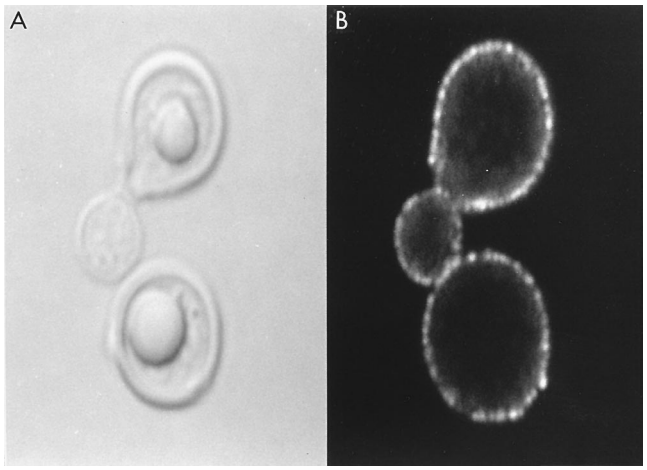

FIG. 2. Immunofluorescent labelling of *C. neoformans* serotype D (ATCC 24067) with GXM MAb 2E9. Labelling was performed as detailed in the text with fluorescent isothiocyanate-conjugated goat anti-human IgM. Bright-field (A) and fluorescent (B) images were collected as detailed in the text with a confocal microscope. A single section from a series of images obtained at 1.6-mm intervals is shown.

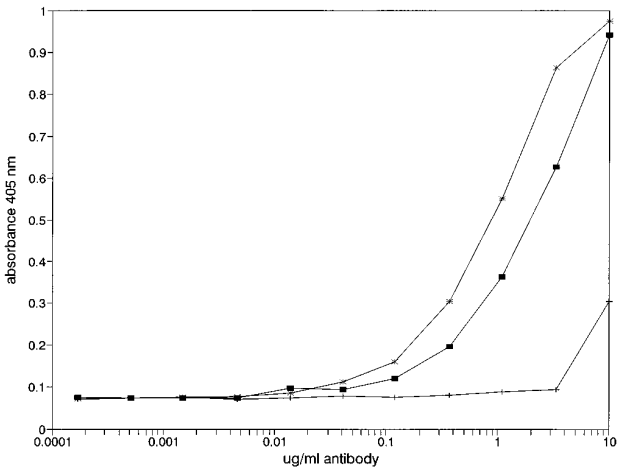

FIG. 3. Reactivity of GXM MAbs 2E9 and 3B6 with SPA. Binding of SPA to the GXM MAbs (2E9 [■] and 3B6 [+]) and polyclonal human IgM (∗) was evaluated by antigen-based ELISA on SPA-coated plates as detailed in the text. Binding, indicated by A_{405} on the *y* axis, is plotted for the concentrations of the antibodies indicated on the *x* axis.

geneous pattern of capsular binding of 2E9 extends to a budding organism in Fig. 2; however, very small budding cells were generally poorly labelled or unlabelled (data not shown). Although fluorescent labelling with both 2E9 and post-GXM-TT vaccination serum was distributed evenly throughout the capsule, serial confocal views showed a stippled pattern. There was no labelling of acapsular *C. neoformans* with 2E9. Postimmunization serum demonstrated labelling of both the acapsular and encapsulated strains. Cell line 3B6 could not be used in immunofluorescence because of extremely low levels of antibody secretion. Immunofluorescence was negative for all negative controls (data not shown).

Polyreactivity of the transformants. Neither of the transformants demonstrated binding to insulin, thyroglobulin, TT, human IgG Fc, or double-stranded DNA (data not shown). *C. lucidilae* binding was negative for both antibodies (data not shown).

SPA and gp120 binding of transformants. Experiments to determine SPA and gp120 binding of the transformants were done because previous reports have suggested that B-cell superantigens bind antibodies that utilize human V_H3 gene elements (5, 48). Both 2E9 and a high concentration of 3B6 bind SPA (Fig. 3). The reactivity of 2E9 with SPA is approximately twofold less than that of polyclonal human IgM and 25 to 50 times greater than that of 3B6 (Fig. 3). 2E9 but not 3B6 binds to both glycosylated and nonglycosylated derivatives of HIV type 1 SF2 gp120 (data not shown). Approximately 50 μg of 2E9 per ml is necessary to demonstrate binding to gp120 (data not shown). This concentration is 50 times greater than has been reported for a myeloma by use of the ELISA described above (5). An enhanced signal ELISA that utilizes biotinylated goat anti-human IgM (Fisher Biotech) followed by streptavidin-alkaline phosphatase (Fisher Biotech) demonstrated binding at slightly lower concentrations (data not shown).

Reactivity of transformants with 2H1αId. 2H1 is one of a large number of protective idiotype-positive murine GXM MAbs (13). As shown in Fig. 4, 2E9 and 3B6 have little homology at the amino acid level with the murine MAb 2H1. The rabbit anti-idiotypic reagent 2H1αId (13) does not react with 2E9 or 3B6 (data not shown). To confirm this result, the ELISA with 2H1αId was repeated with antigen capture using GXM-coated plates and with isotype antibody capture using anti-human IgM (Sigma)-coated plates with 2H1αId as the detection reagent. 2H1αId failed to recognize the human GXM cell lines by each assay. MAb 2E9 was used in competition experiments with protective murine MAbs 2D10 (IgM) and 2H1 (IgG1). MAbs 2D10 and 2H1 recognize the same epitope. There was no competition between 2E9 and the murine GXM MAbs. MAb 3B6 was not used because of low antibody affinity and poor immunoglobulin secretion by the cell line.

2E9 idiotype expression of pre- and post-GXM-TT vaccination sera. The sera of 10 additional GXM-TT recipients were made available by our collaborators to determine idiotype expression. Postimmunization sera of the PBL donor and all 10 additional vaccinees studied (the results of 8 of 10 are shown) demonstrated an increase in idiotype expression compared with that of preimmunization sera (Fig. 5). This rise in idiotype expression was detected by antigen capture ELISA by subtracting the absorbance obtained by detecting serum GXM antibodies with the rabbit preimmune serum from the absorbance obtained with the rabbit 2E9 anti-idiotypic serum. Treatment of sera with β-mercaptoethanol resulted in a 10 to 47% reduction in idiotype expression of both pre- and postimmunization sera (data not shown). Since the rabbit antiserum does not bind human IgM constant regions, this suggests that the 2E9 idiotype is expressed predominantly by antibodies of the IgM isotype. An ELISA in which serum antibodies were captured with human anti-IgM and -IgG (Fisher Biotech) demonstrated the presence of the 2E9 idiotype in all sera tested (data not shown). Some of the binding of 2E9 (idiotype) to GXM (antigen) is inhibitable by the rabbit anti-idiotypic serum (data not shown). Competitive-inhibition ELISAs did not demonstrate inhibition of post-GXM-TT vaccination sera to GXM by the anti-idiotypic serum (data not shown).

Sequence analysis of the transformants. The variable-region gene utilization of 2E9 and 3B6 is shown in Table 1. Both antibodies use the same V_L with different members of the V_H3 family. 2E9 uses J_H6 with an unidentified D_H, and 3B6 uses J_H4b with $D_HDXP'1$. The V_L sequences of 3B6 and 2E9 are shown in Fig. 6, and their V_H sequences are shown in Fig. 7 and 8, respectively. For V_L, the sequences are compared with

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     1                                  CDR1     40                 CDR2                 69         79         89              CDR3              101
2E9  EVQLLESGG GLVQPGGSLR LSCAASGFTF SNYWMIWVRQ APGKGLEWVA NIKQDGGERY YVGSVTGRFT ISRDNARNSL YLQMNSLRAED TAMYYCAR RTAGVSLPYYAM  DVWGQGTAVTVSS
3B6  --------- ---------- ---------- I--G-G---- ---E-----S G-SGS--TSH FAD--K---- ---E-SK-T- S-----Q---- --V----K DSSYYYGSGSYY  TY-----LV----
2H1  D-K-V---- ---KL----K -C-------- -SLF-S---- Y-E-R--L-- T-NSN-DKT- HPDT-K---- ------K-T- ----S-LKS-- --L----- -DGYA-H      YFNY-----TL----
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FIG. 4. Amino acid sequences of the human GXM MAbs compared with that of murine GXM MAb 2H1 (38). Amino acid homology between MAbs is indicated by a dashed line. The amino acids of the CDRs are indicated in boldface type.

that of Humlv117, the $V_\lambda 1$ gene that has the most homology with both 3B6 and 2E9, a genetic element reported in a natural, polyreactive, anti-DNA antibody and a Burkitt's lymphoma (11, 51). The rare changes between the 3E9 and 3B6 coding sequences and the Humlv117 sequence (Fig. 6) could be due to somatic mutation, polymorphism, or the use of a heretofore-unreported $V_\lambda 1$ germ line gene. The nucleotide changes in codons 1, 4, and 5 are encoded by the PCR primer. Therefore, 2E9 and 3B6 are 97% homologous with germ line Humlv117. Subsequent analysis with a PCR primer to the leader sequence revealed that the sequences of 2E9 and 3B6 were identical to that of Humlv117 from codons 1 to 16. The initial nucleotide of codon 17 represents a change from A to G, conferring an amino acid change from lysine to glutamic acid in both 2E9 and 3B6 (Fig. 6). The calculated pIs for the 2E9 and 3B6 V_L regions were 4.40 and 4.12, respectively.

Both 2E9 and 3B6 use members of the V_H3 immunoglobulin gene family (Table 1). DNA was not available from the PBL donor for identification of germ line immunoglobulin genes. Therefore, nucleic acid differences between each cell line and a putative germ line gene identified from the GenBank search were evaluated. After nucleotide 23 (nucleotides 1 to 23 were introduced by the PCR primer), 3B6 is 95% homologous with the V_H3 family member V_H26 (17) (Fig. 7), and 2E9 is 95% homologous with the V_H3 family member HHHG19G (35) (Fig. 8). For 3B6, seven nucleic acid changes occur in the framework regions (FW), one is in complementarity determining region 1 (CDR1), and six are in CDR2 (Table 2 and Fig. 7). Amino acid changes are conferred by both of the changes in CDR1, i.e., serine to asparagine 31 and serine to glycine 35, and five of the six changes in CDR2, i.e., alanine to glycine 50, serine to threonine 57, threonine to serine 58, tyrosine to histidine 59, and tyrosine to phenylalanine 60. In FW3, there are amino acid changes at codon 79, cysteine to serine, and at codon 81, leucine to glutamine. The changes that alter hydrophobicity or charge are the change from alanine to glycine at codon 50, which is a change from a nonpolar to a polar residue; the change from tyrosine to histidine at codon 59, which is a change from a noncharged aromatic residue to a positively charged basic residue; and the change from tyrosine to phenylalanine at codon 60, which is a change from a hydrophilic to a hydrophobic residue. For 2E9, six of the nucleic acid changes are in the FWs, two are in CDR1, and four are in CDR2 (Table 2 and Fig. 8). All of the changes in both CDRs confer amino acid changes, as follows: for CDR1, serine to asparagine 31 and serine to threonine 35; for CDR2, serine to glycine 55, lysine to arginine 57, asparagine to glycine 61, and lysine to threonine 64. For 2E9, amino acid changes are conferred by nucleic acid changes in codon 5 of FW1 (valine to leucine), in codon 75 (lysine to arginine), and in codon 89 (valine to methionine of FW3). The calculated pIs for the coding sequences of the putative germ line genes for 2E9 and 3B6 are compared with those of the expressed V_H and VDJ regions in Table 3. The pI of the 3B6 V_H region (amino acids 1 to 94) increases to

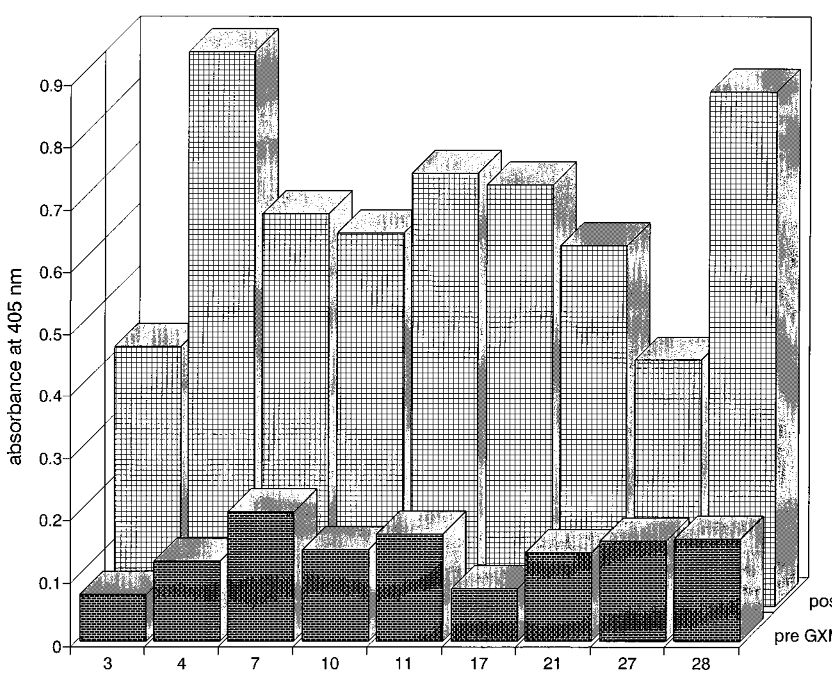

FIG. 5. 2E9 idiotype expression of pre- and postimmunization sera from GXM-TT conjugate vaccine recipients. Binding of a single dilution of each serum to a polyclonal rabbit anti-2E9 antiserum was determined by ELISA as detailed in the text. Binding is indicated on the y axis by A_{405}, and the x axis represents pre- and postimmunization serum samples from the subjects indicated by number designation. Shown are data from 9 subjects. An additional two subjects and the PBL donor were tested with another plate, and these results are not shown.

TABLE 2. R/S ratios of FW and CDRs of GXM antibodies[a]

Cell line	FW1		CDR1		FW2		CDR2		FW3		FWs		CDRs	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
2E9[b]	0	3	2	1	0	0	4	0	2	0	2	3	6	1
3B6[c]	1	1	2	0	1	1	8	2	3	1	5	3	10	2

[a] R, nucleotide base changes that lead to an amino acid replacement; S, nucleotide base changes that do not lead to an amino acid replacement. R and S substitutions have been inferred from comparison with the most-homologous gene identified by GenBank search (see the text). R/S ratios were calculated beginning with nucleotide 24 of FW1 since nucleotides 1 to 23 were encoded by the PCR primer. The number of nucleotides for each region is as follows: FW1, 67; CDR1, 15; FW2, 42; CDR2, 51; FW3, 96.

[b] For 2E9, the R/S ratio for all FWs is 0.97%/1.5% or 0.66 and for all CDRs is 9%/1.5% or 6 (R substitutions: 7.8% CDR1, 13% CDR2).

[c] For 3B6, the R/S ratio for all FWs is 2.4%/1.5% or 1.6 and for all CDRs is 15.1%/3% or 5 (R substitutions: 13% CDR1, 15.6% CDR2).

7.18 from that of the germ line V_H26, namely, 4.68. For 2E9, only the change from lysine to threonine at codon 64 confers a change from a positively charged residue to a noncharged residue. This change lowers the isoelectric point of the 2E9 V_H region (amino acids 1 to 98) to 8.66 from that of germ line V_H family number HHHG19G, which is 9.33.

DISCUSSION

The role of human GXM antibodies in protection against infection with *C. neoformans* remains unknown, although the existence of naturally occurring CPS and/or GXM antibodies in human serum is well documented (20, 23, 25, 28). This paper reports the fine specificity and V_H-V_L gene element utilization of human GXM MAbs 2E9 and 3B6 and (2E9) idiotype expression of GXM antibodies from additional human GXM-TT recipients. The human IgM(λ) MAbs, like GXM-TT-elicited murine GXM MAbs, bind GXM serotypes of *C. neoformans* other than the serotype A strain used for immunization (Fig. 1). The human MAbs have different epitope specificities. They neither compete with murine MAbs for GXM binding nor express the 2H1 idiotype, and they demonstrate different immunofluorescent labelling patterns (39). Our data show that human GXM-TT-elicited GXM antibodies are idiotype restricted like murine GXM antibodies (13, 16) and human *H. influenzae* conjugate-elicited antibodies (36). A cross-reactive idiotype was demonstrated for the latter with a polyclonal goat reagent raised against polyclonal antibodies from an *H. influenzae* conjugate recipient (36). The 2E9 antiserum was raised against an MAb of the IgM isotype; this reagent is unlikely to define a cross-reactive idiotype because it may not react with antibodies that have undergone affinity maturation. The 2E9 idiotype most likely represents GXM antibodies that use V_H3 gene elements (see "Generation of polyclonal rabbit antiserum to 2E9" above) (Fig. 5). This confirms antibody variable-region restriction in another human polysaccharide antibody response (3, 36, 52). The elimination of some binding in all sera following β-mercaptoethanol treatment suggests that the 2E9 idiotype is used predominantly by anti-GXM IgM. The use of different V_H gene elements or somatic mutation-induced changes during affinity maturation of anti-GXM IgG antibodies could result in a lack of binding to the 2E9 antiserum. Both 2E9 (monoclonal IgM) and polyclonal post-GXM-TT vaccination serum (detected with anti-IgG) produced similar fluorescent labelling patterns, suggesting that they could bind a similar epitope (39). Additional idiotypic reagents are necessary to determine whether anti-GXM IgG antibodies arise from 2E9-like IgM precursors.

TABLE 3. pIs of putative germ line V_H and expressed VDJ genetic elements used by GXM MAbs

V-region codons	pI[a]			
	HHHG19	2E9	VH26	3B6
V (codons 1 to 94)	9.33	8.66	4.68	7.18
VDJ (codons 1 to 113)		8.66		5.84

[a] pIs were determined by use of the GCG software (version 7.3) on the basis of published sequences for the coding sequences for germ line VH26 (17) and HHHG19 (35) from codons 1 to 94 and sequences determined in this study for 2E9 and 3B6 (Fig. 6 and 7). For VDJ, the pI values include amino acids 1 to 94 and the D_H and J_H segments.

The V_L and V_H gene elements expressed by the GXM MAbs have also been reported in fetal B lymphocytes (17, 35, 49). Although the J_H genes used by 3B6 and 2E9 are preferentially used in fetal immunoglobulin, they are also the most commonly used J_H genes in human B lymphocytes (27). The CDR3 regions of 2E9 and 3B6 differ markedly on the amino acid level and derive from different D_H and J_H elements (Table 1). CDR3 length and complexity contribute to the generation of antibody diversity (42), and both 2E9 and 3B6 have 12-amino-acid CDR3 regions, longer than most CDR3 regions reported in fetal immunoglobulin (44). Murine GXM antibodies also use V_H gene elements that occur frequently in fetal immunoglobulin (20). Biased murine V_H gene usage has been attributed to the 3′ location of the 7183 family within the immunoglobulin locus (20). However, the V_H3 gene family is not similarly located (57). Since human and murine GXM-TT-elicited MAbs both use V_H genes that derive from the same V_H clan, i.e., V_H3 and 7183, respectively (33), structural features of this group of antibodies could be important for binding GXM.

Antigen-independent selection of murine antibodies (24) and binding of fetal lymphocytes to polysaccharide antigens (51) support the hypothesis that selection by an unknown antigen could produce biased V_H utilization in polysaccharide antibody repertoires. V_H26, the putative germ line gene for 3B6, is also used by anti-DNA, anti-*H. influenzae* type b polysaccharide, anti-insulin, and antiviral antibodies (17, 29, 30). Neither HHHG12G, the putative germ line gene for 2E9, nor FL 13-28 (44), an identical sequence isolated from fetal immunoglobulin (44), has been reported previously in an antibody of known specificity. GXM is a T-cell-independent type 2 antigen (56); however, GXM-TT induces a T-cell-dependent response (15). Somatic mutation and antigen selection appear to be important in the derivation of GXM antibodies (10, 38): in comparison with that of their putative germ line genes, the 2E9 and 3B6 V_H regions both have higher ratios of replacement to silent (R/S) nucleotide substitutions for CDR regions than for FW regions (Table 2). High R/S ratios in CDR2 also occur in murine GXM MAbs (38). Apparent nucleotide substitutions could be attributable to unreported germ line genes or to polymorphism of V_H3 genes (2) rather than somatic mutation; however, we and others have used consensus sequences to infer the location of somatic mutations (38, 43).

The fine specificity of murine anti-DNA and protective human anti-*H. influenzae* CPS antibodies is determined in part by specific charged residues (4, 45). The GXM MAbs do not bind DNA (see Results). However, GXM, like DNA, consists of negatively charged carbohydrate epitopes (9, 34). Since electrostatic interactions have been implicated in the binding of cationic immunoglobulins to negatively charged molecules such as insulin and DNA (29, 45), positively charged residues

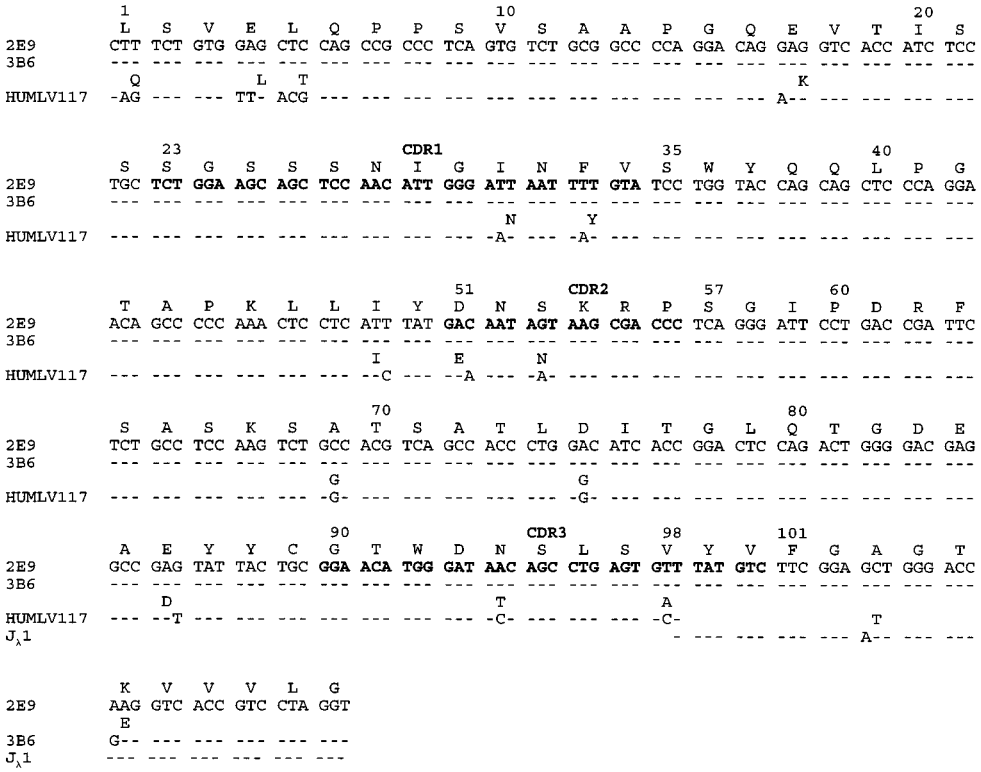

FIG. 6. Nucleotide and amino acid sequence of GXM MAb 2E9 and 3B6 V_L compared with that of putative germ line gene Humlv117 (55) and germ line gene $J_\lambda 1$ (31). Sequences were generated as detailed in the text and compared with the GenBank sequence (21) by use of GCG software. Homology is indicated by a dashed line. CDRs are indicated in boldface type. Single-letter codes for amino acid translation of each codon are displayed above each codon. The codon of the germ line sequence is indicated by a letter only when it differs from that of 2E9 and/or 3B6.

might have enhanced binding to negatively charged polysaccharide capsules such as GXM. For the GXM-binding MAbs, 2E9 has nine V_H arginine residues and 3B6 has five (Fig. 7 and 8). Most of these arginine residues are located in CDR2 and FW3, important regions for antigen binding (1). When compared with its putative germ line HHHG19 sequence, three of nine 2E9 arginine residues are not germ line encoded and could have arisen from somatic mutation, namely, one in CDR2, one in CDR3, and one in FW3 (Fig. 8). For 3B6, all arginine residues appear to be germ line encoded (Fig. 7). Since their V_L sequences are virtually identical, the higher apparent affinity of 2E9 for GXM compared with that of 3B6 (Fig. 1) may be attributable to an increased electrostatic potential of the more-cationic 2E9 V_H region (pI of 8.66 compared with 5.84 for 3B6) (Table 3). Confirmation of the importance of positively charged amino acid residues for binding cryptococcal polysaccharides must await further studies.

SPA, a B-cell superantigen, binds to a subset of human V_H3-expressing antibodies outside the conventional antigen binding site (48). Proposed binding sites for SPA include residues 6 to 24 of FW1, residues 67 to 84 of FW3, and residues at the 3′ end of CDR2, including residue 57 (26). The binding site for HIV gp120 has not been reported. The GXM MAb amino acid sequences are similar in FW1 and FW3 and largely divergent in CDR2, FW3, and CDR3 (Fig. 4). This could account for MAb differences in reactivity with GXM and B-cell superantigens SPA (Fig. 3) and HIV gp120 (see Results). V_H3 genes that contain potential superantigen-binding sites are used in other anti-CPS antibodies (4, 49, 53) and in antiviral antibodies (30); however, the biological significance of superantigen-antipathogen antibody interactions is unknown. At least three explanations for V_H3 gene element restriction of antibodies against pathogenic organisms are plausible. (i) The use of V_H3 is stochastic since it is the largest human V_H family (57). (ii) V_H3 antibodies provide the optimal structure for reactivity with given antigenic determinants such as polysaccharides (33). (iii) Superantigen-mediated selection of V_H3 genes restricts the repertoire of GXM and other T-cell-independent polysaccharide-binding antibodies. The last explanation might clarify why features consistent with both superantigen-driven and antigen-driven responses, demonstrating restriction to V_H3 gene elements and evidence of somatic mutation and antigen selection (10), respectively, occur in antibodies reactive with T-cell-independent antigens. The GXM MAbs have probable somatic mutations and a shared V_L, suggestive of an antigen-driven response (10, 24), yet their use of different V_H3, D_H, and J_H genes is consistent with a superantigen-driven response.

Both expansion and deletion of V_H3-expressing B cells have been reported in HIV-seropositive individuals and AIDS patients, respectively (6, 7). HIV gp120 (superantigen)-mediated deletion of important subsets of V_H3-bearing B cells that encode protective antibodies could contribute to the high rate of infections with polysaccharide-encapsulated pathogens and *C. neoformans* in HIV-infected individuals (14, 54). Our present efforts to analyze more human GXM antibodies and to characterize protective human GXM idiotypes and isotypes will further our understanding of the role of superantigen-mediated selection of the human GXM antibody repertoire.

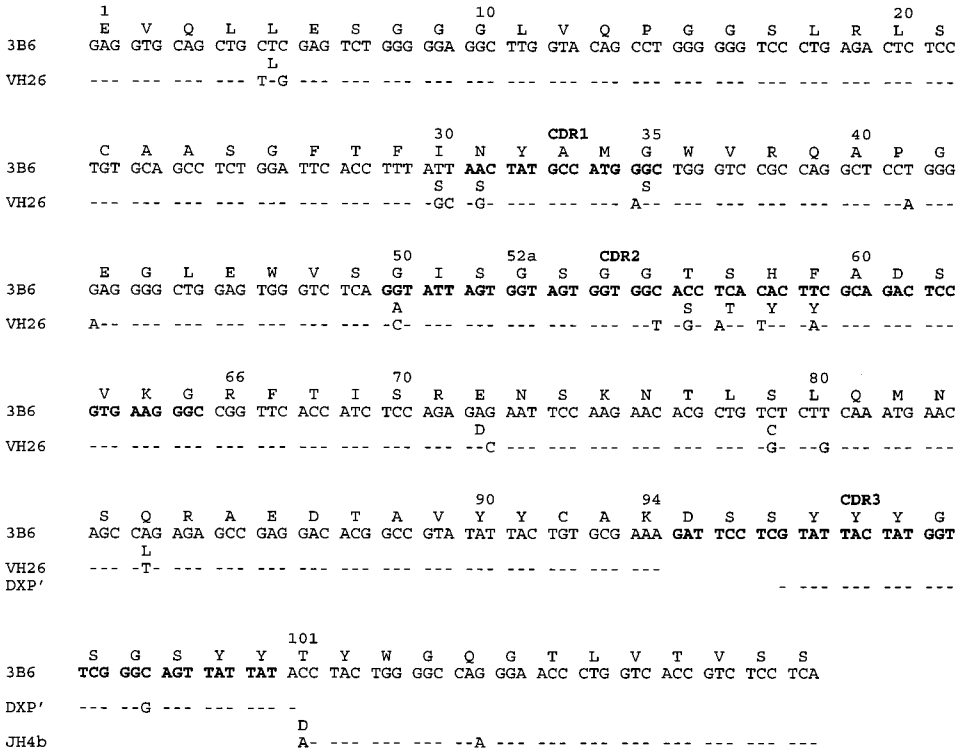

FIG. 7. Nucleotide and amino acid sequences of GXM MAb 3B6 V_H compared with that of putative germ line gene VH26 (17). Sequences were generated as detailed in the text and compared with GenBank sequences (21) by use of GCG software. Homology is indicated by a dashed line. CDRs are indicated in boldface type. Single-letter codes for amino acid translation of each codon are displayed above each codon. The codon of the germ line sequence is indicated by a letter only when it differs from that of 3B6. The D region sequence is compared with that of DXP′, the most-homologous germ line D segment (31). The J region is compared with that of JH4b (31).

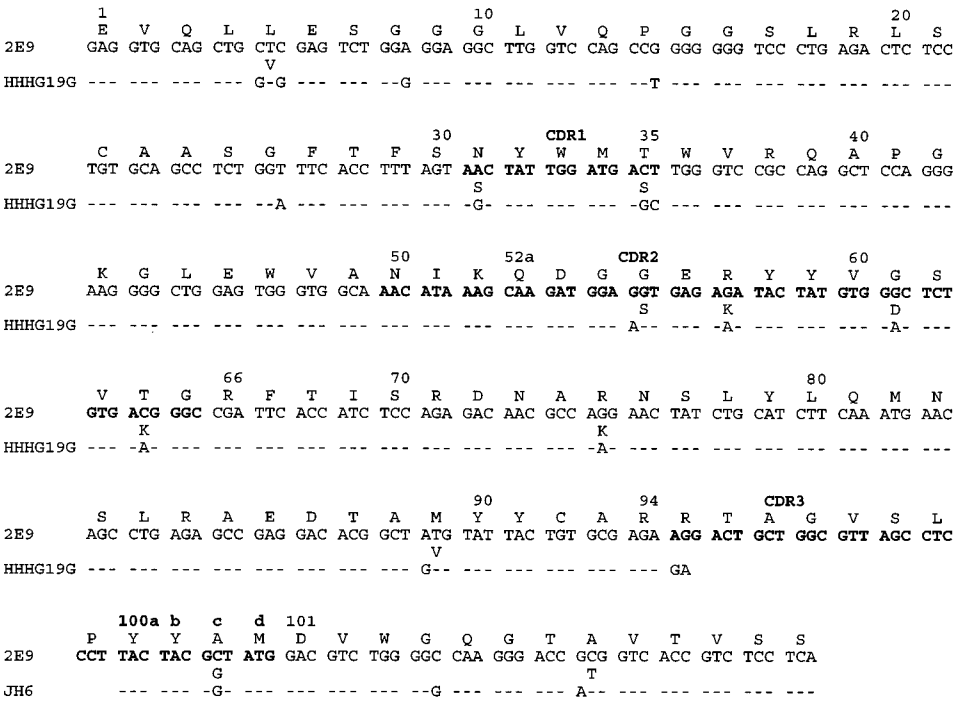

FIG. 8. Nucleotide and amino acid sequences of GXM MAb 2E9 V_H compared with that of putative germ line gene HHHG19G (35). Sequences were generated as detailed in the text and compared with GenBank sequences (21) by use of GCG software. Homology is indicated by a dashed line. CDRs are indicated in boldface type. Single-letter codes for amino acid translation of each codon are displayed above each codon. The codon of the germ line sequence is indicated by a letter only when it differs from that of 2E9. A putative germ line D segment for 2E9 (with homology of greater than 6 nucleotides) cannot be identified from published human D genes (21, 31). The J region is compared with JH6 (31).

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