

Molecular Characterization of the Humoral Responses to *Cryptococcus neoformans* Infection and Glucuronoxylomannan-Tetanus Toxoid Conjugate Immunization

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Summary

The molecular characteristics of the humoral immune response to a serotype A *Cryptococcus neoformans* infection were compared with the response elicited by a cryptococcal glucuronoxylomannan-tetanus toxoid (GXM-TT) conjugate. Anticryptococcal monoclonal antibodies (mAbs) isolated from both responses have previously been shown to recognize the same antigenic determinant of cryptococcal GXM. Southern blot and sequence analyses indicate that the hybridomas isolated from each response arose from only a few precursor B cells. All the mAbs generated from the infected and GXM-TT conjugate-immunized mice utilize the same V_H7183 family member: J_H2/J_H4, v_K5.1, and J_K1; mAbs generated by different B cells had complementarity-determining region 3's (CDR3s) composed of seven amino acids with a common sequence motif. Thus, the molecular analysis of these anticryptococcal mAb-producing hybridomas indicated that the response to both cryptococcal infection and conjugate immunization was oligoclonal and highly restricted with regard to immunoglobulin gene utilization. The GXM-TT conjugate primarily stimulated isotype switching and clonal proliferation, and did not result in hybridomas expressing additional immunoglobulin repertoires. The mAbs from both responses had a number of replacement mutations at the 5' end of CDR2 that appear to be the result of antigen-driven selection. Somatic mutation also resulted in altered epitope specificity for one mAb, 13F1. Passive administration of representative mAbs from different clones generated in response to the GXM-TT conjugate prolonged survival of lethally infected mice.

Cryptococcus neoformans is a ubiquitous organism that is a potential opportunistic pathogen of immunocompromised individuals (1). In recent years cryptococcal infections have become an increasing problem for AIDS patients (2). The major determinant of *C. neoformans* virulence (3) is a capsule composed of a polysaccharide that accumulates in body fluids and tissues during cryptococcal infection (4, 5). *C. neoformans* has been divided into five serotypes (A, B, C, D, and AD) (6, 7), each of which exhibits structural differences within the glucuronoxylomannan (GXM)¹ component of the capsular polysaccharide (8–14). Infected individuals rarely have detectable anticryptococcal antibodies (1). Unresponsiveness to cryptococcal infection and immunization with purified *C. neoformans* capsular polysaccharide (CNPS) has been attributed to immune paralysis, which is probably mediated

by T suppressor cells (15, 16). Moreover, purified CNPS is a T cell-independent (TI) type 2 antigen (16, 17) and does not induce Th cell-mediated amplification of the humoral immune response. In addition, we have shown that the ~5% of mice that produce antibodies during infection with clinical isolate mount a very restricted antibody response with respect to isotype, Ig variable gene utilization, and clonal proliferation (18).

There is considerable evidence that anti-CNPS antibodies contribute to host defense. Such antibodies enhance fungistasis by NK cells (19, 20), promote phagocytosis by macrophages (21, 22) and peripheral blood monocytes (23), and facilitate fungal killing by PBMC (24). Patients with cryptococcosis have a more favorable prognosis if serum anticryptococcal antibodies are present (25). Moreover, AIDS patients lack CNPS-specific IgG, suggesting that a deficiency in antibody immunity may contribute to their marked susceptibility (26). Although purified CNPS is a poor immunogen, conjugates composed of CNPS components and protein carriers have been developed as potential vaccines to stimulate antibody

¹ Abbreviations used in this paper: aka, apparent affinity constant; CNPS, *Cryptococcus neoformans* capsular polysaccharide; GXM, glucuronoxylomannan; R/S, replacement/silent; TD, T cell dependent; TI, T cell independent; TT, tetanus toxoid.

production (12, 27–29). In addition, passive administration of certain mAbs (30, 31) or polyclonal sera (32, 33) prolong survival of lethally infected mice. mAbs have also been shown to improve the efficacy of amphotericin B (34, 35). However, not all antibodies are equally protective (31, 36, 37). Identification of beneficial antibodies and their characteristics is important for determining whether such antibodies are generated in response to conjugate vaccines.

We recently reported the isolation of six IgM κ and one IgG1 κ mAbs from a serotype A *C. neoformans*-infected BALB/c mouse, and eight IgM κ , one IgG3 κ , nine IgG1 κ , and four IgA κ mAbs from a BALB/c mouse immunized with a T cell-dependent (TD) serotype A GXM-tetanus toxoid (GXM-TT) conjugate (38). These mAbs all recognize a common antigenic determinant present on the GXM of the four serotypes of CNPS (38). By determining the primary nucleotide structure of these anti-CNPS mAbs, we have been able to compare the basic antibody structures and gene usage of specific mAbs generated in response to CNPS present during cryptococcal infection and GXM-TT immunization. Examination of the Ig gene rearrangement patterns and CDR3 sequences has enabled us to also determine the clonal origins of the B cells that participated in response to CNPS during infection and immunization with the GXM-TT conjugate. Our results provide insight into the B cell response to cryptococcal antigens and are of fundamental importance with regard to vaccine design and passive mAb therapy of cryptococcosis.

Materials and Methods

***C. neoformans* Strains and Polysaccharides.** Serotype A (strain 24064) and D (strain 24067) *C. neoformans* were obtained from the American Type Culture Collection (ATCC; Rockville, MD). These strains were maintained on Sabouraud's dextrose agar (Difco Laboratories, Detroit, MI) at 4°C and grown with moderate shaking at 37°C in Sabouraud's dextrose broth (Difco Laboratories). CNPS was isolated as described (3).

Hybridomas and mAbs. Hybridomas that produced anticryptococcal polysaccharide-specific mAbs were isolated and characterized as described previously (38) from a serotype A (ATCC 24064)-infected BALB/c mouse and a serotype A (NIH 371) GXM-TT conjugate (27)-immunized BALB/c mouse. These hybridomas were generated at the Albert Einstein College of Medicine Cancer Center Hybridoma Facility (Bronx, NY).

Apparent Affinity Constant (aka) Determination. The *aka* of each mAb for serotype A CNPS (CNPS-A) was determined by the method of Nieto et al. (39). The assay involved measuring the inhibition of mAb binding to CNPS-A-coated microtiter plates in the presence of soluble CNPS-A. 1.5 μ g mAb was incubated 1.5 h at 37°C in the presence of decreasing concentrations of soluble CNPS-A in microtiter plates (25801; Corning Glass Works, Corning, NY) coated with 100 ng CNPS-A and blocked with 1% BSA and 0.5% horse serum. The assay was developed using alkaline phosphatase-labeled goat anti-mouse heavy chain-specific reagents (Fisher Biotech, Orangeburg, NY), followed by addition of alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO). *aka* were calculated assuming a molecular weight of 1.4×10^6 for CNPS-A (40).

Southern Blot Hybridization. Productive and nonproductive rearrangements of the heavy and light chain genes were analyzed as described previously (18). For analysis of Ig heavy chain rearrange-

ment patterns, hybridoma, NSO myeloma fusion partner, and BALB/c liver DNA were digested with the restriction enzyme EcoRI, and blots were probed with the 2-kb BamHI-EcoRI J μ -3-J κ -4 probe, J μ 11 (41). For analysis of Ig light chain rearrangement patterns, hybridoma, NSO myeloma fusion partner, and BALB/c liver DNA were digested with the restriction enzyme HindIII, and blots were probed with the 2.7-kb HindIII-HindIII J κ -1-5 probe (42).

mRNA Sequencing. Total cellular RNA was isolated by CsCl gradient sedimentation of hybridoma lysates prepared in a guanidinium solution (4.23 M guanidine isothiocyanate; (Fluka Bio-Chemika, Buchs, Switzerland), 0.017 M *N*-lauroylsarcosine, 0.025 M sodium citrate, pH 7.0, 0.015 M β -mercaptoethanol, 0.0011% anti-foam A). Poly(A)⁺ mRNA was isolated using an oligo(dT) (Collaborative Research Incorporated, Bedford, MA) affinity column. The nucleotide sequence of hybridoma antibody mRNA was determined using a modification of the method of Geliebter et al. (43), and representative sequence data are available from EMBL/GenBank/DBJ under accession numbers Lo5431 and Lo5432. The following oligonucleotides, synthesized in the DNA synthesis facility of the Cancer Center at our institution, were used as primers: TGGATGGTGGGAAGATG (V κ), GACCCAGAAAATCGGTT (5' V κ), TCTCGCAGGAGACGAGGGGGA (μ), GACGAGGGGGAAGACATT (μ), GGGGCCAGTGGATAGAC (γ ₁), AAGTAGGCCTTTGACAAGGCA (γ ₃), GAGTGTCAGTGGGTAGATGGT (α), and TGTCTTGGCATTTGCTCTG (V μ 7183). Sequence searches and comparisons were performed using the Genetics Computer Group (GCG) Sequence Analysis Software (SAS) Package (44) and the sequence compilation of Kabat et al. (45). Theoretical replacement/silent (R/S) ratios were calculated using the Replacement/Substitution program written by D. Lustgarten and J. Mindell (Albert Einstein College of Medicine).

Protection Studies. Protection studies were performed as described in detail elsewhere (31). Briefly, ascites containing 600 μ g mAbs 10F10, 17E12, or 18B7, or 200 μ l ascites from the non-Ig-producing fusion partner myeloma, NSO, were administered intraperitoneally to 10 female A/J mice in each group. Approximately 30 min later, 10⁸ serotype D (ATCC 24067) *C. neoformans* was administered intraperitoneally. Serotype D cryptococci were used because we wanted to determine whether our mAbs, generated against serotype A and reactive with serotypes A–D (38), would protect against both pathogenic serotypes. Results of each experiment were analyzed by a log-rank analysis program written by Dr. C. J. Chang (Albert Einstein College of Medicine) using the SAS statistical software package.

Results

mAbs. As previously reported (38), two independent sets of anticryptococcal antibody-producing hybridomas were isolated. Seven were derived from a serotype A *C. neoformans*-infected BALB/c mouse, and 22 were derived from a serotype A GXM-TT conjugate-immunized BALB/c mouse. These mAbs were of the IgM, IgG3, IgG1, and IgA heavy chain and κ light chain isotypes (Table 1), and based on competition experiments and reactivity with CNPS, all recognize the same antigenic determinant present on serotype A, B, C, and D CNPS (38). We have now characterized the molecular structure of each of these mAbs and determined their clonal origins and relative binding to CNPS.

Ig Gene Utilization. To determine the molecular structure and variable gene usage, the heavy and light chain Ig mRNA sequence of each mAb was determined for the heavy

Table 1. *Hybridoma Clonal Families and mAb V_H and V_L Usage*

Group	B cell clone	Hybridoma	mAb isotype	aka	Gene usage				
					V _H	J _H	V _L	J _L	
A	1	8H3	μk	* × 10 ⁹ /M	V _H 7183	J _H 4	V _L 5.1	J _L 1	
		1E10	μk	7.0	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		3B10	γ ₁ k	3.0	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		3D1	μk	21	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		4D4	μk	1.5	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		5E9	μk	13	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		7E5	μk	19	V _H 7183	J _H 2	V _L 5.1	J _L 1	
B	3	2D10	μk	209	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		2H1	γ ₁ k	2.5	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		3H5	γ ₃ k	13	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		13G12	αk	1.6	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		18G9	αk	1.3	V _H 7183	J _H 2	V _L 5.1	J _L 1	
	4a	4G9	γ ₁ k	5.2	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		7G7	μk	41	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		9F11	γ ₁ k	1.8	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		12A1	μk	38	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		12F4	γ ₁ k	0.8	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		13F1	μk	39	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		14E1	γ ₁ k	1.3	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		15E8	μk	6.1	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		16E4	μk	0.8	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		18B7	γ ₁ k	5.4	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		25G12	αk	1.6	V _H 7183	J _H 2	V _L 5.1	J _L 1	
		4b	5E4	μk	1.3	V _H 7183	J _H 2	V _L 5.1	J _L 1
			7D8	αk	0.5	V _H 7183	J _H 2	V _L 5.1	J _L 1
	10F10		γ ₁ k	1.5	V _H 7183	J _H 2	V _L 5.1	J _L 1	
	25E12		γ ₁ k	1.7	V _H 7183	J _H 2	V _L 5.1	J _L 1	
	4c	9E11	μk	12	V _H 7183	J _H 2	V _L 5.1	J _L 1	
	4d	17E12	γ ₁ k	11	V _H 7183	J _H 2	V _L 5.1	J _L 1	

Groups A and B refer to hybridomas derived from a serotype A-infected mouse and a serotype A GXM-TT conjugate-immunized mouse, respectively.
* Too low to be measured.

chain sequences (Table 1 and Fig. 1). The mAbs isolated from both the infected and GXM-TT conjugate-immunized mice define a highly restricted response with respect to Ig variable gene usage. 27 of the 29 mAbs isolated from both mice were very homologous to each other and almost certainly use the same germline V_H7183 gene element, have a seven-codon CDR3, and utilize J_H2, V_L5.1, and J_L1 (Table 1). A consensus V_H7183 sequence was derived based on the germline V_H7183:283 sequence (46) by incorporating into the V_H7183:283 sequence those base changes shared by all the mAbs from

both the infected and GXM-TT conjugate-immunized mice. The consensus V_H7183 sequence is 94.9 and 95.6% homologous (44) to the germline V_H7183:283 (46) and V_H7183:10-19 (47) gene sequences, respectively, suggesting the anti-CNPS mAbs are encoded by an as yet unreported V_H7183 family member. When compared with the 29 mAb sequences, 18 mAbs had an A at the first base of codon 54 (Fig. 1), resulting in an asparagine residue. The two described germline V_H7183 (283 and 10-19) sequences (46, 47) each have a G at this position (Fig. 1) encoding an aspartic acid. Since

Table 2. V_H and V_L Ig Gene Rearrangement Patterns

Group	Hybridoma*	H ⁺	H ⁻	L ⁺	L ⁻
				<i>kb</i>	
A	8H3	2.4	-	3.6	-
	1E10, 3B10, 4D4, 5E9	3.2	4.2	3.6	-
	3D1	3.2	-	3.6	-
	7E5	3.2	6.0, 4.2, 2.4	3.6	-
B	2D10, 2H1, 3E5, 18G9, 13G12	3.2	2.8	3.6	-
	4G9, 7G7, 9F11, 12A1, 12F4, 13F1				
	14E1, 15E8, 16E4, 18B7, 25G12	3.2	-	3.6	-
	5E4, 7D8, 10F10, 25E12	3.2	-	3.6	4.1
	9E11	1.8	-	3.6	-
	17E12	3.2	-	3.6	3.3

H⁺ and H⁻ refer to the productive and nonproductive rearrangement fragment sizes as determined with EcoRI-digested DNA probed with J_{H11}. L⁺ and L⁻ refer to the productive and nonproductive rearrangement fragmented sizes as determined with HindIII-digested DNA probed with J_{k1-5}. (-) No hybridizing fragment was detected.

* Hybridomas in groups A and B were isolated from the serotype A-infected and GXM-TT-immunized mice, respectively.

this base difference was not exhibited by all the mAbs and was not present in the known germline sequences, we did not include it in our consensus sequence. However, this G → A change may indeed be germline encoded or, alternatively, may be a somatic mutation that increases the binding of CNPS.

The V_H CDR3 of the anti-CNPS mAbs can be organized into four different consensus nucleotide sequences (Fig. 1). Although CDR3 regions of individual mAbs deviate from the consensus CDR3 sequences, presumably due to somatic mutation, and are thus not identical, each encodes seven amino acids that include 5' arginine and aspartic acid residues followed by a highly conserved pattern of charge, polarity, and phobicity. For example, the CDR3 sequence of the 1E10 group of mAbs from the infected mouse encodes 5' arginine, aspartic acid, glycine, tyrosine, phenylalanine, serine, and histidine 3'. The CDR3 of the 4G9 group of mAbs from the conjugate-immunized mouse encodes 5' arginine, aspartic acid, glycine, threonine, serine, glycine, and threonine 3' (Fig. 1). Unusual reading frames, inversions, deletions, and combinations of reported germline D sequences (45) were examined, but it was not possible to identify a known D segment in our mAbs.

Therefore we do not know whether a germline D element participated in the formation of the V_H CDR3 or whether this region of our antibodies is at least partially due to N sequences introduced enzymatically during VDJ rearrangement (48, 49). The light chains of all the mAbs use the same $V_{\kappa 5.1}$ and $J_{\kappa 1}$ gene elements and have the same V_L - J_L junctions (data not shown), suggesting that these specific light chain sequences are important in formation of a binding site capable of interacting with CNPS.

2 of the 29 mAbs, 8H3 from the infected mouse and 17E12 from the GXM-TT conjugate-immunized mouse, utilize similar Ig gene elements as the other mAbs but have structural differences within these elements. mAb 8H3 utilizes the same D and V_L sequences as the other 27 mAbs, but its V_H 7183 sequence has a two-codon deletion at positions 55 and 56 relative to the V_H 7183 consensus sequence (Fig. 1). It is conceivable that the 8H3 V_H sequence represents another member of the V_H 7183 family or, alternatively, the missing codons are merely due to a deletional somatic mutation. In addition, 8H3 uses J_H4 (Table 1) with a 5' two-codon deletion, resulting in a J_H region that encodes the same number of amino acid residues and displays the same

Figure 1. V_H nucleotide sequences of the anti-CNPS mAbs. The mAbs in the 8H3 and 1E10 groups were isolated from the serotype A-infected mouse. The mAbs in the 2D10 and 4G9 groups were isolated from the serotype A GXM-TT conjugate-immunized mouse. Sequences from clonally related hybridomas are grouped, followed by a space. V_H sequences are compared to the consensus V_H 7183 sequence. The N/D region sequences are compared with consensus sequences determined from the mAbs within each clonally related group. The J_H sequences are compared with J_H2 or J_H4 . Spaces indicate that the sequence is uncertain or has not been determined; dashes indicate identity; (DEL) deletion of a codon; asterisks indicate uncertainty in a base due to compression; capital letters indicate nucleotide changes resulting in amino acid replacements; silent nucleotide changes are indicated by lower-case letters. Framework (FR) and CDR (45) are indicated above the consensus V_H , D, and germline J_H2 and J_H4 sequences. The ACA codon at the 3' end of the hybridoma 3E5 J_H2 sequence is reported by Kabat et al. (45) to be the first 5' codon of the IgG3 heavy chain constant region. However, we (18) and others (Joan L. Press, personal communication) have discovered an intervening GCT codon 3' of J_H and 5' of the ACA codon that is not encoded by any J_H sequence or by the reported IgG3 constant heavy chain gene. V_H 7183:283 (46) and V_H 7183:10-19 (47) are germline BALB/c sequences; V_H 62 is from BALB/c hybridoma mAb 62 (84); and the J_H2 and J_H4 germline sequences were obtained from Kabat et al. (45).

junctional amino acid as that encoded by the J_H2 region used by all the other mAbs (Fig. 1). mAb 17E12 utilizes the same Ig gene elements (the same V_H7183 consensus, J_H2 , $V_{\kappa}5.1$, and $V_{\kappa}1$) and the sequence of its CDR3 is very similar to the other mAbs, except for a one-codon deletion at residue 102 (Fig. 1).

Clonal Origin of Hybridomas. The finding that all the anticytotoxic hybridomas isolated (except 8H3, which uses J_H4 instead of J_H2) utilize the same combination of heavy and light chain Ig gene elements (Table 1) suggests the responses that occurred in both the infected and conjugate-immunized mice were oligoclonal. The clonal origin of each response was studied by examining the heavy and light chain Ig gene rearrangement patterns of each hybridoma by Southern blot hybridization (Table 2). The J_H3 - J_H4 probe (J_{11}) hybridizes to EcoRI germline BALB/c liver DNA restriction fragments of 6.4 and 5.4 kb, and a NSO myeloma restriction fragment of 6.6 kb. The light chain $J_{\kappa}1$ -5 probe hybridizes to HindIII germline BALB/c liver and NSO myeloma DNA restriction fragments of 2.4 and 6.4 kb, respectively. Table 2 summarizes the sizes of the productive and nonproductive J_{11} heavy and $J_{\kappa}1$ -5 light chain hybridizing restriction fragments, but does not include germline (i.e., unrearranged) and myeloma fragments. The productive rearrangement of the V_H7183 consensus and J_H2 sequences used by all the hybridomas (except 8H3) was assigned to the 3.2-kb band, which is the only hybridoma-specific restriction fragment common to the heavy chain J_{11} hybridization patterns of all hybridomas, except 9E11 and 8H3 (Table 2). The 9E11 hybridization pattern lacked the 3.2-kb band, presumably due to a change in a restriction site resulting from somatic mutation. Therefore, the productive 9E11 V_H7183 - J_H2 rearrangement was assigned to the sole hybridoma-specific 1.8-kb band. The productive rearrangement of the V_H7183 consensus and J_H4 sequences used by hybridoma 8H3 was assigned to the single 2.4-kb hybridoma-specific J_{11} -hybridizing band. The productive rearrangement of the $V_{\kappa}5.1$ and $J_{\kappa}1$ light chain sequences used by all the anticytotoxic hybridomas was assigned to the 3.6-kb band present in each $J_{\kappa}1$ -5-hybridizing light chain rearrangement pattern. The remaining heavy and light chain hybridoma-specific rearrangement pattern bands were assumed to be due to nonproductive Ig gene rearrangements (Table 2).

Patterns of heavy chain rearrangement in conjunction with CDR3 sequences were used to determine the clonal relatedness of the hybridomas isolated from each response. Somatic mutations present in several members of a clone are more likely to represent a single inherited mutational event rather than multiple independent identical events, and were thus used along with patterns of light chain rearrangement to confirm clonal assignment and arrange hybridomas from each clone into specific genealogical sublineages (Table 1) (50–53). The hybridomas isolated from the infected mouse exhibit four distinct patterns of heavy chain rearrangement (Table 2), suggesting four progenitor B cells generated the seven B cells isolated as hybridomas from this response. Since its Ig heavy chain uses J_H4 instead of J_H2 (Table 1), hybridoma 8H3 is clearly the product of a separate B cell ancestor, designated clone 1. Since hybridomas 1E10, 3B10, 4D4, and 5E9 share

the same pattern of heavy and light chain productive and nonproductive rearrangements (Table 2), have very similar CDR3 sequences and junctions (Fig. 1), and V_L - J_L junctions, they must have been derived from a second common ancestral B cell (clone 2 in Table 1) (52). Although hybridomas 3D1 and 7E5 each exhibit different heavy chain rearrangements and could have been derived from two separate B cell precursors, nucleotide sequence homology, including CDR3 and its junctions (Figure 1), strongly suggests 3D1 and 7E5 were also derived from clone 2 (52). It is important to note that while nonproductive rearrangements are very useful in establishing clonal relationships, they can easily be lost as hybridomas reduce their chromosome load (54). We cannot explain the multiple fragments in the 7E5 heavy chain rearrangement pattern (Table 2), though it is conceivable 7E5 arose from a triple fusion. Thus, the hybridomas isolated from the infected mouse were probably derived from two, but not more than four, separate B cell precursors, and clone 2 dominated the response.

Three separate heavy chain rearrangement patterns were exhibited by the hybridomas isolated from the GXM-TT conjugate-immunized mouse (Table 2). However, besides the 17E12 six-amino acid D, only two distinct seven-amino acid CDR3 sequences are present in mAbs from the conjugate-immunized mouse (Fig. 1), suggesting these hybridomas are derived from only two different B cell precursors. Based upon common productive and nonproductive heavy chain rearrangement patterns and CDR3 sequence homology, hybridomas 2D10, 2H1, 3E5, 18G9, and 13G12 were derived from a common precursor B cell (clone 3 in Table 1). Similarly, hybridomas 4G9, 7G7, 9F11, 12A1, 12F4, 13F1, 14E1, 15E8, 16E4, 18B7, and 25G12 were derived from a second common precursor B cell (clone 4a). 5E4, 7D8, 10F10, and 25E12 also have the same CDR3 sequence as members of clone 4a, but have an additional, presumably nonproductive, light chain rearrangement (Table 2). As noted above, lack of this nonproductive rearrangement by some members of clone 4 may be due to chromosome loss. We have provisionally assigned hybridomas 5E4, 7D8, 10F10, and 25E12 to a separate branch of clone 4, designated 4b (Table 1), but they may represent a separate clone due to their unique light chain rearrangement pattern and somatic mutational differences (Fig. 1). Although hybridoma 9E11 displays a different heavy chain rearrangement pattern, presumably due to somatic mutation, its CDR3 sequence is the same as that of hybridoma 12F4 and 25E12 (Fig. 1), and its light chain rearrangement is the same as those of clone 4a (Table 2), suggesting but not proving 9E11 was also derived from clone 4. Although hybridoma 17E12 has the same heavy chain rearrangement pattern as clone 4a and 4b members, it does not share the same light chain pattern (Table 2). Moreover 17E12 has a six-, not seven-, amino acid D segment (Fig. 1). However, alignment of the six amino acids within the 17E12 CDR3 relative to others in clones 4a and 4b (Fig. 1) indicates that with the exception of the one-codon deletion, the sequence is identical to that of other mAbs in these clones, including mAbs 5E4 and 7G7. Thus, it is not possible to ascertain whether 17E12 is a member of clone 4 that underwent a deletion and a separate light chain rearrangement, or was the product of a separate B cell

Table 3. R/S Ratios and Incidence of Somatic Mutation in the V_H of Selected Clones

B cell clone	R/S ratios					Incidence of somatic mutation*				
	FRs	CDR1	CDR2 codons		CDR3	FRs	CDR1	CDR2 codons		CDR3
			50-66	50-58				50-66	50-58	
Theoretical [†]	2.76	5.56	3.34	3.24	- [‡]					
2	16/6	2/0	16/0	13/0	4/1	0.014	0.016	0.052	0.08	0.025
3	5/2	2/0	10/2	8/1	2/0	0.0055	0.019	0.047	0.067	0.012
4a,b	29/17	4/2	18/2	17/1	11/2	0.012	0.019	0.026	0.044	0.026

* Incidence of somatic mutation is the ratio of the total number of unique somatic mutations to the total number of bases within a particular region of all the members of a clone.

† The theoretical R/S ratios, determined using the Replacement/Substitution Program, represent the relative number of replacement-to-silent mutations that would be expected if somatic mutation of each were entirely random.

‡ The theoretical R/S CDR3 ratios of the mAbs in clones 2, 3, and 4a,b are 4.0, 4.0, and 4.75, respectively.

precursor, designated clone 4d. Thus, although we may have recovered hybridomas from as many as five different B cell precursors, the conjugate response was dominated by two or possibly three B cell clones.

Somatic Mutations. Somatic mutations relative to consensus V_H and CDR3 and germline J_H , V_L , and J_L Ig sequences were observed for all the anticryptococcal mAbs from both the infected and GXM-TT conjugate-immunized mouse. Most of the light chains had between one and five somatic mutations randomly distributed throughout the frame works and CDRs (data not shown). There were more mutations in the heavy chain (Fig. 1 and Table 3), as has also been observed in other responses (55). Table 3 summarizes the number of unique replacement (R) and silent (S) mutations present in the heavy chain of the mAbs in clones 2-4. Mutations present in more than one member of a clone were considered to be due to an inherited event and were counted only once. The ratio of R/S mutations in each B cell clone can be compared to theoretical values of each variable region segment calculated for that particular segment assuming somatic mutation is a random event. The R/S ratios of V_H CDR2 (codons 50-66) for clones 2, 3, 4a, and 4b are considerably higher than the theoretical R/S ratio for this region (Table 3). Moreover, the bulk of these contributory R mutations are clustered at the 5' end of CDR2 between codons 50 and 58 (Fig. 1 and Table 3). The high R/S ratios and localization of these replacement mutations within a discrete region of CDR2 strongly suggests antigen selection was involved and that these somatic mutations are not the result of random events. The incidence of somatic mutation is also higher in CDR2, especially between codons 50 and 58, than in CDRs 1 and 3 and the frameworks. The incidence of somatic mutation within each region of the mAbs in each clone also indicates that the relative occurrence of somatic mutation in each mAb is approximately the same in each clone (Table 3).

mAb Apparent Affinity. The aka's were compared by determining the amounts of soluble CNPS-A required to inhibit the binding of the antibody to immobilized CNPS-A

(39), as described in Materials and Methods. The aka's of the IgG mAbs were within a 15-fold range. The IgA antibodies exhibited similar aka's, and although IgA molecules are polymeric, their aka's were within the range of aka's exhibited by the IgG mAbs, suggesting the real affinities of the IgA mAbs may be lower than the IgG mAbs. Even within a single clone (Table 1, clone 4a), the IgM mAbs from the response to GXM-TT conjugate immunization had a wider range of relative binding than IgM mAbs isolated from the response to cryptococcal immunization, suggesting affinity maturation occurred in response to the GXM-TT conjugate (see below).

Protection Experiments. We have previously shown that the IgG1 mAb 2H1 from clone 3 effectively prolonged survival of mice lethally infected with *C. neoformans* (31). This prompted us to determine whether mAbs from other B cell clones stimulated by GXM-TT conjugate immunization were also protective. The IgG1 mAbs 18B7, 10F10, and 17E12 are derived from clones 4a, 4b, and 4d, respectively, and have somewhat different aka's (Table 1). Fig. 2 shows survival data

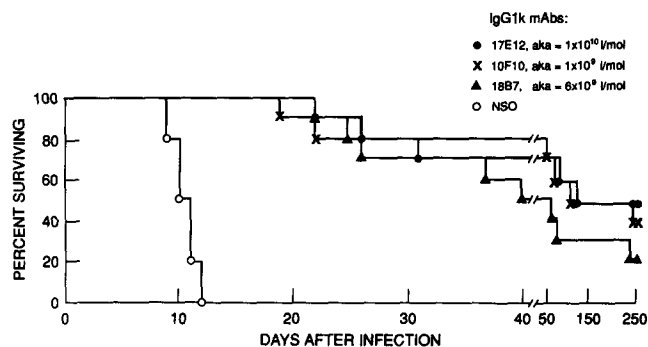


Figure 2. Survival of A/J mice after intraperitoneal treatment with 600 μ g of the IgG1 κ mAbs 10F10, 17E12, or 18B7, or 250 μ l NSO myeloma ascites preceding infection with 10^8 serotype D (ATCC 24067) *C. neoformans*. Average lengths of survival are: 10F10, 146 ± 33 d; 17E12, 80 ± 13 d; and 18B7, 100 ± 31 d.

of A/J mice given 600 μ g mAb 10F10, 17E12, or 18B7 intraperitoneally before intraperitoneal challenge with 10^8 serotype D (ATCC 24067) *C. neoformans*. All three mAbs provided passive protection as evidenced by their ability to prolong survival. Mice given mAbs 10F10, 17E12, or 18B7 lived an average of 146 ± 33 , 80 ± 13 , or 100 ± 31 d, respectively, while control mice lived only 11 ± 0.3 d. However, the results are best reflected in Fig. 2 because these calculated survival values are biased due to the presence of some very long-term survivors in the experimental groups. The ability to prolong survival did not correlate with mAb aka since 17E12 and 10F10, which differ by 10-fold in aka, each give similar levels of protection, and 18B7 with an intermediate aka was the least protective (Table 1 and Fig. 2). However, in conjunction with our previous studies (31), this result does indicate that each B cell progenitor stimulated by the GXM-TT conjugate gave rise to protective antibodies.

Discussion

We have examined the molecular origins of the BALB/c murine humoral response to serotype A cryptococcal polysaccharide in infected and GXM-TT conjugate-immunized mice. On the basis of the hybridomas isolated, only a few progenitor B cells within each spleen proliferated to produce the B cell progeny responsible for each anticryptococcal response. The actual numbers of hybridomas isolated from each response can be used to obtain a rough estimate of the number of B cells activated *in vivo*, for it has been estimated that one hybridoma is isolated for every 10^3 to 10^4 activated B cells (56). Clonal analysis of the hybridomas from the infected mouse suggests that there was one dominant clone represented by five to six hybridomas, whereas the two major clones, 3, and 4a, isolated from the GXM-TT-immunized mouse, were represented by 5 and 11 members, respectively. Based on the number of hybridomas isolated, $\sim 7,000$ – $70,000$ B cells were activated in the spleen of the infected mouse, whereas $\sim 22,000$ – $220,000$ B cells were activated in the spleen of the GXM-TT conjugate-immunized mouse. Comparison of these estimates strongly suggests many more activated B cells were present in the spleen after conjugate immunization, consistent with the fact that GXM-TT conjugate-immunized mice had higher anticryptococcal serum titers than infected mice (38). This indicates the TD activity of the GXM-TT conjugate induced greater clonal proliferation relative to that induced by CNPS during cryptococcal infection.

Restriction with regard to antibody binding site structure was observed at several levels, including utilization of the same Ig heavy and light chain gene elements (the same germline V_H 7183 family member, J_H 2/ J_H 4, V_L 5.1, and J_L 1), joining of these particular genes at the same nucleotides, and a CDR3 with a highly conserved size and pattern of charge and polarity. The need for an antibody molecule with a conserved spatial arrangement of structural elements was emphasized by the finding that mAb 8H3 utilized a J_H 4 gene that had a deletion of two 5' codons, resulting in a J_H region that encoded

the same number of amino acids as that encoded by the J_H 2 gene utilized by all the other mAbs.

Although compared with antiprotein responses antipolysaccharide responses are typically characterized as being "restricted" with regard both to isotype and clonotype (57–64), restriction is a relative term. For example, although mAbs specific for α -(1 \rightarrow 6)-dextran have been shown to use a limited subset of V_H genes, there is considerable junctional diversity and variation in the combinations of V_H and V_L genes utilized (60, 65, 66). Likewise, the response to group A streptococcal polysaccharide has been shown to be characterized by a heterogeneous population of antibodies (59). Similarly, the TI response to NP-Ficoll is characterized by utilization of a variety of V_H , D, and J_H genes and their combinations (67). In contrast, the response to bacterial levan is somewhat more restricted, as indicated by a recently characterized set of mAbs that utilize gene elements from only three or four V_H families (57). The response to 3-fucosyllactosamine is even more restricted and characterized by mAbs composed of V_H 441 and V_L 24B (61, 62). However, with its preferential V_H and V_L gene usage, lack of junctional diversity, and conserved CDR3 sequences, the anticryptococcal response appears to be among the most highly restricted antibody responses described to date (18). The finding that all the anti-CNPS mAbs (except 8H3) utilize the same V region elements suggests very few antigen binding sites are available that will suffice for recognition of cryptococcal polysaccharide and/or that the response was directed by structural features of the antigen. *In vivo*, a single dominant immunogenic epitope of cryptococcal polysaccharide may be recognized. This epitope may also be structurally simple, however, structural simplicity is not sufficient to mediate restriction of Ig gene usage for highly defined epitopes that can be recognized by a diverse set of V_H and V_L regions (68). In fact, even though polysaccharides are composed of fewer types of chemical subunits than proteins, they have greater conformational and rotational freedom than proteins and can thus assume a greater multiplicity of structures (69, 70). Alternatively, only a subset of B cells, defined by their Ig gene usage, may be capable of responding to cryptococcal polysaccharide. Indeed, CNPS may mimic a self-antigen, and the responder B cells may be those that either do not produce a self-reactive antibody or can easily escape clonal deletion or anergy.

Somatic mutations were observed within the Ig V_H and V_L sequences of all the anti-CNPS hybridomas isolated from both infected and conjugate-immunized mice. In particular, V_H CDR2 exhibited a relatively high rate of somatic mutation. This region could be a somatic mutational "hot spot" (71, 72). Alternatively, a selective advantage may be conferred to B cells expressing surface Igs with such mutations since CDRs encode amino acids that contact the antigen (73) and could confer increased affinity and/or changes in epitope specificity (74–77). Indeed, although the Ig gene elements utilized by the anti-CNPS mAbs were the same, differences in both mAb's apparent affinity and fine specificity were observed. The IgM mAbs isolated from the infected mouse all exhibited akas between 1.5×10^9 and 2×10^{10} M, whereas

the IgM mAbs isolated from the GXM-TT conjugate-immunized mouse exhibited a broader range of akas even within a single clone (Table 1). Selection of high affinity clones in response to conjugate immunization is likely since only a small amount (5 μ g) of the antigen was present (38). Conversely, during infection, affinity maturation may not occur simply because there are very large amounts of antigen in circulation, and thus a high affinity Ig receptor may not provide a selective advantage.

The incidence of base changes and the relatively high ratio of replacement mutations in the heavy chain CDR2 suggest the response to CNPS is antigen driven and antigen selected. Since some of the mAbs within each of the clones have higher akas than others of the same isotype (Table 1), somatic mutation must be responsible for the differences in affinity. However, accumulation of somatic mutations did not necessarily lead to higher affinity mAbs, i.e., affinity maturation. For example, one of the highest binding (4.1×10^{10} M) mAbs, 7G7, from clone 4a, contains relatively few somatic mutations and replacement substitutions (V_H : 50, thr \rightarrow ileu, 84, ser \rightarrow thr, 95, tyr \rightarrow phe; V_L : 90, val \rightarrow ileu, 95, gln \rightarrow leu). 7G7 also lacks the asparagine and aspartic acid substitutions at codons 54 and 56, respectively, which are present in most of the mAbs, including mAbs with high akas such as 2D10. Using somatic mutations present in the heavy and light chain sequences, we organized the members of clone 4a into a possible genealogy (Fig. 3). We have tried to compare other members of the 4a genealogy that are of the same isotype but differ with respect to aka. The IgM mAbs 15E8 and 12A1 differ by sixfold in aka (Table 1) and by 14 heavy and light chain amino acid substitutions, five of which are in CDRs (Fig. 1). Similarly, 16E4 and 13F1 differ by 40-fold in aka and by 11 amino acid substitutions, seven of which are in CDRs. In addition, relative to the monomeric IgG1 mAbs, the dimeric IgA mAbs of clones 3 and 4a have unexpectedly low akas (Table 1). Since a single amino acid substitution can result in a 10-fold increase in affinity or complete loss of antigen binding (77, 78), it is difficult to identify specific amino acid substitutions responsible for differences in antigen binding in the absence of a three-dimensional antigen binding site model. These hybridomas almost certainly represent a "snapshot" of a single point during the ongoing process of the immune response in which Igs with a spectrum of affinities are produced. Thus, using hybridomas, it may be difficult to demonstrate that accumulated mutations lead to an increase in affinity. Indeed, as long as a particular threshold affinity is maintained, it may not matter in the response to CNPS whether very high affinities are attained, since lower affinity antibodies may be sufficient to trigger proliferation in response to this polymeric antigen. Apparent affinities may also not accurately reflect the efficacy of antigen-antibody interactions in triggering B cell proliferation (79).

A change in fine specificity was observed with respect to the IgM mAb, 13F1, isolated from the GXM-TT conjugate-immunized mouse. mAb 13F1 is a member of clone 4a, and all the anti-CNPS mAbs, including those of this family, have a fine specificity pattern for the four CNPS serotypes of A

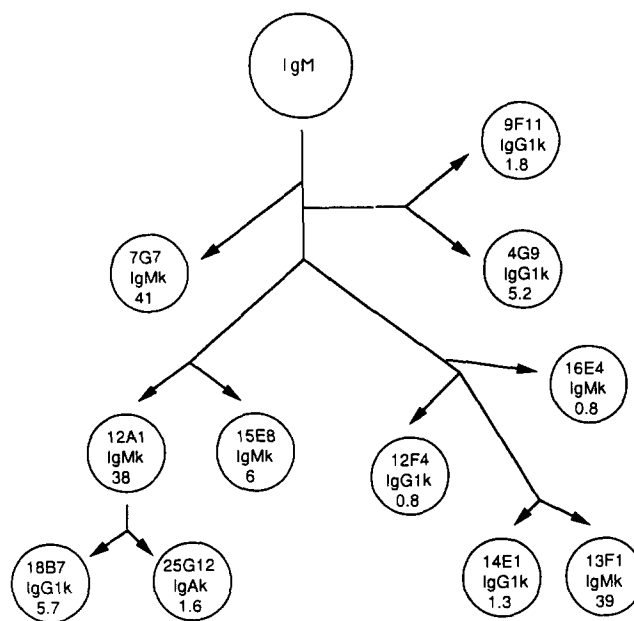


Figure 3. Schematic representation of one possible genealogy for the members of clone 4a. The progenitor B cell is represented by the large circle. Individual hybridomas are indicated by smaller circles designated with the respective mAb name, isotype, and apparent affinity ($\times 10^9/M$).

$> B > D > C$. However, the fine specificity pattern of 13F1 is $A > B > C > D$ (38). Since the gene utilization and combinatorial joining of these elements is identical among the members of clone 4a, somatic mutation must be responsible for the difference in fine specificity, supporting a similar observation by Clarke et al. (80). A unique three-base change at V_H residue 38, which results in an arginine to threonine substitution, may contribute to the change in fine specificity exhibited by mAb 13F1.

Molecular analysis of the antibody response to infection and GXM-TT immunization revealed that both responses are very similar. mAbs from both responses have the same epitope specificity (38), use the same V_H and V_L genes, and have somatic mutations. The most striking difference between the two sets of hybridomas is the isotype distribution. IgM mAbs dominated the response to infection, and IgG mAbs dominated the response to GXM-TT conjugate immunization. The finding that specific mAbs isolated from both responses utilize a highly restricted repertoire of Ig gene elements suggests both infection and conjugate immunization stimulate the same discrete subpopulation of B cells, and moreover indicates preservation of biologically relevant epitopes within the GXM-TT conjugate. Since the response was both oligoclonal and highly restricted, it is likely that only a few progenitor B cells in each mouse exhibited the particular V_HDJ_H combination needed to respond to the cryptococcal antigens presented in association with the appropriate light chain. Since a response was elicited by all mice immunized with the GXM-TT conjugate (38), each mouse must have B cells with the required V_HDJ_H rearrangement capable of associating with the necessary V_LJ_L gene combination. This strongly

suggests that the absence of an antibody response observed for the majority of infected mice (18, 38) is not due to lack of the necessary B cell but rather to immune paralysis induced by the large amounts of CNPS present (81). We have previously proposed that the ~5% of mice that do respond to cryptococcal infection are those that had had prior exposure to a crossreactive TD antigen and that infection stimulated residual memory B cells (18, 38). The finding that the incidence and nature of somatic mutations in CDR2 of the antibodies from the infected mouse were comparable to those isolated from the GXM-TT conjugate-immunized mouse (Fig. 1 and Table 3) is indeed consistent with the view that infection stimulated a secondary response (18).

The results of the study presented here are of fundamental importance not only with regard to understanding the physiology of the B cell populations involved in the humoral response against *C. neoformans*, but also with respect to vaccine design and passive mAb therapy. The GXM-TT conjugate

(or a similar derivative) is a potential vaccine for individuals at risk for cryptococcosis (27). Since individuals with serum anticryptococcal antibodies have a more favorable prognosis (25), the finding that GXM-TT conjugate immunization and cryptococcal infection stimulate the same subpopulation of B cells is important for it implies GXM-TT vaccination will elicit the same type of antibodies occasionally present in infected individuals. Moreover, each B cell clone stimulated by the GXM-TT conjugate produced protective antibodies, as demonstrated by our previous finding that IgM, IgG1, and IgA mAbs from clone 3 (31), as well as the IgG1 mAbs 18B7, 10F10, and 17E12 isolated from clones 4a, 4b, and 4d, respectively, prolong survival of lethally infected mice. This is an important observation, for not all mAbs prolong survival (31, 36). The mAbs described here are potentially useful reagents for cryptococcal polysaccharide detection (82), structural analysis of capsular epitopes (12, 83), and passive immunotherapy of cryptococcosis (30, 31, 35, 36).

We thank Terri Kelly, Yoon Young Jhang, Gerardo Gomez, and Susan Buhl for expert technical assistance in various aspects of this work and Nancy Drenzyk for help in preparing this manuscript. We also thank Dr. Chee Jen Chang for his invaluable assistance in the statistical analysis of the protection data.

This work was supported by National Institutes of Health grants CA-39839-07, CA-13330-20, and AI-10702-20. J. Mukherjee is supported by NIH training grant 2T32C809173-16. M. D. Scharff is supported in part by the Harry Eagle Chair provided by the Women's Division of the Albert Einstein College of Medicine. A. Casadevall was supported by a Pfizer Postdoctoral Fellowship.

The data in this paper are from a thesis submitted by J. Mukherjee in partial fulfillment of the requirements of the Degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University.

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Received for publication 27 August 1992 and in revised form 28 December 1992.

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