Brief Definitive Report

Protective and Nonprotective Monoclonal Antibodies to *Cryptococcus neoformans* Originating from One B Cell

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Summary

Two immunoglobulin M monoclonal antibodies (mAbs) derived from the same B cell recognize different epitopes on the capsular polysaccharide of the pathogenic yeast, *Cryptococcus neoformans.* Their respective epitopes are located in spatially distinct regions of the capsule. Passive administration of one mAb prolonged survival whereas the other mAb did not. The results indicate that specificity is an important determinant of antibody efficacy against *C. neoformans* and that somatic mutations occurring during the antibody response can affect the protective efficacy of antibodies to *C. neoformans.*

In recent years there has been a dramatic increase in the number of *Cryptococcus neoformans* infections as a result of the AIDS epidemic. In New York City there were over 1,200 cases in 1991 with an estimated annual prevalence of 6–8% among AIDS patients (1). *C. neoformans* is unusual among fungal pathogens in that it has a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM) (2). The capsular polysaccharide is a T cell–independent antigen which often fails to elicit an antibody response (3) and the capsule is antiphagocytic (4). During cryptococcal infections, capsular polysaccharide is released into tissues where it may contribute to virulence by inhibiting leukocyte migration (5) and enhancing HIV infection (6).

In AIDS patients, cryptococcosis is often incurable because antifungal drugs usually fail to eradicate the infection despite in vitro susceptibility (7, 8). The difficulties in managing *C. neoformans* infections in severely immunosuppressed patients have stimulated interest in immunotherapy. Two therapeutic strategies under development are passive antibody administration (9) and immunization with GXM-tetanus toxoid (GXM-TT) conjugate vaccines (10). Both strategies rely on antibodies to enhance host nonspecific immunity against *C. neoformans* (11–15). In contrast to GXM, the GXM-TT vaccine is highly immunogenic (10) and elicits antibody responses characteristic of T cell–dependent antigens with a predominance of IgG isotypes (10, 16, 17). The antibody response to the GXM-TT vaccine is highly restricted in BALB/c mice in terms of variable gene utilization. These mAbs all use a V_H from the 7183 gene family rearranged to J_H2 (in one instance J_H4) and $V_k5.1$ rearranged to J_K1 (17). The expressed variable genes have frequent somatic mutations consistent with a T cell–dependent response (17). 11 hybridomas were determined to have originated from one B cell (17). One hybridoma, 13F1, was unusual in that it produced an IgM mAb that differed from the other members in the B cell genealogy in its relative reactivity with capsular polysaccharide from the four *C. neoformans* serotypes, suggesting a change in specificity (16, 17). Here we compare mAb 13F1 with mAb 12A1, another IgM from the same B cell genealogy (17). The V_H sequence of mAbs 13F1 and 12A1 differ by seven amino acids in the first and second CDR and three amino acids in framework regions. The V_L sequences of mAbs 13F1 and 12A1 differ by one amino acid in CDR1 and six amino acids in the V_L frameworks (Fig. 1). Since 13F1 and 12A1 share the same V_H and V_L CDR3 (Fig. 1) and nonproductive variable gene rearrangements, they originated from the same B cell (17).

Materials and Methods

C. neoformans *and Polysaccharide Antigen.* American Type Culture Collection (Rockville, MD) strain 24067 (serotype D) was used for all experiments. GXM was isolated from culture supernatants by cetyltrimethylammonium bromide precipitation as described (18).

mAbs. mAbs 13F1, 2D10, 3E5, and 12A1 have been described (16, 17). Hybridoma ascites was made in pristane-primed BALB/c mice. Ambiguities in the reported (17) V_H CDR3 sequences of

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V_H

	CDR1	FR2	CDR2	FR3	CDR3
		38		80 90	
Consensus	SYFMSWV	R	TINSNGGNTYYPDTVKG	Y D	RDGTFGNYYAM
2D10	-------	R	-V----DR---------	Y D	--SSGSL-L--
12A1	N------	R	MI-I---N-----I---	D D	----T------
13F1	--Y----	T	AI----GS---------	Y N	----T------

V_L

	FR1	CDR1	FR2	CDR2	FR3	CDR3
	14 20 22		51 54		63	
$V_k5.1$	S S S	RSSQSLVHSNGNTYLH	L Y	KVSNRFSG	V	SQSTHVPST
2D10	S * S	----------------	L Y	--------	V	---------
12A1	G S T	---------Y------	V N	--------	*	---------
13F1	S * S	----------------	L Y	--------	V	---------

Figure 1. Amino acid complementarity determining region (*CDR*) and framework (*FR*) sequence differences for mAbs 13F1, 12A1, and 2D10 and a consensus sequence of the V_H gene element (17). The consensus sequence is derived from 29 mAbs (17). mAb 2D10 is derived from a different genealogy than mAbs 13F1 and 12A1 and hence has a different V_H CDR3. Asterisks indicate uncertainty in FR1 position 20 of 2D10 and 13F1 and FR3 position 63 of 12A1.

mAbs 12A1 and 13F1 were resolved. Hybridoma RNA was prepared using an RNA isolation kit (Invitrogen, San Diego, CA), reverse-transcribed using the IgM constant region primer GACGAGGGGGAAGACATT, and amplified by PCR using this primer and the V_H7183 primer GAAGTGAAGCTGGTGGAGTCT. The amplified DNA was cloned into the TA Cloning System (Invitrogen) and sequenced by automated sequencing at the Albert Einstein College of Medicine. The CDR3s of mAbs 13F1 and 12A1 were identical (Fig. 1). Size-exclusion chromatography using a 46 cm Sephadex G-200 (Pharmacia, Piscataway, NJ) column was used to confirm that both mAbs 12A1 and 13F1 are polymeric IgM molecules of the same relative size.

ELISA. Antibody concentrations were determined by ELISA relative to isotype-matched standards of known concentration. mAb binding to GXM was determined by ELISA as described (16, 19, 20). Competition ELISAs were done by mixing mAbs 12A1 or 13F1 with the IgG3 mAb 3E5 and allowing them to compete for binding to plates coated with 10 μg/ml of GXM. Antibody binding was detected by isotype-specific goat anti-mouse alkaline phosphatase reagents.

Agglutination and Indirect Fluorescence. Antibody-mediated agglutination of *C. neoformans* 24067 (American Type Culture Collection) was determined in a 96-well plate containing various concentrations of either mAb 12A1 or 13F1 and 4×10^4 cells/well in 1% BSA in 0.020 M PBS, pH 7.2. Agglutination was determined by microscopic examination. Indirect immunofluorescence was done by adding mAbs 12A1 and 13F1 at 2 μg/ml to stationary phase organisms. mAb binding was detected using FITC-labeled goat anti-mouse IgM. Samples were viewed with a confocal microscope (MRC 600; Bio-Rad Laboratories, Hercules, CA) using a Nikon ×60 normal aperture 1.4 planapochromat optics and a Kr/Ar laser.

Protection Studies. C. neoformans was grown in Sabouraud's dextrose broth (Difco Laboratories, Detroit, MI) at 37°C. Yeast cells were washed three times with PBS, counted in a hemocytometer, and injected intraperitoneally into 6–8-wk-old female A/JCr mice (National Cancer Institute, Rockville, MD). The inoculum per mouse, 5×10^7 yeast, was confirmed by plating on Sabouraud's dextrose agar (Difco Laboratories). Antibody was administered intraperitoneally as ascites ~15 min before infection. Survival data were analyzed by Dr. C.J. Chang, a consultant statistician at our institution, using a log-rank analysis program. To determine the ability of intraperitoneal antibody to decrease serum GXM after infection, three groups of 5 A/JCr mice each were infected i.v. with 1.5×10^5 *C. neoformans* and 24 h later each group was given either 1.0 mg of mAb 13F1, 12A1, or 0.25 ml of PBS (untreated control) i.p. Serum GXM levels were measured before mAb administration on day 1 and then on days 2, 3, and 5 of infection by capture ELISA (19). Serum samples were treated with proteinase K and boiled (15, 21) before use to eliminate potential antibody–antigen complexes and other interfering substances.

Results and Discussion

mAbs 12A1 and 13F1 have similar apparent affinities when binding to constant amounts of polystyrene-immobilized antigen as measured by antigen inhibition ELISAs (16). However, when the concentration of GXM is varied, mAb 12A1

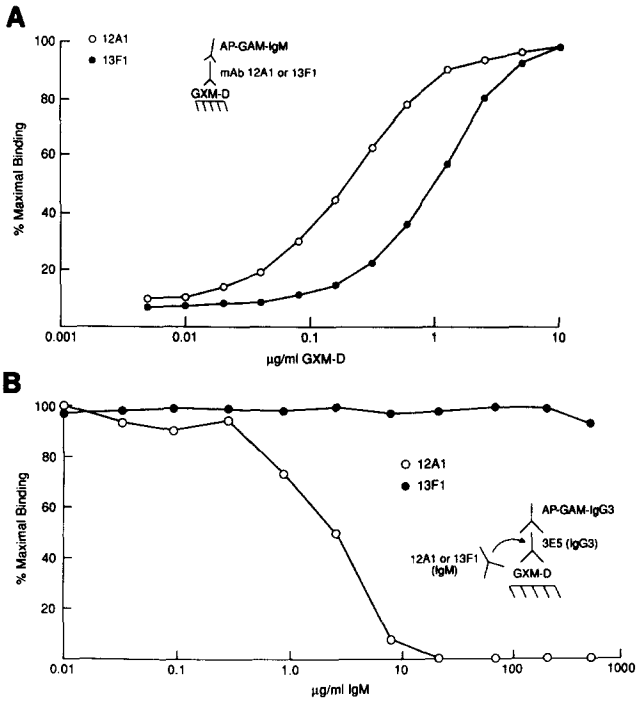

Figure 2. *A* shows binding of mAbs 13F1 and 12A1 to different concentrations of GXM bound to polystyrene plates. *B* shows a competition ELISA between IgM mAbs 12A1 and 13F1 versus the IgG3 mAb 3E5. Points show the binding of mAb 3E5 detected with alkaline phosphatase labeled goat anti-mouse IgG3 (*AP-GAM-IgG3*) and are the average of four measurements. At IgM concentrations >1 μg/ml the binding of mAb 3E5 is inhibited by mAb 12A1 but not mAb 13F1. The ELISA configurations used are diagrammed. GXM-D is serotype D GXM.

binds more strongly than 13F1 at the lower GXM concentrations (Fig. 2 *A*). Presumably, varying the GXM concentration alters the spatial distribution of polysaccharide binding sites such that mAb 12A1 binds more strongly. Serotype D polysaccharide was previously found to be more effective than serotype C polysaccharide in inhibiting mAb 12A1 binding to serotype A polysaccharide whereas polysaccharide C was more effective than D for mAb 13F1 (16). This implied that mAbs 13F1 and 12A1 differed in epitope specificity and this was confirmed in this study by antibody competition assays (Fig. 2 *B*). The competition ELISA tested the ability of mAbs 13F1 and 12A1 to inhibit an IgG3 mAb (3E5) and was designed to avoid IgM steric hindrance (Fig. 2 *B*). The binding of mAb 3E5 to GXM was inhibited by 12A1 but not 13F1 at IgM concentrations > 1 μg/ml. Inhibition of binding indicates competition for the same epitope or steric hindrance whereas absence of competition indicates binding to a different epitope. mAb 12A1, but not 13F1, inhibited mAb 3E5 binding to GXM, confirming that mAbs 12A1 and 13F1 differ in specificity.

Indirect immunofluorescence provided direct visual evidence for differences in binding to the *C. neoformans* capsule by mAbs 13F1 and 12A1. Serial sections of organisms analyzed by confocal microscopy revealed mAb 13F1 was distributed throughout the yeast cell capsule in a punctate pattern, whereas mAb 12A1 produced a homogenous annular fluorescence pattern (Fig. 3). Comparison of phase contrast and fluorescent images confirmed the annular fluorescence pattern of mAb 12A1 was located on the external border of the capsule. The punctate fluorescence pattern observed with mAb 13F1 revealed a previously unsuspected heterogeneity in capsular antigenic structure. Both mAbs 12A1 and 13F1 agglutinated *C. neoformans* indicating that some of their respective epitopes are present at exposed locations in the capsule. However, the agglutination end-points for mAbs 13F1 and 12A1 were 9 and 1 μg/ml, respectively. This difference in agglutination end-points is consistent with the fluorescence data which suggested that even in antibody excess mAb 12A1 remained on the surface of the capsule whereas mAb 13F1 penetrated the capsule.

When mAbs 13F1 and 12A1 were administered intraperitoneally similar serum concentrations were measured within 4 h and both mAbs disappeared from the circulation at approximately the same rate (data not shown). However, intraperitoneal administration of mAb 13F1 shortly before

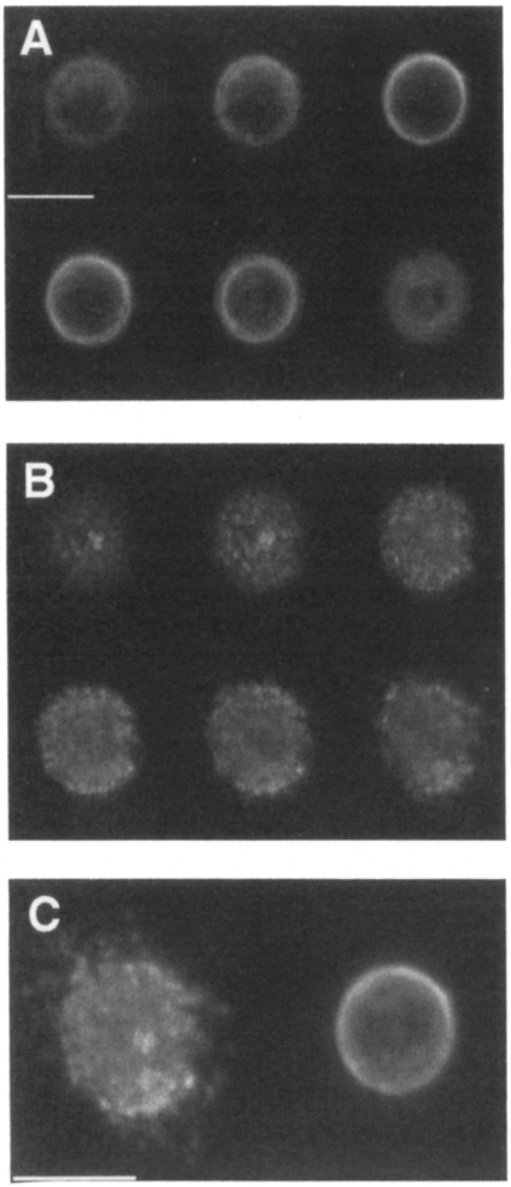

Figure 3. Indirect immunofluorescence of stationary phase organisms. Single optical sections obtained at 1.6-μm intervals with mAb 12A1 (*A*) and mAb 13F1 (*B*). Scale bar in *A* represents 10 μm in *A* and *B*. A composite of 16 optical sections of the organisms shown in *A* and *B* were collected at 0.8-μm intervals and imaged using the maximum projection method (*C*). Scale bar in *C* represents 10 μm.

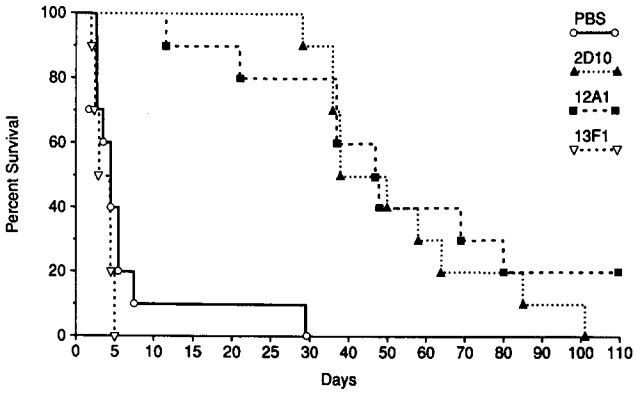

Figure 4. Survival of A/JCr mice given 1.0 mg of either mAbs 2D10, 12A1, or 13F1 or PBS (*control*) i.p. and infected i.p. with 5×10^7 *C. neoformans* 24067 (American Type Culture Collection). Average survival and standard deviation for the PBS, 2D10, 12A1, and 13F1 groups ($n = 10$ per group) were 6.75 ± 2.53, 53.4 ± 7.55, 51.1 ± 8.09, and 3.65 ± 0.37 days, respectively. *p* values obtained by log-rank analysis for the comparison of the PBS group versus 2D10, 12A1, and 13F1 groups were 0.0001, 0.0001, and 0.1104, respectively. The *p* value for the comparison between 12A1 and 13F1 groups was 0.0001.

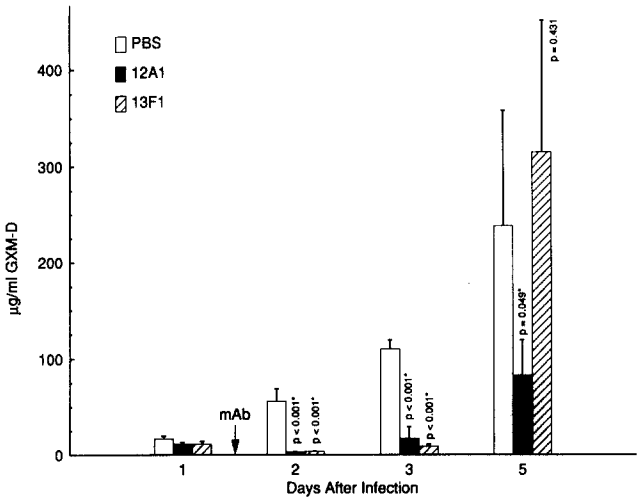

Figure 5. Serum GXM levels of mice infected i.v. with 1.5×10^5 *C. neoformans*. 1 d after infection mice were bled and then given 1.0 mg of either mAb 12A1, mAb 13F1, or 0.25 ml of PBS (*control*) i.p (*arrow*). Mice were then bled on days 2, 3, and 5 after infection. Points are the average of five mice; brackets denote SD. *P* values were calculated by *t* test relative to the PBS control using Primer of Biostatistics: The Program Version 3.01 (McGraw-Hill Inc., New York).

lethal infection of A/JCr mice had no effect on survival, whereas mAb 12A1 significantly prolonged survival relative to the control group (Fig. 4). Survival of mice given mAb 12A1 was similar to that of mice given mAb 2D10, a protective IgM (22) included in the experiment as a positive control. Thus the specificity difference between mAb 12A1 and 13F1 resulted in a marked difference in protective efficacy against *C. neoformans*. Murine cryptococcal infections result in high levels of serum capsular GXM antigen which is a reflection of fungal load, the amount of polysaccharide released into the circulation, and its rate of clearance. Passive antibody administration can reduce serum GXM levels (15). Both mAbs 13F1 and 12A1 were initially effective at reducing the concentration of serum GXM (Fig. 5). At day 3, the GXM levels of mice receiving either mAb 13F1 or 12A1 were ~10-fold lower than the control group. However, the effect was temporary and by day 5 there was a significant increase in serum GXM for both mAb 13F1 and 12A1 treated mice. These results suggest that mAb 13F1 was active in vivo despite its ineffectiveness in prolonging survival.

Somatic mutation of immunoglobulin variable regions is responsible for affinity maturation in antibody responses and generating neutralizing antibodies to viral pathogens (23). Somatic mutations can result in antibodies with different specificities to proteins (24) and haptens (25). Our results confirm this observation and extend it to the polysaccharide antigen of a fungal pathogen. Somatic mutation is generally believed to provide the host with a mechanism for increasing genetic diversity of the antibody response which may be useful in countering antigenic variation by pathogens. However, mAb 13F1 provides an example in which somatic mutation generated an antibody of markedly reduced protective efficacy despite retaining its avidity for antigen. The finding that protective efficacy of antibodies to the polysaccharide capsular antigen is dependent on specificity suggests a potential explanation for the contradictory observations obtained with passive administration of polyclonal antisera (26, 27). The structural heterogeneity of the capsule revealed by these two mAbs provides a potential explanation for the decreased efficacy of mAb 13F1. The immunofluorescence data indicate that most of mAb 13F1 is sequestered within the capsule. As has been demonstrated with mAbs to group A streptococci, the location of mAb binding is important for mAb function (28).

Polyclonal responses are likely to contain both protective and nonprotective antibodies and the preponderance of each type may be responsible for the effect observed. Similarly, susceptibility of individuals to cryptococcosis may vary depending on the type of antibody produced. Since mAbs 12A1 and 13F1 were elicited by GXM-TT immunization, it appears that this vaccine can elicit nonprotective, as well as protective, antibodies. The difference in protective efficacy of mAbs 13F1 and 12A1 also illustrates the need for careful epitope mapping of genetically engineered antibodies that are candidates for passive immunotherapy.

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