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The common *Cryptococcus neoformans* glucuronoxylomannan M2 motif elicits non-protective antibodies

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ABSTRACT

The *Cryptococcus neoformans* capsular glucuronoxylomannan (GXM) is a potential vaccine antigen that can elicit protective and non-protective antibodies. In an attempt to focus the immune response on a single antigenic component, a heptasaccharide oligosaccharide representing the major structural motif (M2) of the most common clinical isolate was synthesized and conjugated to human serum albumin (HSA). Monoclonal antibodies (mAbs) generated from mice immunized with M2–HSA produced the characteristic punctuate immunofluorescence associated with non-protective mAbs. None of the mAbs elicited by M2 immunization was opsonic. Passive administration of mAbs elicited by M2–HSA was not protective and there was no difference in the survival of mice immunized with M2–HSA and HSA. Hence, we conclude that the M2 motif represents an antigenic determinant in *C. neoformans* GXM that elicits non-protective responses and is not a suitable vaccine candidate. Furthermore, the results illustrate the first molecular assignment of a *C. neoformans* polysaccharide epitope and suggest a general strategy for the identification of GXM epitopes.

1. Introduction

Cryptococcus neoformans is a yeast-like fungus that is a major cause of meningoencephalitis individuals with impaired immunity, such as patients with AIDS [1]. Cryptococcosis is almost always fatal without therapy, yet the available therapies remain unsatisfactory, since current treatment requires prolonged administration of antifungal drugs and even with treatment there is high mortality and morbidity [1]. Other populations at risk include organ transplant recipients, individuals receiving immunosuppressive therapy, and occasional children with hyper-IgM syndrome. Studies in rats have raised the possibility that the spectrum of *C. neoformans*-related disease may extent to the immunologically normal population where the primary infection is often asymptomatic and lifelong [2]. Rats infected with *C. neoformans* are asymptomatic but manifest immune dysregulation in the lungs that translates into hyper-reactive airways, leading to the suggestion that cryptococcal infection may be a contributing factor to the pathogenesis of asthma [2]. Given the problem of cryptococcosis in immunosuppressed populations and the possibility that this infection is a co-factor in pulmonary disease of normal individuals there is considerable interest in development of an effective vaccine [3].

Studies in laboratory animals show that it is possible to protect against experimental *C. neoformans* infection with vaccines that elicit antibody or cell-mediated immunity [3–8]. Given that this fungus is encased in a polysaccharide capsule that is a major virulence determinant, that polysaccharide epitopes can be found in fungal spores [9], that the capsular polysaccharide can elicit protective antibodies [10], there is considerable effort to generate a suitable vaccine that targets the capsule. The *C. neoformans* capsular polysaccharide is composed of two major components, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). GXM is a large molecular weight polymer that comprises most of the mass of the capsule [11]. All polysaccharide vaccine-related work for *C. neoformans* to date has focused on GXM and a conjugate vaccine composed of GXM linked to tetanus toxoid was shown to elicit a protective antibody response in mice and humans [12,13]. However, a prior vaccine composed of total polysaccharide conjugated to protein was immunogenic but not protective [14,15]. GXM is structurally complex [16], and studies using monoclonal antibodies (mAbs) generated from mice vaccinated with GXM-containing vaccines have shown that this molecule can elicit protective, non-protective and even disease-enhancing antibodies [17,18]. Furthermore, the capsular polysaccharide has potent immunosuppressive properties raising concern as to its suitability

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as a vaccine component [19–21]. Consequently, interest in vaccines that elicit antibodies to GXM has evolved to focus on the suitability of peptide mimotopes of GXM and oligosaccharide representing discrete domains of polysaccharide structure [22–25]. An alternative approach is to synthesize oligosaccharides representing the major structural motifs of GXM to focus the response on a few epitopes. The recent report that oligosaccharide-based vaccines can protect against *C. albicans* infection provides encouragement for the development of this strategy against cryptococcosis [26].

The basic structural unit of GXM is a tri-mannose repeat with a glucuronic acid residue in the first mannose [11]. This structure is further modified in individual strains by the addition of xylose substitutions on the mannan backbone. Cherniak et al. defined six triads known as M1–M2 that are found in various proportions in GXM from the various serotypes [27]. M2 is the most common triad in serotype A GXM [27]. Given that serotype A strains are the most common clinical isolates and that serotype A GXM can elicit protective antibodies, a heptasaccharide oligosaccharide representing the M2 structural motif was chemically synthesized, conjugated to human serum albumin (HSA) and used to immunize mice. A preliminary study revealed that the M2–HSA motif was immunogenic but, surprisingly, elicited antibodies that produced a punctuate immunofluorescence pattern on the *C. neoformans* capsule reminiscent of that associated with non-protective mAbs [28]. To investigate the functional efficacy of antibodies elicited by M2–HSA we generated mAb and challenged immunized mice with *C. neoformans* infection. The results indicate that M2 elicits a non-protective antibody response.

2. Materials and methods

2.1. C. neoformans and glucuronoxylomannan

Strain 24067 (serotype D) was obtained from the American Type Tissue Collection (Rockville, MD). GXM was produced from culture supernatants with minor modifications of standard protocols. Strain 24067 was used in all immunofluorescence, phagocytosis and challenge experiments. Strain H99 (serotype A) was obtained from Dr. John Perfect (Duke University, NC). Strain NIH 3939 (serotype B) was obtained from Dr. Kwong Chung. Strain NYS 1343 (serotype C) was obtained from Dr. T.G. Mitchell (Duke University, NC). These strains were grown in Sabouraud media (Difco Laboratories, Detroit, MI) at 30 °C in a rotary shaker at 150 rpm.

2.2. Mice and immunization

Female BALB/c mice (6–8 weeks old) obtained from the National Cancer Institute (Bethesda, MD) were immunized intraperitoneally (i.p.) with 10 μg of an oligosaccharide representing motif 2 conjugated to human serum albumin (M2–HSA) in complete Freund's adjuvant (Sigma, St. Louis, MO). The synthesis of this oligosaccharide has been previously reported and it described as compound 17 in that publication [28]. Mice were bled and serum tested at 2-week intervals and boosted as required with 10 μg M2–HSA conjugate in incomplete Freund's adjuvant.

2.3. ELISA

Serum Ab titers and mAb binding to GXM were tested by ELISA using methods previously described [29]. Briefly, 1 μg/ml of GXM in phosphate buffered saline (PBS) was used to coat polystyrene microtiter plates. The plate was then blocked with 1% bovine serum albumin in PBS. Primary antibody binding from mAb or serum was detected using alkaline-phosphatase labeled goat anti-mouse antibody reagents (Southern Biotechnology, Birminham, AL). Plates were developed with P-nitrophenol phosphatase substrate (Sigma, St. Louis, MO). All incubations were carried at 37 °C for 1.0 h. Absorbances were measured in a microtiter plate reader at 405 nm (Labsystems Multiskan).

2.4. Generation of monoclonal antibodies

Antibody-producing hybridomas were generated by fusing splenocytes from M2–HSA immunized mice to NSO myeloma cells, at a ratio of 4:1 as described [30]. Hybridoma supernatants were screened by ELISA as described above for production of mAbs to GXM. Clones that tested positive were selected and stabilized by cloning twice in soft agar. ELISA was used to determine the isotypes of the murine mAbs by using isotype-specific reagents.

2.5. Survival studies

BALB/c mice (6–8 weeks old) were immunized intraperitoneally with 10 μg of oligosaccharide representing motif 2 conjugated to human serum albumin (M2–HSA) in complete Freund's adjuvant (Sigma, St. Louis, MO) (described in [28]). After three doses at intervals of 2 weeks, mice were infected intravenously (i.v.) with 6×10^5 *C. neoformans* strain 24067 cells. For passive protection experiments 6–8 weeks old BALB/c mice were given 0.5 mg mAb intraperitoneally 30 min prior infection with a 100 μl suspension of 1×10^6 *C. neoformans* cells in PBS injected intravenously into the tail vein. Mice were monitored daily for mortality.

2.6. V_H and V_L sequences

Total RNA was isolated from hybridomas cells using Trizol reagent (Gibco BRL, Gaithersburg, MD). cDNA was generated using reverse transcriptase and an oligonucleotide primer. DNA containing the V_H and the V_L region was amplified using the primers 5′Vhuni, TGAGGTGCAGCTGGAGGAGTC; 5′Vkuni, GACATTCTGATGACCCAGTCT; 3′MsCgamma, AGACCTATGGGGCTGTTGTTTTGGC; 3′MsCkappa, TGGATACAGTTGGTGCAGCATCAGC; 3′MsCmu, AGACATTTGGGAAGGACTGACTCTC. The amplified DNA was sequenced at the Sequencing facility of the Cancer Center at the Albert Einstein College of Medicine.

2.7. Immunofluorescence

A suspension of *C. neoformans* cells in PBS was incubated with 10 μg/ml of mAb or immune sera diluted 1/100 in PBS at 37 °C for 30 min. The cells were then washed three times with blocking solution (1% PBS, 0.05% horse serum in PBS) and incubated with 10 μg/ml fluorescein-labeled goat anti-mouse isotype-specific Ab (Southern Biotechnology, Birmingham, AL) at 37 °C for 30 min. After three washes with blocking solution the cells were suspended in mounting medium (0.1 M n-propyl gallate), placed on a slide and viewed with an AX70 microscope (Olympus, Melville, NY).

2.8. Phagocytosis assay

J774.16 cells were incubated in DMEM supplemented with 10% heat inactivated fetal calf serum (FCS), 10% NCTC-109 medium (life technologies), and 1% none essential amino acids (Cellgro, Washington, DC). Cells were plated at a density of 10^5 in 96-well culture plate and stimulated with 50 U/ml murine IFN-Gamma (Genzyme, Cambridge, MA) and 1 μg/ml of LPS (Sigma–Aldrich). Briefly, macrophages were incubated overnight at 37 °C in medium containing IFN-Gamma and LPS. After overnight incubation, the medium was replaced with fresh medium and *C. neoformans* cells were added at ratio of 5:1 macrophage to fungi. Then, the cells were incubated for 2.0 h at 37 °C, washed three times with sterile PBS, fixed with cold methanol and stained with 1/20 dilution of Giemsa

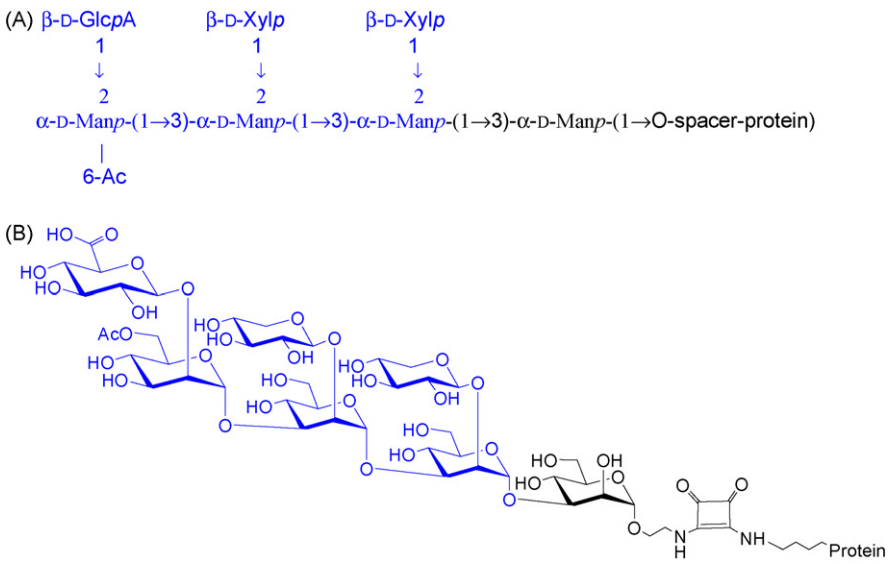

Fig. 1. Structure of motif 2 conjugated to human serum albumin (M2–HSA) compound used in murine immunization studies. (A) The M2–HSA structure written in the format as described by Cherniak et al. [27]. (B) Chemical structure of the M2–HSA compound previously described by Oscarson et al. [28]. M2 motif is shown in blue and applies to both panels.

stain. Phagocytosis was analyzed in presence or absence of mAbs and respective controls. The phagocytic index was determined by PI = $P \times F$, where P is the percentage of phagocytic macrophages and F is the average of yeast cells per macrophage. Experiments were done in triplicate and four different fields were counted per well.

2.9. Statistical analysis

Survival data was done with the Student's *t*-test and by log rank analysis (Sigmastat, Chicago, IL).

3. Results

The synthesis of M2–HAS (Fig. 1) was described in an earlier publication [28]. In this study, we describe the isolation and characterizations of GXM-binding mAbs from M2–HAS immunized mice and ascertained the ability of this conjugate to protect immunized mice against *C. neoformans* challenge.

3.1. MAbs and V region analysis

Five GXM-binding mAbs were recovered from the spleen of a mouse immunized with M2–HSA (Table 1). Four mAbs were IgM and one was IgA. All mAbs had kappa light chains. The molecular structure of four of the IgM mAbs was inferred from sequencing V_H and V_L mRNA (Table 1). The four sequenced mAbs all had a V_H comprised of V_H7183 and J_H4 but each used a different light chain variable region. None of the mAbs reacted with an anti-idiotypic mAb that recognizes Class II mAbs such as mAb 12A1 (data not shown).

3.2. Reactivity of mAbs with C. neoformans serotype D

The M2 motif was initially chosen for synthesis because it is commonly found in serotype A strains which are the most common clinical isolates. All five mAbs reacted with GXM from strain 24067 (serotype D) by ELISA although their apparent affinity was lower than the IgM mAbs 12A1 and 13F1 recovered previously [31] from a mouse immunized with a GXM–tetanus toxoid vaccine (Fig. 2). Immunofluorescence of all five mAbs with *C. neoformans* serotype D strain revealed a punctuate pattern that was very similar to that observed with mAb 13F1 (Fig. 3). Moreover, mAbs were tested for binding with strains H99 (serotype A), NIH 3939 (serotype B) and NYS 1343 (serotype C), but no immunofluorescence reactivity was detected with any of these strains (data not shown).

3.3. Active and passive immunization studies

Mice immunized with the M2–HSA conjugate in complete Freund's adjuvant were challenged with a lethal dose of *C. neoformans* intravenously and compared with mice given mock immunization with PBS or HSA in complete Freund's adjuvant (Fig. 4B). Mice that received either M2–HSA or HSA alone survived longer than mice given PBS alone but this most likely reflects non-specific immune activation by the complete Freund's adjuvant. However, mice immunized with M2–HSA manifested a reduction in average

Table 1
Isotype and molecular characteristics of GXM-binding hybridomas.

Hybridoma	Isotype	Heavy chain		Light chain		Genbank accession numbers	
		V_H	J_H	V_L	J_L	V_H	V_L
1H1	IgA	V_H7183	J_H4	V_K21	J_K2	EU817851	EU817852
5H8	IgM	V_H7183	J_H4	IgV$_K$ rf	J_K2	EU817845	EU817846
3F10	IgM	V_H7183	J_H4	IgV$_K$ 19-15	J_K2	EU817847	EU817848
1D6	IgM	V_H7183	J_H4	IgV$_K$	J_K4	EU817849	EU817850
1E3	IgM	ND[a]	ND	ND	ND	NA[b]	

[a] ND means not done.
[b] NA means not applicable.

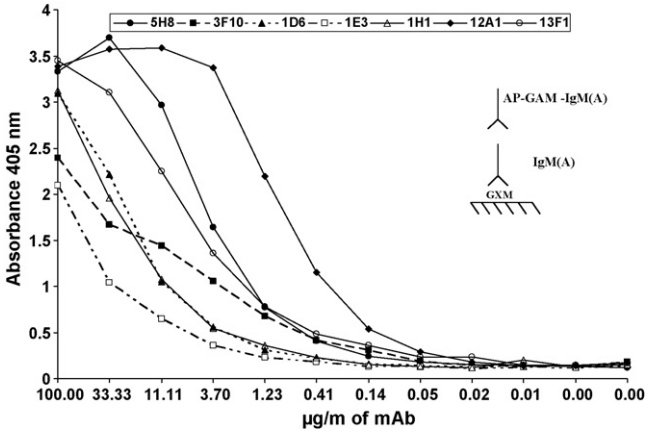

Fig. 2. Binding of mAbs generated from mice immunized with M2–HSA to GXM as measured by ELISA. Serotype D GXM (1 μg/ml) is absorbed onto polystyrene plates and specific antibody binding to GXM is detected with alkaline-phosphatase conjugated goat anti-mouse IgM or IgA (GAM-IgM/A-AP). Plates were developed with P-nitrophenol phosphatase substrate. All incubations were carried at 37 °C for 1 h. Inset shows ELISA configuration.

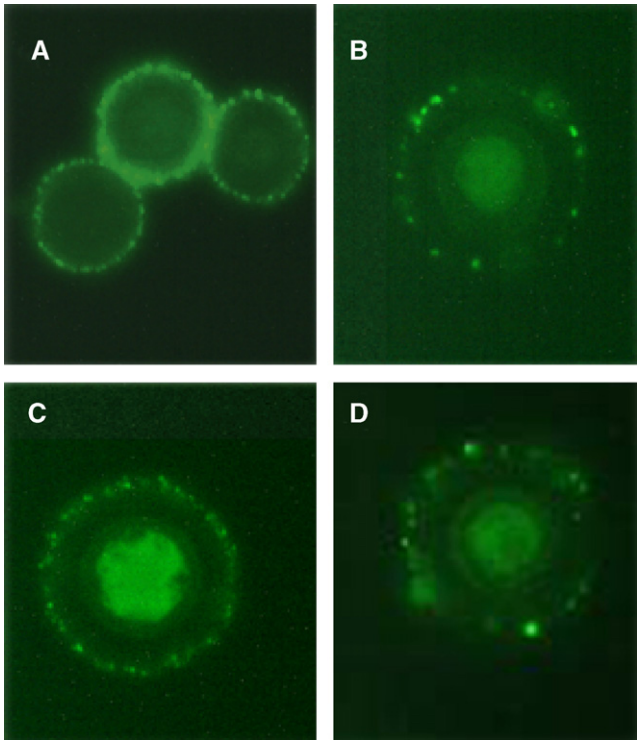

Fig. 3. Indirect immunofluorescence analysis of *C. neoformans* ATCC 24067. Yeast cells were incubated with mAbs 13F1 IgM (panel A), mAb 5H8 IgM (panel B) and 1H1 IgA (panel C) and immune serum (panel D). Cells were then incubated with 10 μg/ml of fluorescein isothiocynate (FITC)-labeled goat anti-mouse IgG and suspended in mounting media (0.1 M n-propyl gallate in PBS). Slides were viewed with an Olympus IX 70 microscope equipped with standard FTTC filters. MAbs 3F10 IgM and 1D6 IgM results in the same binding pattern as mAb 5H8 (not shown). Magnification 250×.

survival at day 200 relative to mice receiving HSA alone, suggesting that the antibody response to the oligosaccharide–protein conjugate was detrimental. In a separate experiment, the protective efficacy of the five mAbs was evaluated in the same model of i.v. lethal infection. None of the mAbs prolonged survival and several were associated with significantly shorter survival (Fig. 4). None of the mAbs was opsonic for *C. neoformans* in vitro using J774.16 cells (data not shown).

4. Discussion

Immunization of mice with a heptasaccharide oligosaccharide representing the major structural motif M2 conjugated to HSA elicited antibodies that produced a punctuate immunofluorescence pattern that is associated with non-protective mAbs [28]. This observation and the fact that the M2 heptasaccharide bound best to non-protective mAbs suggested that this polysaccharide structure represented an epitope that elicited non-protective antibodies. However, to establish conclusively that the M2 motif elicited non-protective antibodies it was necessary to challenge mice immunized with M2–HSA conjugate with *C. neoformans*. Since a negative result with polyclonal sera would not be conclusive, we also made mAbs and evaluated them for serological properties, molecular structure and protective efficacy. Furthermore, this approach provided the opportunity to study the molecular genetics of the immune response to a defined GXM epitope since the V region usage of the elicited mAbs could be compared to the large database of mAbs to GXM [32–34].

From a mouse immunized with the M2–HSA conjugate, we recovered four IgM and one IgA mAbs. Although these mAbs were

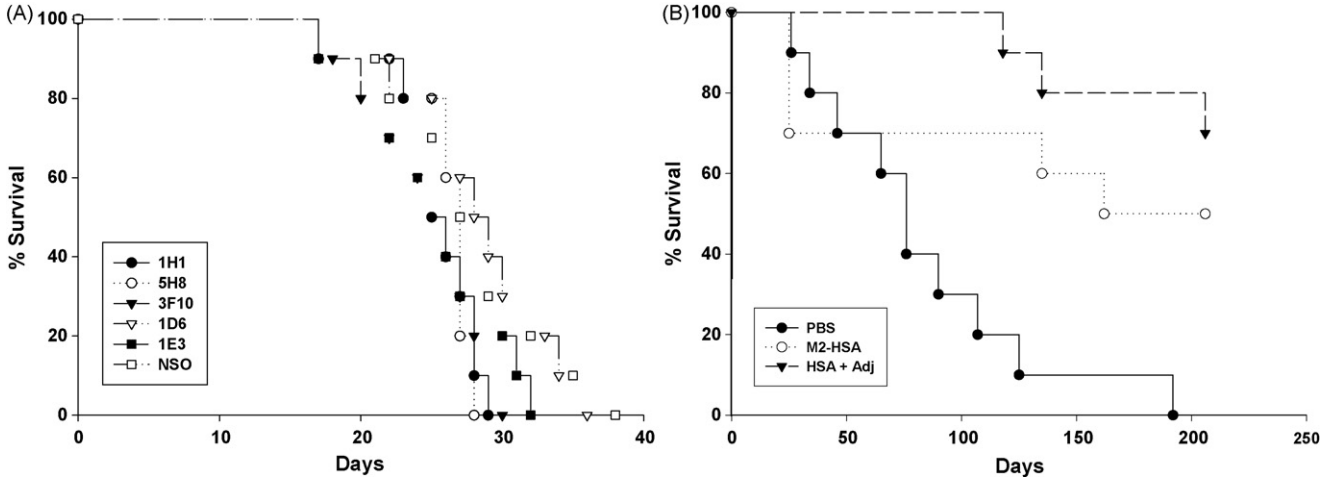

Fig. 4. Survival studies of BALB/c mice lethally infected with *C. neoformans* ATCC 24067. (Panel A) MAbs 1H1 (IgA), 5H8 (IgM), 3F10 (IgM), 1D6 (IgM), and 1E3 (IgM) were each administered 30 min prior to infection with 10^6 yeast cells. By intraperitoneal injection. NSO ascites generated from the nonproducing hybridoma partner, was used as a control. (Panel B) Survival of mice immunized with M2–HSA (10 μg/ml), HSA (10 μg/ml), or PBS and then challenged with *C. neoformans*. Mice were immunized three times prior to infection with 6×10^5 yeast cells. In panels A and B, mice were infected with *C. neoformans* intravenously.

not protective prior work has shown that IgM and IgA can protect against systemic infection in mice [17,18]. Despite the fact that the M2 oligosaccharide reacted with mAb 13F1 [28], which had been previously generated from GXM–tetanus toxoid immunized mouse [31], the serological properties of these mAbs were unlike those mAbs described for other mAbs to GXM [31,34–39]. This antibody set reacted only with the serotype D strain in a punctuate pattern. Molecular analysis of four mAbs revealed that all used a V_H composed of V_H7183 and J_H4. Hence, these mAbs use a gene element of the same family as the Class II mAbs that bind GXM [40]. However, unlike Class II mAbs that use exclusively a V_L composed of VK5.1, these mAbs each used a different light chain gene element. Despite the sequence similarities to Class II mAb, none of the mAbs expressed the Class II idiotype, consistent with a different antigen binding structure. Consequently, we assign these mAbs to a new molecular class than previously described mAbs [40,41]. The molecular basis for the punctuate immunofluorescent specificity also appears to be different from other Class II mAbs. For the mAb 13F1 which also gives a punctuate pattern with serotype D strains this binding has been associated with Y33 and S57 [42]. However, for this antibody set the residues at positions 33 and 57 were glycine and threonine, respectively.

Despite the presumed high prevalence of the M2 motif in the GXM of many cryptococcal strains we observed reactivity only with strain 24067 (serotype D). Since the M2 motif is small and comprises a linear heptasaccharide, the absence of reactivity in the other strains could be explained by the different conformation of this motif in the native capsular GXM of those strains. Alternatively, this epitope may not be accessible in native capsular GXM. In this regard, it is now apparent that GXM has a propensity for self-aggregation [43] and the formation of complex structures and it is conceivable that the M2 motif is blocked and/or exists in a different conformation not recognizable by mAbs in the native capsule. In this regard, it is noteworthy that the serotype D GXM is the least substituted of the four serotypes [11], which may allow a more open or flexible structure for mAbs to find the M2 linear epitope. Since the mAbs reacted only with strain 24067, and this strain has been used extensively in prior protection studies, we tested the M2–HSA vaccine and mAbs in a mouse model of infection using this strain. We observed no protection against *C. neoformans* infection in mice immunized with M2–HSA as measured by survival time. Immunization with M2–HSA was associated with increased survival relative to the saline control but this likely a result of stimulation of non-specific immunity by adjuvant, given that vaccination with killed bacteria has been shown to have some protective effect against challenge with *C. neoformans* [44,45]. In fact, there was a suggestion that the antibodies elicited by M2 were disease enhancing given that immunized mice lived less time than controls. Similarly, passive administration of mAbs generated by M2–HSA immunization was not protective. Since neither active nor passive immunization was protective, we conclude that M2 represents a non-protective epitope that is an unsuitable vaccine candidate.

M2 by virtue of its relatively small size must represent a linear type of epitope in GXM. All protective mAbs to *C. neoformans* lose binding to GXM when the polysaccharide is de-O-acetylated. A comparative analysis of protective mAb binding to wild type and de-O-acetylated mutants of *C. neoformans* has led to the conclusion that many, if not all, bind to conformational epitopes that are unlikely to be represented in the M2 structure.

In summary, we provide the first molecular description of a GXM epitope and show that a synthetic heptasaccharide motif of GXM is immunogenic when conjugated to protein carrier. Unfortunately, despite the enormous effort involved in the chemical synthesis of M2–HSA, this compound did not elicit protective antibodies, yet was used to generate an unusual set of mAb reagents that may be useful in the future studies of GXM structure. The approach of making a synthetic oligosaccharide vaccine that elicits a protective immune response remains to be proven for *C. neoformans* despite the recent success of an oligosaccharide vaccine in protecting against candidiasis in mice [26]. However, there is a suggestion in the literature that protective epitopes in GXM are conformational and if that were the case, a synthetic vaccine would need to use larger oligosaccharide chains.

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