Synthesis and immunological studies of glycoconjugates of Cryptococcus neoformans capsular glucuronoxylomannan oligosaccharide structures

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Received 25 October 2004; received in revised form 24 January 2005; accepted 16 February 2005

Available online 24 March 2005

Abstract

Antibodies to the glucuronoxylomannan (GXM) component of the polysaccharide capsule of Cryptococcus neoformans are protective and GXM–protein conjugate vaccines can elicit protective immune responses. We report the synthesis of a heptasaccharide oligosaccharide representing the putative dominant motif of serotype A GXM and demonstrate that it is recognized by some monoclonal antibodies (mAbs) generated to GXM. Conjugation of the heptasaccharide to human serum albumin (HSA) resulted in an immunogenic compound that elicited high-titer IgG responses in mice when given with complete Freund’s adjuvant. The antibody response elicited by the oligosaccharide conjugate vaccine had characteristics of a T-cell-dependent response. The availability of an immunogenic oligosaccharide representing a structural motif of GXM will prove useful in studies of antibody epitope specificity and represents a potential synthetic oligosaccharide vaccine against this fungal pathogen.

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Keywords: Antibody; Conjugate; Polysaccharide; Vaccine; Immune response; Cryptococcus

1. Introduction

Cryptococcus neoformans is a fungus that is the causative agent of cryptococcosis, a disease that affects primarily individuals with impaired immune system, such as those with advanced HIV infection, organ transplants, and immunosuppressive therapies. C. neoformans is unusual among eukaryotic pathogens in having a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM). For C. neoformans, the polysaccharide capsule is recognized as a critical attribute for pathogenicity [1]. One remarkable feature about C. neoformans infections is that they are associated with the release of large amounts of capsular polysaccharide in tissue [2]. This soluble polysaccharide is believed to also contribute to virulence by interfering with leukocyte migration and causing dysregulation of inflammatory responses [3].

Cryptococcosis is associated with high mortality and morbidity even with antifungal therapy and there is considerable interest in the generation of vaccines to prevent disease in individuals at high risk. Given the importance of the capsular polysaccharide in virulence and the precedence of successful polysaccharide-based vaccines against encapsulated bacterial pathogens, the potential utility of antibody responses to the capsule in host defense have attracted much interest. Numerous studies have established that antibodies to GXM are protective in mouse models of infection [4–7]. Unfortunately, cryptococcal infection elicits either low levels of capsule binding antibodies and/or the antibodies produced have characteristics of non-protective antibodies [8–10]. Hence, the contribution of the natural antibody response to host defense is uncertain, although there is evidence that in certain hosts antibody responses of certain molecular types are associated with improved outcome (reviewed in [11]). Conjugation of C. neoformans capsular polysaccharide to proteins results in highly immunogenic compounds that can elicit high-titer antibody responses. In the 1960s, Goren described a conjugate vaccine composed of capsular polysaccharide–protein complexes, which was highly immunogenic but not protective [12,13]. However, a
subsequent vaccine composed of GXM conjugated to tetanus toxoid was both immunogenic and protective [14–16]. The discovery that GXM can elicit protective and non-protective antibodies provided a potential explanation for the discrepant results observed in vaccination studies and the difficulties involved in consistently associating antibody responses with resistance to infection [6,17]. Recent studies have identified antibody isotype, amount, and specificity as critical attributes for antibody efficacy in mouse models [17–20].

The difficulties associated in consistently eliciting protective antibody responses using GXM-based vaccines have stimulated alternative approaches based on employing peptide mimotopes as immunogens. A peptide mimetic of GXM isolated using a protective murine mAb elicited a protective antibody response [21]. However, other peptide mimetics isolated using protective murine mAbs did not elicit protective immune responses [22,23]. Detailed studies revealed that the glycosylation structures in the protein carrier had determinants that could elicit non-protective mAbs by antigenic mimicry with GXM [23]. Another approach is to synthesize oligosaccharide-based vaccines to identify motifs that are immunogenic and potential vaccine candidates. In this study, we report the synthesis and immunogenicity of oligosaccharides containing a GXM repeat motif common in Serotype A GXM. The results illustrate the feasibility of generating synthetic oligosaccharide vaccines for C. neoformans.

2. Materials and methods

2.1. General methods

Thin-layer chromatography (TLC) was carried out on Merck precoated 60 F 254 plates using UV-light and/or 8% sulfuric acid for visualization. Column chromatography was performed on silica gel (0.040–0.063 mm, Amicon). NMR spectra were recorded in CDCl3 (internal Me 4 Si, δ = 0.00) or D2O (internal acetone 13 C, δ = 31.0, 1 H, δ = 2.21) at 25 °C on a Varian 300 or 400 MHz instrument. Organic phases were dried over Na2SO4 and filtered before concentration, which was performed under reduced pressure.

2.2. Synthesis of 2-azidoethyl [tert-buty] (2,3,4-tri-O-acetyl-3-O-allyl-4-O-benzyl-α-d-glucopyranosyl)uronate

A solution of compound 2 (21 mg, 0.010 mmol) and compound 1 (14 mg, 0.015 mmol) in dry diethyl ether (5 mL) containing powdered molecular sieves (4 Å) was stirred at room temperature in an argon atmosphere for 30 min. DMTST (11 mg, 0.040 mmol) was added to the mixture and the stirring was continued for 6.5 h. After neutralization with NEt3, the mixture was filtered through Celite and concentrated. The residue was purified on a silica gel column (toluene-EtOAc 9:1) to yield compound 4 (20 mg, 70%). Selected NMR data: 13C, δ 97.5 (reducing d-Man), 99.5, 100.5, 101.2 (α-Man), 103.1, 103.5, and 103.6 (β-Xyl and β-GlcA).

2.3. Synthesis of 2-aminoethyl [tert-buty] (2,3,4-tri-O-benzyl-β-d-glucopyranosyl)uronate

(1 → 2)-(6-O-acetyl-3-O-allyl-4-O-benzyl-α-d-mannopyranosyl)-(1 → 3)-(2,3,4-tri-O-benzyl-β-d-sialosylpyranosyl)-(1 → 2)-(4,6-di-O-benzyl-α-d-mannopyranosyl)-(1 → 3)-(2,3,4-tri-O-benzyl-β-d-sialosylpyranosyl)-(1 → 2)-(6-O-acetyl-4-O-benzyl-α-d-mannopyranosyl)-(1 → 3)-2,4,6-tri-O-benzyl-α-d-mannopyranoside (compound 5)

Compounds 3 (20 mg, 0.010 mmol) and 1 (14 mg, 0.015 mmol) were coupled as described for compounds 2 and 1 above to give compound 5 (20 mg, 70%). Selected NMR data: 13C, δ 97.4 (reducing α-Man), 99.6, 100.4, 101.2 (α-Man), 103.1, 103.3, and 103.6 (β-Xyl and β-GlcA).

2.4. Synthesis of 2-aminoethyl [tert-buty] (β-d-glucopyranosyl)uronate

(1 → 2)-(6-O-acetyl-α-d-mannopyranosyl)-(1 → 3)-(β-d-glucopyranosyl)-(1 → 2)-(α-d-mannopyranosyl)-(1 → 3)-α-d-mannopyranoside (compound 8)

PdCl2 (60%, 1 mg, 3.2 μmol) was added to a solution of compound 4 (40 mg, 0.014 mmol) in EtOH-MeOH (1:1 4 mL). The mixture was stirred for 2 h, filtered through Celite, concentrated and purified on a silica gel column (toluene-EtOAc 9:1) to yield crude compound 6 (29 mg, 74%) which was dissolved in EtOAc:AcOH:H2O (4:1:1, 6 mL). Palladium hydroxide on carbon was added and the mixture was hydrogenolyzed at 8 atm overnight. Some more catalyst was added and hydrogenolysis was continued for another 48 h. The mixture was centrifuged and the pellets were washed with water and centrifuged and the supernatants were combined and concentrated. Partition between water/diethyl ether followed by freeze-drying of the aqueous phase gave compound 8 (12 mg, 93%) as a white solid. Selected NMR data: 1H, δ 4.75 (1H, s, reducing α-Man), 5.08 (2H, s, α-Man), 5.14 (1H, s, α-Man). MALDI-TOF: m/z 1270.6 (M + Na)⁺.
2.5. Synthesis of 2-aminoethyl (tert-butyl) ((β-D-glucopyranosyl)uronate) (1\(^\to\)2)-(6-O-acetyl-α-D-mannopyranosyl) (1\(^\to\)3)-(β-D-xylopyranosyl) (1\(^\to\)2)-(6-O-acetyl-α-D-mannopyranosyl) (1\(^\to\)3)-α-D-mannopyranoside (compound 9)

Compound 8 (41 mg, 0.014 mmol) was deallylated as described for compound 4 above to give crude 7 (27 mg, 67%) and thereafter, hydrolyzed as above to yield compound 9 (10 mg, 80%) as a white solid. Selected NMR data: 
- 3H, δ 4.78 (1H, s, reducing Hα of the sugar) and 5.15 (1H, s, α-Man).
- MALDI-TOF MS. Purification on a Biogel P2 column eluted with H\(_2\)O (1% H\(_9251\). 4.78 (1H, s, reducing Hα of the sugar) and 5.15 (1H, s, α-Man).

2.5.1. Procedure for biotin-labeling of spacer glycosides

Biotin-3-sulfo-N-hydroxysuccinimide ester sodium salt (2 mg, Sigma) in H\(_2\)O (200 µL) was added to a solution of the spacer glycoside compounds 8 or 9 (3 mg) in phosphate buffer (1 mL, 0.1 M, pH 7.0). After 48 h, TLC on silica gel (EtOAc–HOAc–MeOH–H\(_2\)O, 12:3:3:2) and MALDI-TOF MS, the reaction mixture was neutralized by adding a solution of Dowex H\(^+\) ion-exchange resin, filtered, and concentrated. Purification of the residue on a Biogel P2 column eluted with H\(_2\)O (1% n-BuOH) and lyophilization gave the oligosaccharide–biotin conjugate was dissolved in deionized water and lyophilized to afford compound 17.

2.6. Mice and immunization studies

BALB/c mice (6–8 weeks) were obtained from the National Cancer Institute (Bethesda, MD, USA). BALB/c mice were selected for studying immune responsiveness because this strain had been used in prior studies of GXM-conjugate immunogenicity and thus, provided a basis for comparison [14,16]. The mice were immunized and boosted at intervals of 2 weeks. Mice were bled at regular intervals for antibody assays. Mice were immunized by injecting the candidate oligosaccharide intraperitoneally at a dose of 2.5 and 10 µg per mouse in either PBS or in suspension with complete Freund’s adjuvant (Sigma, St. Louis, MO, USA), respectively. For the mice that received oligosaccharide in CFA, booster doses were administered in incomplete Freund’s adjuvant.

2.7. Monoclonal antibodies (MAbs)

MAbs 21D2 (IgM), 2H1 (IgG1), 12A1 (IgM), 13F1 (IgM), 7B13 (IgM), and 18B7 (IgG1) have been described [14]. MAb E1 was generated from mice immunized with serotype A GXM [4]. With the exception of mAb 21D2, which had been recovered from an infected mice [8], each of the mAbs was made from hybridomas generated from splenocytes of GXM-TT immunized mice [14]. MAb E1 was generated from mice immunized with serotype A GXM [4]. MAbs were used from concentrated hybridoma supernatants and their concentrations were determined by ELISA relative to isotype-matched standards.

2.8. Antibody binding ELISA

Antibodies were tested for reactivity to oligosaccharides by ELISA. Oligosaccharides conjugated to biotin were immobilized in polystyrene 96-well plates coated with 1 µg/ml of streptavidin at 37°C for 1 h. The plates were then blocked for non-specific binding with blocking solution (1% bovine serum albumin in PBS, pH 7.4) for 1 h. After blocking a solution, the oligosaccharide solution (10 µg/ml) was added, the solution was serially diluted and allowed to incubate by incubating for 1 h. MAbs were tested for reactivity by adding 2 µg/ml for 1 h at 37°C. Bound MAb was detected using alkaline phosphatase-conjugated (isotype matched)
2.9. Serum reactivity ELISA

The ability of oligosaccharide vaccines to elicit antibody to GXM was measured by ELISA using assays previously developed [25]. Briefly, a 1–100-dilation serum was incubated in a 96-well plate coated with 1 μg/ml of glucuronoxymannan (GXM) from either strain 24067 (serotype D) or H99 (serotype A). After 1 h, a mixture of goat anti-mouse reagent specific for murine IgM, IgG, IgA and IgE conjugated with alkaline phosphatase was added and incubated for 1–1.5 h. The presence of bound antibody was then detected colorimetrically with p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, USA). Plates were developed with goat anti-mouse (Southern Biotechnology, Birmingham, AL, USA) for 30 min at 37 °C in a rotary shaker at 150 rpm. The cells were washed and incubated with 10 μg/ml of fluorescein isothiocyanate-labeled rabbit antibody specific for the various antibodies bound to the plate with mAb 6A10, a murine mAb that recognizes the 2H1 epitope, which is common in antibodies to GXM [26].

2.10. Immunofluorescence

A suspension of 10⁸ C. neoformans cells was incubated with either pre-immune or immune serum diluted 1/100 in blocking solution (1% BSA, 0.5% horse serum in PBS) and incubated at 37 °C for 30 min. For immunofluorescence studies, we used C. neoformans strains H99 (serotype A), NIH3939 (serotype B), NY1343 (serotype C), and 24067 (serotype D) grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI, USA) for 24–48 h at 30 °C in a rotary shaker at 150 rpm. The cells were washed and incubated with 10 μg/ml of fluorescein isothiocyanate-labeled goat anti-mouse mix of IgG and IgM (Southern Biotechnology, Birmingham, AL, USA) for 30 min at 37 °C. After three washes, cells were suspended in mounting medium (0.1 M n-propyl gallate in PBS). The cells were viewed with an AX 70 microscope (Olympus, Melville, NY, USA) under a fluorescence isothiocyanate filter.

3. Results

3.1. Synthesis of oligosaccharide–biotin and oligosaccharide–HSA conjugates

DMTST-promoted couplings of donor 1 [27] with pentasaccharide acceptors 2 or 3 [28,29] gave after work-up heptasaccharide compounds 4 and 5 in 70% yields (Fig. 1). Removal of the alkyll group in compounds 4 and 5 gave compounds 6 (74%) and 7 (67%), hydrogenolysis of which to remove benzyl protecting groups and concomitantly reduce the azido-group to form the amino-group needed for subsequent conjugation to proteins, afforded the tert-butyl ester protected heptasaccharides compounds 8 and 9, corresponding to C. neoformans CPS serotype A trisaccharides in 93 and 80% yield, respectively.

Biotinylation of the spacer glycosides was performing using a commercial hydroxyxuccinimid ester reagent (Fig. 2). Mixing of the compounds 8 or 9 with this reagent in a phosphate buffer afforded the biotinylated compounds 10 and 11, respectively, from which the tert-butyl ester protecting group was removed by acid (TFA) hydrolysis to give compounds 12 and 13 as identified by MALDI-TOF MS. Treatment of compound 13 with sodium methoxide in methanol yielded the deacetylated compound 14.

Conjugation to HSA was performed using squareate technology (Fig. 3) [30,31]. Heptasaccharide compound 8 was reacted with dimethyl sulfate at neutral pH to give the intermediate compound 15, from which the tert-butyl ester was removed by acid (TFA) treatment (→16). After purification by gel filtration, the squareate compound 16 was conjugated to HSA at pH 10. The obtained glycoconjugate was purified by ultrafiltration. MALDI-TOF MS of compound 17 showed an average loading of seven oligosaccharides/protein (Figs. 4 and 5).

3.2. MAb binding to oligosaccharides

ELISA was used to investigate whether mAbs that bind whole GXM bound to the synthetic oligosaccharides. The IgM mAbs 13F1 and 7B13 demonstrated strong reactivity for compounds 12 (monoacetylated), 13 (de-O-acetylated), 14 (non-acetylated) and the Man-Xyl-Man derivative (Figs. 2 and 3) while mAbs 12A1 and 21D2 exhibited only weak reactivity. Each of the IgG1 mAbs 18B7, 2H1, and E1 demonstrated strong reactivity for compounds 12 and 13 as identified by MALDI-TOF MS. Treatment of compound 13 with sodium methoxide in methanol yielded the deacetylated compound 14.

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3.3. Immunization studies with heptasaccharide–protein conjugates

Mice were immunized with either 2.5 or 10 μg of heptasaccharide–protein conjugate compound 17 alone or in CFA. Groups A and B received 2.5 and 10 μg/mouse without adjuvant, respectively. Groups C and D received 2.5 and 10 μg/mouse with CFA, respectively. Each group had four mice and antibody responses to GXM were observed only with mice immunized with compound 17 in CFA (Fig. 6). After three doses, all mice immunized with 10 μg of compound 17 in Freund’s adjuvant had significant antibody titers to GXM, regardless of whether serotype A or D GXM was used in the ELISA (Fig. 6 for serotype D GXM; data not shown for serotype A GXM). In contrast, only 25% of mice...
given 2.5 µg of compound 17 in CFA manifested a significant increase in antibody titers to GXM. None of the mice given heptasaccharide–protein conjugate compound 17 without adjuvant demonstrated a significant increase in antibody to GXM (Fig. 6). Mice were boosted with the same dose at 13 months after the first immunization and serum titer to GXM were measured again, 2 weeks later. GXM-binding antibody was measured in three surviving mice that received 10 µg/mouse and one of four mice that received 2.5 µg/mouse (Fig. 7A). These mice were boosted again with the same dose at 16 months and all had appreciable levels of serum GXM-binding antibody (Fig. 7B). These results indicate that antibody responses can be boosted consistent with induction of immunological memory.

Analysis of isotype composition in mice immunized with the oligosaccharide–protein conjugate revealed significant mouse-to-mouse variation in the response to GXM (Fig. 8). IgG isotypes predominated in all mice and at least two mice responded with significant titers of IgG2a and IgG2b (Fig. 8). This isotype composition was significantly different from the response to the GXM-TT conjugate vaccine [14,16], which includes primarily IgM, IgG1, and IgG3 (Fig. 8, inset). Antibodies to GXM elicited by the oligosaccharide–protein conjugate were not reactive with the anti-idiotypic mAb 6A10 (data not shown). In contrast to the strong reactivity observed by ELISA with serotype A GXM (data not shown), indirect immunofluorescence studies revealed that the antibodies to GXM elicited by the oligosaccharide–protein conjugate were primarily reactive with serotype B and D cells, and the staining pattern on the capsule was uneven and often punctate (Fig. 9).

4. Discussion

Given the biological importance of C. neoformans GXM, there have been several attempts to synthesize oligosaccharides that reproduce its structural repeat [28,29,32–34]. Many of these structures are synthesized as methyl glycosides, which do not allow subsequent conjugation to a protein.
Fig. 2. Syntheses of biotin-labeled compounds 12–14

Key: 1) Biotin succinimidyl ester, pH 7 buffer; 2) TFA, H2O; 3) NaCMe, MeOH.

Fig. 3. Synthesis of Man-Xyl-Man biotin-labeled compound.

Key: 1) Biotin succinimidyl ester, pH 7 buffer.
Fig. 4. Synthesis of oligosaccharide–protein compound 17.

Key: (i) dimethyl squarate, MeOH, Et$_3$N; (ii) TFA, H$_2$O; (iii) HBA, pH10-buffer.

Fig. 5. Reactivity of seven mAbs with four oligosaccharides. For corresponding structures, see Figs. 2 and 3. This experiment was done three times with similar results. Diagram illustrates the ELISA configuration.
Furthermore, the synthetic approach rarely allows continued elongation of the structures or presence of acetyl substituents in the target products. The largest oligosaccharide reported to date is a hexasaccharide [33,34]. However, for immunological studies larger structures, with and without O-acetylation are desirable, since this increases the likelihood of reproducing the epitopes found in the natural polysaccharide. Because of the presumed repetitiveness of the GXM polysaccharide structures [35], a block synthesis approach is most attractive. We have now synthesized all the necessary di- and trisaccharide blocks as thioglycosides [28,29] to allow synthesis of any GXM structure even with acetates. Here, we show the efficient subsequent couplings of disaccharide blocks to obtain the heptasaccharide compounds 8 and 9, differing only in their acetylation pattern. The heptasaccharide consists of two M2 triad repeats and mannose residue. The M2 repeat was selected because it is commonly found in GXM of serotype A strains, although this repeat is also found in other serotypes [36]. This successful synthesis proves the feasibility of the thioglycoside block synthetic approach for GXM structures and implies that most epitopes should now be available by chemical synthesis. Furthermore, these heptasaccharides represent the largest oligosaccharide motifs of C. neoformans GXM synthesized to date.

Seven mAbs were analyzed for binding to various heptasaccharides by ELISA that were selected for different serological characteristics. Of the seven, only the IgMs mAb 13F1 and 7B13 bound strongly to the heptasaccharides, including the O-acetylated and de-O-acetylated moieties. Prior studies have shown that mAbs 2H1, 18B7, and 13F1 bind to GXM epitopes that are dependent on O-acetylation while mAbs 21D2 and 12A1 can bind to de-O-acetylated GXM [37]. Hence, we were surprised to note that both 13F1 and 21D2 were reactive with both the O-acetylated and de-O-acetylated heptasaccharides. One potential explanation for this phenomenon is that the role of O-acetylation in the maintenance of epitope structure is to provide a favourable conformation for binding. Consistent with this hypothesis, O-acetylation is important in polysaccharide structure in other antigenically important microbial polysaccharides [38]. In this scenario, the reactivity of both O-acetylated and de-O-acetylated moieties in our mAb set could reflect the ability of the relatively small and unconstrained heptasaccharide to adopt the suitable conformation to bind into the antibody-binding packet. The reactivity of mAbs 21D2 and 7B13 with all the oligosaccharides structures including Man–Xyl–Man was surprising, specially since this structural motif is not part of the basic structure proposed by Cherniak for GXM [35]. However, GXM-binding mAbs have also shown to react with the glycan structures in KLH [23] and peptide mimetics [39], which are structurally different from GXM. In this regard, the reactivity with the compounds 12–14 and Man–Xyl–Man oligosaccharides could reflect reactivity with a GXM motif or cross-reactivity with the carbohydrate structures displayed on the oligosaccharide. There is increasing evidence that antibody reactivity to oligosaccharides and polysaccharides may follow different rules. For example studies of the reactivity of a mAb to Candida albicans (1→2)-beta-mannopyranan with synthetic oligosaccharides have shown the paradoxical result that di- and trisaccharides react more strongly than tetra-, penta- and hexasaccharides [40]. In fact, crystallographic studies of monoclonal Fab bound to Shigella flexneri oligosaccharides of different sizes revealed that the smaller trisaccharide was buried more snugly and made more molecular contacts than the larger pentasaccharide [41].

Fig. 6. Antibody response of mice immunized with oligosaccharide–HSA compound 17 as measured by ELISA after three doses. Groups A and B received 2.5 and 10 μg/mouse without adjuvant, respectively. Groups C and D received 2.5 and 10 μg/mouse with CFA, respectively. After initial immunization, subsequent doses were administered in incomplete Freund’s adjuvant. For this experiment, serum was diluted 1:100 and assayed by ELISA for reactivity to GXM. Diagram illustrates the ELISA configuration.
heptasaccharide–HSA in the absence of adjuvant. The response elicited by compound 17 was long lasting and could be boosted, consistent with the induction of immunological memory. This, together with the predominance of IgG iso-
types, implies that the oligosaccharide conjugate elicited a T-cell-dependent antibody response to the carbohydrate epi-
topes. The lack of antigenicity of heptasaccharide–HSA in the absence of adjuvant contrasts with the observation that the GXM-TT vaccine was immunogenic without adjuvant [14,16]. This difference may reflect the antigenic complexity of GXM-TT, which is composed of large fragments of sonicated GXM conjugated to protein or protein conjugate effects [42]. In fact, chain length has been shown to be an im-
portant determinant of immunogenicity for polysaccharide conjugate vaccines [43]. Alternatively, the fragments of native GXM may be of sufficient size and complexity to interact with Toll-like receptors and provide an adjuvant effect since GXM can interact with TLR 4 [44], a property that may not reside in the smaller synthetic oligosaccharide.

The protective efficacy of the antibody response to the oligosaccharide vaccine is unknown and will be the subject of future studies. The antibodies elicited by the oligosaccharide–HSA that reacted with GXM were primarily IgG and in at least two mice, there were significant amounts of IgG2a and IgG2b. This isotype distribution is unusual since prior studies using GXM conjugates have reported that the
Fig. 8. Isotype composition of antibodies to GXM in the sera from mice immunized with five doses of compound 17. Inset shows isotype composition of serum antibodies to GXM elicited by the GAM-TT vaccine. Serum was tested at a dilution of 1:100. Diagram illustrates the ELISA configuration.

Fig. 9. Indirect immunofluorescence showing reactivity of immune serum with C. neoformans strains 24067 (serotype D) and NIH 3939 (Serotype B). Left panel reveals immunofluorescence while the right panel shows the cell in light microscopy. The scale bar represents a distance of 5 μm.
majority of antibodies reactive with GXM are IgM, IgG1, and IgG3 [14,16,26]. From the viewpoint of protective efficacy, the presence of IgG3 is encouraging since this isotype is the most protective against C. neoformans [45]. On the other hand, the immunofluorescence pattern was punctate and this has been associated with non-protective antibodies [17,46]. In this regard, it is noteworthy that oligosaccharide compounds were strongly reactive with mAbs 13F1, which produces a punctate immunofluorescence pattern and is not protective [17,46]. The antibodies to GXM elicited by the oligosaccharide–HSA vaccine were not reactive with a mAb to the 2H1 idiotype which is found in both protective and non-protective mAbs to GXM, and is the dominant idiotype in antibodies to GXM elicited by the GXM–TT conjugate vaccine [26]. The absence of the 2H1 idiotype in GXM-binding antibodies elicited by the oligosaccharide–HSA suggests that the antibodies made to the vaccine may be qualitatively different from those elicited to GXM–TT [26] or C. neoformans infection [14,47]. We noted strong reactivity of antibodies elicited by the oligosaccharide–HSA by serotype A GXM by ELISA, but not by indirect immunofluorescence. Interestingly, serum was most reactive with serotype D and B cells by immunofluorescence. Variability in reactivity of antibodies to GXM with the different serotypes as a function of the antibodies and those presented in the assay [37]. Although conformational epitopes found in GXM may not be present in the oligosaccharide by virtue of its small size, the antibodies elicited in response to vaccination with compound 17 may react with conformational epitopes in GXM.

In summary, we report the first synthetic oligosaccharide vaccine to a fungal pathogen. The vaccine was shown to be immunogenic in mice although the antibodies elicited appeared to be qualitatively different than those elicited by GXM-derived immunogens. The ability to synthesize heptameric oligosaccharide motifs of GXM that are immunogenic will allow new strategies for mapping epitope recognition in mAbs and for vaccine design.

Acknowledgement

A.C. is supported by NIH grants R01AI033142, AI033774, and HL059842.

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