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Cryptococcus neoformans Serotype A Glucuronoxylomannan-Protein Conjugate Vaccines: Synthesis, Characterization, and Immunogenicity

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We synthesized *Cryptococcus neoformans* serotype A glucuronoxylomannan (GXM) conjugate vaccines under conditions suitable for human use to prevent disseminated cryptococcosis. The purified, sonicated GXM was derivatized with adipic acid dihydrazide through either hydroxyl or carboxyl groups and then covalently bound to tetanus toxoid (TT) or *Pseudomonas aeruginosa* exoprotein A (rEPA). The immunogenicity of these conjugates was evaluated in BALB/c and general purpose mice by subcutaneous injection in saline. The conjugates elicited higher GXM antibody responses than GXM alone. Booster immunoglobulin G (IgG) and IgM responses were elicited by all conjugates in BALB/c mice. The conjugates prepared through hydroxyl activation (GXM-TT2 and GXM-rEPA) were more immunogenic than the one prepared through carboxyl activation (GXM-TT1). GXM antibody response was enhanced by the administration of monophosphoryl lipid A 2 days following the injection of GXM-TT2 ($P < 0.03$). The conjugates also elicited IgG antibodies to the carrier proteins. Gel diffusion tests using conjugate-induced hyperimmune sera and chemically modified GXMs suggested that the specificity of GXM-TT1-induced antibodies was conferred by the O-acetyl groups. Hyperimmune sera generated by GXM-TT2 precipitated with the chemically unmodified and the de-O-acetylated GXMs but not with the carboxyl-reduced and de-O-acetylated GXM. GXM-TT2-induced hyperimmune serum also precipitated with the capsular polysaccharides of *C. neoformans* serotypes D, B, and C. The conjugate vaccines prepared through hydroxyl activation of the GXM are sufficiently immunogenic and appear to be suitable for clinical evaluation.

Cryptococcus neoformans is an encapsulated yeastlike fungus which causes systemic infections, including fatal meningoencephalitis, in normal, diabetic, and immunocompromised subjects, particularly in patients with reticuloendothelial malignancy, kidney transplantation, and AIDS (14, 32, 34, 43). The incidence of cryptococcosis is high (~10%) in patients with AIDS (11). The morbidity, mortality, and relapse rates are unusually high despite advancement in antifungal therapy. Survivors suffer from permanent neurological damage, including visual loss, cranial nerve palsies, and dementia (29). The prevention of disseminated cryptococcosis through vaccines is considered a worthwhile objective.

The capsular polysaccharide (CP) of *C. neoformans* is antiphagocytic (6, 39) and serves as an important virulence factor. In vivo studies show that the acapsular mutants are less virulent in laboratory animals (35). The glucuronoxylomannan (GXM) component of the CP confers serotype specificity, and its detection in cerebrospinal fluid or serum is used for diagnosis and monitoring of the host response to treatment (3). Serotype A of *C. neoformans* accounts for about 80% of the clinical isolates in the United States (40). GXM of serotype A isolates consists of a linear α(1→3)-linked mannan backbone singly substituted with nonreducing β(1→2) xylose and β(1→2) glucuronic acid side branches (Fig. 1); O-acetyl groups are present at C-6 of the mannosyl residues (4, 8).

Both cellular and humoral immune responses offer resistance to cryptococcosis. In addition to occurring in patients with impaired cellular immunity, disseminated cryptococcosis occurs in subjects with no recognized immune deficiency (13, 43). In humans, the development of anticapsular antibodies is correlated with improved prognosis; patients who survive cryptococcosis have higher anticryptococcal antibody titers than those who succumb (5, 13). Similarly, an association has been established between the disappearance of serum capsular antibodies and death in lethally infected mice (52). There is in vitro evidence to show that anti-GXM immunoglobulin G (IgG) antibodies potentiate opsonophagocytosis in complement-deficient systems (31, 37, 50), antibody-dependent cell-mediated killing (12, 42, 44), or antibody-mediated cryptococcal growth inhibition by natural killer cells (45, 47).

In addition to its antiphagocytic activity (6, 39), cryptococcal GXM is reported to be tolerogenic (38, 46), poorly immunogenic, and T cell independent in humans and experimental animals (7, 30, 38). Attempts to induce anticapsular antibodies in mice by active immunization with killed cryptococcal cells have yielded low or marginal responses (22, 23). Injection of the CP alone, as a complex with an ion exchange resin, or as a mixture with Freund's adjuvant

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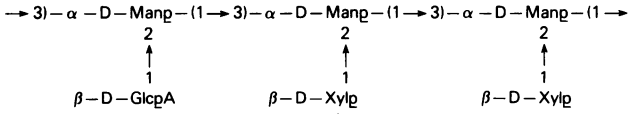

FIG. 1. The structure of GXM of *C. neoformans* serotype A (4).

elicited transient and low titers of antibodies, mostly of the IgM class, only in a small proportion of mice (7, 22, 36, 38, 46). Low levels of IgG antibodies have been elicited only in high responder mice strains by immunization with an optimal dose of GXM (19, 20). Attempts have been made to render cryptococcal CP immunogenic in animals by coupling it to bovine serum albumin (36, 54), bovine gamma globulin (36), and sheep erythrocytes (21). Goren and Middlebrook (26) were the first to couple the unfractionated cryptococcal CP (containing GXM and probably galactoxylomannan and mannoprotein) to bovine gamma globulin by nitrocarbanilation and diazotization. Some of the conjugates induced high levels of cryptococcal agglutinins in mice when injected with Freund's complete adjuvant. These methods of conjugation and immunization are not suitable for clinical use.

We conjugated the purified, sonicated *C. neoformans* serotype A GXM to two different protein carriers after introducing the spacer, adipic acid dihydrazide (ADH), through its carboxyl or hydroxyl groups. The immunogenicity of the resultant soluble conjugates, alone or with monophosphoryl lipid A (MPL), was studied with three strains of mice. The saline solutions of conjugates induced high levels of antibodies to both GXM and the protein carriers, the GXM response being better in BALB/c mice than in general purpose mice. The immunodeterminants of the conjugates prepared by two different synthetic schemes were identified by using chemically modified GXMs.

MATERIALS AND METHODS

Chemicals and reagents. Yeast extract and neopeptone were from Difco Laboratories, Detroit, Mich., and hexadecyltrimethylammonium bromide (Cetavlon) and trinitrobenzene sulfonic acid (TNBS) were from Fluka Biochemika, Ronkonkoma, N.Y. Cyanogen bromide (CNBr), ADH,1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), sodium borohydride ($NaBH_4$), thimerosal, avidin, *N*-hydroxysuccinimidobiotin, bovine serum albumin, acetylcholine chloride, *p*-nitrophenyl phosphate, and agarose were from Sigma Chemical Co., St. Louis, Mo. Sepharose 2B-CL was from Pharmacia Inc., Piscataway, N.J. Alkaline phosphatase-labeled goat anti-mouse IgG and IgM antibodies were from Kirkegaard and Perry Inc., Gaithersburg, Md. Tetanus toxoid (TT), lot GYA, was a gift from Dominique Schulz, Pasteur Merieux Serums and Vaccines, Lyon, France. *Pseudomonas aeruginosa* exoprotein A (rEPA), a nontoxic but antigenic equivalent of exotoxin A, was prepared from a genetically engineered strain (52a). MPL was prepared by RIBI Immunochem Research Inc., Hamilton, Mont., from the Re mutant of *Salmonella minnesota* (R595) as described previously (2).

Antisera. Rabbit hyperimmune serum to cryptococcal CP (serotype A) was produced as described previously (55). Murine IgG monoclonal antibodies (MAbs) to *C. neoformans* CP were donated by T. Kozel, University of Nevada School of Medicine, Reno, Nev. (21), and F. Dromer, Institut National de la Santé et de la Recherche Medicale U13, Paris, France (19). Cryptococcal IgM MAb (6b) was donated by A. Casadevall, Albert Einstein College of Medicine, Bronx, N.Y.; a quantitative precipitin test was performed on this MAb to determine the antibody content to serotype A GXM (33).

Preparation of CP. *C. neoformans* (serotype A, strain NIH 371) was grown in neopeptone dialysate containing 2% glucose at 35°C for 72 h on a gyratory shaker at 150 rpm (55). After inactivation with 0.25% Formalin, the liquid culture was centrifuged two times at 4°C for 2 h at 16,000 × *g*. The supernatant was treated with 2.5 volumes of absolute ethanol, left overnight at 3 to 8°C, and centrifuged at 16,000 × *g* for 2 h at 4°C. The precipitate was dissolved in and dialyzed against deionized water and freeze-dried. The CPs of *C. neoformans* serotypes B, C, and D were prepared similarly.

Purification of native GXM. Native GXM of serotype A *C. neoformans* was purified by precipitation with Cetavlon (9). Briefly, the CP, dissolved in 0.2 M NaCl, was mixed with 10% Cetavlon to a final concentration of 0.39% with constant stirring at room temperature. The precipitate was collected by centrifugation at 16,000 × *g* for 1 h, and the supernatant was reprecipitated with 0.05% Cetavlon. The precipitates were dissociated in 1 M NaCl, deproteinized by cold phenol extraction (27), dialyzed extensively against sterile pyrogen-free water, and freeze-dried. This material was denoted native GXM.

Chemical analysis. GXM and protein were measured by the anthrone reaction and Lowry's assay, with the purified GXM and bovine serum albumin, respectively, as the standards (33, 56). The O-acetyl contents of GXM, GXM-AH, and chemically modified GXMs were quantitated by Hestrin's method, with acetylcholine chloride as the standard (33). The adipic acid hydrazide (AH) contents of the derivatized GXMs were measured by the TNBS assay, with ADH as the standard (10). The glucuronic acid contents of unmodified and carboxyl-reduced GXMs were measured by the carbazole reaction (33).

Depolymerization of native GXM. Attempts at conjugating the native GXM resulted in precipitation. Therefore, it was depolymerized by ultrasonic irradiation (Heat System Ultrasonicator, model w225R) at a power setting of 2 and a pulse of 90% for 1.5 h in an ice bath (53). The sonicated GXM was subjected to gel filtration through a Sepharose 2B-CL column (1.5 by 30 cm) (41). The GXM-containing fractions eluting at about the middle of the column (Fig. 2A and B) were collected, dialyzed against pyrogen-free water at 3 to 8°C, filter sterilized (0.45-μm-pore-size filter), and freeze-dried. This sonicated material was assigned the general term GXM.

NMR spectroscopy. The ^{13}C nuclear magnetic resonance (NMR) spectra of GXMs were recorded at 125.2 MHz with a JEOL GSX-500 NMR spectrometer. Carbon spectra were collected with complete proton decoupling, using a 30 kHz sweep width and 32 k data points; prior to Fourier transformation, the free induction decay signal was zero filled to 64 k data points and exponentially multiplied so as to result in a 10-Hz line broadening in the frequency domain spectrum. Approximately 15 mg of the unmodified or chemically modified GXMs was dissolved in 0.6 ml of D_2O, and the sample was heated to 65°C for data collection. A 90° pulse width was used with a 2.5-s repetition time.

Derivatization of GXM. GXM was derivatized by the following two methods. (i) In method 1, ADH was introduced into GXM by the activation of carboxyl groups with EDAC. GXM (5 mg/ml of 0.2 M NaCl) was derivatized with 0.5 M ADH and 0.1 M EDAC at pH 4.85 for 3.5 h at room temperature, using a pH Stat (51). After extensive dialysis

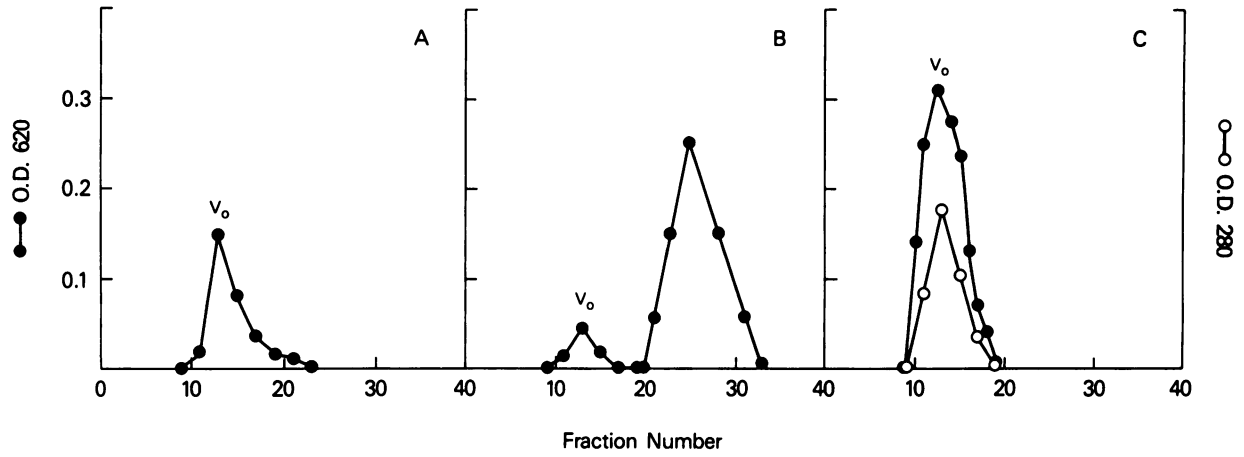

FIG. 2. Gel filtration profile of GXM-TT2 conjugate passing through Sepharose 2B-CL column (1.5 by 30 cm) equilibrated in 0.2 M NaCl. The GXM in the fractions was monitored by anthrone reaction (optical density at 620 nm [●]), and protein was monitored by A_{280} (○). The fraction size was 1 ml. (A) Native GXM; (B) GXM or GXM-AH; (C) GXM-TT2 conjugate. V_0, void volume.

against 0.2 M NaCl, the reaction mixture was passed through a 2B-CL Sepharose column (1.5 by 30 cm) equilibrated in 0.2 M NaCl. The fractions containing GXM were pooled and concentrated to the original volume. (ii) In method 2, ADH was introduced into GXM by the activation of hydroxyl groups with CNBr. GXM (5 mg/ml of 0.2 M NaCl) was activated with an equal weight of CNBr at pH 10.5 for 6 min at 4°C, using a pH Stat (10). An equal volume of 0.5 M $NaHCO_3$ (pH 8.5) containing 0.5 M ADH was added. The reaction mixture was tumbled at 3 to 8°C for 18 to 20 h, dialyzed against 0.2 M NaCl, and passed through a 2B-CL Sepharose column (1.5 by 30 cm). The fractions containing GXM were pooled and concentrated to the original volume.

Synthesis of conjugates. The reaction mixture, containing equal concentrations (3.0 to 7.5 mg/ml) of GXM-AH (derivatized by either method) and TT or rEPA in 0.2 M NaCl, was brought to pH 5.6 with 0.05 N HCl, and 0.05 to 0.1 M EDAC was added; the pH was maintained at 5.6 in a pH Stat for 1 to 3 h at 4°C (10). The reaction mixture was dialyzed against 0.2 M NaCl at 3 to 8°C and passed through a Sepharose 2B-CL column (1.5 by 30 cm) equilibrated in 0.2 M NaCl. The void volume fractions containing the GXM and the protein (Fig. 2C) were pooled and stored in 0.01% thimerosal at 3 to 8°C.

The conjugate GXM-TT1 was prepared through carboxyl activation (method 1), and GXM-TT2 and GXM-rEPA were synthesized through hydroxyl activation (method 2).

Immunization of mice. Groups of 10 general purpose or BALB/c mice (NIH colony), 4 to 5 weeks old, were injected subcutaneously three times, 2 weeks apart, with 2.5 μg of native GXM, GXM alone, or GXM as a component of the conjugate (51). The mice were bled a week after each immunization. Mice that were bled before the first immunization served as normal controls.

In another experiment, the adjuvant effect of MPL on the immunogenicity of GXM and GXM-TT2 in mice was examined (2). Groups of 20 ICR (Harlan Sprague Dawley) female mice, 5 to 7 weeks old, were injected intraperitoneally with 2.5 μg of GXM alone or with 2.5 μg of GXM-TT2 admixed in 50 μg of MPL in saline (2). Another group of mice was immunized with the conjugate in saline on day 1 and then with MPL intraperitoneally 2 days later. Ten mice in each group were sacrificed after 21 days, and the remaining mice in each group were immunized again with the same dose on day 21 and sacrificed a week later. Mice injected with saline alone served as controls.

Hyperimmune sera against GXM-TT1 and GXM-TT2 (5.0 μg each) and the protein carriers were generated by using Freund's adjuvants in adult BALB/c mice and general purpose mice, respectively (41).

Chemical modification of GXM. Serotype A GXM was de-O-acetylated by treating it with NH_4OH (4). GXM was reduced by repeated treatments with EDAC at pH 4.7 followed by treatment with $NaBH_4$ (8).

Serology. Double immunodiffusion was performed with rabbit antiserum to CP and mouse hyperimmune antisera to carrier proteins in 0.5% agarose in saline. The immunodeterminants of GXM-TT1 and GXM-TT2 were defined by gel diffusion with conjugate-induced hyperimmune sera and the unmodified, carboxyl-reduced and/or de-O-acetylated GXMs of *C. neoformans* serotype A. GXM-TT2-induced hyperimmune serum was reacted with the CPs of *C. neoformans* serotypes A, B, C, and D. CPs and GXMs were used at a concentration of 0.2 mg/ml.

An enzyme-linked immunosorbent assay (ELISA) was performed, with GXM (10 μg/ml) as the coating antigen and alkaline phosphatase-labeled goat anti-mouse IgG and IgM as reagents (19). GXM antibody concentrations were calculated by parallel-line analyses, comparing their linear curves with those of monoclonal standard sera. Concentrations of IgG to protein carriers were quantitated by using murine hyperimmune sera to TT and rEPA as standards; these sera were assigned a value of 100 ELISA units.

Statistical analysis. Data analysis was performed by using the Statistical Analysis System. The logarithms of the concentrations were used for all statistical calculations. Antibody concentrations (geometric means) that were below the sensitivity limit of the ELISA were assigned values equal to one-half of that value (0.1 μg/ml for GXM antibodies and 0.02 ELISA units for TT and rEPA antibodies). Comparisons of geometric means were performed with the two-sided *t* test.

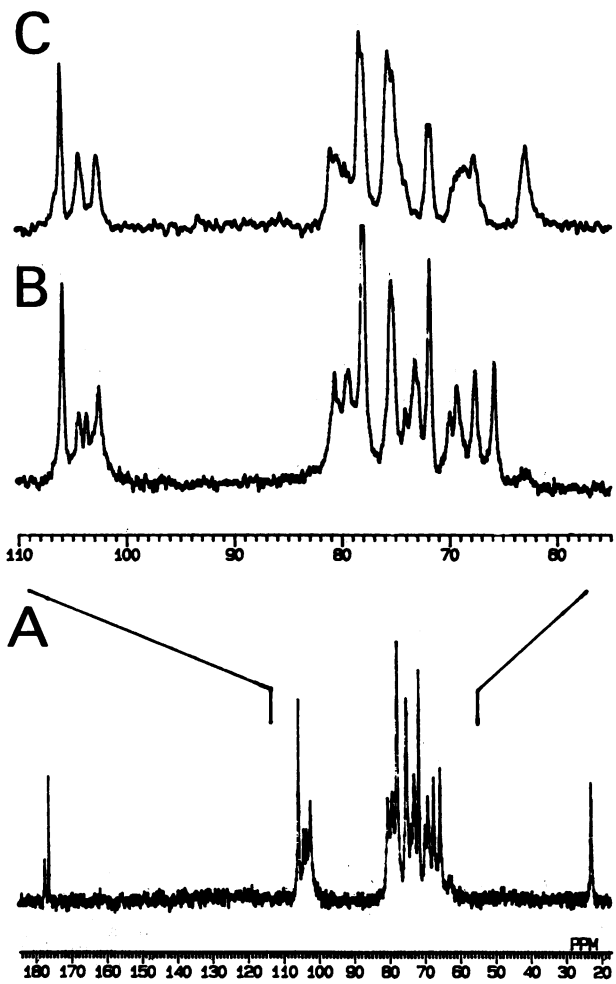

FIG. 3. The ^{13}C NMR spectrum of the GXM of *C. neoformans* serotype A, strain NIH 371 (~15 mg dissolved in 0.6 ml of D_2O, pH 7.0, 35°C). Chemical shifts are in parts per million and are relative to the O-acetyl -CH_3 resonance, which was set to 23.0 ppm. (B) Expansion of the region indicated in panel A. (C) The region between 55 and 110 ppm of the ^{13}C spectrum of the GXM following NH_4OH hydrolysis of the O-acetyl groups and neutralization with HCl.

RESULTS

GXM. *C. neoformans* serotype A GXM contained less than 1% protein and nucleic acid and 15.2% O-acetyl (wt/wt). The ^{13}C NMR spectrum of GXM (Fig. 3A) showed a carboxylic acid resonance (~178 ppm) and O-acetyl signals (23.0 and 177 ppm). The spectrum was similar in overall appearance to that reported by Bhattacharjee et al. (4). A comparison of the signals in the 63- to 67-ppm region of the GXM and de-O-acetylated GXM (Fig. 3B and C, respectively) indicated that, in the GXM, each of the mannosyl residues was O-acetylated at C-6. The signal at ~63 ppm was due to nonacetylated -CH_2OH groups of mannosyl residues (Fig. 3C) and was essentially absent in the GXM (Fig. 3B), indicating that each mannosyl residue was O-acetylated at C-6. Assuming that the mannose-xylose-glucuronic acid ratio of serotype A GXM (strain NIH 371) is 3:2:1 (4) and that the C-6 position of mannose is the only site of acetylation, then the O-acetyl percentage calculated from the ^{13}C NMR spectrum was 15.5% by weight, a value that was similar to that determined by Hestrin's method. The slight difference in O-acetyl content observed between our GXM and the one reported previously (4) for the same strain, NIH 371 (12.2%), may be due to the differences in the methods of cryptococcal cultivation and GXM purification.

The ^{13}C NMR spectra of de-O-acetylated and/or carboxyl-reduced GXMs showed the absence of carboxylic acid and/or O-acetyl resonances (not shown). This was confirmed by the colorimetric assays, which revealed the degree of de-O-acetylation and carboxyl reduction to be 100 and 98%, respectively.

Derivatized GXM. Derivatization of GXM by method 1 did not alter the O-acetyl content, whereas derivatization by method 2 resulted in a slight loss (from 15.2 to 12.5 and 12.8%) of the O-acetyl content (Table 1). The AH content of GXM derivatized by method 1 was slightly higher (4.0%) than that of those derivatized by method 2 (1.9 and 2.8%). Both GXM and GXM-AH had a K_d of ~0.32 in Sepharose 2B-CL (Fig. 2A and B). GXM derivatized by both methods precipitated with the rabbit anticryptococcal serum by immunodiffusion (not shown).

Characteristics of the conjugates. The compositions of the conjugates are given in Table 1. The protein/polysaccharide ratio of various conjugates ranged between 1.0 and 6.1. The conjugates eluted in the void volume of the Sepharose 2B-CL column (Fig. 2C). Continuing the conjugation reaction beyond 1 h resulted in precipitation and a poor yield of the conjugate. The conjugates reacted with rabbit anti-CP and mouse anti-TT or anti-rEPA hyperimmune sera, producing a single continuous precipitin line (Fig. 4a).

GXM antibodies. The immunogenicity of GXM, GXM-TT1, and GXM-TT2 was evaluated in both BALB/c and general purpose mice; for GXM-rEPA, the immunogenicity was evaluated only in general purpose mice (Table 2). There were no detectable levels of GXM antibodies in the preimmunization sera. Native GXM elicited low levels of IgG and IgM antibodies in BALB/c mice and only IgM antibodies in general purpose mice. GXM induced low levels of only IgM antibodies in both strains of mice. Neither native GXM nor GXM elicited a booster response.

Conjugates were better immunogens than native GXM or GXM alone and produced booster antibody responses. BALB/c mice were better responders to the conjugates than

TABLE 1. Composition of GXM-protein conjugates

Conjugate	% AH in GXM	% O-acetyl in GXM-AH	GXM (μg/ml)	Protein (μg/ml)	Protein/GXM ratio	% GXM yield
GXM-TT1[a]	4.0	15.2	74.0	272.1	3.7	6.4
GXM-TT2[b]	2.8	12.5	49.8	294.7	6.1	7.4
GXM-rEPA[b]	1.9	12.8	172.4	165.9	1.0	48.7

[a] GXM derivatized by carboxyl activation (method 1).
[b] GXM derivatized by hydroxyl activation (method 2).

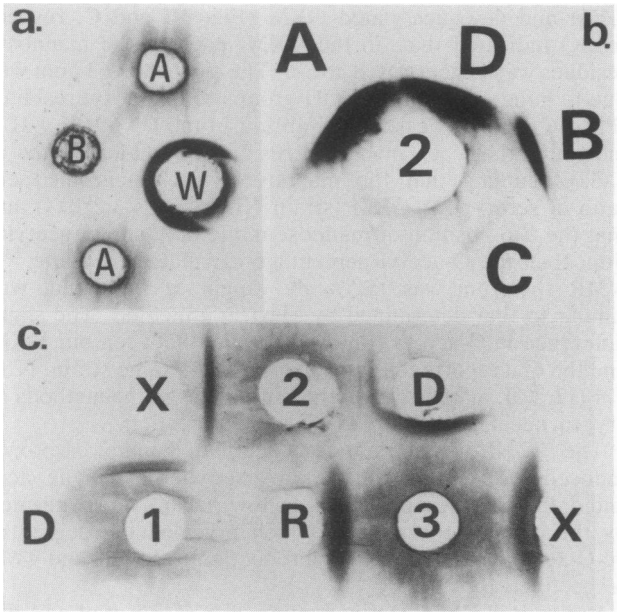

FIG. 4. (a) Double immunodiffusion showing the reactivity of GXM-TT2 conjugate with anticryptococcal and anti-tetanus toxoid hyperimmune sera. Center well (W), GXM-TT2; wells A, rabbit anticryptococcal serum; well B, mouse tetanus toxoid antiserum. (b) Double immunodiffusion showing the cross-reaction between the CPs of *C. neoformans* serotypes A, D, B, and C with GXM-TT2-induced mouse hyperimmune serum (well 2). (c) Double immunodiffusion showing the reactivity of conjugate-induced mouse hyperimmune antisera with the unmodified and chemically modified GXMs of *C. neoformans* serotype A. X, chemically unmodified GXM; D, de-O-acetylated GXM; R, reduced and de-O-acetylated GXM; 1, GXM-TT1-induced mouse hyperimmune antiserum; 2, GXM-TT2-induced mouse hyperimmune antiserum; 3, rabbit anticryptococcal serum.

general purpose mice. All conjugates elicited an IgM response after the first injection in both strains of mice. IgG antibodies were detected after the second immunization with all conjugates except GXM-TT1 in general purpose mice. The highest IgG antibody levels were elicited by GXM-TT2, followed (in descending order) by GXM-rEPA and GXM-TT1. The antibody response produced by GXM-TT1 in general purpose mice was similar to that induced by GXM alone. However, this conjugate elicited a booster IgG and IgM response in BALB/c mice. GXM-TT2 and GXM-rEPA elicited booster antibody responses in both strains of mice ($P = 0.001$). GXM-rEPA induced higher levels of IgM antibodies than the other two conjugates after each injection ($P = 0.0005$).

Effect of MPL on the immunogenicity of GXM-TT2. Mice immunized with GXM admixed in MPL failed to produce detectable levels of IgG antibodies regardless of the time of administration of MPL (Table 3). However, this mixture induced a rise in the level of IgM antibodies after the second immunization ($P = 0.001$). GXM-TT2, when injected alone or concurrently with MPL, elicited a significant rise in the level of IgG antibodies after the first injection and booster IgG and IgM responses following the second injection ($P = 0.03$). The antibody response observed after the administration of MPL, 2 days following the injection of GXM-TT2, was higher than the response produced by the conjugate alone or by the conjugate injected concurrently with MPL.

Serotype and epitope specificities of conjugate-induced antisera. Pooled murine hyperimmune serum produced against GXM-TT2 formed intense precipitin bands with the CPs of *C. neoformans* serotypes A and D, a less intense band with serotype B CP, and a faint line with the CP of serotype C (Fig. 4b).

Murine hyperimmune sera generated against GXM-TT1 and GXM-TT2 were used to define the immunogenic determinants of GXM. GXM-TT2-induced antiserum precipitated with the chemically unmodified and the de-O-acetylated GXMs of serotype A, whereas the antiserum generated by GXM-TT1 reacted only with the unmodified GXM (Fig. 4c). None of these two antisera precipitated with the carboxyl-reduced GXM, which also lacked O-acetyl groups. These results suggested the preservation of the O-acetyl determinant in GXM-TT1 and of both O-acetyl and glucuronyl epitopes in GXM-TT2. Rabbit anticryptococcal serum precipitated with the unmodified and the de-O-acetylated GXMs and also with the carboxyl-reduced and de-O-acetylated GXM.

TABLE 2. Antibody response to GXM elicited in mice by GXM alone or by GXM conjugated to TT and rEPA[a]

Mouse strain and vaccine	Postimmunization geometric mean[b,c]					
	IgG			IgM		
	1st injection	2nd injection	3rd injection	1st injection	2nd injection	3rd injection
BALB/c mice						
Native GXM	0.13 (0.1, 0.15)	0.13 (0.1, 0.15)	0.23 (0.21, 0.25)	0.14 (0.1, 0.19)	0.29 (0.25, 0.37)	0.22 (0.17, 0.29)
GXM	<0.1	<0.1	<0.1	0.11 (0.1, 0.11)	0.13 (0.1, 0.16)	0.16 (0.1, 0.27)
GXM-TT1	<0.1	0.14 (0.1, 0.22)	1.34* (0.26, 7.97)	0.12 (0.1, 0.13)	0.35 (0.1, 0.74)	0.19† (0.1, 0.32)
GXM-TT2	<0.1	0.83 (0.46, 1.52)	23.91‡ (16.31, 42.55)	<0.1	6.81 (3.96, 9.11)	7.07§ (5.32, 9.44)
General purpose mice						
GXM	<0.1	<0.1	<0.1	0.12 (0.1, 0.12)	0.18 (0.1, 0.32)	0.21 (0.1, 0.57)
GXM-TT1	<0.1	<0.1	<0.1	0.12 (0.1, 0.12)	0.12 (0.1, 0.12)	0.22 (0.1, 0.37)
GXM-TT2	<0.1	1.61 (0.54, 3.67)	12.02‡ (6.69, 27.31)	0.84 (0.56, 1.16)	2.63 (2.06, 4.87)	5.30§ (0.99, 22.95)
GXM-rEPA	<0.1	0.52 (0.27, 0.78)	7.05‡ (4.61, 15.74)	2.57 (1.69, 4.07)	15.60 (8.88, 28.77)	17.33# (4.92, 60.1)

[a] Groups of ten mice were injected subcutaneously three times, 2 weeks apart, with 0.1 ml of the vaccines (see Materials and Methods). Their blood samples were collected a week after each immunization, and GXM antibodies were measured by ELISA (19).

[b] In micrograms of antibody per milliliter. Values in parentheses are for 25th and 75th percentiles, respectively.

[c] P values are as follows: for figures marked ‡ versus figures marked *, $P = 0.0001$; for § versus †, $P = 0.001$; for # versus §, $P = 0.005$.

TABLE 3. Murine antibody response to GXM and GXM-TT2 used alone and admixed in MPL[a]

Vaccine	Postimmunization geometric mean[b,c]			
	IgG		IgM	
	1st injection	2nd injection	1st injection	2nd injection
Saline	<0.1	<0.1	1.57 (1.26, 1.94)	1.43 (0.97, 2.01)
GXM	<0.1	<0.1	8.02 (3.60, 14.36)	3.33 (1.86, 5.08)
GXM + MPL	<0.1	<0.1	1.76 (1.23, 2.61)	4.36 (3.26, 5.31)
GXM and MPL 2 days later	<0.1	<0.1	3.14 (1.48, 6.25)	19.82 (12.37, 25.68)
GXM-TT2	4.60 (1.3, 25.95)	19.42# (9.90, 35.17)	19.03 (11.2, 32.64)	78.61‡ (69.69, 121.01)
GXM-TT2 + MPL	5.82 (2.02, 9.88)	37.72 (13.13, 144.11)	14.08 (7.25, 26.19)	90.97‡ (21.58, 257.10)
GXM-TT2 and MPL 2 days later	19.25 (11.91, 27.03)	122.91* (74.48, 388.88)	27.39 (20.87, 36.66)	314.20† (179.64, 496.41)

[a] Two groups of 10 ICR mice were injected intraperitoneally with 0.1 ml of the immunogens in saline on days 0 and 21, as outlined in Materials and Methods. Blood samples were collected on days 21 and 28, and GXM antibodies were determined by ELISA (19).
[b] In micrograms of antibody per milliliter. Values in parentheses are for 25th and 75th percentiles, respectively.
[c] *P* values are as follows: for figures marked * versus figures marked #, $P = 0.008$; for † versus ‡, $P = 0.03$.

IgG response to protein carriers. All conjugates elicited booster IgG responses to the carrier proteins following the second immunization ($P = 0.001$) (Table 4).

DISCUSSION

Development of serum capsular antibodies is correlated with protection in subjects who recover from disseminated cryptococcosis (5, 13). About 40 to 50% of immune-competent subjects with cryptococcal infections produce GXM antibodies, and the concentrations are low (13, 30, 48). Mice experimentally infected with *C. neoformans* also produce low levels of antibodies, mostly of IgM class (7, 52). Normal subjects have low levels of anticapsular antibodies to *C. neoformans*, probably because of natural exposure to this ubiquitous fungus (30). Subjects with AIDS and AIDS-related complex have been shown to have much lower levels of serum IgG antibodies to GXM than healthy controls (15), probably because of an intrinsic B-cell deficiency in these patients. These data indicate that the induction of sufficiently high levels of anti-GXM antibodies in immune-sufficient or immune-deficient subjects may confer protective immunity against cryptococcal infections. For this reason, we synthesized the conjugate vaccines of GXM of serotype A *C. neoformans* to prevent disseminated cryptococcosis, particularly among high-risk populations.

GXM was covalently bound to TT or rEPA after introducing the spacer, ADH, through its carboxyl or hydroxyl groups. The conjugates prepared through hydroxyl activation (GXM-TT2 and GXM-rEPA) elicited higher levels of GXM antibodies than the one synthesized through carboxyl activation (GXM-TT1) regardless of the protein carrier used. Our finding that BALB/c mice produced low levels of IgG antibodies to native GXM alone is in agreement with published data (19). BALB/c mice also responded better to both types of conjugates than did general purpose mice. The conjugates also elicited booster IgG responses to the protein carriers. The immunogenicity of GXM-TT2 was further enhanced in mice by the administration of MPL. The adjuvant effect of MPL was more pronounced when it was given 2 days after the administration of the conjugate than when given concurrently. A similar phenomenon has been observed with the CP of type III *Streptococcus pneumoniae* when it is given with MPL (1). The mechanism of this enhanced antibody response is explained as inactivation by MPL of antigen-specific suppressor T cells, whose activity is maximal 2 to 3 days after the injection of the antigen. MPL, a nontoxic immunomodulator, deserves clinical evaluation, particularly in risk populations with decreased immune capabilities.

TABLE 4. Serum IgG response to the respective protein carriers elicited by GXM-protein conjugate vaccines[a]

Vaccine	Postimmunization IgG geometric mean[b,c]		
	1st injection	2nd injection	3rd injection
GXM	<0.02	<0.02	<0.02
GXM-TT1	0.04† (0.02, 0.08)	0.20* (0.12, 0.52)	1.47‡ (0.83, 2.78)
GXM-TT2	0.03† (0.02, 0.04)	0.16* (0.08, 0.27)	0.81‡ (0.46, 1.22)
GXM-rEPA	0.04† (0.02, 0.08)	1.11# (0.57, 2.02)	1.54‡ (1.07, 1.96)

[a] General purpose mice (NIH), 4 to 5 weeks old, were injected subcutaneously three times, 2 weeks apart, with 0.1 ml of the immunogens. Blood samples were collected a week after each immunization, and TT and rEPA antibodies were measured by ELISA.
[b] In ELISA units per milliliter. Values in parentheses are for 25th and 75th percentiles, respectively.
[c] *P* values are as follows: for figures marked ‡ or * versus figures marked †, for ‡ versus *, and for # versus †, $P = 0.001$.

Glucuronyl and O-acetyl groups are recognized to be the major immunodeterminants of serotype A GXM (9, 54). Hyperimmune serum generated against GXM-TT1 precipitated with the chemically unmodified GXM but not with the de-O-acetylated GXM, whereas the antiserum raised against GXM-TT2 reacted both with the unmodified and the de-O-acetylated GXMs. These observations suggest that the less immunogenic GXM-TT1 retained the O-acetyl determinant and that GXM-TT2 retained both the glucuronyl and the O-acetyl determinants. The latter was significantly more immunogenic and induced booster IgG and IgM GXM responses both in BALB/c and general purpose mice. These observations confirm the published reports about the importance of the glucuronopyranosyl side chain of GXM in immunogenicity (9, 54). The carboxyl groups may therefore form an important part of the reactive epitope of GXM-TT2 and render it more immunogenic.

Serotypes A and D of *C. neoformans* account for almost all isolates from AIDS patients, whereas serotypes B and C are commonly recovered from nonimmunocompromised patients in certain confined areas (40). Murine antibodies induced by GXM-TT2 precipitated with the CPs of serotypes

A = D > B > C (order of intensity of precipitation is indicated). The role of these antibodies in cross-protection against cryptococcal infections caused by different serotypes will be investigated further.

Protection of mice by passive or active immunization, as assayed by prolonged survival, has been reported by some investigators (22, 23, 28) but not by others (25, 26). The passive protection experiments using anticapsular MAbs have yielded discrepant results, perhaps because of the difference in animal models and experimental designs. Anticapsular IgG1 and IgM MAbs were effective in delaying significantly the death of DBA/2 and A/J mice, respectively, which were infected with lethal doses of *C. neoformans* (6a, 17). Sanford and coworkers (49), using a different murine model, observed a reduction in the number of cryptococci in the lungs and spleens but not in the brains of mice passively immunized with capsular IgG2 MAbs. These workers have also demonstrated the efficient opsonic properties of these MAbs in vitro (18, 50). Further, passive immunization with cryptococcal capsular antibodies has significantly potentiated the chemotherapeutic effectiveness of amphotericin B in infected mice (16, 24). The protective efficacy of our conjugates prepared through hydroxyl activation is being evaluated in animal models. By inducing elevated levels of IgG antibodies to GXM, these immunochemically defined conjugate vaccines may serve as effective prophylactic regimens against disseminated cryptococcosis, especially in high-risk populations such as subjects with AIDS-related complex and diabetes and those in the initial stages of human immunodeficiency virus infection or malignancy.

In conclusion, the conjugation of cryptococcal GXM to TT or rEPA enhanced the immunogenicity and conferred T cell-dependent properties to GXM. The best conjugates were obtained by sonicating the GXM, derivatizing it with ADH by CNBr activation, and then conjugating this derivative to TT or rEPA. This method preserves both the glucuronyl and O-acetyl epitopes. The conjugate vaccines prepared by this method appear suitable for clinical evaluation against cryptococcosis.

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