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Antibodies to Keyhole Limpet Hemocyanin Cross-React with an Epitope on the Polysaccharide Capsule of Cryptococcus neoformans and Other Carbohydrates: Implications for Vaccine Development

Rena J. May,* David O. Beenhouwer,**† and Matthew D. Scharff*†

Cryptococcus neoformans causes a life-threatening meningoencephalitis in AIDS patients. Mice immunized with a glycoconjugate vaccine composed of the glucuronoxylomannan (GXM) component of the cryptococcal capsular polysaccharide conjugated to tetanus toxoid produce Abs that can be either protective or nonprotective. Because nonprotective Abs block the efficacy of protective Abs, an effective vaccine must focus the Ab response on a protective epitope. Mice immunized with peptide mimetics of GXM conjugated to keyhole limpet hemocyanin (KLH) with glutaraldehyde developed Abs to GXM. However, control peptides P315 and P24 conjugated to KLH also elicited Abs to GXM. GXM-binding Abs from mice immunized with P315-KLH were inhibited by KLH treated with glutaraldehyde (KLH-g), but not by P315. Furthermore, KLH-g inhibited binding of GXM by serum of mice immunized with GXM-TT, indicating that glutaraldehyde treatment of KLH reveals an epitope(s) that cross-reacts with GXM. Vaccination with KLH-g or unmodified KLH elicited Abs to GXM, but did not confer protection against C. neoformans, suggesting the cross-reactive epitope on KLH was not protective. This was supported by the finding that 4H3, a nonprotective mAb, cross-reacted strongly with KLH-g. Sera from mice immunized with either native KLH or KLH-g cross-reacted with several other carbohydrate Ags, many of which have been conjugated to KLH for vaccine development. This study illustrates how mAbs can be used to determine the efficacy of potential vaccines, in addition to describing the complexity of using KLH and glutaraldehyde in the development of vaccines to carbohydrate Ags. The Journal of Immunology, 2003, 171: 4905–4912.

*ncapsulated organisms, such as Streptococcus pneumoniae, Haemophilus influenzae type B, Neisseria meningitidis, group B streptococcus, and Cryptococcus neoformans, are responsible for many serious infections and for most deaths from meningitis (1). Given their medical significance, there is a great need for improved vaccine strategies against these organisms. The polysaccharide capsule of these organisms is the primary virulence factor and, consequently, acapsular strains do not cause significant disease. Purified capsular polysaccharide vaccines of S. pneumoniae and N. meningitidis can prevent disease with homologous serotypes (reviewed in Ref. 2). However, purified polysaccharides are T cell-independent Ags that elicit an immune response lacking immunologic memory (3, 4). Furthermore, capsular vaccines have not been effective in protecting those individuals most susceptible to infection, such as infants, people over age 65, and those with impaired immunity (5).

Currently, glycoconjugate vaccines are the most effective means of protecting individuals against infection by encapsulated organisms. Glycoconjugate vaccines are composed of capsular polysaccharide conjugated to an immunogenic protein carrier, such as tetanus toxoid (TT),4 or keyhole limpet hemocyanin (KLH) and generate a T cell-dependent Ab response to the polysaccharide Ags (4, 6, 7). Despite the success of the Haemophilus influenzae type B glycoconjugate vaccine (8), there are still a number of potential problems with the use of glycoconjugates. First, these vaccines may not be sufficiently immunogenic in immunosuppressed and elderly individuals (reviewed in Ref. 4). Second, glycoconjugates are comprised of the entire capsular polysaccharide, which may contain epitopes that elicit both protective and nonprotective Abs (9–11). In the case of C. neoformans, these epitopes have been defined based on their reactivity with mAbs that do or do not prolong the life of mice lethally infected with C. neoformans (12, 13). In addition, there is evidence that Abs directed against nonprotective epitopes of the polysaccharide capsule of Schistosoma mansoni, N. meningitidis, and C. neoformans can block the efficacy of protective Abs and even enhance infection (11–15).

To address these issues, we and several others (4, 16–21) have searched for a method of generating a vaccine to encapsulated organisms, such as C. neoformans, that is T cell dependent, highly immunogenic, and will specifically direct the Ab response to a protective epitope. mAbs against the glucuronoxylomannan (GXM) component of the capsular polysaccharide can protect

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mice against infection with *C. neoformans* (22). Although glycoconjugates using oligosaccharides that reacted with protective mAbs might serve this purpose, such oligosaccharides have not been identified. Peptide mimetics of polysaccharides offer an attractive alternative because they: 1) induce a T cell-dependent response, 2) are small and present few epitopes, and 3) can be identified using high throughput methods, and 4) are easy and cost effective to produce (reviewed in Ref. 21). Therefore, we sought to create an effective vaccine by identifying a peptide mimic of a protective epitope of GXM (23). Phage display peptide libraries were screened with 2H1, a protective anti-GXM mAb, and a number of peptides were isolated (19–21, 23).

To study their efficacy as vaccines, peptide mimetics were conjugated to KLH, and mice were then immunized with these conjugates. Carrier proteins such as TT, KLH, OVA, and BSA are commonly conjugated to small Ags, thereby providing the hapten with a strong T cell epitope (24). The hemocyanin of the keyhole limpet marine mollusk (KLH) has a remarkable immunostimulatory effect because it can activate both humoral and cellular immunity (25). Due to these properties, many vaccine studies have used KLH as the immunogenic carrier. The haptons used together with KLH have varied, from capsular polysaccharides (26) to peptide Ags (19, 27), anti-idiotypic Abs (28, 29), as well as a number of tumor-associated carbohydrates (30, 31). We report in this study that peptide mimetics of GXM did not elicit a strong response to GXM when they were conjugated to KLH. Rather, KLH contained polysaccharide that elicited an anti-GXM response to nonprotective epitopes. These results have significant ramifications for vaccine development.

**Materials and Methods**

**Peptides**

The identification of peptides PA1 (LQYTPSWMLV), P206 (FGGETFTPDWMMEVAIDNE), P206N (FGGETFTPDDWMMEV), and P206C (AFPDWMMEVAIDNE) have been described (19, 23). As illustrated in Fig. 1A, two of these peptides (PA1 and P206N), and P206C and their biotinylated counterparts were synthesized by the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine (AECOM). Peptide preparations were analyzed by HPLC and mass spectroscopy. Control peptide P315 (CKVMVHDPHSLA), the carboxyl-terminal domain of murine MHC class I H2Kk (32), has been identified. Peptide mimetics of polysaccharides offer an atypical epitope. These results have significant ramifications for vaccine development.

**Immunizations**

Peptides (1 mg) were conjugated to KLH (3 mg; Pierce, Rockford, IL) in 0.25 ml PBS, pH 7.5, with the slow addition of 120 μg of GXM (Sigma-Aldrich, St. Louis, MO), as described (33). Conjugates were then dialyzed at 4°C against sterile PBS for 48 h with daily changes. KLH and BSA were used for in vitro studies and control immunizations were treated with glutaraldehyde in the same manner (termed KLH-g or BSA-g). Six-week-old BALB/c mice (National Cancer Institute, Bethesda, MD) were maintained in a pathogen-free barrier facility and immunized via i.p. injection with 100 μg of each peptide-KLH conjugate or 0.5 μg of GXM-TT (gift of J. Robbins, National Institutes of Health, Bethesda, MD) in emulsion with CFA (Difco, Detroit, MI) on day 0 and with IFA (Difco) on day 28. Mice were bled before the first immunization and on days 14 and 42 postimmunization. Protective efficacy of immunizations was assessed by infecting mice with 1 × 10^6 CFU of *C. neoformans* strain 24067 i.v. on day 56, and survival was monitored.

**Carbohydrates**

*C. neoformans* strain 24067 (serotype D) was provided by A. Casadevall (AECOM). Total cryptococcal polysaccharide was prepared by ethanol precipitation from whole *C. neoformans* by cetyltrimethylammonium bromide precipitation, and did not contain detectable protein contaminants (35). *Mycobacterium tuberculosis*-derived lipoparabumin (LAM) was a gift from A. Freedman (AECOM). All other carbohydrate reagents were obtained commercially: Lewis Y tetrasaccharide (Calbiochem, San Diego, CA), LPS from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich), *S. pneumoniae* vaccine containing capsular polysaccharide of 23 different serotypes (Pnu-immune 23; Lederle, Pearl River, NY), and GD3 disialoglucosamine salt extracted from bovine buttermilk (Calbiochem).

**Periodate treatment**

KLH (Pierce) was treated with 0.2 M sodium periodate in 0.01 M sodium acetate buffer (pH 4.5) overnight at 4°C (36). The reaction was stopped by the addition of an equal volume of 0.05 M sodium borohydride for 30 min at room temperature. Periodate-treated KLH (KLH-p) was then dialyzed against sterile PBS for 48 h with daily changes.

**ELISAs**

Sera from immunized animals were treated with 0.1 M 2-ME at 37°C for 1 h to dissociate pentameric IgM. GXM ELISAs were performed by coating plates with 10 μg/ml of GXM; KLH ELISAs were prepared by coating plates with 10 μg/ml of KLH (KLH-g or KLH-p described above). To analyze binding to various carbohydrates, ELISA plates were coated with either 10 μg/ml GD3, 10 μg/ml Lewis Y, 10 μg/ml LPS, 10 μg/ml PC, or 5 μg/ml LAM. BSA-coated wells served as controls on each microtiter plate. For all ELISAs, sera were incubated in serial dilutions starting at 1/25 or 1/50. GXM and KLH binding were detected with a mixture of alkaline phosphatase-conjugated anti-mouse IgG isotypes (Southern Biotechnology, Birmingham, AL), or Abs to each individual isotype. Microtiter plates were developed with 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich) in 1 M diethanolamine and 0.25 mM MgCl_2, pH 9.8, and the absorbance was measured at 405 nm. Ab reactivity to peptides was assayed on biotinylated peptide (1 μg/ml) bound to streptavidin-coated (1 μg/ml) ELISA plates. Binding of GXM mAbs (12, 37–39) (0.5 μg/well) to KLH-g coated plates was detected using a mixture of alkaline phosphatase-conjugated anti-mouse IgG isotypes, and developed as described above. Control ELISAs were performed by first incubating serum dilutions with different concentrations of peptide, BSA-g, KLH-g, or KLH-p, fully solubilized in PBS, for 2 h at 37°C. Concentrations of KLH and KLH-g greater than 5 mg/ml come out of solution, thus limiting the competition assay. Samples were then transferred to an ELISA plate, and GXM binding was detected as described above. In all cases, a dilution was considered positive when the OD observed (minus the BSA-reactive background) was 3 times greater than background. The 4H3 competition ELISA was performed in a similar manner; Serum dilutions were incubated on a GXM-coated microtiter plate at 37°C for 1 h. mAb 4H3 (1 μg/ml) was then added to each well, and the plates were incubated at 37°C for an additional hour. Binding of 4H3 to GXM was detected using alkaline phosphatase-conjugated anti-mouse antibody and developed as described above.

**Statistical analysis**

Data were analyzed with StatView statistical software (SAS Institute, Cary, NC). ELISA data were compared using the Mann-Whitney U test for nonparametric data. Survival data were subjected to Kaplan-Meier analysis, and statistical significance was determined by the log rank (Mantel-Cox) test. A p value of <0.05 was considered statistically significant. Error bars represent an SE of 1.

**Results**

**KLH-g elicits Abs to GXM**

We have previously screened phage peptide display libraries and identified peptide mimetics of GXM that bound with high affinity to protective, but not to nonprotective, mAbs to GXM (20, 21, 23). Two of these peptides, PA1 and P206 (20), were conjugated to KLH with glutaraldehyde and injected into mice. As a negative control, mice were immunized with KLH conjugated to an irrelevant peptide, P315, which does not bind any mAb to GXM (20, 23). As illustrated in Fig. 1A, immunization with P315-KLH resulted in IgG titers against GXM comparable to those in mice immunized with P206-KLH, the highest affinity peptide mimic of GXM yet identified (20), and greater than those elicited by PA1, a lower affinity peptide mimic of GXM (21). In contrast, the anti-peptide response to P315 was significantly lower than that of animals receiving other peptide-KLH conjugates (Fig. 1B).

To rule out the possibility that P315 was playing a unique role in eliciting Abs to GXM, a second experiment was done using
P24A, a peptide mimic of phosphorylcholine (17), as an alternative negative control. Additional mice were immunized with two other high affinity peptide mimetics of GXM, P206N, and P206C (20) conjugated to KLH, and with native KLH as an additional control. The two control Ags, P24A-KLH and native KLH, elicited IgG titers to GXM that were equal, and in some cases better than the Ab response elicited by the high affinity peptide mimetics (Fig. 1C). The animals immunized with the different peptides all mounted a significant Ab response to the peptide Ag (Fig. 1D). Competition studies revealed that soluble peptides inhibited binding of sera to homologous peptide-coated ELISA plates; soluble peptides did not inhibit binding of sera to GXM (data not shown and see below).

Because KLH alone could elicit an Ab response to GXM, the ability of KLH to inhibit the binding of Abs to GXM in the serum of a P315-KLH-immunized mouse was analyzed (Fig. 2A). Native KLH weakly inhibited the binding of serum Abs to GXM. However, KLH-g effectively inhibited binding of serum Abs to GXM. The difference between native and glutaraldehyde-treated KLH implies that glutaraldehyde treatment changes the structure of KLH, creating or revealing cross-reactive epitopes to GXM. Although it is difficult to make quantitative comparisons between molecules that may have different epitope densities, KLH-g inhibited the binding of serum Abs to GXM more efficiently than GXM itself (Fig. 2A). The average m.w. of KLH is twice that of GXMs, but even if this difference was corrected for, the inhibition would be comparable. KLH-g did not bind to GXM-coated plates (data not shown), ruling out the possibility that KLH-g was merely acting as a lectin. P315 alone did not inhibit the binding of serum Abs to GXM (data not shown). These data suggested that KLH, the carrier used in these immunizations, was eliciting the anti-GXM response. This was confirmed when mice immunized with KLH-g, in the absence of any peptide, mounted an Ab response to GXM that was even greater than animals immunized with the various peptide conjugates or KLH alone (compare Figs. 1C and 2B).

**GXM and KLH share a carbohydrate epitope**

Animals immunized with the various peptide conjugates mounted an anti-KLH-g IgG response that was 10-fold lower than the anti-peptide response (data not shown). To further establish that the anti-GXM Abs elicited by the peptide-KLH conjugates were stimulated by KLH, competition studies were conducted with sera from mice immunized with the various conjugates. KLH-g inhibited an average of 60% of GXM binding in the serum of mice immunized with the different peptide mimetics conjugated to KLH (Fig. 3). KLH-g also inhibited an average of 70% of the serum GXM-binding IgG from mice immunized with GXM-TT (Fig. 3), even though these animals had never been exposed to KLH.

To examine whether the glycosylated regions of KLH were responsible for the cross-reactivity between KLH and GXM, KLH was treated with periodate (KLH-p), which cleaves the vicinal hydroxyl groups of carbohydrates on the surface of KLH (36). KLH-p also inhibited the binding of sera to GXM, but was less efficient than KLH-g (Fig. 3). BSA-g does not inhibit the binding of GXM-reactive Abs to the same extent as KLH-g (Fig. 3). These
results further support the conclusion that KLH surface carbohydrates contribute to the cross-reactivity between KLH and GXM, but also suggest that glutaraldehyde can modify proteins to produce an epitope(s) that cross-reacts with GXM.

**KLH cross-reacts with a nonimmunodominant, nonprotective epitope of GXM**

If KLH-g shares an epitope(s) with GXM, then immunization with KLH-g should boost the anti-GXM titer of mice previously immunized with GXM. However, when mice were immunized with GXM-TT and then boosted with either GXM-TT or KLH-g, the GXM titer was not significantly enhanced in those animals that received KLH-g, compared with mice that were primed and boosted with GXM-TT (Fig. 4). These data imply that although Abs to KLH-g can cross-react with GXM, the cross-reactive epitope on KLH-g is not recognized by a sufficient number of memory B cells elicited by GXM-TT to boost the anti-GXM response.

Due to the lack of structural data with regard to GXM and KLH epitopes, the easiest way to determine which epitope of GXM cross-reacted with KLH-g would be to screen the sera of immunized mice on a panel of KLH- and GXM-derived oligosaccharides. However, such oligosaccharides do not exist, so we chose to identify the cross-reactive epitope by studying the binding of a panel of mAbs to GXM to KLH-g (Fig. 5). mAbs 2H1, 18B7, 2D10, and 12A1 did not bind to KLH-g or KLH-p. These mAbs recognize the same, or a closely related, protective epitope on GXM, and are members of the group II anti-cryptococcal mAbs that express V_{H} 7183 and VK_{s} 5.1 (12, 39). These Abs prolong the life of lethally infected animals (11, 38, 40, 41). In addition, KLH-g did not bind to either 13F1 and 2D12, two nonprotective group II mAbs (11, 12). However, mAb 4H3 bound strongly to KLH-g, and very weakly to KLH-p, providing evidence that a GXM cross-reactive epitope on KLH that binds to 4H3 is a polysaccharide. The 4H3 is encoded by V_{H} 441 and V_{K} 11, members, reacted weakly with KLH-g and more strongly with KLH-p, confirming that the glycosylated regions of KLH may cross-react with other carbohydrate Ags. This was confirmed when mice immunized with KLH-g were not protected against lethal *C. neoformans* infection (38, 42). mAb 3E5, another group II member, reacted weakly with KLH-g and more strongly with KLH-p, suggesting that it was recognizing still another epitope on KLH. Sera from mice immunized with either native KLH or KLH-g could inhibit the binding of mAb 4H3 to GXM (Fig. 6). These findings suggest that at least some of the epitopes on KLH that cross-react with GXM can elicit nonprotective Abs. This was confirmed when mice immunized with KLH-g were not protected against lethal *C. neoformans* infection (Fig. 7), despite the appreciable titers of Ab to GXM stimulated in these animals (Fig. 2B).

**KLH shares epitopes with many carbohydrate Ags**

The existence of a shared epitope between GXM and KLH alerted us to the possibility that the glycosylated regions of KLH may cross-react with other carbohydrate Ags. The serum from mice immunized with KLH-g was screened against a number of carbohydrates, some of which have been conjugated to KLH and used successfully as vaccines (Fig. 8) (7, 30, 31, 43). None of the pre-immunization sera reacted with Lewis Y tetrasaccharide, LAM, GXM, GD3, LPS, or Pnu-immune 23 (data not shown). In addition to binding to GXM, the sera from the secondary immunization with KLH-g bound each of these carbohydrates (Fig. 8). To determine whether this finding was limited to epitopes of KLH-g, we immunized mice with unmodified KLH and their sera reacted with all the carbohydrate Ags, albeit with lower Ab titer (Fig. 8).
result implies that regardless of whether KLH is treated with glutaraldehyde or not, there are epitopes on the surface of KLH that cross-react with a variety of different carbohydrate Ags.

Discussion
In an attempt to create effective peptide vaccines that will prevent infections, we have used the GXM capsular polysaccharide of C. neoformans as our model system. mAbs were generated that identified protective and nonprotective epitopes of GXM. One such Ab that reacted with a protective epitope was used to screen phage peptide libraries for peptide mimetics. Mice immunized with the GXM mimetic peptides conjugated to TT (20) did not generate significant anti-GXM Ab responses. However, when mice were first primed with the GXMTT glycoconjugate to generate memory B cells to GXM and then boosted with the P206-TT conjugate, high titers of IgG Ab to protective epitope(s) of GXM were obtained (20). We have attempted to extend those studies by developing strategies that would allow us to immunize naive mice with the peptide mimotope conjugates. Because it is now difficult to obtain carriers such as TT or diphtheria toxin, a more readily available carrier such as KLH was tested. KLH is a large (>2000 kDa), highly glycosylated protein frequently used as a carrier protein in animals and humans because of its potent immunogenicity. Several groups developing carbohydrate vaccines have reported success when using KLH as the carrier protein (7, 30, 31, 43).

In the studies described in this work, peptide-KLH conjugates were prepared with a glutaraldehyde conjugation method commonly used with candidate vaccines (17, 29, 44). Glutaraldehyde is a bifunctional coupling reagent that links two compounds primarily through their amino groups. The peptides we used did not contain internal lysines, and therefore, the main reactive group should be at the amino terminus. We used P315 and P24A as control peptides, and were surprised to find that naive mice immunized with these irrelevant peptides conjugated to KLH mounted an Ab response to GXM. This response was composed primarily of the IgG1 and IgG2a isotype (data not shown), and these Abs were inhibited from binding to GXM by KLH-g. The results from this and other inhibition studies led us to conclude that neoepitopes generated by treating KLH with glutaraldehyde are cross-reactive with GXM. Further studies revealed that native KLH also had epitopes that cross-reacted with GXM.

It has been known for some time that KLH, which is itself a highly glycosylated protein, can cross-react with a number of carbohydrate Ags. Although the nature of the oligosaccharides on the surface of KLH has not been completely elucidated, those identified to date have been shown to contain unique oligosaccharide patterns, resulting in novel structures (45, 46). These novel structures, together with the high levels of cross-reactivity with a variety of carbohydrate Ags, may contribute to the immunogenicity of KLH (47). In 1974, Olsson et al. (48) discovered that patients immunized with KLH had a reduction in recurrence of bladder carcinomas. The cross-reactive epitope was identified (49), and clinical trials with Immucytol, a KLH subunit, are reported (50). In addition, a fucose epitope on the surface of S. mansoni has been found to be cross-reactive with KLH (46, 51). Immunobilized KLH or anti-KLH Abs have been used to diagnose acute vs chronic infections of S. mansoni (52).

Because these studies indicate that the cross-reactive nature of KLH is not limited to GXM, sera from mice immunized with KLH-g and KLH were screened against a variety of different carbohydrate Ags, a number of which have been conjugated to KLH (7, 30, 31), and are either being studied as candidate vaccines or are in clinical trials (43). The average titers to the various carbohydrate Ags were greater in the sera of mice immunized with KLH-g compared with untreated KLH, possibly because cross-linking the KLH with glutaraldehyde and modifying the primary amines may either reveal more cross-reactive epitopes or render KLH more immunogenic. It is unlikely that the glutaraldehyde structure alone is responsible for the cross-reactive nature of KLH-g, and carbohydrates on the surface of KLH must cross-react with GXM because BSA-g, an irrelevant protein, cannot inhibit the binding of GXM-specific Abs as effectively as KLH-g (Fig. 3), and mice immunized with native KLH induce anti-GXM Abs (Fig. 1C) that are inhibited by mAb 4H3 (Fig. 6). It is possible that glutaraldehyde treatment of other heavily glycosylated carrier proteins may result in the induction of cross-reactive Abs to carbohydrate Ags.

To determine whether the cross-reactivity observed with GXM was also due to a carbohydrate epitope on native KLH, KLH was subjected to periodate oxidation, which has been shown to disrupt the polysaccharide structure on the surface of KLH (36). If carbohydrates are subjected to one round of oxidation and reduction, this procedure will destroy the immunogenicity of the carbohydrate, while not disturbing the amino acids or linear stretches of internal sugar residues (53). This method has been used in other systems to determine whether carbohydrate epitopes bind to mAbs (53).
Treating KLH with periodate reduced its ability to inhibit serum binding to GXM by ~50%, indicating that some of the cross-reactive epitope(s) must be carbohydrate in nature. KLH-p does not inhibit all of the serum reactivity to GXM, and there are a number of explanations for this finding. First, it is possible that a protein epitope of KLH also contributes to the cross-reactivity with GXM, because periodate treatment does not disturb protein epitopes (53, 54). Second, while most of the reactivity of mAbs to carbohydrate is lost following periodate treatment (53, 54), polyclonal sera retain some binding to the carbohydrate Ag even after oxidation (55, 56). Third, it is possible that, as is seen with 3E5 IgG1 (Fig. 5), and as documented in other cases (57, 58), periodate oxidation may unmask other carbohydrate residues, thus exposing epitopes that can bind to Abs in polyclonal sera.

Because oligosaccharides of KLH and GXM were unavailable, and simple carbohydrate molecules representing the known carbohydrate motifs of KLH did not inhibit the binding of KLH-specific Abs to GXM (data not shown), we used a panel of anti-GXM mAbs to further characterize the cross-reactive epitope between KLH and GXM. Of all the anti-GXM mAbs screened against KLH-g and KLH-p, mAb 4H3 bound most strongly to KLH-g and did not bind to KLH-p. In addition, the polyclonal sera from mice immunized with either KLH-g or KLH inhibited the binding of 4H3 to GXM, indicating that the 4H3 epitope is at least one of the epitopes that is cross-reactive between KLH and GXM. mAb 4H3 is encoded by different V regions than the dominant group II Ab epitopes (53, 54). Second, while most of the reactivity of mAbs to carbohydrate is lost following periodate treatment (53, 54), polyclonal sera retain some binding to the carbohydrate Ag even after oxidation (55, 56).

The immune system's continual exposure to ubiquitous carbohydrate Ags may result in a population of memory B cells that are nonreactive to KLH, and the possibility that by the time these mice were bled (day 14 after immunization), T cells specific for KLH had been activated, sufficient amounts of T cell memory may not have been induced, providing an additional explanation to the weak secondary response mounted in these immunized animals.

Taken together, these findings lead us to conclude that caution needs to be taken when creating vaccines for carbohydrate Ags using KLH as the carrier. This is not the first study showing that one needs to be careful when dealing with the cross-reactive nature of KLH. Recently, there have been reports cautioning against the use of KLH in the diagnosis of S. mansoni because Abs to KLH have been detected that are not specific to this parasite and cross-react with other worms, leading to false positives (60). In addition, KLH cross-reacts with nonprotective S. mansoni epitopes, and not the protective ones (10). Just as GXM contains protective and nonprotective epitopes, similar findings have been reported with other carbohydrate Ags (14, 15). There is a possibility that other, yet unknown, nonprotective epitopes that cross-react with KLH exist on carbohydrates of other organisms, possibly resulting in deleterious effects.

There is also the possibility that the carbohydrate residues on KLH could act as a polyclonal activator, as has been shown with other carbohydrate epitopes (61). Whether or not KLH activates B cells by binding to Toll-like receptors or by some other method, the Abs to KLH generated in such a response would derive from a diverse group of variable genes and limit the ability to generate a focused response to the Ag of choice (reviewed in Ref. 62). Due to its strong immunogenicity and highly cross-reactive nature, KLH also has the potential of causing carrier-induced suppression. The immune system's continual exposure to ubiquitous carbohydrate Ags may result in a population of memory B cells that are cross-reactive to KLH. The existence of KLH-specific B cells may result in an inhibitory effect on the production of Abs to a new Ag that has been coupled to KLH (reviewed in Ref. 63). Whether this suppression is a result of clonal dominance (64), T cells (65), or macrophages (66), a pool of circulating KLH-reactive B cells could result in a weakened Ab response to any Ag that has been
coupled to KLH. Taken together, these findings have important implications for the future role of KLH in the development of new vaccines.

The potential problems with KLH revealed by the studies reported in this work and those of others discussed above indicate the need for a strategy that would make it possible to identify potentially useful Ags and carriers before in vivo studies and clinical trials. If a panel of mAbs is available that identifies protective and nonprotective epitopes, these Abs can be used in a preliminary screen to identify those Ags that are more likely to stimulate a protective immune response. In the example shown in this work, prior screening of KLH-g and KLH would have shown strong reactivity with 4H3, a mAb to a nonprotective epitope (42), and little reactivity with mAbs to protective epitopes. This would have led us to search for other carriers that did not cross-react with GXM. Screening Ags and carriers of potential vaccines with mAbs to protective and nonprotective epitopes could provide a surrogate indication for predicting the efficacy of new vaccines before animal and clinical studies.

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