Polysaccharide structure dictates mechanism of adaptive immune response to glycoconjugate vaccines

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Glycoconjugate vaccines are among the most effective interventions for preventing several serious infectious diseases. Covalent linkage of the bacterial capsular polysaccharide to a carrier protein provides CD4+ T cells with epitopes that facilitate a memory response to the polysaccharide. Classically, the mechanism responsible for antigen processing was thought to be similar to what was known for hapten-carrier conjugates: protease digestion of the carrier protein in the endosome and presentation of a resulting peptide to the T cell receptor on classical peptide-recognizing CD4+ T cells. Recently, an alternative mechanism has been shown to be responsible for the memory response to some glycoconjugates. Processing of both the protein and the polysaccharide creates glycopeptides in the endosome of antigen-presenting cells. For presentation, the peptide portion of the glycopeptide is bound to MHCII, allowing the covalently linked glycans to activate carbohydrate-specific helper CD4+ T cells (Tcarbs). Herein, we assessed whether this same mechanism applies to conjugates prepared from other capsular polysaccharides. All of the glycoconjugates tested induced Tcarb-dependent responses except that made with group C \textit{Neisseria meningitidis}; in the latter case, only peptides generated from the carrier protein were critical for helper T cell recognition. Digestion of this acid-sensitive polysaccharide, a linear homopolymer of \(\alpha(2 \rightarrow 9)\)-linked sialic acid, to the size of the monomeric unit resulted in a dominant CD4+ T cell response to peptides in the context of MHCII. Our results show that different mechanisms of presentation, based on the structure of the carbohydrate, are operative in response to different glycoconjugate vaccines.

\textbf{Significance}

Helper T cell responses to glycoconjugate vaccines are regulated through mechanisms dependent upon the structure of the polysaccharide. We show that three of the four important conjugate vaccines tested induced antibody responses regulated primarily by carbohydrate-recognizing helper T cells. However, the adaptive immune response to meningococcal group C (MenC) conjugate was restricted to peptide-recognizing helper T cells. We show that MenC is degraded to a monomeric sialic acid residue that cannot be recognized by T cell receptor as an independent antigen. The structure of the saccharide constitutes a critical factor in determining the processing and presentation of glycoconjugate vaccines. An understanding of the mechanisms underlying the immune responses to glycoconjugates will be crucial in the production of highly protective knowledge-based vaccines.
Results
Various Glycoconjugates Induce T Helper Cells Recognizing Different Epitopes. We had previously shown that a clear biomarker for Tcarb-dependent responses was a polysaccharide-specific antibody response after priming with a polysaccharide covalently linked to a carrier protein and boosting with the same polysaccharide linked to a different and unrelated carrier protein. To investigate the involvement of Tcarbs in the humoral immune response to different glycoconjugates, we performed priming and boosting immunization experiments with glycoconjugates made with Vi, GBSIb, Hib, and MenC CPSs. BALB/c mice were primed at the beginning of the experiment and boosted 14 d later with different antigen combinations. One week after the boost, serum levels of polysaccharide-specific IgG were determined. For Vi, GBSIb, and Hib glycoconjugates, boosting with a glycoconjugate containing the same polysaccharide but a heterologous carrier protein induced polysaccharide-specific IgG titers of the same magnitude as those seen after priming and boosting with glycoconjugates containing the same carrier (Fig. 1A). As reported with GBSIII and Pn3P (5, 6), this result supports a Tcarb-dependent mechanism for all three of these glycoconjugates.

In marked contrast to the above results, priming and boosting with MenC glycoconjugates containing heterologous carrier proteins induced significantly lower levels of MenC-specific IgG than did primary and secondary immunization with either a MenC-OVA (ovalbumin) conjugate or a MenC-CRM197 (non-toxic mutant of diphtheria toxin) conjugate. This result was confirmed with a combination of different carriers, including MenC-TT (tetanus toxoid), MenC-HEL (hen egg lysozyme), MenC-OVA, and MenC-CRM197 (Fig. 1B). We also found that MenC-specific IgM levels were similar in groups immunized with different conjugate combinations, a result suggesting a similar level of B cell activation independent of the carrier (SI Appendix, Fig. S1A). To exclude the possibility that we were seeing a T cell-independent response, we treated mice with CD4-specific blocking antibody during the interval between priming and boosting with MenC-CRM197. The excellent booster IgG response observed in mice treated with isotype control antibody was significantly reduced in mice treated with anti-CD4 (SI Appendix, Fig. S1B). The failure of MenC conjugates prepared with different carrier proteins to induce a booster IgG response suggested that perhaps these glycoconjugates were not able to induce Tcarbs and that Tcarbs were not essential in regulating MenC polysaccharide-specific IgG responses.

Carrier-Specific T Cell-Mediated Adaptive Immune Response to MenC Conjugates. On the basis of the previous results, we theorized that carrier protein/peptide-specific CD4+ T cells may be the only T cell subset essential to regulate the response to MenC conjugate vaccines (9, 10). To further test this hypothesis, we primed mice with a combination of MenC-CRM197 plus unconjugated OVA protein, and we boosted these mice with MenC-OVA. We hypothesized that priming with this combination would yield MenC-specific memory B cells resulting from MenC-CRM197 as well as OVA-specific memory helper T cells generated from the OVA protein component. Interestingly, after boosting, with MenC-OVA, mice primed with the mixture of MenC-CRM197 and OVA protein had MenC IgG levels similar to those in mice primed and boosted with MenC-OVA. The MenC IgG levels were significantly lower in mice primed with MenC-CRM197 without OVA (Fig. 2A and SI Appendix, Fig. S2 A and B). This result suggests that in MenC glycoconjugates carrier protein-activated T cells are essential in inducing a humoral immune response and antibody class switching.

To further explore the requirements for cooperation between B and T cells, we performed another series of immunization experiments. We first primed mice with MenC-CRM197 and then boosted them with either MenC-CRM197 or MenC and CRM197 protein (either alone or physically mixed but not conjugated). Booster IgG responses occurred only in mice that received the
MenC-CRM197 conjugate for both primary and secondary immunization. Similarly, priming of mice either with unconjugated MenC or CRM197 protein alone or with a mixture of unconjugated MenC and CRM197 protein did not support a robust secondary IgG response upon boosting with the MenC-CRM197 conjugate (SI Appendix, Fig. S2C). This result suggested that a conjugate is required to stimulate MenC-specific memory B cells.

To directly examine the contribution of carrier-specific T cells to MenC-specific booster IgG responses, we performed adoptive transfer experiments (Fig. 2B). Donor groups of BALB/c mice were immunized with either MenC-CRM197 or OVA, and splenic and lymph node B and CD4+ T cells from each group were purified. Three groups of recipient mice all received B cells from the MenC-CRM197-immunized donors. Groups A and B also received CD4+ T cells from MenC-CRM197-immunized donors, while group C received CD4+ T cells from OVA-immunized donors. One day after adoptive transfer, group A recipient mice were actively immunized with MenC-CRM197, and groups B and C were immunized with MenC-OVA. The resulting IgG and IgM antibody titers in the three groups are shown in Fig. 2C. Groups A and C had significantly higher MenC-specific IgG titers than group B, while MenC-specific IgM levels were similar in the three groups. In a previously reported experiment supporting the role of Tcarrs in the immune response to glycoconjugates (6), Middleton et al. adoptively transferred B cells and CD4+ T cells from mice immunized with Pn3P-KLH (keyhole limpet hemocyanin) to recipient mice and then immunized the recipient mice with either Pn3P-KLH or Pn3P-OVA. The similar IgG levels in these two groups supported a Tcarr-mediated response. In contrast to these results with the Pn3P conjugate, our observations with MenC glycoconjugates suggested that a mechanism in which carrier protein/peptides are essential in recruiting T cell help is operative.

Conjugation Chemistry Is Not the Critical Factor in the Induction of Tcarrs. The structures of the GBSIII and MenC glycoconjugates were very different, though in both the polysaccharides were conjugated to carrier proteins through reductive amination. Whereas the GBSIII conjugate was cross-linked by oxidative creation of aldehydes on a fraction (≈30%) of the side-chain terminal sialic acid residues of the repeating unit, the MenC conjugate was linked at a single site on the reducing end of the polysaccharide (Fig. 3A). In addition, MenC depolymerization by mild acid hydrolysis and oxidation resulted in a size of 10–30 kDa, while GBSIII used for conjugation maintained its original size (>100 kDa). Thus, we hypothesized that conjugation chemistry might affect T cell recognition of carbohydrates. To increase the resemblance to the structure of a cross-linked glycoconjugate, we conjugated MenC through the carboxylic acid to
derivative carrier protein treated with adipic acid dihydrazide (ADH) linker (SI Appendix, Fig. S3) (11). This method activated a fraction of repeating units along the chain of MenC without reducing its molecular size (>100 kDa). The cross-linked MenC conjugate was significantly larger than the end-linked MenC conjugate, as shown by size exclusion chromatography (Fig. 3B).

We performed priming and boosting immunization experiments with the cross-linked MenC glycoconjugates containing either the same or different carrier proteins. Mice primed and boosted with MenC glycoconjugates containing the same carrier protein—either MenC-ADH-CRM197 or MenC-ADH-TT—had strong booster MenC-specific IgG responses. However, priming and boosting with MenC conjugates containing different carrier proteins induced significantly lower titers of MenC-specific IgG (Fig. 3C). This result was similar to that observed with MenC linked at a single reducing end of the polysaccharide to either the same or different proteins (Fig. 1B). Therefore, conjugation chemistry does not seem to explain the failure of MenC glycoconjugates to induce Tcarbs.

**Processing and Presentation of MenC Polysaccharide.** We previously reported that, following immunization with a glycoconjugate vaccine, a depolymerized form of GBSIII (−10 kDa) bound to an MHCII binding peptide can be presented and the carbohydrate recognized by the T cell receptor (TCR) (5). T cell recognition of carbohydrates was also reported for zwiterionic polysaccharides (12), which presumably bind directly to MHCII by electrostatic interactions. In this study, we speculated that MenC polysaccharide might fail to be presented in the context of MHCII on the antigen-presenting cell (APC) surface. One possible underlying reason could be the sensitivity to acidic hydrolysis of MenC, which is a linear homopolymer of α(2 → 9)-linked sialic acid. It is possible that the ketosidic linkage between sialic acid repeating units on MenC is hydrolyzed to an extremely small size in the endolysosome and therefore cannot be presented to or recognized by TCRs.

We conducted a flow cytometry analysis to assess this possibility by determining whether antigenically active MenC carbohydrates can be presented on the APC surface. Bone marrow-derived dendritic cells (BMDCs) from wild-type mice were incubated with unconjugated or conjugated GBSIII or MenC for 18 h. The cells were collected and stained with a monoclonal antibody specific for GBSIII or MenC at 4 °C and then incubated with a fluorophore-conjugated secondary antibody. Consistent with previously published results, the control saccharide (GBSIII) was detected on the surface of the cells incubated with the GBSIII conjugate. However, no BMDC surface presentation of MenC was detected after incubation of the cells either with unconjugated MenC or with conjugated MenC-TT or MenC-CRM for 18 h. The cells were collected and stained with a monoclonal antibody specific for GBSIII or MenC at 4 °C and then incubated with a fluorophore-conjugated secondary antibody. Consistent with previously published results, the control saccharide (GBSIII) was detected on the surface of the cells incubated with the GBSIII conjugate. However, no BMDC surface presentation of MenC was detected after incubation of the cells either with unconjugated MenC or with conjugated MenC-TT or MenC-CRM (Fig. 4A).

We performed parallel experiments with a control polysaccharide—polysaccharide A (PSA) from *Bacteroides fragilis*—that is known to be processed to a size of ∼15 kDa (12). We compared the molecular size of the endosomally depolymerized MenC to that of similarly processed [3H]-MenC on a size exclusion HPLC column: ProSEC 300S (MW range, 1,500–800,000) or Aquagel-OH 20 (MW range, 100–20,000). (C) Raji B cells were treated with either 4-OH TEMPO (a superoxide inhibitor) or bafilomycin A1 (BFA, an endosomal acidification inhibitor) for 1 h before incubation with [3H]-MenC. The molecular size distributions of the endolysosomal lysates were analyzed on a size exclusion column (ProSEC 300S). SA, sialic acid.

**Fig. 4.** Processing and presentation of MenC. (A) Flow cytometry analysis of BMDCs after incubation (18 h) with unconjugated GBSIII or GBSIII-TT (Left) or with unconjugated MenC, MenC-TT, or MenC-CRM197 (Right) followed by surface staining with monoclonal antibody to GBSIII or MenC. (B) Elution profile of lysates of Raji B cell endolysosomes after 18 h of incubation with [3H]-MenC on a size exclusion HPLC column: ProSEC 300S (MW range, 1,500–800,000) or Aquagel-OH 20 (MW range, 100–20,000). (C) Raji B cells were treated with either 4-OH TEMPO (a superoxide inhibitor) or bafilomycin A1 (BFA, an endosomal acidification inhibitor) for 1 h before incubation with [3H]-MenC. The molecular size distributions of the endolysosomal lysates were analyzed on a size exclusion column (ProSEC 300S). SA, sialic acid.

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less depolymerization of the polysaccharide was detected in the endolysosome after incubation with either inhibitor. In bafilomycin-treated cells, a small peak was seen at the void volume of the column that was not observed with 4-OH TEMPO treatment. When treated with a combination of the two inhibitors, a similar upward size shift was observed (Fig. 4C). This result suggested that both oxidative depolymerization and acidic hydrolysis contribute to MenC depolymerization, with acid hydrolysis perhaps being somewhat more important.

**Discussion**

Antibodies to CPSs mediate protection against encapsulated bacteria (2). Several highly effective glycoconjugate vaccines have been created using a hapten-carrier protein conjugation strategy (14, 15). It has become standard practice to couple CPSs from bacterial targets to T cell-dependent carrier proteins to create glycoconjugate vaccines (16–19). Immunization with glycoconjugates, as opposed to pure polysaccharides, elicits T cell help for B cells that produce IgG antibodies to the polysaccharide component (2, 4). In addition to inducing polysaccharide-specific IgM-to-IgG switching, glycoconjugate immunization elicits both B and T cell memory responses (2).

Other than polysaccharides with a zwitterionic charge motif (e.g., PSA of *B. fragilis* or the polysaccharide of type 1 *S. pneumoniae*), which bind directly to MHCII through electrostatic interactions, most bacterial polysaccharides fail to bind to MHCII and therefore are not presented to the TCR. As a result, most pure polysaccharides induce immune responses that are T cell independent. The traditional explanation for the mechanism by which glycoconjugates induce humoral immune responses is that the carrier protein portion of the conjugate activates CD4+ T cells to help carbohydrate-specific B cells produce long-lasting IgG antibodies through both cognate and cytokine-mediated interactions (2, 4). The classical hypothesis of immune activation by glycoconjugate vaccines suggests that only peptides generated from the polysaccharide-linked carrier protein can be presented to and recognized by T cells. This view, however, ignores the synthetic linkage of carbohydrates to proteins by strong covalent bonds that are unlikely to be broken within the endosome. Thus, we previously raised the possibility of glycopeptide presentation to T cells. We considered whether T cells could recognize carbohydrates linked to another molecule (e.g., a peptide) whose binding to MHCII allows carbohydrate presentation on the APC surface.

Our earlier work uncovered a key feature of the cellular and molecular mechanisms underlying adaptive immune responses mediated by some glycoconjugate vaccines (5). We showed that glycoconjugate immunization induces CD4+ T cells, designated Tcarbs, that recognize only the carbohydrate portion of the glycoconjugate vaccine. Upon endosomal uptake by APCs, a GBSIII glycoconjugate undergoes depolymerization, yielding a glycan of reduced size (∼10 kDa) that is chemically bound to a peptide (glycan–peptide) fragment. Glycan–peptide is displayed on the surface of APCs in the context of MHCII to the CD4+ T cells. We successfully generated T cell clones and validated the existence of T cells that recognize only the processed carbohydrate portion of the glycoconjugate vaccine—i.e., Tcarbs (8). These findings suggested that Tcarbs contribute to the protection induced by the GBSIII glycoconjugate vaccine. Similar mechanisms involving Tcarb responses were recently reported for type 3 *S. pneumoniae* glycoconjugates (6).

In the present study, we sought to determine whether the mechanisms involved in processing and presenting GBSIII and Pn3P glycoconjugates are also present for glycoconjugates of other bacterial polysaccharides. We show that a Tcarb-dependent response is induced by glycoconjugates made with the Vi polysaccharide of *Salmonella* Typhi, the type b polysaccharide of *H. influenzae*, and the type Ib polysaccharide of group B *Streptococcus*. In contrast, we found that a glycoconjugate made with the group C polysaccharide of *N. meningitidis* induces carrier-specific helper T cells, not Tcarbs. Active immunization with various vaccine constructs and adoptive transfer experiments clearly showed that carrier peptide-specific CD4+ T cells are sufficient to induce adaptive immune antibody responses to the MenC conjugate. Given that the covalent linkage between the linking sialic acid residue and the lysine group on the protein generated during chemical conjugation would not be broken down in the endolysosome, it is likely that some processed sugars from MenC are still presented on the surface along with the conjugated peptide. However, our findings indicate that these sugars do not constitute an antigenic epitope and do not sufficiently mask the peptide in the MHCII binding cleft to prevent its recognition; thus they fail to induce Tcarb helper responses. We did show that the MenC polysaccharide is digested to a reduced size comparable to that of a single sialic acid residue in the endosome. Although these sialic acids linked to the MHCII binding peptide are too small to fit in the TCR pocket independent of the peptide, the attachment of the monosaccharide could modify the TCR specificity to the peptide. Detailed studies on CD4+ T cell recognition of glycopeptides showed that the TCR makes specific contact with both the sugar moieties and peptide residues (20–22). In the case of the MenC conjugate, it is possible that peptide modified with a single sialic acid might initiate the activation of a novel T cell subset that collaborates as helper T cells with the dominant peptide-specific T cells.

*N. meningitidis* is a major cause of bacterial meningitis worldwide, especially in the African meningitis belt, and has a high associated mortality. MenC conjugate vaccine has been shown to be safe and immunogenic and to be capable of priming infants, toddlers, young children, and adults for immunologic memory (23–26). However, preliminary surveillance data in England and Wales suggest a waning of effectiveness from 1 y after three-dose priming in infancy (27). A better understanding of the mechanisms involved in the immune response to MenC glycoconjugate immunization could lead to better vaccines with improved efficacy. Clearly, more than one mechanism is responsible for the induction of immune responses to glycoconjugates. It is reasonable to assume that glycoconjugate vaccine design and scheduling might be optimized if we had a fuller understanding of the mechanisms underlying this “decision-making” process. We show that, to induce more sustainable memory responses with MenC glycoconjugates, booster immunization is more effective when the polysaccharide is linked to the same carrier protein rather than to a heterologous protein. Our findings support a recent controlled trial conducted in the United Kingdom and Malta, wherein boosting children with Hib-MenC-TT vaccine after priming them with a single MenC-TT dose in infancy resulted in a more robust bactericidal antibody response than boosting with MenC-CRM197; the response elicited by the MenC boost with the same carrier protein used for priming resulted in antibodies persisting at 24 mo of age (28).

Overall, our study suggests that different mechanisms are involved in immune responses to immunization with different glycoconjugates and that the structure of the polysaccharide is critical to the mechanisms used by APCs to present antigen to CD4+ T cells. An understanding of these differences is an important factor in the specific design of each glycoconjugate and optimization of the vaccination schedule.

**Materials and Methods**

**Mice.** Six-week-old female BALB/c mice were purchased from Taconic Biosciences. All mouse experiments were approved by the Harvard Medical Area Standing Committee on Animals (Animal Protocol 00000636).

**Antigens.** Purified MenC, the Men-C-CRM197 conjugate, Vi polysaccharide, the Vi-CRM197 conjugate, Hib polysaccharide, the Hib-CRM197 conjugate, and CRM197 protein were obtained from GSK Vaccines and GSK Vaccines Institute.
for Global Health. The Hib-OMPC (outer membrane protein complex) conjugate was purchased from Merck. GSBlb was isolated and purified from type Ib group B Streptococcus. Two conjugation methods were applied: cross-linked GSBlb and end-linked MenC conjugates were made through reductive amination, as previously described (8); cross-linked MenC and Vi conjugates were made using EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide], with an ADH linker to couple the carbohydrate residues from the protein and the carbohydrate.

Immunizations and Antibody Responses. Groups of BALB/c mice were primed on day 0 and boosted on day 14 by i.p. injection of the antigen of interest (4–6 µg as polysaccharide content) in PBS mixed with 0.5 mg of alum hydroxide gel adjuvant. At least four mice were immunized in each experiment. Mice were bled from the tail vein 1 wk after boosting immunization. Levels of carbohydrate-specific antibodies in the serum were determined by solid-phase ELISA, as previously described (29).

Adoptive Transfer. Groups of donor BALB/c mice were primed and boosted with 4 µg of MenC-CRM197 as saccharide content given i.p. at 3-wk intervals. Mice were killed 5 d after boosting immunization. CD4+ T cells were isolated from spleens and lymph nodes of mice immunized with either MenC-CRM197 or OVA and negatively selected with a mouse CD4+ T cell isolation kit (130-104-454, Miltenyi Biotech). B cells from mice immunized with MenC-CRM197 were isolated with a mouse B cell isolation kit (130-104-463, Miltenyi Biotech). CD4+ T cells (10^6) from mice immunized with MenC-CRM197 or OVA and B cells (10^5) from MenC-CRM197–immunized donors were adoptively transferred to recipient mice. The recipient mice were immunized 1 d after adoptive transfer with MenC-CRM197 or MenC-OVA.

Antigen Presentation by BMDCs. BMDCs from wild-type mice were incubated with antigen (GSBlb or the GSBlb-TT conjugate, MenC or the MenC-CRM197 or MenC-TT conjugate) for 18 h at 37 °C. After incubation, cells were collected, washed five times with PBS, and labeled at 4 °C first with either GSBlb-specific monomolecular antibody or MenC-specific monoclonal antibody and then with a fluorophore-labeled secondary antibody. Surface staining was assessed by flow cytometry (MACSQuant Analyzer).

Cell Fractionation and in Vitro Processing Assays. Raji B cells (10^5) were cultured in the presence of 1 µg of [3H]-MenC for 18 h at 37 °C. Cells were then washed five times with PBS to remove unreacted [3H]-MenC. Cell lysis was performed by passage of cells through a 27-gauge needle in 250 mM sucrose with 10 mM Tris-HCl, pH 7.5. Differential centrifugation was then used to fractionate the lysed cells into endolysosome and cell-membrane fractions, as previously described (5, 12, 30). Endolysosomal fractions were solubilized by boiling for 20 min in 1% SDS and analyzed by size exclusion chromatography on either Agilent ProSEC 300S (MW range, 1,500–800,000) or Aquagel-OH 20 (MW range, 100–20,000) with an UltiMate 3000 system.

Statistical Analysis. Statistical significance was determined with the ordinary one-way ANOVA, using GraphPad Prism 7.0c. Data with P values of ≤0.05 were considered statistically significant (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).

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