# Carbohydrate Moieties as Vaccine Candidates

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**Carbohydrate epitopes or glycotopes are structurally diverse, occur in a variety of chemical contexts, and are present on the surfaces of cells in the body and on the surfaces of pathogens. These various structures and modes of presentation affect how they are perceived and processed by the body and dictate the outcome of the immune response directed against them. This review focuses on mechanisms of carbohydrate immunity, with an emphasis on carbohydrate vaccines that have been or are being developed for protection against encapsulated bacterial pathogens. We discuss the cellular basis of carbohydrate immunity, newly identified glycotope processing pathways and recognition capabilities, and the synthetic and microarray technologies that are being developed that will permit new experimental approaches to carbohydrate vaccine development and the exploration of the interaction of the immune system with self and nonself glycans.**

This article focuses primarily on mechanisms of carbohydrate immunity, with an emphasis on carbohydrate vaccines that have been or are being developed for prevention of diseases caused by encapsulated bacterial pathogens. Data on the use of recent technological advances in immunology, genomics, and glycomics in vaccine development are also presented.

## IMMUNITY TO ENCAPSULATED PATHOGENS AND PS VACCINES

It has long been known that bactericidal and/or opsonic antibodies directed against capsular polysaccharide (PS) glycotopes protect against invasive diseases caused by encapsulated bacteria, and, accordingly, vaccine development has focused on the elicitation of these antibody specificities. Purified microbial PS vaccines have been in use for >40 years, but they have proven to be variably immunogenic and variably efficacious in protecting susceptible populations against invasive meningococcal, pneumococcal, and *Haemophilus influenzae* type b (Hib) diseases [1, 2]. When administered as purified PSs, their effectiveness generally is limited by the modest nature of the immune response, the lack of a memory response, and the inability to stimulate responses in young children who are at the greatest risk for serious infections due to encapsulated organisms. The demonstration that infants could produce protective anticapsular antibody responses following immunization with Hib PS-protein conjugates represented a major advance in public health and laid the groundwork for the subsequent development of new conjugate vaccines [3].

An effective pneumococcal heptavalent capsular PS conjugate vaccine has been developed and is licensed in the United States for use in infants and children [4]. Use of this vaccine has led to a significant reduction in serious pneumococcal disease caused by pneumococci expressing capsular serotypes contained within the vaccine. The vaccine has also been shown to reduce the incidence of *Streptococcus pneumoniae* otitis media [5, 6]. Studies from Israel have indicated that this vaccine may impact favorably by reducing the number of antibiotic-resistant strains in the vaccinated population [7, 8]. However, emerging evidence demonstrates that serotype replacement with nonvaccine capsular types may be a future problem necessitating changes in the vaccine spectrum. An 11-valent pneumococcal PS conjugate vaccine is presently undergoing evaluation.

Meningococcal serogroup A PS is unusual because it is immunogenic in infants as young as 3 months of age, and administration of repeated doses elicits booster antibody responses. Reimmunization at 2 and 6 years of age maintains a high degree of protection. Older children and adults require only 1 injection. Vaccines against group A meningococci have been highly effective in preventing disease in a number of regions. In Finland, herd immunity to group A meningococci was induced by vaccinating approximately one-third of the

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population. Use of vaccines has significantly reduced the prevalence of disease in China. Household contact vaccination has proven to be highly effective in Africa. However, attempts at preparing protein conjugates of group A meningococcal capsular PS have not increased immunogenicity.

Group B meningococcus is the major cause of serious meningococcal disease in the United States [9]. Group B capsular PS is nonimmunogenic, presumably because of its identity with self-antigens [10]. The potential for induction of autoimmunity and the lack of immunogenicity have discouraged commercial development of PS-based group B vaccines. Alternative approaches have focused on other antigens, such as the outer membrane vesicle. The outer membrane–vesicle vaccine has good efficacy in older children and adults; however, the data for infants are mixed [11]. Following extensive testing, an OMV vaccine prepared from an epidemic group B strain in New Zealand has been provisionally licensed. This vaccine has been introduced into the population in an attempt to control the epidemic [12]. Investigators at Wyeth and Oxford University, England, are studying detoxified meningococcal lipopolysaccharide as a conjugate vaccine. The inner core of the meningococcal lipopolysaccharide is relatively highly conserved, and the use of appropriate conjugation procedures can result in a broadly cross-reactive meningococcal lipopolysaccharide vaccine.

The meningococcal group C PS conjugate vaccine has proven to be an excellent vaccine [13]. In the United Kingdom, a decrease in the rate of group C disease was observed after vaccination with this conjugate. In children who were vaccinated during infancy at 2, 3, and 4 months of age, the effectiveness of the vaccine decreased to very low levels after 1 year [13]. There was a much smaller decline in efficacy in infants who were vaccinated at 5–11 months of age, suggesting that a booster dose administered at 13–15 months of age might protect against the loss of efficacy and is now recommended. Follow-up studies also demonstrated that there was no evidence of a switch of meningococcal capsular serogroup from serogroup C to other serogroups caused by vaccine pressure.

## ONTOGENY OF PS-SPECIFIC ANTIBODY REPERTOIRES

As noted above, with the exception of the group A meningococcal PS, children aged <2 years generally are unresponsive to vaccination with purified PS antigens. One early explanation for this anergy was that infants lacked the relevant PS-specific B cells. However, numerous studies have demonstrated that an insufficient antibody repertoire cannot account for the refractoriness of the infant to PS vaccination. Infants can produce anti-PS antibodies after PS conjugate vaccination, and studies of the Hib PS antibody repertoire have shown that infants can utilize the same variable (V) region genes as those used by adults [14]. Despite intensive investigation, the mechanism underlying the unresponsiveness of the infant to PS vaccination has not been precisely identified. The mechanism is likely multifactorial and may involve lacking expression of sufficient costimulatory molecules and/or their respective receptors and immaturity of dendritic cells and/or marginal zone B cells.

Cell surfaces in the body are replete with glycan molecules, and it is clear that self glycotopes can function as tolerogens and thus negatively shape the expressed antibody repertoire. The absence of antibodies to A and B blood–group substances in individuals expressing the respective blood groups represents a classical example. However, tolerance is imperfect, and antibodies against self-glycan structures can be present in healthy subjects. Infectious agents or vaccines expressing glycotopes structurally related to self glycans can elicit antibodies reactive with self antigens. For example, neutralizing antibodies elicited by an inactivated severe acute respiratory syndrome coronavirus vaccine cross-react with the human serum glycoprotein asialoorosomucoid [15]. What is less clear is the role of self glycans in the positive selection of the B cell repertoire. A growing body of evidence indicates that positive selection occurs during murine–B cell development [16] and can involve selection against the self glycoprotein Thy-1 [17]. The recent discovery of an endogenous CD1-restricted glycosphingolipid, isoglobotrihexosylceramide, suggests that self glycans may mediate the positive selection of T cells and/or NK cells [18].

## CELLULAR MECHANISMS OF GLYCONCONJUGATE VACCINE IMMUNOGENICITY

Despite our lack of understanding of the mechanism, it is well established that the refractoriness of infants to PSs, a class of antigens designated as thymus-independent (TI) type 2, can be overcome when PS glycotopes are presented in the context of an immunogenic carrier protein. Coupling of protein is thought to convert glycotopes into T cell–dependent immunogens. Immunization with PS protein–conjugate vaccines likely involves activation of the follicular B cell population. Follicular B cells take up PS protein conjugates via their PS-specific B-cell receptor, process the carrier protein moieties, and present the resulting peptides on cell surface major histocompatibility complex class II molecules where they may engage peptide-specific T cells. PS-specific B cells thereby receive signals via cognate T cell–B cell interactions and CD40-CD40L engagements that are sufficient for activation, differentiation into antibody secreting cells, germinal-center formation, class switch recombination, and somatic hypermutation. Memory B cells generated during this T cell–dependent process can be activated by subsequent stimulation with either PS-protein conjugates or by multi-epitope PS.

Different carrier proteins have been used in the formulation

of PS-conjugate vaccines, and although they are thought to convert the glycotope into a T cell–dependent form, they possess disparate immunological properties. For example, Hib PS–conjugate vaccines utilizing different carriers elicit distinct antibody populations, as has been discerned by idiotypic [19] and avidity analyses [20]. Furthermore, studies of humans indicate that both TH1 and TH2 cytokine responses are elicited following immunization with pneumococcal conjugate vaccines [21]. The outer membrane protein complex derived from *Neisseria meningitidis* is used as a carrier protein in some PS-conjugate vaccines. The Hib PS–conjugate vaccine, having an outer membrane protein complex as the carrier, is uniquely capable of stimulating a serum anti-Hib PS–antibody response in 2-month-old infants after a single immunization. In contrast, conjugates utilizing bacterial toxoids as carriers usually do not elicit significant seroconversion until the second immunization [22]. These properties of the outer membrane–protein complex carrier may be related to its ability to engage Toll-like receptor–2 [23]. Thus, carrier proteins function not only as a source of peptide epitopes permitting the delivery of T cell help, but they also can possess mitogenic and adjuvant-like properties that stimulate the innate immune response.

The immunological variability of different carriers and the increasing use of multivalent glycoconjugates utilizing the same carrier protein has generated concern about potential complications arising from high carrier doses, such as antigenic competition and carrier-induced epitope suppression. This concern has prompted the search for alternate carrier-protein strategies that include the synthesis of a peptide carrier that functions as a universal T helper–cell epitope by binding promiscuously to human leukocyte antigen–DR molecules [24] and the synthesis of recombinant carrier proteins containing strings of T cell epitopes derived from several pathogen-derived antigens [25].

## PS AS TI-2 ANTIGENS

PSs have been classified as TI-2 antigens on the basis of their ability to stimulate antigen-specific B cells in the absence of T cells [26]. PS antigens, by nature of their multivalent, repeating glycotope structure, engage a sufficient number of B cell receptors that result in B cell activation and maturation to antibody secretion. The inability of PS immunization to lead to anamnestic responses suggests that memory B cells are not generated by this type of antigenic stimulation, presumably because of failure of the immunogen to associate with MHC-II molecules and recruit T cell help. Although B cell activation by TI-2 antigens can occur independently of T cells, it is becoming increasingly clear that accessory signals, in addition to those generated by B cell–receptor cross-linking, are required to generate antibody responses to TI-2 antigens. In vitro, highly purified B cells do not respond to TI-2 antigens. Accessory cells, such as dendritic cells and macrophages, and costimulatory signals are required for an effective response to TI-2 antigens. In addition, many TI-2 antigens are able to fix complement, and the formation of these complexes in vivo could facilitate B cell activation by providing costimulation through complement receptors. Engagement of the complement receptor lowers the antigen signaling threshold for B cell activation [27], and the coupling of complement C3d fragments enhances PS immunogenicity [28]. Several molecules—such as the transmembrane activator and its ligand B lymphocyte stimulator, tyrosine kinase Pyk-2, Bruton tyrosine kinase, and phospholipase C-$\gamma$2—have been identified as costimulatory signals and/or receptors necessary for the generation of antibody responses to TI-2 antigens (reviewed in [29]). Understanding the mechanisms by which these molecules exert their effects will not only suggest new ways to enhance responses to carbohydrate-based vaccines, but also new ways to suppress autoimmune responses directed against self glycans.

## IMMUNE RESPONSE TO ENCAPSULATED BACTERIA

The immune response to glycotopes presented in the context of the microbe is important for understanding carriage, the acquisition of naturally acquired immunity, and the response to infection [30]. The majority of older children and adults possess serum antibodies specific to a plethora of bacterial capsular glycotopes, and these antibodies mediate protective immunity. Presumably, exposure to the homologous organism or to organisms expressing cross-reacting glycotopes drives the formation of these antibodies. These natural antibodies appear to derive from memory B cells, because molecular analyses of adult pneumococcal antibodies show that they have undergone both a class switch and hypermutation [31–33]. These findings suggest that exposure to glycotopes presented in the context of the bacterial surface generates both PS-specific B cell antibody secretion and memory development. In mice, $CD4^+$ T cells and engagement of the Toll-like receptor–2 and its adaptor protein, MyD88, have been implicated in the response to pneumococcal glycotopes elicited by intact bacteria [34]. Unlike conjugate vaccination, which usually occurs by the intramuscular route, natural exposure occurs predominantly across mucosal surfaces. Thus, understanding the induction of natural immunity and the maintenance of the carrier state must take into account the migration patterns between secondary lymphoid tissues and the homeostatic mechanisms operating at mucosal surfaces [29].

It is likely that bacteria, purified PS, and glycoconjugate vaccines address overlapping as well as distinct subsets of B cells. As discussed above, follicular B cells are probably the major subset activated by PS-protein conjugates, whereas blood-borne bacteria and PS antigens are thought to stimulate marginal-zone B cells (and B-1 B cells in the mouse) [35, 36]. Marginal-

zone B cells differ from follicular B cells in their rapid proliferative response and elaboration of IgM antibodies early in the course of bacterial infection, and it has been suggested that these B cells represent an innate-like response that mediates immediate immunity before the slower adaptive response has developed [35]. Human marginal-zone B cells express high levels of CD1c, which suggests that they might be well suited for presentation of bacterially derived lipid antigens. In humans, there is a subpopulation of circulating B cells that have the phenotype of marginal-zone B cells. These cells have been designated "IgM memory B cells" because they express both cell-surface IgM and the memory cell marker CD27. The V genes of peripheral blood IgM memory B cells are mutated [37], and a recent report indicates that these cells are mobilized in response to PS vaccination [38]. IgM memory B cells with mutated V genes are present in patients with hyper IgM syndrome who lack functional CD40–CD40 ligand interactions [39]. This finding has prompted the suggestion that 2 pathways of somatic hypermutation exist in humans: the traditional pathway dependent on T cells and CD40 receptor–CD40L receptor interactions, and another pathway independent of CD40 receptor-CD40L receptor interactions [39].

## ANTIBODY RECOGNITION OF GLYCOTOPES

Molecular modeling and x-ray crystallographic studies have verified the original proposal of Kabat et al. [40] that antibody-combining sites recognizing glycotopes can exist as either shallow grooves or as deep clefts, with the former binding internal epitopes expressed along the length of the glycan polymer and the latter binding to epitopes on the nonreducing ends of the glycan chain. Some glycotopes are expressed by short oligomers, whereas others require longer chain lengths for expression. Studies of the type 14 pneumococcal PS have shown that the induction of osponically active antibodies is critically dependent on chain length and the expression of a conformational epitope [41]. Similarly, the protective glycotope of type III group B streptococcal PS is a conformationally dependent, helical structure requiring extended chain lengths for expression [42]. Thus, antibodies prepared against short oligomeric glycotopes may show the requisite antigen-binding specificity, but they may not recognize native capsular glycotopes and mediate protection. This issue is particularly important in the design of vaccines using synthetic oligosaccharides.

Avidity has been shown to be a critical determinant of the efficacy of anticapsular antibody protection against Hib [19, 20], pneumococcus [43] and meningococcus [44]. Variations in antibody avidity occur during the course of the immune response and can be influenced by vaccine type [20] and age [45]. The structural basis of avidity variation has been studied in detail in the human response to Hib PS, in which a structurally conserved, canonical combining site dominates the repertoire. Regions in the combining site that drastically impact Hib PS–binding avidity have been localized to areas thought to represent antigen contact sites, and they include allelic polymorphisms in complementarity region (CDR)–2 of the heavy chain V region [46] and polymorphisms in the light chain CDR-3 that are generated by differential J-region use and utilization of different insertional residues at the V-J junction [14, 47].

Recently, a new mechanism of antibody glycotope recognition was described elsewhere [48]. A monoclonal antibody that is specific for a mannose glycotope of HIV was found to have exceptionally high affinity and potent neutralizing activity against numerous viral isolates. x-ray crystallographic analysis demonstrated that the fragment antigen binding dimer possesses 4 binding sites for the mannose determinant. Two combining sites constitute the traditional site created by the association of variable heavy and light chain domains, and 2 additional sites are created by domain exchange at the VH-VH interface. This capability for multivalent interaction provides a structural explanation for the high avidity of this antibody. Thus, targeting glycotopes in addition to protein epitopes may be a worthwhile strategy in the development of an effective multivalent HIV vaccine that elicits broadly neutralizing antibody responses [49].

Molecular mimicry has been a major area of investigation in carbohydrate immunology. Numerous reports have shown that peptides binding to carbohydrate combining sites can be isolated from peptide libraries [reviewed in 50]. These peptides show apparent specificity in that their binding is peptide-sequence dependent but is usually limited to the combining site against which they were selected. A fundamental issue is whether these mimetics, when presented in an immunogenic form, are able to elicit carbohydrate-specific antibody responses. Some studies suggest that this, indeed, is the case. Peptide mimics have been shown to induce cross-reactive responses to carbohydrate antigens expressed on bacteria [51, 52], fungi [53], HIV envelope protein [54], and tumor cells [55], to name a few. In a mouse-tumor challenge model, immunization with a DNA construct encoding a mimetic inhibited tumor growth [56] and antimimotope antibodies block cell adhesion of HIV gp120–expressing cells and human dendritic cells [57]. One could imagine a number of advantages of using peptidyl mimics rather than glycotopes. These include the ease of synthesis and purification, the ability to be formulated in a variety of ways not amenable to glycotopes, such as multiple antigen peptides and DNA vaccines [58], and the ability to engage cellular processing and activation pathways not normally within the capability of glycotopes.

## T CELL RECOGNITION OF GLYCOTOPES

The dogma that the recognition of glycotopes is the exclusive provenance of antibody combining sites has been overturned by studies in the past decade that demonstrate that T cell receptors are able to recognize glycotopes if they are presented in the appropriate MHC-binding context [59–61]. Functional and structural studies of glycopeptide antigens have demonstrated that MHC molecules tether the glycopeptide by binding the peptidyl moiety in the MHC-binding pocket, such that the glycotope can directly engage the T cell receptor. Cytotoxic T cell responses to carbohydrate antigens can be induced by immunization with either naturally occurring or synthetic glycopeptides [62, 63].

Glycolipid antigens can also be recognized by T cells and NK cells via a CD1-dependent mechanism [64]. CD1 molecules, which exist in multiple isoforms, are structurally related to MHC class I molecules. Examples of CD1-restricted antigens include mycobacterial glycolipids and the self-derived ganglioside GM1. The T cells recognizing CD1-presented lipid antigens may utilize a variety of T cell receptor–$\alpha$ and T cell receptor–$\beta$ chains. In addition, NK cells expressing an invariant T cell receptor–$\alpha$ chain paired with various $\beta$ chains (in both mouse and man) also are CD1-restricted, and they recognize a glycosphingolipid known as $\alpha$-galactosylceramide, a glycolipid originally identified in marine sponges. $\alpha$-Galactosylceramide binds with high affinity to CD1d and has potent immunoregulatory properties. Administration of $\alpha$-galactosylceramide induces production of IFN-$\gamma$ and IL-4 and activates NK cells, T cells, and B cells. A synthetic analogue of $\alpha$-galactosylceramide has potent protective activities in murine models of malaria and melanoma. CD-1 restricted T cells may produce either TH1-type or TH1/TH2–type cytokine responses that could be important in antitumor and antimicrobial immune responses. Although the precise role of CD1-restricted T cells in adaptive immune responses requires further study, some evidence suggests that CD1-restricted NKT cells are involved in immunity to *Mycobacterium tuberculosis, Pseudomonas auruginosa, Plasmodium yoelii, Trypanosoma cruzi,* and *Francisella tularensis* (reviewed in [64, 65]).

A recent study of the capsular PS of *Bacteriodes fragilis* [66] identified a new pathway of glycotope processing and presentation. This work originated from earlier studies demonstrating that PS-specific $CD4^+$ T cells were involved in protection against sepsis caused by *B. fragilis* [67]. The capsular PS of *B. fragilis* (and some pneumococcal capsules) is zwitterionic and forms a helical structure. The PS is capable of inducing protective PS-specific T cells in an antigen presenting cell-dependent fashion, and this effect depends on the zwitterionic character of the PS. The *B. fragilis* PS is processed by antigen presenting cells via an endosomal-lysozomal pathway that involves chemical cleavage by a nitric oxide–mediated mechanism that generates low–molecular weight carbohydrates capable of binding to MHC-II and being presented to $CD4^+$ T cells. Given the diversity of glycan structures and their chemical contexts, it is likely that additional carbohydrate-processing pathways will be discovered. For example, the C-type lectin, known as specific intracellular adhesion molecule-grabbing nonintegrin–R1 (SIGN-R1), which is expressed on splenic marginal-zone macrophages, binds with high affinity to the pneumococcal type 14 capsular PS and mediates uptake of type 14 pneumococci [68].

The discoveries that foreign PS antigens and glycotopes can be processed by endosomal pathways, can be presented by MHC-I, MHC-II, and CD-1 molecules, and can be recognized by T cells and endogenous lectins hold great promise for designing new glycan-based adjuvants and vaccines against tumors, bacteria, and parasites.

## NOVEL TOOLS FOR STUDYING CARBOHYDRATE ANTIGENS

Research in carbohydrate immunology is being accelerated by the development of new technologies, such as carbohydrate microarrays, automated syntheses of oligosaccharides, and biophysical and computational methods for studying antigen-antibody interactions. Carbohydrate microarrays can be used to investigate lectin and antibody specificities, to identify new lectins, to screen blood for disease patterns, or to identify lead structures for vaccine design [69, 70]. A Consortium for Functional Genomics has been established and has developed a novel glycan-array format that has been demonstrated to have applicability for profiling the specificity of a wide variety of carbohydrate-binding molecules [71]. These new technologies will contribute to the design of better-defined carbohydrate vaccines and will serve as essential tools in our understanding of the glycome and its interaction with the immune system.

Progress is being made in the development of chemical methods and instruments capable of efficient, high-capacity oligosaccharide synthesis [72], and these advances are being applied to basic research and to the development of synthetic carbohydrate vaccines. Experimental vaccines utilizing synthetic carbohydrates have been developed for a number of pathogens, including *S. pneumoniae* [73], *Vibrio cholera* [74], *Shigella flexneri* [75], and *Plasmodium falciparum* [76]. A Hib vaccine containing a fully synthetic oligomer of the Hib capsular PS has been tested in humans and has been demonstrated to elicit functionally active antibodies [77].

## FUTURE PERSPECTIVES

Carbohydrate antigens represent a significant resource for the development of vaccines against infectious agents, particularly against pathogens for which traditional protein and/or whole

organism–based approaches have not been successful. Although we do not discuss it in this article, there is a large body of work exploring carbohydrate-based vaccines for the elicitation of both humoral and cellular immunity against tumors [78]. New carbohydrate-based vaccines are being developed for pathogens such as HIV and malaria, and we might expect to see clinical trials evaluating new adjuvants, such as $\alpha$-galactosylceramide.

Aside from the need for research, the need for a variety of resources is essential. The availability of carbohydrate microarrays and appropriate animal models, including primate models and transgenic mouse platforms, should be enhanced. Also, tetramers and analytical chemistry are needed to aid in the identification of glycolipid antigens recognized by CD-1. Other infrastructural needs include good laboratory practice resources for the production of synthetic carbohydrate vaccines and for testing the safety of vaccine candidates in animals and more opportunities (i.e., computer capacity) to model carbohydrate antigen-antibody interactions. Resources will be needed to ensure compliance with regulatory standards, such as a defined chemical composition and structure, the ability to manufacture with consistent physical and chemical characteristics, the lack of inherent toxicity, and the capability to induce protective immune responses.

It is becoming increasingly apparent that the various structures and modes of glycotope presentation affect how they are perceived and processed by the body and dictate the outcome of the immune response directed against them. New glycotope-processing pathways and recognition capabilities are being identified, the signals and molecules involved in cell activation are being elucidated, and new synthetic and microarray technologies are being developed that will permit new experimental approaches to explore the interaction of the immune system with self and nonself glycans. These discoveries can be expected to translate into new therapeutics and vaccines to combat disease and improve human health.

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