Carbohydrate Based Vaccines

B. Kuberan and R. J. Linhardt*

Division of Medicinal and Natural Products Chemistry, Department of Chemistry, Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa, 52242, USA

Abstract: Vaccination is the most cost-efficient and the most powerful medical intervention in the control, prevention and eradication of many diseases that affect human population. Capsular polysaccharide based vaccines have been in use for many decades providing immunity against various bacterial infections. The advent of new efficient and sensitive analytical and synthetic methods in carbohydrate chemistry, together with our increased understanding of glycobiology and glycoimmunology, makes the development of carbohydrate based vaccines against various cancers possible. This review article describes the biological basis of vaccination and summarizes the recent progress in the development of carbohydrate based glycoconjugate vaccines with particular emphasis on structure, function and chemistry of glycoconjugates.

The central nervous system and the immune system are the only systems in the body that have memory. This unique, intricate and important property of immune system provides the opportunity for the most cost-efficient of all medical interventions, preventive vaccination. In vaccination a pathogen or toxin is isolated and a specific immunogen is prepared that is both antigenic and innocuous. This immunogen is then formulated into a vaccine that maintains and ideally augments immunogenicity.

Our increased understanding of the immune system, the molecular architecture, and the biology of pathogens have lead to a tremendous improvement in vaccine efficacy. It is now quite common to prepare vaccines that consist of highly purified antigenic molecules or haptens derived from or modeled on the most immunogenic domains of a pathogen. Recent advances in molecular biology have also opened new avenues to educate and stimulate the immune system. One of the most remarkable advances involves the transfection of relevant genes from pathogens directly into a human host.

Most common infections causing morbidity and mortality are predominantly found in the poorer portions of the human population. It is in these populations that vaccines offer the most promise, as high technology drugs are simply not affordable. Thus, preventive medicine based on effective and relatively inexpensive vaccines offer the best hope against a wide variety of diseases. The development of immunizing biologicals has become a top priority in the research community worldwide.

**IMMUNIZATION**

The aim of vaccination (immunization) is to utilize infectious agents prophylactically to produce

---

*Address correspondence to this author at the University of Iowa, Phar S 328, Iowa City, IA, 52242, USA; Fax 319-335-6634; email robert-linhardt@uiowa.edu

1385-2728/00 $19.00+00 © 2000 Bentham Science Publishers B.V.

---
resistance to infection within a population. The different types of immunization procedures are summarized in Fig. (1).

Immunoglobulins (Ig or antibodies) derived from human (or humanized) serum are used in passive immunization. This immunization approach is used when: a) there is no active vaccine available; b) immunocompromised person is at risk or susceptible to endemic infection; and c) after exposure occurs or when there is no time for active immunization. Passive immunization provides rapid protection for a short period of time, up to 3 months, after which, the levels of Ig drops below a minimum level required for protection.

The immune system requires several months after active immunization to provide adequate rapid protection against pathogens. However, the resultant immunity is long lasting, persisting for many years or even a lifetime. Any recurrence of infection by the same pathogen results in a very fast and vigorous antibody response, called immunological memory.

There are 3 main types of first generation vaccines against bacteria and viruses that are still currently under use. Live vaccines consist of attenuated (weakened) microorganisms, which multiply in the body and mimic natural infection as far as antibody production is concerned but without causing symptoms. A single dose provides long-lasting immunity and subsequent boosters (re-immunization) provide reinforcement of immunological memory. Killed vaccines, while posing less risk of infection than live vaccines, may not lead to long lasting immunoprotection. Finally, toxoid vaccines are used to protect against infections whose pathology or symptoms are caused by exotoxins, shed by the microorganism (i.e., Diphtheria and Tetanus; toxoid vaccines).

The development of second-generation vaccines represents an important advance in immunization and consists of immunogens having a more defined composition, affording highly reproducible biological properties. The preparation of vaccines based on purified sub-units and the production of attenuated strains containing programmed genetic modifications has become possible because of advent of: 1) improved synthetic methodology 2) advances in analytical methods (NMR, MS, etc); and 3) the advent of molecular biology.

**IMMUNE SYSTEM**

The main function of the immune system is to protect against non-self. All immune responses must begin with the recognition by the host that a pathogenic microorganism is a foreign agent. The immune system can be divided into innate (natural) immunity and acquired (adaptive) immunity. Innate immunity offers non-specific protection against infectious agents through mechanical, chemical and biological barriers and does not involve immunological memory. Acquired immunity is specific for a particular antigen and on re-exposure to that antigen produces a rapid and heightened response due to immunological memory.

**INNATE IMMUNITY**

Innate immunity involves four broad groups of biological responses: 1) phagocytosis; 2) complement activation; 3) cytokine release; and 4) inflammation.

Phagocytes are cells capable of engulfing and killing the whole microorganisms in a process called phagocytosis. Phagocytes include monocytes, macrophages and neutrophils. Engulfed microorganisms are killed intracellularly by reactive oxygen species such as superoxide ions, peroxide ions and hydroxyl free radicals and non-oxidatively by a variety of enzymes including lysozymes, hydrolases and peroxidases.

The complement system is comprised of a series of plasma proteins that: 1) lyse gram-negative bacteria; 2) opsonize; and 3) promote an inflammatory response. These complement proteins exist in an inactive form in the plasma and require proteolytic cleavage to become active. Such activation triggers a cascade of reactions, initiated by xenobiotic substances, organisms, immunoglobulins and polysaccharides. The cascade can occur through the classical, the alternative or MBL pathways. Most bacteria are activators of the alternative pathway. A complex between antigen (Ag) and antibody (Ig) also activates the complement pathways leading to a membrane attack complex (MAC) that can lyse a bacterial cell.

Cytokines are proteins involved in intercellular communication. Interferons (INF), for example, are a group of cytokines produced in a response to viral infections. Responding to cytokines, immune cells and their active products accumulate at the site of infection or tissue damage in a process known as inflammation. The accumulation of these immune cells enhances the immune response.

**ACQUIRED IMMUNITY**

The responses in acquired immunity can be divided into antibody-mediated immunity (humoral)
and cell-mediated immunity (cellular). After sensitization, humoral responses are fast, their purest form is an allergic response leading to anaphylaxis, while cellular responses are slow and include the killing of cancer cells and transplant rejection. The components of acquired immunity include antigens (Ags), antibodies (Igs), B-cells, T-cells and killer-cells.

An antigen is a foreign substance, usually large proteins or carbohydrates that binds to an antibody. The antibody produced in response to an antigen binds only to a very small site on the antigen referred to as an antigenic determinant (epitope). Antibodies, which are small molecules, often fail to elicit an active immune response. Very small antigens, known as haptens, only become immunogenic when chemically conjugated to larger carrier molecules. Large antigens or hapten-carrier conjugates capable of provoking an active immune response are called immunogens. As antigens become cleaner (i.e., a pure recombinant protein subunit might now be used in place of an attenuated or inactivated virus) the immune response often decreases. The use of adjuvants or additives often becomes necessary to promote a stronger immune response. Adjuvants work by enhancing non-humoral cell-mediated response such as the involvement of T-cells.

T-cells (thymus derived cells) are able to recognize and differentiate non-self antigens through T-cell receptor (TCR) with the help of the major histocompatibility complex (MHC) a membrane-bound protein complex that defines self. T-cells undergo maturation in thymus and migrate to lymph nodes and spleen. B-cells (bone marrow derived), migrate directly to lymph nodes and spleen after maturation. B-cells recognize antigen through antibodies on their surface that are specific for one antigen and are responsible for the production of antibodies in an active immune response.

T-cells and B-cells are activated by a substance that has been recognized as antigen and has been processed and presented by dendritic cells or macrophage, antigen presenting cells (APC). The antigen, normally protein, is degraded into peptides and anchored to MHC class I or class II molecules for presentation at the cell surface. The class II pathway activates T-helper cells, whereas, class I pathway activates cytotoxic or T-killer cells.

HUMORAL IMMUNITY

Five different types of immunoglobulins (Ig) or antibodies (Ab), are produced by activated B-cells. These immunoglobulins are pentameric IgM, dimeric IgA and monomeric IgG, IgD and IgE. Antibodies consist of an antigen binding region (Fab) and a cell-binding domain (Fc). On exposure to an antigen, B-cells are activated, clonally expanded to produce antibody secreting plasma cells and memory B-cells. The level of antibody reaches a peak, and after some time declines as the plasma cells die. This is called the primary response, characterized by the B-cells producing pentameric IgM antibody. The IgM antibody generally binds less tightly and with less specificity to its antigen than does the IgG antibody. On subsequent exposure to the same antigen, the antibody secretion is rapid and massive and longer lasting, as a consequence of clonal expansion of the memory B-cells. This is called the secondary response, characterized by the B-cells producing monomeric IgG (in blood and tissues) and dimeric IgA (at mucosal surfaces) through class switching from IgM. The Fab region of Ig recognizes and binds to the specific antigenic determinant to form an Ig-Ag complex. Ig-Ag complex results in: a) blocking the properties of that antigen b) preventing the antigen from binding to its receptors: c) aggregating bacteria or viruses and thereby initiating phagocytosis (phagocytes possess Fc receptors and hence are able to engulf antibody-coated micro-organisms efficiently); and d) activating the complement cascade leading to lysis of the bacteria by the MAC.

CELL MEDIATED IMMUNITY

T-cells are activated by antigens to produce cytokines, following presentation of the antigen by the MHC complex to the TCR. APC-T-cell binding requires the help of other cell-adhesion molecules and corresponding receptors. T-cells also perform specialized functions including facilitation of B-cell response (T-helper); amplification of B-cell response (T-amplifier); suppression of B-cell response (T-suppressor); and killing of cells recognized by antibodies (T-cytotoxic or T-killer). These specialized responses generally take place in hours, days or sometimes months after the initial humoral response.

BACTERIAL INFECTIONS

Carbohydrates contain an enormous amount of structural information as a result of their multiple stereocenters, anomeric linkages, ring size (pyranose or furanose), and chemical substitution (i.e., sulfation, phosphorylation, esterification, etc). Even a simple disaccharide contains many
more structural permutations than a considerably larger polypeptide. Their large content of structural information, along with their strategic location on the cell surface, unquestionably makes carbohydrates the major player in cell-cell recognition processes. Most carbohydrates in eukaryotic cells are conjugated with proteins (glycoproteins) or lipids (glycolipids). Carbohydrate capsules in prokaryotes are characteristically composed of repeating units of oligosaccharides and are found on the surface of many bacteria. These extensively hydrated, polyanionic capsular polysaccharides serve several functions, such as specific transport of ions and molecules, promotion of bacterial adhesion to the surfaces and the stimulation of microcolonization.

In the early stages of acute infections, invading encapsulated bacteria can be eliminated from the circulation by phagocytosis, complement activation and humoral response. Capsular polysaccharides are implicated in interfering with this early response, thereby enhancing the virulence of bacteria. Thus, the polysaccharide capsule is often referred to as a virulence factor.

Experimental observations suggest that the hydrophilic nature of polysaccharide capsules, resulting from their high level of hydration, reduce the surface tension at the interface between the phagocyte and the bacterium [1]. This impairs the phagocytosis. Furthermore, unfavorable interactions between the bacteria and the phagocytes result from the net negative surface charge on each.

The complement component, C3b, is of critical importance in fighting against invasion by encapsulated bacteria (Fig. 2) [2]. C3b can be generated independently by both classical and alternative pathways. Many capsular polysaccharides fail to activate complement because their surfaces favor inactivation of C3b [3]. Capsular polysaccharides containing N-acetyl neuraminic acid, such as Group B and C meningococi, E.coli K1 and Group B Streptococci, bind to complement factor H, a regulator protein of the alternative pathway that binds to C3b. The deposition of H-C3b on the bacterial cell surface, instead of C3bBb (alternative pathway amplification factor) and the inactivation of C3b by factor I results in a collapse of the amplification cycle [4]. In the case of Pneumonococci, C3b deposits underneath the capsule on the cell surface and thus, recognition of C3b by the phagocytes is sterically hindered [5].

Both classical and alternative pathways can function in tandem in the host defense against invading organisms. Capsular polysaccharides are able to suppress the activation of the faster alternative pathway mechanisms, thereby forcing the immune system to utilize the slower classical pathway, which requires the presence of specific IgG antibody. This represents yet another important virulence factor of bacteria. The alternative pathway is activated by lipopolysaccharides of Gram-negative bacteria and the teichoic acids of Gram-positive bacteria [6,7].

Capsular polysaccharides are poor immunogens and the immune response to these molecules is strongly age, dependent [8]. From birth to 3 months of age human newborns are protected by maternal antibodies through passive immunization.
(Fig. 1). Human infants of less than two years possess immature immune systems. Therefore, after 3 months of age they are highly susceptible to bacterial infections. In older children and adults, the shedding of capsules from the bacterial surface is an important mechanism for the immunological evasion of these pathogens. The capsular polysaccharide of group B meningococci is an α(2-8) linked homopolymer of N-acetyl neuraminic acid [9]. The poor immunogenicity of this homopolymer is due to immuno-tolerance. The immune system falsely recognizes this polysaccharide as "self" since a similar polysaccharide is a component of the glycoprotein, called Neural Cell Adhesion Molecule (NCAM) [10]. NCAM is expressed in spatiotemporal fashion during fetal brain development and play significant roles in organogenesis and neural cell growth.

GLYCOCONJUGATES

Bacterial polysaccharides are T-cell independent (TI) antigens [11]. The multivalency and large size of these antigens causes the B-cell receptors to cluster and induce immunoglobulin synthesis [12]. Thus, booster injections bring falling antibody levels back to original post immunization levels, but fail to produce a massive response [13]. Most importantly, booster immunization fails to promote switching from IgM to IgG class. In contrast, most proteins are univalent and require T-cell participation to induce antibody synthesis and thus, are T-cell dependent (TD) antigens [14]. Hence, proteins produce heightened antibody response on booster injection and promote antibody class switching from IgM to IgG class. This is due to the participation of T-helper cells. In addition, in a T-cell dependent response the B-cells differentiate into a subset of B-memory cells, imparting an immunological memory of the specific antigen involved. In high risk populations, (i.e., infants, elderly people and immunocompromised people) it is highly desirable to convert TI polysaccharide antigens into TD antigens for use in vaccines. This is accomplished by covalently binding carbohydrate antigens to proteins, resulting in the preparation of glycoconjugate vaccines [15].

The importance of effective bacterial glycoconjugate vaccines has grown with the increased incidence of antibiotic resistant bacterial strains in recent years. Both antibiotic resistance and the necessity to prevent disease in infants, geriatric adults and immunocompromised persons have fostered the development of new carbohydrate-protein based conjugate vaccines.

RATIONAL CONJUGATE DESIGN

The tremendous wealth of information on structure-immunogenicity relationships has been obtained for carbohydrate-protein conjugates during the commercial development of glycoconjugate vaccines. The immune response to carbohydrate-protein conjugates depends on several factors: a) the size of the carbohydrate epitope; b) the carrier
protein; c) the nature and number of covalent bonds between the carbohydrate and carrier protein; d) the nature of the linker arm (bridging molecule), and e) the ratio of carbohydrate to protein. Furthermore, all of these factors contribute to the three dimensional structure of a glycoconjugate vaccine.

The syntheses of glycoconjugates involve the use of either random or defined activation sites within the polysaccharide for potential protein attachment sites. The choice of activation method is primarily governed by the molecular size of the polysaccharide. Large polysaccharides are usually randomly activated whereas oligosaccharides typically are selectively activated at the reducing-end.

The single-ended model in which the protein is linked to the oligosaccharides through its reducing-end, mimics the structural aspects of glycoproteins (Fig. 3) [16]. This model differs from natural one with regard to carbohydrate chain length, structure and attachment points. In this neoglycoprotein model, the carbohydrate's antigenic sites are readily accessible to antibodies. The antigenic activity of such neoglycoproteins primarily depends on the density of carbohydrate hapten on the carrier protein.

When both antigenic carbohydrate and carrier protein have multiple attachment sites, conjugation will lead to a cross-linked lattice matrix (Fig. 4) [16]. Decreasing the number of cross-link sites on a long polysaccharide chain, by decreasing cross-linking and size of the conjugate matrix improves vaccine solubility. Regardless of the degree of cross-linking, the large number of accessible antigenic sites present on the surface of the cross-linked lattice matrix ensures its high level of immunogenicity.

CONJUGATE CHEMISTRY

Conjugation chemistry of carrier protein with carbohydrate antigens is often limited by the propensity of carrier protein to undergo denaturation. While a denatured protein usually retains its immunogenicity, any modifications in the tertiary structure can affect the specificity of antibodies produced. The conjugation of carbohydrate antigen with carrier protein generally relies on modification of carboxyl, hydroxyl, phenoxy, hemiacetal, mercapto/disulfide and amino/imino functional groups. Conjugation chemistry involving reductive amination utilizes sodium cyanoborohydride to selectively reduce intermediate imine adducts, known as Schiff bases (Fig. 5a) [17,18]. Even though the formation of Schiff base is a disfavored equilibrium process in water, reduction drive the equilibrium to completion providing the stable adducts. The formation of

![Complex model](image-url)
glycosylamine is a straightforward reaction in which carbohydrates are treated with saturated ammonium bicarbonate solution at room temperature for 5-7 days. Trapping the glycosyl amine by forming the peptide with iodoacetic acid and reaction of the cystein residue of a protein to the thiol reactive iodoacetyl group affords neoglycoconjugates (Fig. 5b) [19-21]. A spacer arm, such as allyl group introduced at the reducing-end can be used to generate aldehyde, through ozonolysis, which can be coupled with greater ease to the amine groups of protein through reductive amination (Fig. 5c). To increase the accessibility of reactive centers and functional groups, thiol reactive malimide group can be attached to carbohydrates through a spacer arm (Fig. 5d) [22]. Amide forming chemistry using DCC, water soluble EDC, or sulfo-NHS activation of carboxylic groups for coupling with amino functionality in protein or carbohydrate has been widely used to form

Fig. (5). Carbohydrate-protein conjugation chemistry.
neoglycoproteins (Fig. 5e and 5f). [23,24] p-Nitrophenyl glycosides can be transformed to the very reactive diazonium salts to form adducts with the electron rich aromatic tyrosine or tryptophane residues of proteins (Fig. 5g) [25]. Though considered harsh by modern standards, diazo coupling provides strongly immunogenic conjugates, such as *pneumococcal* type 3 CPS vaccine, and has been widely used in the preparation of conjugate vaccines. Cyanogen bromide, which non-specifically activates hydroxyl groups, through the formation of reactive cyanate esters, has been used to conjugate carbohydrates to the amino groups of proteins in aqueous alkaline solution through a stable O-alkyl isourea linkage (Fig. 5h) [26].

**STRUCTURES OF CAPSULAR POLYSACCHARIDES**

*Streptococcus pneumoniae* is the main cause of middle ear infection in children and lower respiratory tract infection in adults. This Gram-positive organism has capsular polysaccharides of different sero types and a common group antigen called C-substance. The structures of *pneumococcal* polysaccharides are presented in Fig. 6 [27-39]. Because of their number, structural diversity and complexity, for the purposes of this review only the structures of the polysaccharides of *pneumococci* that affect the US population and that have been used in recent vaccines will be discussed. With the exception of type 14 and type 37 (not shown), all *pneumococcal* polysaccharides are acidic. The acid functionality include uronic acid residues (types 1, 2, 9V), phosphate groups (types 6A, 6B, 18C, 19F and 23F) and pyruvate groups (type 4). Structural homology of capsular polysaccharides leads to extensive serologic cross-reactivities. This favors the development of multicomponent (multivalent) vaccines with smaller numbers of capsular polysaccharides. There are, however, ample examples where cross-reactivity and cross-protection has failed, requiring the inclusion of additional polysaccharides to provide adequate protection/immunity. For example, the type 9V and 9N polysaccharides differ structurally by the
Fig. (6). Structures of capsular polysaccharides of *Streptococcus pneumoniae*.

Position of an O-acetyl substituent and 19A and 19F polysaccharides differ by only one glycosidic linkage. A multivalent vaccine containing 19F and 9N failed to provide protection against *pneumococcal* infections with type 19A and 9V. Hence, these two polysaccharides are now included in the current 23-valent pneumonia vaccines [40]. Other issues, in addition to cross-reactivity, are important criteria in vaccine formulation. For example, type 6B polysaccharide is preferred over type 6A as a component of the 23-valent pneumonia vaccines, since 6B is more stable than 6A [41].

Fig. (7). Repeating unit of C-substance.
Polysaccharides 6A and 6B only differ in the position of their linkage between α-L-rhamnopyranosyl residues and D-ribofuranosyl residue and d-ribofuranosyl residue. The neighboring group participation of the 4-OH group of D-ribofuranosyl residue in 6A causes greater instability to the phosphodiester linkages, whereas in 6B, such participation is not possible since the 4-OH group is glycosidically linked to an adjacent residue. In addition to structural issues, complex problems are also encountered including differences in pneumococcal disease isolates in different parts of the world and differences in age-related immune responses that severely limits the development of universal vaccine for mankind. These concerns have prompted efforts to develop an alternative vaccine based on C-substance (Fig. 7), a common subcapsular carbohydrate antigen found in pneumococci. Although the results obtained to date with a structurally simple, low molecular weight carbohydrate antigen are not promising, the development of a C-substance based vaccine still remains an attractive approach.

*Haemophilus influenzae* are Gram-negative organisms that are classified into six serotypes (types a, b, c, d, e, f) based on the structure of their capsular polysaccharides [42-48]. Type b H. *influenzae* is the cause of meningitis, the most serious infection among infants, leading to severe and permanent neurological defects in survivors [49]. The structures of the repeating disaccharide units of capsular polysaccharides are shown in Fig. (8). In types a, b, c and f, the repeating disaccharide units are joined through phosphodiester linkages, whereas in types d and e are linked through a glycosidic linkage. All the polysaccharides are acidic, either due to phosphoric diester groups (types a, b, c and f) or by the presence of carboxylic acids (types d and e). Types a and b differ from the rest in containing ribitol, types c and f contain 2-acetamido-2-deoxy hexose. Type f is also O-acetylated as are some type c strains, but only in about 80% of the repeating units. These O-acetyl groups are known to be immunodominant features. Type d and e capsular polysaccharides possess an unusual 2-acetamido-2-deoxy-D-mannose uronic acid that has been proposed to be of phylogenetic significance. Type d polysaccharides carry amide linked amino-acid substituents, including L-serine, L-threonine or L-alanine, at the C6 of the uronic acid residue however, this substituent appears to be of little immunologic consequence. Type e polysaccharides can carry fructose substitution at 3

![Fig. (8). Structure of capsular polysaccharides of *Haemophilus influenzae*.](image-url)
position but the biological significance of this substitution is largely unknown. The pure *H. influenzae* type b polysaccharide vaccine is protective in children older than 18 months, whereas no protection was realized in younger infants. The recent developments in polysaccharide-protein conjugate based vaccines have afforded immunoprotection in infants below the age of 18 months [50].

*Neisseria meningitidis* causes meningitis worldwide in children and adults. The mortality rate is very high without antibiotic treatment and the infection causes severe and permanent neurological defects. *Neisseria meningitidis* is a Gram-negative organism and has been serologically classified into several types, A, B, C, 29e, W-135, X, Y and Z, based on their capsular polysaccharide (Fig. 9) [51-54]. The group A polysaccharide is partially O-acetylated (1→6) linked homopolymers of 2-acetamido-2-deoxy-D-mannopyranosyl phosphate. The group B and C polysaccharides are homopolymers of α(2→8) linked sialic acid. The presence of ulosonic acid glycosidic and phosphodiester linkages, labile under mildly acidic conditions, has hampered the application of conventional chemical techniques for the structural elucidation of these polysaccharides. As a result, the structures of the meningococcal polysaccharides, with the exception of group A, were deduced entirely by NMR spectroscopy. Groups A, B and C are responsible for most of the meningococcal infections and hence, a simplified tetravalent polysaccharide vaccine comprised of group A, C, W135 and Y polysaccharides, has been successful in the worldwide prevention of these forms of meningococcal meningitis [55]. Group B meningococcal infection is of epidemiologic importance but poses a unique problem. The group B polysaccharide is poorly immunogenic in humans. The reasons for poor immunogenicity are: a) the rapid depolymerization

![Structure of capsular polysaccharides of *Neisseria meningitidis*.](image-url)
of α(2→8) linked sialic acid homopolymer in human tissue due to its extreme lability under mildly acidic conditions and through the action of ubiquitous neuraminidases; and b) polysialic acids are recognized as a "self"-antigen, due to their spatiotemporal expression in NCAM during early fetal development, and thus, is non-immunogenic [10].

Chemical conjugation of polysialic acid to protein also failed to induce immunoprotection. Efforts were next focused on direct chemical modification of the α(2→8) linked polysialic acid to generate epitopes capable of stimulating immune response through augmenting the production of cross-reactive B-polysaccharide specific antibodies. The most successful chemical modification, which preserves the antigenicity of the modified group B specific polysaccharide antibodies, was replacement of N-acetyl groups at C-5 of the sialic acid residues with N-propionyl groups [56]. Covalent conjugation of N-propionylated polysialic acid to protein elicited much higher levels of B-group specific polysaccharide antibodies and in addition, a significant booster effect is obtained following three injections [56]. However, it should be kept in mind that the success of this vaccine came at the expense of breaking immune tolerance and the biological consequence of this action is not yet determined.

Group B Streptococci are Gram-positive organisms, which are classified into different serotypes (Ia, Ib, II, and III), based on their capsular polysaccharide (Fig. 10) [51,57,58]. Streptococci type III accounts for most of the infections in the newborn. The structures of the repeating units of the type Ia, Ib, II and III polysaccharides have terminal sialic acid α(2→3) linked to β-D-galactopyranosyl (constituent of human M and N blood group substances) residues.

Fig. (10). Structure of capsular polysaccharides of group B Streptococcus.
and also contain Glc and GlcNAc residues. Common structural motifs of this kind are believed to be an evolutionary acquisition by the bacteria to evade the human immune system. The core asialo-
oligosaccharide structure type III, while structurally identical to type 14 *Streptococci pneumoniae*, fails to provide cross protection [59]. This suggests that the presence of a non-immunogenic sialic acid can control the 3-dimensional structure of the determinants that are responsible for the production of protective antibodies. Group B *Streptococcus* causes infections in newborns who fail to mount an immune response following vaccination, due to their underdeveloped immune system. Young women can be effectively vaccinated prior to pregnancy, so that infants can acquire passive immunity by the placental transfer of IgG isotype specific antibodies [60,61].

**ALTERED GLYCOSYLATION PATTERNS IN CANCER**

Malignancy is often associated with profound alterations in cell surface bound carbohydrate components of glycoconjugates [62-66]. Such structural changes are due to incomplete glycosylation or novel glycosylation by tumor cells, which in turn arise from either down-regulation or up-regulation of certain glycosyl transferases (Fig. 11). The tumor associated carbohydrate antigens (Fig. 12) are comprised of the:

1) Globo series found in melanoma
   Gal β1-3 GlcNAc β1-4 Gal or GalNAc β1-3 Gal α1-4 Gal

2) Lacto series structures found in various adenocarcinoma cancers
   Gal β1-3 GlcNAc β1-3 Gal (type 1) and Gal β1-4 GlcNAc β1-3 Gal (type 2)

3) Tn, sialyl Tn (sTn) and T antigens found in carcinoma-associated mucins

4) Lewis X (Le*) and sialyl Lewis X (sLe*) expressed on polymorphonuclear leukocytes, increased β1-6 GlcNAc branching of N-linked core glycans and loss of sulfation or sialic acid O-acetylation.

5) GM2, GD2 and GD3 gangliosides are over expressed on melanomas, sarcomas and neuroblastomas

6) Polysialic acids expressed in brain and bronchial epithelial cells

---

**Fig. (11).** O-glycosylation pathways: The structures, shown in the box, are found primarily on the surface of tumor cells.
Fig. (12). Tumor associated antigens.

Our growing knowledge of tumor glycobiology and available evidence suggests that altered glycosylation is correlated with increased metastatic behavior and decreased immune response towards the cancer cells due to altered cell adhesion, migration and communication with immune system.

IMMUNOGENICITY OF TUMOR ASSOCIATED ANTIGENS

Vaccines containing various bacterial carbohydrate-based antigens provide protection from subsequent bacterial challenges. Similarly, tumor associated carbohydrate antigens are normally anticipated to be good vaccine candidates, because of the presence and abundance of these epitopes on the surface of tumor cells. However, there are many difficulties associated with the development of cancer vaccines [67]. Most importantly, many tumor antigens are auto-antigens or self-antigens and hence, they are poor immunogens. This immunotolerance is substantiated by the lack of immunological interference in the presence of growing tumors and antigen shedding. In addition, even if these tumor
associated antigens are not self-antigens or auto-antigens, they are surrounded by auto-antigens providing a disguise (or camouflage) that reduces the immune response. Another obstacle to the development of cancer vaccines is that antigenic heterogeneity is an inherent feature of malignancies. The immune response against carbohydrate antigens is further complicated by the T-cell independent response to carbohydrate antigens. All of these factors represent obstacles that need to be addressed when designing carbohydrate-based tumor vaccines [68,69].

The immunogenicity of GD3 ganglioside-based glycoconjugate vaccines was the first one studied in detail [70]. These studies correlated immunogenicity with different carrier proteins and adjuvants. Keyhole limpet hemocyanin (KLH) is among the best of all carrier proteins promoting a strong response to conjugated carbohydrate haptens [71]. QS21, a saponin fraction obtained from the bark of the Quillaja saponaria molina tree [72], is among the most potent adjuvants for eliciting an immune response against various ganglioside-KLH conjugates.

The immunogenicity of Tn, sTn, TF, and globo H antigens were tested in various clinical trials [73-77]. The sTn-KLH vaccines showed remarkable immunogenicity, resulting in the production of IgM antibodies and the induction of IgG antibodies. Unfortunately, the sTn-KLH vaccine failed to evoke the desired reactivity against sTn motifs expressed on the tumor associated mucins (Fig. 13). The recent success of studies, using a sTn trimer vaccine, in which three sTn antigens are clustered in a peptide backbone suggest the importance of multivalency to closely mimic the cell surface sTn motif [78]. Globo H-KLH conjugate with the QS21 adjuvant has been tested as a vaccine for patients with prostate cancer. This vaccine successfully induced specific high-titer IgM and IgG antibodies.

There was concern that antibodies induced against tumor antigens (i.e., GM3, GM2, GD2, GD3) might result in auto-immunity against some of the non-cancerous tissues that normally express these self-antigens. Although there was no evidence of local or systemic toxicity associated with the use of these vaccines, high doses of monoclonal antibodies (mAb) against GD2 led to peripheral neuropathy in melanoma patients [79]. The administration of mAb against Le^a and SLe^a also results in short lived neutropenia in some patients.

**Fig. (13).** Normal mucins (A) and tumor associated mucins (B). The branches represent the oligosaccharide chains attached to the core protein (central line). Tumor associated mucins have a fewer and truncated oligosaccharides.
Polysialic acid is spatially and temporally expressed during development, disappearing soon after birth [80,81]. This molecule is also present in group B Meningicocci. Polysialic acids can reappear in adulthood diseases such as Wilms tumor of the kidney [82,83], small cell lung carcinoma [84] and various malignant neuroendocrine tumors [85-88], such as neuroblastoma, pheochromocytoma and medullary thyroid carcinoma. The most well demonstrated functional property of polysialic acids is in cell-cell interaction and adhesion. It has been postulated that alteration of the polysialic acid glycans of NCAM reduces cell adhesion and may be involved in invasive metastasis [84]. Polysialic acids are known to exhibit both chemical and enzymatic hydrolytic instability [89]. The glycosidic linkages of polysialic acids are very labile and subject to self-cleavage under mildly acidic conditions [89]. This instability results from the direct donation of a proton, from an internal carboxyl group to an adjacent glycosidic oxygen, as a result of the unusually high pKa of the carboxyl group. The unusual lability of and structural mimicry of polysialic acids result in immune tolerance, attenuating host-tumor and host-pathogen immune reactions [83,90] and pose the most difficult challenge toward developing both passive and active immunotherapy against various cancers and Group B Meningicocci.

TUMOR-ASSOCIATED MUCIN CARBOHYDRATES AS TARGET ANTIGENS

Cancer cells differ from the normal epithelial cells with regard to expression of mucin associated carbohydrate molecules (Fig. 13). Firstly, the cell surface mucins on the normal epithelial cells are found on the luminal surface and hence are not recognized by the immune system. Tumor cells lose their ability to express mucins in a similar polarized fashion and instead express them uniformly in an orientation that is easily recognizable by the immune system [91]. In addition, there are significant differences in glycosylation of mucins associated with tumor cells and normal cells. Mucins associated with tumor cells are underglycosylated and have truncated carbohydrate structures such as sTn and Tn, whereas the mucins of non-cancerous cells have fully extended and branched complex carbohydrate structures. In the mucins of normal tissues, the internal sugar units and the core peptide sequences remain hidden by the extended carbohydrate structures [92]. In contrast, mucins associated with tumors have smaller exposed, and unusual sugar residues and peptide sequences. Thus, tumor associated mucins can potentially be recognizable by immune system [76,77,93].

TUMOR ASSOCIATED GLYCOSPHINGOLIPIDS AS TARGET ANTIGENS

The tumor associated gangliosides, recognized by monoclonal antibodies, can also be found on normal cells. There are no structural differences between glycosphingolipid (GSL) antigens expressed by tumor cells and normal cells. However, GSL density, defined as the number of antigens per unit area of cell membrane, is considerably higher in tumor cells than in normal cells. Furthermore, GSL antigens are not expressed homogeneously at the cell surface, but rather are assembled as microclusters through a process known as self-assembly. When GSL antigens are organized in clusters, with a density above a threshold value, they are immunogenic [94,95]. Thus, while GSL antigens in tumor cells are immunogenic, in the case of normal cells the GSL density falls below the threshold value and are not immunogenic and do not bind antibodies. Many tumor associated GSLs, especially gangliosides (GM3, Gb4, nLc4, and GalGb4), are normally assembled very closely to transducer molecules, such as c-src, Ras, Rhö and FAK, on the tumor cell surface [96-98]. These transducer molecules are involved in signal transduction and are oncogene or proto-oncogene products. Tumor associated GSL antigens can also promote tumor metastasis and invasion, primarily through binding of tumor cells to micro vascular endothelial cells with the connivance of GSL-GSL or GSL-lectin interactions [99,100]. Ganglioside antigens (GD3, GD2, GM3, GM2, extended GM2 and fucosyl GM1) and their metabolites also appear to regulate cell growth through modulation of key molecules in signal transduction [101,102]. Experimental proof is still required to definitively establish: a) that the organization of GSL antigens in the membrane distinguishes immunogenicity of tumor cells from normal cells; b) the functional role of GSL antigens as adhesive molecules in tumor metastasis and invasion; c) the role of GSL and their metabolites as modulators of signal transduction in tumor biology; and d) the significance of the presence of oncogenes and proto-oncogene products embedded in the GSL clusters with respect to growth signaling.

Livingston and coworkers have presented impressive results on augmentation of immune responses by active immunization with GM2,
GM3, GD2, and GD3 glycoconjugates or passive immunization against those GSL antigens with specific antibodies [103,104]. GSL antigens were covalently linked to the carrier protein, KLH by ozonolysis of double bond of the sphingosine moiety to an aldehyde groups that could be coupled via reductive amination. Immunization of patients with these glycoconjugate vaccines resulted in significantly enhanced IgM and IgG antibody titers without significant toxicity or cross-reactivity against GM2 and GD3 [70,105]. Large scale clinical trials with melanoma patients are currently underway.

**sTn SYNTHESIS**

The synthesis of sialyl Tn antigen (sTn) both in monomeric and trimeric form, has been reported by several groups (Scheme 1) [106-109]. The critical steps involved in the synthesis are: a) azidonitration; b) stereoselective glycosylations with serine derivative acceptor; and c) stereoselective glycosylation of Tn derivative with sialic acid donor. The O-glycosidic bonds to Ser/Thr is sensitive to both acid and base. The glycosidic linkage of sialic acid to GalNAc residue is also labile toward mild acid conditions. Hence

Scheme 1. sTn synthesis.
for any successful synthetic strategies, the protection group chemistry and the point of introduction of these sensitive groups must be taken into account. The presence of an azide group as a non-participating substituent at C-2 position is essential in stereoselective formation of α-O-glycosidic linkage between GalNAc and Ser/Thr. Originally, STn synthesis used a C-3 thiophenyl derivative of sialic acid as glycosyl donor for coupling with appropriately protected Tn acceptor. The C-3 thiophenyl is a directing group enlisted to ensure the desired stereoselective α-glycosylation. More recent studies have demonstrated that phosphite or chloride donor affords the desired α-
glycosidic linkage without any participating group (Scheme 1) [110]. The STn trimer was synthesized employing standard peptide coupling chemistry. The immunogenicity and cancer vaccine studies of STn are underway [73,75-77].

**GLOBO H SYNTHESIS**

The breast tumor associated hexasaccharide antigen, globo H, has been synthesized using glycal assembly approach, originally developed by Danishefsky and coworkers (Scheme 2) [111]. The selectively protected galactosyl fluoride donor was

---

Scheme 2. Globo H synthesis.
prepared from galactal and coupled with appropriately protected lactal derivative under modified Mukayama conditions [112] to provide trisaccharide glycal. The DDQ mediated deprotection of PMB group afforded trisaccharide acceptor ABC. The synthesis of the trisaccharide donor DEF is as outlined in Scheme 2. Since glycosylation with iodo sulfonamide donor failed, the thioglycoside donor was used for coupling with trisaccharide acceptor ABC leading to β-linkage formation and fully protected globo H hexasaccharide derivative ABCDEFG. The formation of β-linkage was assisted by neighboring group participation of sulfonamide group at C-2. The coupling of hexasaccharide with ceramide or allyl alcohol afforded corresponding glycosides. The ozonolysis of the allyl group, followed by reductive coupling with lysine residues of KLH carrier protein provided a globo H conjugate vaccine for clinical evaluation [113-115].

Scheme 3.
KH-1 ANTIGEN SYNTHESIS. SYNTHESIS OF KH-1 ANTIGEN

KH-1 antigen is one of the many antigens commonly found in colonic adenocarcinoma [95,116,117]. The synthesis of KH-1 antigen relied on the glycal assembly approach and is very similar to the chemistry applied toward globo H antigen (Scheme 3) synthesis [118]. It is noteworthy that three α-L-fucose residues were introduced simultaneously in a single glycosylation step, affording the nonasaccharide in a reduced number of steps.

SYNTHESIS OF GM2 BASED NEOGLYCOLIPID AS A POTENTIAL CANCER VACCINE

The ganglioside, GM2 conjugated to the carrier protein, KLH, and has been under clinical trials in melanoma patients [103-105]. Schmidt and coworkers designed a fully synthetic conjugate vaccine in which the carbohydrate epitope, GM2, is linked through a linker arm (spacer) to an immunostimulant (B-cell stimulatory glycolipid BAY R100G) as shown in Scheme 4 [119]. This immunostimulant has been shown to amplify the proliferation of B lymphocytes in response to stimulation with antigens. This neoglycoconjugate showed reactivities with several antibodies generated against GM2 as assessed by enzyme-linked immunosorbent assay (ELISA) and immune thin-layer chromatography (ITLC).

SYNTHESIS OF GLYCOCONJUGATE VACCINE AGAINST NEISSERIA MENINGITIDIS

Geert-Jan Boons and co-workers have developed a synthetic methodology to construct a fully synthetic glycopeptidolipid for use as a vaccine against Neisseria meningitidis (Scheme 5) [120]. A glycoconjugate containing L-glycerol-D-manno-heptose that acts as a B epitope (MHC class II), a peptide (derived from an outer membrane protein of N. meningitidis and the lipopeptide, S-[(R/S)-2,3-dipalmityloxy-proply]-N-palmitoyl-R-Cysteine (Pam3Cys) that functions as a immunostimulant and activates B cells and macrophages, were each synthesized. The N-terminus of T-cell epitope peptide bound to a solid support was coupled to the immunostimulant, Pam3Cys. After release from the solid support by TFA treatment, the glycopeptide was coupled to the amine-containing spacer moiety of the heptio-sugar unit to afford the fully synthetic glycoconjugate vaccine against N. meningitidis. The use of such glycoconjugates as this one have several advantages over conventional carbohydrate-protein conjugates as vaccines. They are well-defined, can easily be

purified to homogeneity and chemically are more stable than carbohydrate-protein conjugates. In addition, such conjugates should be more effective than carbohydrate-carrier protein conjugates, particularly when patients have already been immunized with (exposed to) the carrier-protein.

**SYNTHETIC SHIGELLA VACCINES**

*Shigella dysenteriae* type 1 is a Gram-negative bacteria that causes endemic and epidemic dysentery worldwide [121,122]. There is no vaccine available against this bacterium, which is increasingly becoming resistant to antibiotic treatments in various countries. The repeating unit of the O-specific polysaccharide contains α-linked D-galactose, N-acetyl-D-glucosamine and L-rhamnose residues [123]. Pozsgay has reported the synthesis of the tetrascarbohydrate repeating unit and dodecamer obtained from subsequent repetitive glycosylation with tetramer units (Scheme 6) [124]. A heterobifunctional linker arm was appended to the reducing end of the oligosaccharide for covalent attachment to human serum albumin as a model carrier protein [125]. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was used to estimate the average number of saccharide chains attached per carrier protein molecule. The synthesis relies heavily on the Schmidt glycosylation method and the principles of protecting group chemistry on donor/acceptor reactivities, developed by Paulsen [126].

**FUTURE DIRECTIONS**

Carbohydrate based vaccines have been used very successfully to protect humans against various pathogens and diseases. Current advances in carbohydrate synthesis should facilitate the chemical or enzymatic preparation of well defined, homogeneous carbohydrate antigens for glycoconjugate vaccine development. Analogs of the various carbohydrate antigens, such as C-glycosides and S-glycosides that are metabolically stable, might improve both the antigenicity and the immunogenicity of the glycoconjugate vaccines. Furthermore, such analogs should improve our understanding of carbohydrate recognition by antibodies, antigen processing and presentation, and the stimulation of immune system. This improved understanding of glycoimmunology should facilitate the construction of ideal glycoconjugate-based vaccines for specific infection or disease. Toward this goal, our laboratory has prepared C-glycoside analogs [127,128] of tumor

associated antigens for vaccine development. These synthetic, metabolically stable, non hydrolyzable carbohydrate antigens should also improve batch to batch consistency in vaccine manufacturing and reduce the requirement for cold storage that is usually necessary to maintain and assure potency of currently used carbohydrate based vaccines. The clinical success of new and improved tumor specific carbohydrate based vaccines should encourage the scientific community working in this area of research, to tailor polyvalent vaccines containing multiple tumor specific carbohydrate antigens against many cancers to provide global protection.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Fab</td>
<td>Antibody binding domain</td>
</tr>
<tr>
<td>Fc</td>
<td>Cell binding domain</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen processing cell</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>TI</td>
<td>T-cell independent</td>
</tr>
<tr>
<td>TD</td>
<td>T-cell dependent</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
</tbody>
</table>

REFERENCES

Carbohydrate Based Vaccines


[84] Scheidegger, E. P.; Lackie, P. M.; Papay, J.; Roth, J. Lab Invest., 1994, 70, 95.


