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Minireview

Carbohydrate based vaccines

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Abstract In the past decades, a gradual increase in the resistance to antibiotics has been observed, leading to a serious thread for successful treatment of bacterial infections. This feature in addition to difficulties in developing adequate drugs against (tropical) diseases caused by parasites has stimulated the interest in vaccines to prevent infections. In principle, various types of cell surface epitopes, characteristic for the invading organism or related to aberrant growth of cells, can be applied to develop vaccines. The progress in establishing the structure of carbohydrate immuno-determinants in conjunction with improvements in carbohydrate synthesis has rendered it feasible to develop new generations of carbohydrate-based vaccines.

Keywords: Glycoconjugate vaccines; Cell surface antigens; Infections; Cancer; Oligosaccharide synthesis

1. Introduction

The development of vaccines based on carbohydrates has a long history. As early as 1923, Heidelberger and Avery [1] described a 'soluble specific substance', of pneumococci to consist most likely of polysaccharides (PSs) and being typical for the serotype. Francis and Tillett [2] noted that intradermal injections of type-specific polysaccharides induced the development of circulating antibodies for heterologous types of Pneumococci. Later, Heidelberger et al. [3] established that pneumococcal capsular polysaccharides could be used as vaccines, providing a long lasting immunity. Despite the potential to apply such compounds as vaccines, the development of chemotherapeutics and antibiotics has led to a loss of interest in this application. Renewed interest for preventive vaccination was induced by the steady increase in resistance towards antibiotics. In 1983, Pneumovax™ was introduced, being a capsular polysaccharide vaccine derived from 14 *pneumonia* serotypes. Subsequently, Pneumovax™ 23 was presented containing isolated polysaccharides from 23 serotypes out of the about 90 known. This vaccine gives in healthy adults (short term) protection for about 90% of the infections by these microorganisms. However, polysaccharides are poorly immunogenic in persons of high-risk groups: (i) neonates and children until the age of two; (ii) elderly and chronically ill people; (iii) splenectomised patients; (iv) immuno-compromised people e.g. HIV infected. The age-related response to plain polysaccharides may also be structure dependent: in contrast to other capsular polysaccharides, those of group A *Neisseria. meningitidis* and pneumococci type 3 and 18C are good immunogens in infants from 3 to 6 months. They induce protective IgG antibodies. In fact vaccines were prepared from their capsular polysaccharides, e.g. against meningococcal infections vaccines containing capsular polysaccharides from the meningococcal types: A + C, A + C + W135 or A + C + Y + W135. Polysaccharides are considered to give an immune response independent of T cells; they stimulate B-cells to produce antibodies without the involvement of T-cells. However, some zwitterionic capsular polysaccharides can activate $CD4^+$ T cells. These polysaccharides are processed to low molecular weight carbohydrates by a nitric oxide-mediated mechanism and presented to T cells through the MHCII endocytic pathway [4]. Furthermore, tolerance could become a problem.

In contrast to polysaccharides, glycoproteins are T-cell dependent (TD) antigens, having a larger immune response for the same antigens. Already in 1931, Avery and Goedel [5] reported that covalent attachment of carbohydrates to a suitable protein induced an enhanced immunogenicity compared to the polysaccharides as such. In general, immunization with neoglycoproteins consisting of capsule-derived carbohydrates coupled to an immunogenic protein provides a long lasting protection to encapsulated bacteria for adults as well as for persons at high risk and young children (for reviews see: [6–8]). In addition to isolated polysaccharides, oligosaccharides derived from the capsular polysaccharides by degradation or obtained by total synthesis are effective compounds for the preparation of glycoconjugate vaccines. Such oligosaccharides have the advantage that they can be purified to single molecular species and thereby being good starting compounds for the preparation of well-defined vaccines. In general polysaccharides are intrinsically polydisperse in molecular mass. Conjugation of these macromolecules will yield heterogeneous mixtures of compounds.

In this mini review, the emphasis will be on some of the main aspects of glycoconjugate vaccines, rather than comprising all developments in this fast moving area.

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Abbreviations: CRM_{197}, cross-reactive material of diphtheria toxoid; KLH, key hole limpet emocyanin; PS, polysaccharide; GPIs, glycosylphosphatidylinositol anchors; CEA, carcino-embryonic antigen; STn, Sialyl Tn; LPG, lipophosphoglycan

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2. Preparation of neoglycoproteins

2.1. Carrier protein

Any protein, to be used in conjugation procedures for the preparation of vaccines or medical treatment, has as a general prerequisite that it should be allowed and safe for human administration. Proteins that have been applied to couple with carbohydrates are e.g. tetanus and diphtheria toxoids, cross-reactive material of diphtheria toxoid (CRM_{197}) [9], key hole limpet hemocyanin (KLH) and the outer membrane complex of *Neisseria meningitides* [10]. A few other bacteria-derived proteins were so far studied in the laboratory, only.

2.2. Coupling

For coupling of polysaccharides to a protein, chemical activation of the polysaccharide and sometimes of both compounds is necessary. The procedure to activate the carbohydrate depends on the structure e.g.: reactive aldehyde groups can be created from *vicinal* hydroxyl groups by periodate oxidation; carboxyl groups can be activated with carbodiimide followed by appropriate conversion; *N*-acetylamino functions can be (partially) de-*N*-acetylated followed by activation with nitrous acid. Most of these reactions give rise to a random creation of reactive centers in the polysaccharide. Main reactive groups in proteins are the terminal amino group and the ε-amino functions of lysine. Random activation of the reaction partners will allow the formation of conjugates, however the structure of the reaction product may be rather undefined.

To create neoglycoconjugates having well defined structures, the introduction of the linkage between carbohydrate and protein should be as specific as possible. To avoid steric hindrance between protein and glycan and to expose the immunogenic epitopes, bifunctional spacer molecules can be introduced in one or both of the reaction partners. Spacer molecules may contain as reactive functions amino, carboxyl or thiol groups [11].

In general, polysaccharides rarely present a singe molecular species, but rather a family of closely related compounds, differing in degree of polymerization. Since the immunogenic epitope comprises only part of the molecule, carbohydrate chains as short as oligosaccharides can be used for the preparation of effective vaccines. Oligosaccharides can be obtained in pure state through organic synthesis or by degradation of polysaccharides. Coupling of oligosaccharides to proteins can be performed in a rather specific route, affording well-defined conjugates in a reproducible way [6–8].

3. Glycoconjugate vaccines against bacterial infections

For several types of bacterial infections glycoconjugate vaccines can be based on fragments of capsular polysaccharides. In particular, the following bacteria should be mentioned:
Streptococcus pneumoniae
Neisseria meningitidis
Haemophilus influenzae
Salmonella typhi
Shigella dysenteriae
Group B Streptococcus
Klebsiella pneumoniae

The availability of methods to prepare specific oligosaccharide structures opened the possibility to explore the relation between the oligosaccharide chain length and the potency of the glycoconjugate as vaccine. We investigated the potency of synthetic polysaccharide type-3 related di- tri- and tetrasaccharide-CRM_{197} conjugates to provide protection against *S. pneumoniae* type 3 infection. To this end the synthetic oligosaccharides were coupled via the squarate method [12] to cross-reactive material (CRM_{197}) of modified diphtheria toxin in various oligosaccharide/protein ratios. The products were analyzed for protein and carbohydrate content. The protective immunity in mice was investigated in mice after two subcutaneous challenges with a three-week interval of 2.5 μg oligosaccharide per mouse. All mice immunized with the tri- or tetrasaccharide conjugates had IgG antibodies that bound the capsular polysaccharide. After intraperitoneal injection of a dose *S. pneumonia* lethal for control mice, all immunized mice having formed polysaccharide type 3 specific antibodies survived. Here, a correlation was found between polysaccharide specific antibodies and protective capacity of the conjugates [13] (see Table 1).

The results fit well to previous studies [14,15], wherein it was shown that a hexasaccharide derived from polysaccharide type 3 coupled without spacer as a pentasaccharide to KLH was capable of inducing protective immunity against type 3 pneumococci in mice.

In a study on the immunogenicity and protective capacity of *S. pneumoniae* 6B capsular polysaccharide derived di-(Rhaα1-4-Rib-ol-5*P*-), tri- (Rib-ol-5*P*-2Galα1-3Glc-) and tetrasaccharide (Rhaα1-4-Rib-ol-5*P*-2Galα1-3Glc-) conjugated to KLH, we have found that the di- and tetrasaccharide (one repeating unit) conjugates contain already epitopes capable of inducing 6B-specific, fully protective antibodies in rabbits and mice, respectively [16].

In an investigation of a vaccine against *S. dysenteriae* type 1, a series of specific, synthetic oligosaccharides was prepared on the basis of the tetrasaccharide repeating unit of the O-specific polysaccharide. The oligosaccharides were coupled to human serum albumin and the conjugates were applied as vaccines [17]. A clear influence was observed of chain lengths. The octa-, dodeca-, and hexadeca-saccharides were immunogenic, without adjuvant. Interestingly, the oligosaccharide conjugates induced higher anti O-specific IgG levels than the conjugate of the native O-specific polysaccharide. However, the tetrasaccharide representing a single repeating unit was not effective. Also oligosaccharide loading of the protein is a relevant factor, it should not be too high, nor too low.

The development of a synthetic conjugate vaccine against *H. influenzae* type B provides an interesting example of the potency of present day methodology. Peeters et al. [18] had shown that conjugates of tri- and tetrameric 3-β-D-ribose-(1-1)-ribitol-5-phosphate and tetanus or diphtheria toxoid afforded anti capsular-polysaccharide responses with an increasing IgG/IgM ratio in adult mice and monkeys. Chong et al. [19] have chosen a different approach. They coupled oligosaccharides consisting of repeating units of the *H. influenzae* type B polysaccharide to synthetic peptides containing potent T-helper cell determinants and B-cell epitopes. The conjugate provided T-help and the carbohydrate hapten became T-cell-dependent. In an infant rat model, the raised antibodies were protective against *H. influenzae* type B infection. Optimal results were obtained for the trimeric repeating unit. An break-

Table 1
Groups of 4 female BALB/c mice were immunized subcutaneously with 2.5 μg saccharide as a protein conjugate

Conjugate	^{10}log IgG serum titers before infection in individual mice				Survival time in days of individual mice			
CRM	<2	<2	<2	n.t.	2	3	3	n.t.
CRM-Glc	<2	<2	<2	<2	3	3	3	3
CRM-GlcA	<2	<2	<2	<2	3	>14	>14	3
Buffer	<2	<2	<2	n.t.	3	3	3	n.t.
CRM-Di 1	4.0	4.3	<2	4.2	>14	>14	4	>14
CRM-Di 2	2.8	3.1	<2	3.2	>14	>14	2	>14
CRM-Tri 1	4.6	4.0	3.4	3.7	>14	>14	>14	>14
CRM-Tri 2	4.7	4.0	4.7	4.5	>14	>14	>14	>14
CRM-Tri 3	4.5	4.3	4.4	4.3	>14	>14	>14	>14
CRM-Tetra 1	4.5	4.6	4.1	4.3	>14	>14	>14	>14
CRM-Tetra-2	3.4	4.1	4.7	3.4	>14	>14	>14	>14
CRM-Tetra 2 (0.25 μg)	n.t.	<2.0	2.6	3.4	n.t.	>14	>14	>14
CRM-Tetra-3	4.5	4.4	3.9	3.8	>14	>14	>14	>14

Control mice received CRM_{197} alone, CRM_{197} coupled to the monosaccharides Glc and GlcA or buffer solution. One group received 1/10 of the dose of CRM-Tetra 2 (0.25 μg). A similar booster was given 3 weeks after the first injection. Blood samples were taken 2 weeks before the challenge and each sample was tested for IgG recognizing PS3. After 7 weeks a peritoneal challenge with 20 LD_{50} dose of type 3 *S. pneumoniae*. Survival was recorded daily for 14 days. n.t. = not tested [13].

through was achieved by Verez-Bencomo et al. [20]. They succeeded in preparing a synthetic, commercial vaccine consisting of synthetic oligosaccharides with an average of eight repeating units conjugated to human serum albumin, being safe for human administration. The vaccine was evaluated in extensive clinical trials in Cuba. The long-term protection was adequate. This result indicates that for the production of vaccines the synthesis of oligosaccharides is commercially feasible.

In summary, various oligosaccharide-conjugate vaccines based on the capsular or O-specific polysaccharides exhibit good immunogenic properties. However, this feature cannot easily be related to chain length and/or saccharide density on the carrier molecule. These aspects remain problems that require a large number of comparative, empirical approaches. Anyhow, the synthesis of clearly defined molecular entities will render possible the study of the influences of these parameters as well as of the characterization of the effects of different carrier molecules.

4. Glycoconjugate vaccines against protozoan parasites

The principle of attacking invaders on the basis of unique cell surface carbohydrates is in principle universally applicable. In practice, it is not easy to develop vaccines that provide complete protection to any kind of invader. The situation for anti parasites vaccines is more complicated than for vaccines directed against encapsulated bacteria. These parasites are genetically and biologically complex organisms. Fundamental studies are needed to gain insight into the processes like the control of immune responses and the induction of appropriate immunological memory [21]. In the framework of this review, only some studies will be mentioned that are directed towards glycoconjugate vaccines. Accepting these restrictions, interesting developments can be noted with regard to diseases like malaria, toxoplasmosis and leishmaniasis that are caused by protozoan parasites. For malaria caused by *Plasmodium falciparum*, the attention is focused on the involvement of glycosylphosphatidylinositol anchors (GPIs) in signal transduction processes and on the possibility to employ such specific compounds as targets [22–24]. In this context the following GPI-anchor-related compound was synthesized: NH_2CH_2-CH_2PO_4(Manα1-2)6(Manα1-2Manα1-6Manα1-4)GlcNH_2α1-6*myo*-ino-sitol1,2 cyclic phosphate.

The product was then coupled to ovalbumin or to KLH and the obtained conjugate was applied for administration to mice to be studied as potential vaccines. Significant protection was observed against malarial acidose, pulmonary oedema, cerebral syndrome and mortality. It seems thereby that the stage of lead compounds for vaccine development has been attained. Furthermore, the synthetic compounds offer the possibility to investigate various basic immunological problems in relation to malaria.

Toxoplasma gondii is a protozoan parasite that is responsible for toxoplasmosis in man. Also for this organism the GPIs are interesting targets. The parasite contains in addition to the more common compound: ($NH_2CH_2CH_2PO_4$)Manα1-2Manα1-6(GalNAcβ1-4)Manα1-4GlcNH_2α1-*myo*-inositol-P0_4-lipid, the interesting novel GPI related compound [25]: (NH_2CH_2-CH_2PO_4)Manα1-2Manα1-6(Glcα1-4GalNAcβ1-4)Manα1-4GlcNH_2α1-*myo*-inositol-P0_4-lipid.

Both compounds occur with and without terminal ethanolamine. Remarkably, only GPIs carrying the Glcα1-4GalNAcβ1-4 side chains are immunogenic in humans. By experiments that comprise also synthetic compounds [26], it was shown that *T. gondii* GPIs are bioactive compounds that participate in the production of TNFα during toxoplasmal pathogenis. It is worthwhile to investigate to which extent a vaccine prepared on the basis of these GPIs can be suitable to prevent infection.

The leishmania parasite has very complex cell surface glycoconjugates [27], containing phosphosaccharide repeat units at all stages of their life cycle. Lipophosphoglycan (LPG) represents one of these compounds and is considered to be a virulence factor [28]. Since previous studies [29–31] had indicated that purified LPG has a protective effect on mice on challenge with *Leishmania major*, this feature was further investigated by Routier et al. [27]. They synthesized neoglycoproteins and neo-

glycolipids containing *Leishmania* phosphosaccharide repeats of various lengths viz.: (i)Galβ1-4Manα-PO_4H-6Galβ1-4Manα-PO_4H-6Galβ1-4Manα-O-$(CH_2)_8CH{=}CH_2$, (ii) a similar compound, but extended at the penultimate Gal with a Glcβ1-3 residue and a PO_4H inserted before the spacer and (iii) one elongated with a Manα1-2Manα-PO_4H unit. Immunological studies are still being carried out.

In a different approach to develop a synthetic vaccine against Leishmanisasis, the target was another part of the lipophosphosaccharide, namely the tetrasaccharide cap. This saccharide was synthesized [32,33] and coupled through a spacer to KLH: Manα1-2Manα1-2(Galβ1-4)ManO$(CH_2)_6$NH–CO–CH_2-S-CH_2CO-NH{KLH}.

In another attempt, the tetrasaccharide was linked to a spacer and then conjugated to the immunostimulator tripalmitoyl-*S*-glycerylcysteine. These potential vaccines are tested in an animal model. However, the World Health Organization states in its report on 'Parasitic Diseases' that there is up to now no vaccine for prevention of any form of Leishmaniasis [34]. Apparently, there is still a long way to go for obtaining vaccines that provide adequate immunity towards infections.

5. Glycoconjugate vaccines against cancer

Cancer vaccines are intended either to treat cancers (therapeutic vaccines) or to delay or preferably prevent the development of a relapse of cancer (prophylactic vaccines) after any other form of (radical) therapy. Furthermore, prevention of metastasis is an important aspect. Therapeutic vaccines are designed to stimulate the immune system to recognize and attack human cancer cells, without doing harm to normal cells. Obviously, the attack of self-antigens should be avoided. The only cancer-related vaccine licensed so far by the FDA is a prophylactic vaccine against hepatitis B virus. Infection, with this virus is associated with liver cancer.

Cancer cell antigens may be unique to individual tumors, shared by several tumor types, or expressed by the normal tissue from which the tumor grows. Some of these antigens are carbohydrates and several trials are being carried out to explore their possibilities to be used for the development of vaccines. For example, on breast cancer cells Mucin-1 (MUC-1), NER-2/*neu*, carcino-embryonic antigen (CEA), p53, Sialyl Tn (STn) and Globo H have been found. The MUC-1 STn epitope: Neu5Acα2-6GalNAcα1-O conjugated to KLH (Biomira Inc. Edmonton, Alta., Canada), given in combination with the adjuvant DETOX-B is currently under investigation [35,36]. Although the results show the formation of STn-specific IgM and IgG antibodies, so far no correlation was found between antibody formation and clinical aspects [37]. In addition, Tn-KLH is on a large scale being tested in breast cancer patients. (For a review on anti-breast cancer vaccines see [38].) NeuGcGM3 is another example of a compound forming the basis for a possible vaccine against breast cancer [39].

Globo H is a prominent epitope on prostate cancer cells. On this basis a potential vaccine was prepared consisting of the synthetic hexasaccharide chain of Globo H, linked to KLH. The effect is monitored by checking the prostate specific antigen (PSA) concentration [40].

Ganglioside molecules (e.g. GM2, GD2, GD3) are expressed on several cancer cells. A vaccine that contains GM2 and GD2 is evaluated for the induction of antiGM2 and antiGD2 antibodies, specifically directed towards melanoma cells (Progenics Pharmaceuticals, Tarrytown, NY, USA) [41]. Furthermore, a GM3 ganglioside conjugated vaccine is on trial for anti-melanoma effects [42].

CEA is found in high levels in colorectal, lung, breast and pancreatic cancers. Therefore, it could be a good starting point for the development of a (semi) synthetic vaccine.

The examples given above represent just a small selection of the wealth of studies that are currently in progress. They have in common the search for anticancer vaccines based on carbohydrate epitopes.

6. In summary

In this mini review on glycoconjugate vaccines, the emphasis is put on the character of the epitopes and on proteins as carrier molecules. It should be noted that liposomes [30,43] and dendrimers [44,45] can be applied as carriers, but the detailed discussion of these systems is beyond the scope of this review. Since the large-scale synthesis of carbohydrates is no longer a limiting factor, the preparation of carbohydrate-based and structurally well-defined vaccines has become feasible [20]. The screening for the optimal length of the carbohydrate chain, the selection of the most suitable spacer and most effective (protein) carrier are now realistic targets. The stability of carbohydrates in biological systems is a point of continuous attention. In this perspective, the synthesis can be mentioned of analogues of GM2 and GM3 containing S-linked neuraminic acid and an amino terminated, truncated ceramide homologue [46]. After conjugation to a protein these compounds are tested as immunogens. The obvious advantage of these compounds is their resistance against hydrolytic degradation.

For vaccines directed against bacterial polysaccharides significant progress has been made, leading to wide spread clinical applications. With regard to a number of other pathogens, breakthroughs can be expected.

The prevention of parasitic infections through immunization is urgent, in particular in tropical regions. However, despite many trials an effective vaccine is not yet available. The development of functional anticancer vaccines is even less straightforward. The immunological problems are complex and will require further multidisciplinary research. The cancer specific carbohydrate antigens are in principle good targets. To which extent it could be profitable to induce antibodies against an ensemble of antigens remains to be investigated [47]. The individual differences and biological variation could be limiting factors in the clinical use. It should be noted that there might exist other epitopes that are better suited to be used as starting compounds for the preparation of anti cancer vaccines. The selection of effective adjuvants is an important aspect in the actual application of the glycoconjugate vaccines that needs further consideration.

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