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Special Feature

Carbohydrate vaccines as immunotherapy for cancer

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Summary Carbohydrates have established themselves as the most clinically relevant antigens of those tested and subsequently developed for vaccines against infectious diseases. However, in cancer patients, many of the defined carbohydrate antigens are really altered 'self' antigens and for unclear reasons, the body does not react to them immunologically. Although these self antigens have been found to be potentially suitable targets for immune recognition and killing, the development of vaccines for cancer treatment is actually more challenging compared with those for infectious diseases mainly because of the difficulty associated with breaking the body's immunological tolerance to the antigen. These antigens lack the inherent immunogenicity associated with bacterial antigens and, therefore, methods to enhance immunological recognition and induction of immunity *in vivo* are under investigation. These include defining the appropriate tumour-associated antigen, successfully synthesizing the antigen to mimic the original molecule, inducing an immune response, and subsequently enhancing the immunological reactivity so that all components can work together. This has been successfully accomplished with several glycolipid and glycoprotein antigens using carriers such as keyhole limpet haemocyanin (KLH) together with a saponin adjuvant, QS-21. Not only can high titre IgM and IgG antibodies be induced, which are specific for the antigen used for immunization, but the antibodies can mediate complement lysis. The approaches for synthesis, conjugation, clinical administration and immunological potential are discussed.

Key words: cancer vaccine, carbohydrate vaccine, immunotherapy.

Introduction

Carbohydrate antigens

Carbohydrate antigens can be categorized into two major groups: (i) glycolipids such as GM2, GD2, GD3, and fucosyl GM1 (gangliosides), and Lewis^y (Le^y) and globo H (neutral glycolipids); and (ii) glycoproteins such as the mucin-related epitopes Tn (GalNAcα-O-Ser/Thr), TF (Thomsen-Friedenreich, Galβ1→3GalNAcα-O-Ser/Thr) and STn (NeuAcα2→6GalNAcα-O-Ser/Thr). Natural and vaccine-induced antibodies against GM2 and STn have been detected in patients with cancer, and have been associated with prolonged disease-free periods and overall survival.[1]

Upon infection by pathogens, antibodies are the primary immune mechanism for active elimination of early tissue invasion and circulating pathogens from the bloodstream. This is clearly an 'active' response by which the body generates its own defences in comparison to a more 'passive' approach whereby immune globulin or commercially prepared monoclonal antibodies (MoAbs) can passively confer immunity without involvement of the body's own defences. Of the many antigens studied, the carbohydrate antigens have been proven to be the most suitable and clinically relevant antigens. The antibodies against these antigens have been shown to correlate with protection from subsequent bacterial invasion.[2–4] The selection of antigens for use in vaccines against infectious disease has been relatively straightforward: they are identified by post-infection ('immune') sera. They are generally immunogenic because they are seen as foreign by the immune system. In contrast to infectious diseases, the choice of antigens for the development of cancer vaccines has proven to be problematic because natural antibodies are not generally detected. The exception has been in melanoma, where naturally occurring antibodies to the ganglioside GM2 have been shown to correlate with improved survival. However, of all the antigens studied as cancer vaccines for induction of humoral immunity to date, carbohydrate antigens have proven to be the most effective targets for immune recognition and attack. The importance of antibodies in mediating protection from tumour recurrence has been proven in tumour-bearing mice using the MoAb 3F8 against melanoma antigens such as GD2. Mice were subsequently protected from tumour growth. Significant protection was also seen when antibody was administered for the treatment of micrometastases as long as 2–4 days after tumour challenge. This timing may be comparable to antibody induction in the adjuvant setting that occurs after surgical resection of primary melanoma or lymph node metastases in humans. Similar protection was also seen when a GD2-KLH conjugate vaccine actively induced anti-GD2 antibodies. Based on studies of bacterial vaccines, the most likely mechanism for this protection was complement-mediated attack and lysis coupled with possible antibody-dependent cell-mediated cytotoxicity of tumour cells, with the tumour antigens expressed on the cell surface serving as targets. If antibodies against cell surface antigens of sufficient titre can be induced in cancer patients to

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Table 1 Summary of carbohydrate antigens as targets for vaccine construction

Tumour	Antigens
B cell lymphoma	GM2, GD2
Breast	GM2, globo H, TF(c), Le^y
Colon	GM2, TF(c), STn(c), Le^y, Tn, sialyl Le^a
Lung	GM2, globo H, Le^y
Melanoma	GM2, GD2, GD3L, GD3
Neuroblastoma	GM2, GD2, GD3L, polysialic acid
Ovary	GM2, globo H, TF(c), STn(c), Le^y
Prostate	GM2, globo H, Tn(c), TF(c), STn(c), Le^y
Sarcoma	GM2, GD2, GD3L, GD3
Small cell lung cancer	GM2, FucGM1, globo H, polysialic acid, sialyl Le^a
Stomach	GM2, Le^y, Le^a, sialyl Le^a

eliminate tumour cells from blood and lymphatic systems and to eradicate micrometastases, this would dramatically change our approach to one of treating the cancer patients earlier in the disease, before the cancer becomes visibly metastatic.

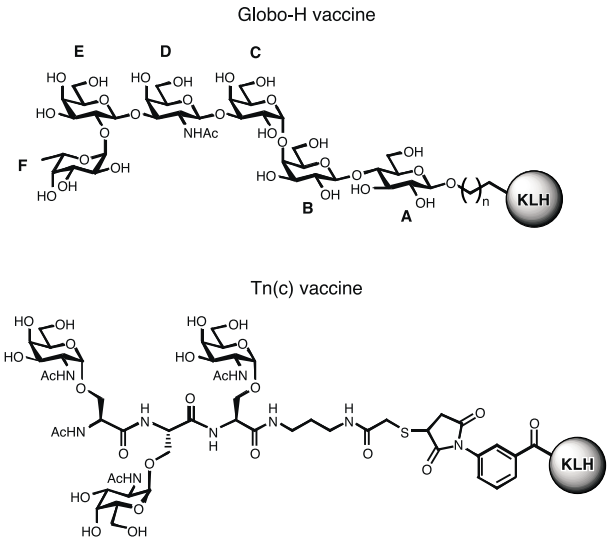

Figure 1 Structures of the monovalent vaccine Globo H, and the trimer formation for Tn (cluster) vaccine.

Structural presentation of antigens

On the surface of cancer cells, the carbohydrates are displayed as constituents of either glycolipids or glycoproteins. In the case of glycosphingolipids, it is believed that the lipid portion inserts itself into the plasma membrane and then self-associates to form glycosphingolipid microdomains on the surface of cells, appearing as large, heavily glycosylated patches.[5] Additionally, the antigens can be components of glycopeptides in which the glycan is linked through either oxygen (*O*-linked) or nitrogen (*N*-linked) atoms of amino acid side chains. When the *O*-linked glycoproteins contain an *N*-acetylgalactosamine residue at the site of linkage to the protein, they are called mucins.

Antigen screening: defining the target

Carbohydrate cancer antigens expressed on the surface of tumour cells are mostly upregulated (except on mucins) because of changes in the expression of enzymes or mutation in the DNA. These antigens are present on the cell surface as glycolipids or glycoproteins.[6–8] Zhang *et al*. screened a variety of malignant and normal tissues by immunochemistry using panels of MoAbs against carbohydrate antigens.[7,8] The carbohydrate antigens were expressed on 50% or more of tumour cells in 60% or more of biopsy specimens of some of the most common types of cancers, which are listed in Table 1. Melanomas, sarcomas, and neuroblastomas expressed a broad range of carbohydrate antigens, as well as GD2 or GD3. GM2, GD2 and GD3 are all expressed in the brain, especially GD2, which is also expressed on some peripheral nerves and, unexpectedly, a on subpopulation of B-lymphocytes in the spleen and lymph nodes. GM2 was expressed at the secretory borders of most epithelial tissues. GD2 and GD3 were also expressed at low levels in connective tissues of multiple organs and GD3 was expressed on some human T lymphocytes. Fucosyl GM1 was expressed on occasional cells in the islets of Langerhans and in some bronchial epithelial cells. Globo H, Le^y, TF, Tn and STn were expressed at the secretory borders of a variety of epithelial tissues. Le^x and sialyl Le^x were expressed at the secretory border of many epithelial tissues but also on polymorphonuclear leucocytes.

Glycolipids such as GM2, GD2, GD3, 9-*O*-acetyl GD3, fucosyl GM1, Lewis^y (Le^y), and globo-H are attached to the lipid bilayer at the cell surface by hydrophobic forces through the ceramide moiety. In glycoproteins, carbohydrate antigens such as Thomsen–Friedenreich (TF), sialylated Tn (STn), Tn, Le^y and globo H are generally attached to the OH (hydroxyl) group of serine or threonine in mucins through a glycosidic linkage (*O*-linked), but may also be *N*-linked to other proteins through asparagine. Whether expressed as glycolipids or glycoproteins, these antigens are more abundantly expressed at the cell surface than any protein antigens, and the immune response appears to be predominantly against the carbohydrate moiety.

The broad expression of most of these carbohydrate antigens on normal tissues raises concern over their suitability as targets for immunotherapy. However, there is now sufficient experience from clinical trials with MoAbs against GD2, GD3, Le^x, and STn, and with vaccine-induced antibody responses against GM2, GD2, GD3, TF, Tn, STn, globo H and Le^y to draw conclusions about the consequences of antigen distribution on various normal tissues. Of the various carbohydrate antigens studied as cancer vaccines, the gangliosides against melanoma have been studied most intensively. In this review, the experience with each antigen is summarized as it is presented within the context of a different vaccine preparation and its immunological impact.

Vaccine design and function

The first carbohydrate vaccines investigated were monomeric, containing a single carbohydrate moiety conjugated to a carrier protein. Vaccines that contain the complex hexasaccharide globo H (Fig. 1) have been investigated in a number of different cancers.[9–11] Truncated, synthetic versions of the globo H antigen were made and investigated for their

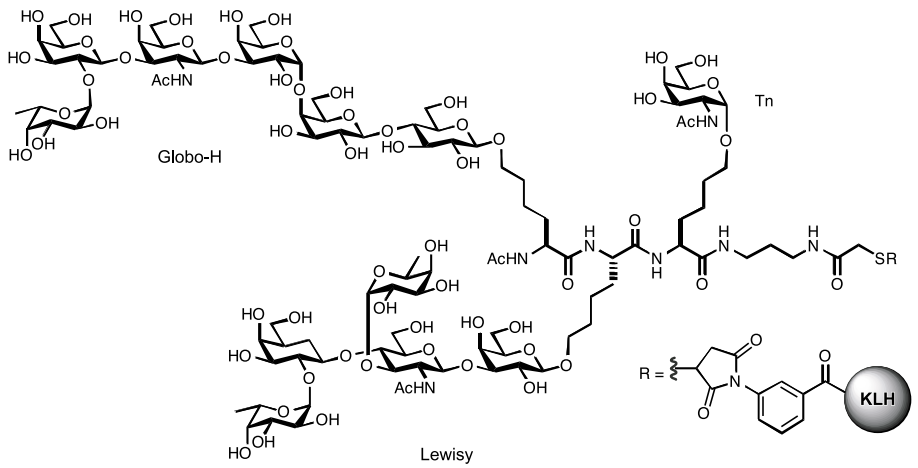

Figure 2 Vaccine constructed to present three different antigens (globo-H, Lewis^y and Tn) to the immune system.

ability to bind the MBr1 MoAb. The terminal fucosyl residue (ring F) is critical for antibody recognition, whereas the A and B rings were found to be less significant.[10] This suggests that the binding domain is localized in the CDEF ring system. This phenomenon was also seen for vaccines containing the KH-1 antigen, a nonasaccharide comprising both the Lewis^y tetrasaccharide and the Lewis^x trisaccharide epitopes. The antibodies elicited by the KH-1 vaccine constructs recognized the KH-1 antigen in addition to the Lewis^y antigen, which is similar to the four saccharides found at the non-reducing end of the KH-1 nonasaccharide.[12]

The second generation of glycopeptide-based vaccines was developed to mimic the tumour cell surface, which is characterized by mucins (large glycoproteins consisting of high percentages of serine and threonine residues, which are often found in repeating arrays ranging from two to five). It was discovered that the smaller tumour-associated mucin antigens (e.g. STn, TF and Tn) were required to be displayed in a clustered format (Fig. 1) in order for a robust immune response to realized.[13] In another example, nuclear magnetic resonance (NMR) spectroscopy was used to determine the solution conformation of the *N*-terminal amino acid segment STTAV (serine-threonine-threonine-alanine-valine) of the cell-surface glycoprotein CD43 with four different tumour-associated antigens (STn, α-TF, β-TF, α-Tn).[14,15] The striking feature of these glycopeptides was the elongated secondary structure of the isolated mucin, which is reminiscent of and approaching the stability of motifs found in folded proteins. In order for this degree of order to emerge, the *O*-glycosylation linkage must be in α-configuration. Immunological characterization of constructs very closely related to those used for the NMR experiments have been shown to elicit robust antibody responses that cross-react with tumour cells displaying the corresponding antigen.[16] This strongly suggests that the observed conformational epitopes represent distinct architectural features found on tumour cell surfaces.

Some of the more recent vaccines of interest have focused on targeting the heterogeneity of antigens associated with certain cancers. Mixtures of existing monomeric and clustered vaccines have been investigated.[17,18] Unimolecular multivalent vaccines have been developed that contain as many as five different tumour-associated carbohydrate antigens displayed on a peptide backbone and conjugated via a linker to two different carriers.[19] The antigens incorporated into a multivalent vaccine (globo H, Lewis^y, STn, Tn and TF) for prostate cancer were all commonly found on prostate cancer tumour cells and cell lines. The vaccine shown in Fig. 2, which contains three different antigens (globo H, Lewis^y and Tn), has been evaluated in murine hosts and it has been shown that antibodies were generated that recognized each of the antigens of interest.[19] Unique features of these unimolecular vaccines are the non-natural amino acid side chains linking the peptide backbone to the antigenic carbohydrate domains as well as the fact that large antigens such as Lewis^y and globo H can be successfully incorporated into the glycopeptide motif.

Vaccine development

Clinical responses have been reported in patients treated with MoAbs against GD2, GD3, STn. Therefore, cell surface carbohydrate antigens have shown their value as suitable targets for immune attack against cancers by both active and passive immunotherapies. Various approaches, including vaccination with whole or lysed tumour cells, purified or synthetic carbohydrates, immunogenic carbohydrate derivatives, or carbohydrates conjugated to immunogenic carriers and administered with immunological adjuvants have been adopted to induce and enhance immune responses against these carbohydrate antigens. Compared with other methods of vaccination, carbohydrate conjugate vaccines have consistently induced the highest titre IgM and IgG antibodies against their immunogens as well as tumour cells expressing these antigens. Preclinical and clinical studies with carbohydrate conjugate vaccines have induced higher titre IgM and IgG antibodies and complement-mediated cytotoxicity of tumour cells *in vitro*, protection from tumour challenge in mice, and prolonged disease free periods and overall survival in patients.

Vaccines designed to induce an optimal antibody response have several components, each of which must be maximized. Our experience in developing 'conjugate' vaccines has suggested that certain criteria must be met in order to successfully disrupt immunological tolerance. The first component is the antigen itself (carbohydrate), which must closely resemble

its expression on the target, which can be a glycolipid or a glycoprotein. The second component found to be necessary for an optimal antibody response mandates that the antigen be covalently conjugated to an immunogenic carrier protein. We have found keyhole limpet haemocyanin (KLH) to be the optimal carrier in clinical studies for antibody induction, irrespective of the antigen used. The conjugation must be achieved in a manner that does not interfere with the antigenic epitope itself and that achieves as high an antigen/carrier ratio as possible. This has been achieved using the MBS (m-maleimidobenzoyl-*N*-hydroxysuccimide ester) heterobifunctional cross-linker, which links the terminal cysteine group of the cluster backbone to amino groups on KLH. The final necessary component is the immunological adjuvant. Our studies in mice and in patients with prostate, ovarian, melanoma and breast cancers have shown that saponin adjuvants such as QS-21 have been the most potent for augmenting the antibody response against conjugate vaccines, although a semisynthetic analogue, GPI-0100, is slightly less optimal and has fewer local reactions. The 100 μg dose level of QS-21 used in clinical studies in prostate, ovarian and breast cancer has been found to be optimal, with higher doses resulting in excessive local and systemic toxicity and lower doses resulting in decreased immunogenicity.

Early experience with vaccines

Cancer vaccines evolved from whole cell vaccines. Initially, mice and patients were immunized with a series of irradiated autologous or allogenic melanoma cells or cell lysate vaccines mixed with various adjuvants, and the resulting immune responses were analysed.[20–24] Vaccination of mice with irradiated melanoma cells was able to induce low levels of IgM antibodies against GD3. In contrast to mice, all patients treated in this manner developed strong serological responses against irrelevant antigens on melanoma cells such as HLA antigens or FCS (present in the media), but several responses with relative specificity for melanoma cells were induced. The gangliosides GM2 and GD2 were the only melanoma antigens recognized by more than one patient. These antibodies were primarily IgM. Others also found GM2 and GD2 to be particularly immunogenic; 10 of 26 (39%) patients vaccinated with a mix of irradiated allogenic melanoma cell lines produced detectable IgM antibodies against GM2, and two patients produced antibodies against GD2.[20] Livingston *et al*. demonstrated that anywhere from 0 to 80% of patients were able to generate antibodies to GM2 depending on the cell lines chosen for vaccine production.[26] However, these vaccines induced only moderate titres of IgM antibodies. They were difficult to prepare consistently and administer, and it would have been necessary to select additional cell lines if antibodies against other antigens were desired. The occasional anti-GM2 antibody responses induced by vaccination with melanoma cells provided a base for testing the immunogenicity of purified GM2 ganglioside.[20] Two independent studies also showed that patients with antibodies against GM2 occurring either naturally or induced by vaccine had a significantly longer disease-free period and overall survival rate compared with antibody-negative or untreated patients.[1,25]

Non-conjugate carbohydrate vaccines (purified GM2, GD2 or GD3 plus Bacillus Calmette Guerin vaccines)

Based on the early data using whole cell preparations as vaccines, a series of pilot clinical trials using purified GM2 were reported. No antibodies were induced when mice and patients were immunized with purified GM2 alone.[18,26] Immunization of mice with GM2 mixed with a variety of different adjuvants showed monophosphoryl lipid A (MPLA)-containing liposomes and *Salmonella minnesota* mutant R595 to be the most effective adjuvants in mice, but Bacillus Calmette Guerin (BCG) was shown to be less effective.[20,26] Similar immunizations in man, however, showed that BCG was significantly more effective than *Salmonella minnesota* R595 or MPLA-containing liposomes and resulted in the induction of IgM antibodies in the majority of patients.[18–20] The antibody response induced by purified GM2 with adjuvant was primarily an IgM response of moderate titre and short duration, irrespective of whether it was in either mice or in men. No secondary immune response was observed even with repeated immunizations. These antibodies demonstrated effective cell surface reactivity with tumour cells expressing GM2, and in many cases were able to mediate complement-dependent cytotoxicity.

A randomized clinical trial was conducted at the Memorial Sloan-Kettering Cancer Center (MSKCC), New York, with 122 patients with the American Joint Commission on Cancer (AJCC) stage III melanoma who received GM2/BCG vaccine.[27] In this trial, patients who were free from disease after resection of metastatic melanoma in regional lymph nodes were randomized to receive five vaccinations over a 6 month period with either GM2 adherent to BCG (58 patients) or BCG alone (64 patients) after pretreatment with a low dose of cyclophosphamide (to decrease suppressor cell reactivity). The serological analysis showed that anti-GM2 antibodies were detected in 50 of 58 patients treated with GM2/BCG (median titre of 1/160). GM2 antibodies were detected in seven of 64 patients treated with BCG alone (median titre of 1/80). A highly significant increase in the disease free interval ($P = 0.004$) and a 17% increase in overall survival ($P = 0.02$) were observed in these 57 G2 antibody-positive patients with a minimum follow-up of 51 months. When comparing all patients in the two treatment groups, a 14% improvement in disease-free interval and 11% improvement in overall survival were seen in the GM2/BCG group. Although these results did not achieve statistical significance, they provided a basis for attempts to further augment the immunogenicity of GM2 ganglioside vaccines.[27]

Several attempts were made to immunize patients with GD2/BCG or GD3/BCG vaccines, which resulted in only occasional low titre IgM antibodies against GD2 but no antibodies induced against GD3. A series of approaches were developed in an attempt to overcome the non-immunogenic nature of GD3 and augment GD3 immunogenicity by making minor structural modification to GD3. These modifications included GD3 lactones, GD3 amide, GD3 gangliosidol and a series of *O*-acetyl GD3 derivatives acetylated at various sites. These preparation were adherent to *Salmonella minnesota* mutant R595 in mice or to BCG in patients with melanoma.[28–30] The congeners were used based on the assumption that because these were (foreign) antigens, they would be more

immunogenic, and those antibodies cross-reactive with native GD3 would be induced. The GD3 derivatives all proved more immunogenic in mice than native GD3, and induced consistent IgM and low titre IgG antibodies,[30,31] but even these antibody responses were short-lived and the antibody titres could not be boosted. Unfortunately, the antibodies produced by patients in response to immunization with these GD3 congeners were specific for the particular congener. No cross-reactivity was detected with GD3, nor did these immunizations prime the patients for subsequent immunization with GD3/BCG. Even immunization with a mix of GD3 congeners inducing antibodies against each of these derivatives did not induce detectable antibodies against GD3 itself. The immunological tolerance to GD3 was probably due to expression of GD3 on a variety of normal tissues in humans, including T lymphocytes. On the other hand, GD3 was more immunogenic in the mouse because of its more restricted expression on normal tissues.[32]

Carbohydrate conjugate vaccines: evolution of covalent conjugate ganglioside vaccines

On the basis of exciting progress with bacterial polysaccharide conjugate vaccines, several laboratories explored the use of gangliosides and related carbohydrates conjugated to BSA or tetanus toxoid for the production of antibodies and monoclonal antibodies.[33,34] The effect of the covalent conjugation of protein carriers on ganglioside GD3 and their immunogenicity was systematically established by Helling *et al*.[35] Of many methods of conjugation, and the diverse carriers and adjuvants studied, covalent attachment of GD3 with KLH by reductive amination and immunological adjuvant QS-21 (purified saponin fraction obtained from the bark of the tree *Quillaja saponaria*) proved optimal.[36] High-titre IgM and IgG responses against GD3, which could mediate complement lysis of human melanoma cells expressing GD3, were seen in most mice. Striking findings in these studies were the absolute necessity of conjugation of ganglioside to the immunogenic carrier KLH, the importance of the method of conjugation, and the need for a potent immunological adjuvant such as QS-21. The same GD3-KLH conjugate with other immunological adjuvants, other conjugation procedures, or immunization with ganglioside alone or covalently attached to other carriers plus QS-21, resulted in a minimal response.[35]

Clinical trials with GM2-KLH conjugate vaccine

The demonstration of immunogenicity of GM2 in man was documented in a series of clinical trials conducted with the GM2-KLH conjugate alone or GM2-KLH mixed with the immunological adjuvants BCG, Detox (containing BCG cell wall skeletons and monophosphoryl lipid A) or QS-21 in patients with melanoma.[37] The covalently conjugated GM2-KLH without adjuvant or with BCG or Detox was moderately immunogenic. GM2-KLH plus QS-21 not only induced the highest IgM titres, but also induced durable IgG antibodies in most patients. The IgG antibodies were of the IgG_1 and IgG_3 subclasses and, similar to IgM antibodies, were able to induce complement-mediated lysis of GM2-positive tumour cells and, in most cases, the IgG antibodies mediated antibody-dependent cell-mediated cytotoxicity (ADCC) as well.[37–39] These trials established the optimal dose of the adjuvant QS-21 as 100 μg. There was no evidence that higher doses were associated with increased adjuvanticity and immunogenicity compared with the 100 μg dose, and higher doses were clearly more toxic.[40]

The dose of GM2 in GM2-KLH required to induce optimal antibody titres against GM2 was studied in additional phase I trials using 1, 3, 10, 30 and 70 μg of GM2 in GM2-KLH plus a constant dose of QS-21 (100 μg). No clear dose–response relationship was observed between 3 and 70 μg, although doses of 1 μg or lower were incapable of inducing a consistent immune response. No difference was seen when patients were pretreated with low-dose cyclophosphamide. The toxicity of GM2-KLH plus QS-21 in patients remained restricted to local erythema and induration at injection sites lasting 2–4 days, plus occasional flu-like symptoms.[40]

A single-institution phase III trial conducted at the Memorial Sloan-Kettering Cancer Center, New York,[41,42] served as the impetus for an intergroup adjuvant E1694/S9512/C509801 trial, which enrolled a total of 880 patients. This led to a multi-institutional cooperative group phase II study,[43] which was designed to evaluate the combination of GMK (GM-2) and IFN-α2b. The E2696 trial was undertaken to evaluate the toxicity and other effects of the established adjuvant high-dose IFN-α2b regimen in relation to immune responses to GM-2, and to evaluate the potential clinical and immunological effects of the combined therapies. One hundred and seven patients with resectable high- or very high-risk melanoma (AJCC stages IIB, III and IV) were included. IFN-α2b did not significantly inhibit IgM or IgG serological responses to the vaccine, and the combination of high-dose IFN-α2b and GM2 were well tolerated in this patient population. A Cox analysis demonstrated that the combination of GM2 with IFN-α2b produced an improvement in the relapse-free survival of patients with very high-risk melanoma (including those with resectable M1 disease). Based on these results, randomized phase II trials with the GM2-KLH plus QS-21 vaccine were initiated by Progenics Pharmaceuticals (Tarrytown, NY) within a multi-institutional cooperative setting. These trials are based on the higher-titre, longer-lasting antibodies against GM2 induced by the GM2-KLH vaccine plus QS-21. A phase II trial comparing the standard treatment (high-dose interferon) with GM2-KLH plus QS-21 vaccine is being conducted in patients with AJCC stage III disease by the Eastern Cooperative Oncologic Group (ECOG), the South-west Oncology Group (SWOG), the North Central Treatment Group, the Memorial Sloan-Kettering Cancer Center, and the Cancer and Leukaemia Group B. The Melanoma Group (MG) of the European Organization for Research and Treatment of Cancer (EORTC 18961) have initiated a phase III multicentre trial for stage II melanoma, accruing 1300 patients to compare GM2-KLH/QS21 with observation. The trial is currently ongoing and the results are eagerly awaited.

Clinical trials with GD2, GD3, GD3-lactone or fucosyl GM1 conjugated with KLH plus QS-21 vaccines

Based on the findings of studies on the GM2-KLH conjugate vaccine, various clinical trials have been conducted with other gangliosides. Small groups of melanoma patients were vaccinated with GD2-KLH or GD3-KLH conjugate plus QS-21.

The GD2-KLH vaccine induced a moderate anti-GD2 IgM antibody titre in all patients vaccinated.[27] As seen with purified GD3, the GD3-KLH conjugate vaccine was unable to induce antibodies against GD3.[20] Other attempts have been made to induce anti-GD3 antibodies with a GD3-lactone conjugated with KLH.[44,45] Six melanoma patients were immunized with GD3-lactone-KLH (GD3-L-KLH) conjugates containing 30 μg of ganglioside plus 100 μg of QS-21 at 0, 1, 2, 3, 7, and 19 weeks. After immunization, IgM and IgG antibodies were detected against both GD3 and GD3-lactone. The GD3-L-KLH vaccine induced IgM titres against GD3-L of 1/40–1/1280 in all patients and IgG titres of 1/160–1/1280 in four patients. These antibodies cross-reacted with GD3. ELISA reactivity was confirmed by immune thin-layer chromatography on GD3 and melanoma extracts. Sera obtained from four of six patients showed cell surface reactivity by using FACS and two showed strong cell surface reactivity by using immune adherence (IA) and complement lysis against the GD3 positive cell line SK-Mel28.[44,45]

Fucosyl-GM1 (Fuc-GM1) (Fucα1→2Galβ1→3GalNAcβ1→ 4(NeuAcα2-3) Galβ1 →4Glcβ1→*O*-cer) is a small cell lung cancer (SCLC)-associated ganglioside initially defined by the murine MoAb F12. Recently, we have established the immunogenicity of Fuc-GM1 in mice that were given either the antigen alone, or the antigen mixed with KLH or covalently linked to KLH plus immunological adjuvant QS-21. As seen in the other ganglioside vaccine in mice, the Fuc-GM1-KLH conjugate plus QS-21 was optimal for IgM and IgG antibody induction. These antibodies were strongly reactive with fucosyl-positive cells and mediated complement-dependent cell lysis.[46]

Fucosyl GM-1 is expressed more frequently and more abundantly on SCLC.[47,48] than are two other gangliosides, GD3 and GM2. The presence of fucosyl GM1 has been demonstrated by HPLC immunostaining in culture media from SCLC cell lines and in tumour extracts and serum of nude mouse xenografts. It was also detected in the serum of 4 of 20 patients with SCLC, all of whom had extensive stage disease.[49] Fucosyl GM-1was not detected in the sera of 12 patients with non-small cell lung carcinoma or in 20 healthy donors.[48] A recent study by Krug *et al*. tested the immunogenicity of three different doses of a synthetic version of fucosyl GM-1 in patients with SCLC after a major response to initial therapy.[50] Five of six patients at a dose of 30 μg and three of five patients at a dose of 10 μg generated IgM responses of 1:80 or greater. Antibodies were confirmed by flow cytometry in seven of eight cases. These observations have led to a larger study combining synthetic fucosyl GM1 vaccine at a dose of 30 μg with vaccines against three other antigens, GM2, globo H and polysialic acid post chemotherapy.

Fully synthetic carbohydrate vaccines

Globo H

Globo H (Fucα1→2Galβ1→3GalNAcβ1→3Galα1→ 4Galβ1→ 4Glcβ1→*O*-cer) is a human breast cancer-associated carbohydrate antigen identified and defined by murine MoAb MBr1 generated by immunization of mice with human breast cancer cell MCF-7.[51,52] Lack of a suitable natural source of globo H, a hexasaccharide, has led to its chemical synthesis using the glycal assembly method as an allyl glycoside and as a glycolipid attached to ceramide.[53,54] Pre-clinical studies in mice were conducted using the vaccine prepared with globo H ceramide alone or with adjuvants such as *Salmonella minnesota* mutant R595 or globo H allyl glycoside covalently conjugated with KLH or BSA by reductive amination, plus QS-21. Similar to ganglioside again the globo H-KLH plus QS-21 was proven to be the optimal vaccine, inducing high IgM and IgG titres against globo H by ELISA.[55] The antibodies induced against the synthetic globo H were strongly reactive with the natural globo H expressed on the globo H-positive MCF-7 cells but not a globo H-negative melanoma cell-line by FACS and an immune adherence rosetting assay. The polyvalent immune response could be completely inhibited by synthetic globo H hexasaccharide, and partially inhibited by a range of truncated synthetic globo H compounds, but there was no inhibition of this reactivity with tumour cells with unrelated compounds.[55]

Four groups of patients with biochemically and radiographically relapsed prostate cancer were vaccinated with globo H-KLH vaccines containing 3, 10, 30 and 100 μg of globo H.[56] Twenty patients were enrolled, of whom 18 were evaluable. Of the nine patients who were radiographically free of disease but had rising prostate-specific antigen (PSA) levels as the sole biomarker of disease progression, six had a change in the PSA logslope. The vaccines were safe, no significant toxicity was detected, and they induced specific high titre IgM antibodies against globo H. This was the first demonstration of a complex synthetic carbohydrate antigen inducing an immune response in humans. Of four doses tested, the 30 μg dose was the most immunogenic dose. In most patients, after immunization, both IgM and IgG antibodies were detected against synthetic globo H by ELISA, and against synthetic and prostate and breast cancer-derived globo H extract by immune thin-layer chromatography (ITLC). In prevaccinated patients no serological reactivity against synthetic globo H was detected. These antibodies also strongly reacted with the globo H-positive cell-line MCF-7 but not with a globo H-negative melanoma cell-line SK-mel-28 as assessed by flow cytometry and complement-mediated cytotoxicity assays. The antibodies separated with a globo H affinity column showed that the vaccine induced specific antibodies that reacted with globo H and other truncated forms of globo H, but not with structurally related synthetic and natural compounds.[33] The affinity-purified IgG antibodies were mostly IgG_1 and IgG_4. The impact of the vaccine effect on PSA levels was also seen in one-third of patients. A possible clinical impact of these antibodies was suggested by declines in pretreatment versus post-treatment PSA log slopes over time. Five patients had stable PSA levels in the absence of any radiographic evidence of disease progression for longer than 2 years.[56,57] In some patients the antibody titres were present 2 years after the last vaccination.

Globo H was also studied in patients with breast cancer.[9] A trial compared the globo H-KLH vaccine prepared by direct reductive amination or by using a bifunctional cross-linker 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH). Groups of nine patients received 10 μg of globo H in a globo H-KLH conjugate prepared by direct conjugation or cross-linked through MMCCH and the vaccine was stored lyophilized or not, and mixed with 100 μg QS-21 prior

to injection. The antibody response in all groups was similar to that described above in the prostate cancer patients.

Lewis^y

Lewis^y (Le^y, Fucα1→2Galβ1→4[Fucα1-3]GlcNAcβ1→ 3Galβ1→ *O*-cer) is a blood group epitope overexpressed by many cancers of epithelial origin. Mice immunized with synthetic Le^y pentasaccharide allyl glycoside[58,59] coupled to KLH or BSA by reductive amination or with the bifunctional cross-linker[34] showed that the Le^y-KLH conjugate prepared by reductive amination was the most effective in mice for eliciting both IgM and IgG antibody responses. These antibodies reacted with synthetic as well as with the natural forms of Le^y epitopes carried on mucins and glycolipids. The induced antibodies were also reactive with and cytotoxic to Le^y-expressing cancer cell-lines.[34]

A phase I trial of varying doses of Le^y-KLH conjugate plus 100 μg QS-21 carried out in patients with ovarian cancer. In that trial, the majority of patients produced anti-Le^y antibodies, with antitumour cell reactivity assessed by flow cytometry and complement-dependent cytotoxicity. One patient's serum reacted with glycolipids but not with glycoproteins or mucins expressed by ovarian cancer cell line OVCAR-3. It was well-tolerated without any gastrointestinal, haematological, renal, or hepatic toxicity related to the vaccine.[59]

TF(c)-KLH, Tn(c)-KLH and STn(c)-KLH conjugate vaccines

Thomsen-Friedenreich (Galβ1→3GalNAcα-O-Ser/Thr), Tn (GalNAcα-O-Ser/Thr) and STn (NeuAcα2→6GalNAcα-O-Ser/Thr) are simple carbohydrate antigens expressed by mucins on most epithelial cancers.[60–65] The immunogenicity of TF and STn antigens has been studied in a series of small clinical trials in colon, breast and ovarian cancer patients by immunization with TF-KLH and STn-KLH conjugate vaccines plus different adjuvants.[61,66] Patients with rising PSA levels following surgery or radiation therapy were treated on a dose-escalating phase I trial with TF(c)[67] conjugated to KLH and given with QS21. Following six vaccines given over 24 weeks, the median IgM antibody titre by week 7 was 1/1280 at 10 μg, 1/320 at 30 μg, 1/1280 at 3 μg and 1/1280 at 1 μg dose groups. The IgM titres from all groups remained greater than 1/320 by week 32 and beyond, through to week 50. There also appeared to be a small correlation between antibody titre and time to radiographic progression of disease in bone with patients with the highest treatment dose. As had been seen in prior trials, dose escalation did not correlate with greater antibody response. Similar observations were seen using a Tn(c) plus KLH and QS-21 vaccine in a similar cohort of patients.[68]

STn(c)

STn (NeuAcα2→6GalNAcα-*O*-Ser/Thr) is a simple disaccharide antigen associated with the MUC1 mucin core peptide that is present on a number of cancers. Apart from the ganglioside GM2, STn is an intensively studied antigen as a cancer vaccine. The percentage of immunoreactive carcinoma positive for STn has been reported to vary from 25 to 75% in breast cancer and from 31 to 100% in ovarian cancer.[60–64]

A variety of STn-KLH conjugate vaccines mixed with immunological adjuvants have been tested in clinical trials.[69,70] Immunization of breast cancer patients with STn-KLH plus Detox in combination with low-dose cyclophosphamide induced high titre IgM and IgG antibodies (median titre 1/1024) against synthetic STn in most cases. IgM and IgG reactivity against ovine submaxillary mucin (OSM) was lower (1/64) and titres were 2–4 fold lower in patients not pretreated with low dose cyclophosphamide. In separate trials, patients with colon cancer were immunized with STn-KLH plus Detox or QS-21 (without cyclophosphamide) in the adjuvant setting. Median IgM and IgG antibody titres against synthetic STn were 2- and 8-fold higher, respectively (1/5120 and 1/2560) with QS-21 than with Detox but IgM and IgG titres against OSM were only 1/80 and 1/40. Although STn-KLH plus QS-21 vaccines clearly augmented IgM antibodies and induced IgG antibodies against STn expressed on tumour cells or natural mucins, they induced far higher titres of antibodies against synthetic STn.[61,70–72] The definitive phase III trial comparing the outcomes of patients with metastatic breast cancer receiving vaccinations with Theratope vaccine (Biomira, Edmonton, Alberta, Canada) versus vaccination with the non-specific immune stimulants such as KLH and Detox-B (RIBI ImmunoChem Research Institute, Hamilton, MT, USA) stable emulsion (now called Enhanzyn Immunostimulant; Corixa, Seattle, WA, USA) was closed to enrolment on 30 March 2001. More than 1000 women with distant metastatic breast cancer were enrolled into the program. The trial completed accrual in 2003 and an interim analysis from the trial has confirmed the safety of the STn-KLH vaccine. However, the final data possibly demonstrating a survival benefit have not yet been presented.

Attempts to improve immunogenicity: clustering (c)

The use of monovalent antigens in clinical trials led us to explore the use of alternative configurations of these antigens for vaccine construction. Although Tn, STn and TF are also expressed on normal epithelial cell mucins, they are far more abundant on cancer mucins that are glycosylated with shorter, simple carbohydrates. Consequently, although normal and cancer mucins contain series of 2–5 serines or threonines that are glycosylated, only cancer mucins are likely to express 2–4 Tn, STn, or TF molecules in a row. We have termed these grouping, clusters (c). The number of these clusters conjugated to each KLH is generally 350–450. Our study with MoAb B72.3 and mouse antibodies induced against STn-KLH and STn-cluster-KLH conjugate vaccines demonstrated that these cluster antigens were more relevant targets than the single epitopes.[61,73]

More recently, breast cancer patients were immunized with an STn(c)-KLH vaccine plus QS-21.[72–74] Median IgM titres against synthetic STn and against OSM were the same (1/2560–1/1520), and IgG titres were 1/1280 and 1/640, respectively.[74] Consistent reactivity with STn expressed at the cell surface of cancer cells was demonstrated by flow cytometry with these sera. These results confirmed that STn(c) more closely resembled STn as it was expressed at the tumour cell surface than individual STn epitopes. A similar increase in relevant immunogenicity has been described using single STn epitopes when they were packed more tightly onto the KLH surface, that is, when the STn/KLH ratio exceeded 2000/1.

Based on these studies, prostate cancer patients have recently been vaccinated with Tn(c)-KLH plus QS-21 and TF(c)-KLH plus QS-21 vaccines. IgM and IgG antibody titres against OSM and desialylated OSM were 10–100-fold higher than seen with the previous vaccines. Antibody reactivity was demonstrated by flow cytometry against tumour cells expressing TF and Tn.

A large multicentre randomized phase III trial with STn-KLH plus Detox prepared by Biomira (Edmonton, Alberta, Canada) was initiated in late 1999 in North America and Europe. The STn-KLH plus Detox vaccine (Theratope)[75] is being compared to no treatment in patients with metastatic breast cancer who have had a complete or partial response to combination chemotherapy. This trial is based on the consistent immunogenicity of this vaccine, the correlation of the vaccine-induced antibody response to more favourable clinical outcomes, and in preclinical models the ability of this vaccine to protect against tumour challenge.[76]

Multivalency: can immunogenicity be enhanced?

Monovalency does not take into account the heterogeneity associated with most cancers. As illustrated in Table 1, multiple antigens are found in high percentages in various cancers. By combining multiple antigens in a single molecule, thereby forming a unimolecular multivalent vaccine, there is the possibility that the immune system will generate a multifaceted immune response in which antibodies to each antigen are produced. This type of response has a higher likelihood of targeting a greater percentage of cancerous cells than the traditional single-antigen containing vaccines. It is also believed that these unimolecular multivalent vaccines may be a more accurate representation or mimic of the highly glycosylated tumour cell surface. One can envision the surfaces of transformed cells to contain regions of glycosylation that are not limited to a single antigen, but to a variety of antigens. The vaccine shown in Fig. 2, which contains three different antigens (globo-H, Lewis^y and Tn), has been evaluated in murine hosts and it has been demonstrated that antibodies were generated with specificity for each of the antigens of interest.[19] The best method to develop vaccines of this type is to incorporate the carbohydrate antigens by appending them to amino acid side chains. The individual glycosylamino acids can then be put together in a modular fashion via standard and robust peptide coupling reactions. Unique features of these unimolecular vaccines are the non-natural amino acid side chains linking the peptide backbone to the antigenic carbohydrate domains as well as the fact that large antigens such as Lewis^y and globo-H can be successfully incorporated into the glycopeptide motif. Additionally, unimolecular multivalent vaccines have been developed that contain as many as five different tumour-associated carbohydrate antigens displayed on a peptide backbone and conjugated via a linker to two different carriers.[77] The antigens incorporated into the multivalent vaccine (globo-H, Lewis^y, STn, Tn and TF) are all commonly found in prostate cancer.

A bivalent[78] vaccine with globo H-KLH and glycosylated MUC-2-KLH was studied in prostate cancer patients with rising PSA levels using two batches of a semisynthetic preparation of QS-21, GPI-0100. GPI-0100 was tested as two preparations, one of which was intensively purified with doses ranging between 100 mg and 5000 mg in groups of five patients. All doses were well tolerated and antibody titres against globo H and MUC-2 escalated with the increasing dose levels. At the 5000 mg dose level in this patient population, toxicity remained minimal, with only occasional grade II local toxicity at vaccination sites and occasional sporadic grade I elevations in liver enzymes. Compared with a subsequent trial with the same bivalent vaccine plus QS-21 at the maximal tolerated dose of 100 μg, the 5000 μg dose of GPI-0100 produced comparable antibody titres with diminished local toxicity at vaccination sites.[78]

A six-antigen multivalent trial has been studied in a high-risk cohort of prostate patients who were free of disease but whose biomarker, PSA, was doubling in less than 6 months.[79] Prognostically, these patients are at greater risk for metastases compared with patients whose doubling times are greater than 6 months. The vaccine that contained glycosylated MUC-1-32mer, GM-2, globo H, Tn(c), TF(c) and Le^y was well-tolerated but the antibody titres against each antigen were lower in the multivalent vaccine compared with the titre of each, respective, antibody generated in a monovalent trial. Whether this has to do with immunosuppression of antibody induction by the presence of a larger molecule such as MUC-1 remains under investigation, but clearly this approach warrants further investigation.

Future directions

Cancer carbohydrate antigens such as gangliosides (GM2, GD2, GD3, 9-*O*-acetyl GD3 and fucosyl GM1), neutral glycolipids (Le^y and globo H), and mucin related epitopes (TF, Tn, and STn), are suitable targets for both active and passive immunotherapies because they are overexpressed at the cell surface of malignant cells and poorly expressed or not accessible on most normal cells. Although there is every indication that immunization with single antigen vaccines may prove beneficial when administered in the adjuvant setting, multivalent vaccines may offer greater promise. As described earlier in this review, we have now induced consistent antibodies against GM2, fucosyl GM1, Tn(c), TF(c) and STn(c). Conjugate vaccines against GD3, GD2, globo H, Le^y and TF have induced antibody responses in 60% or more of patients. These antibodies have been demonstrated to react strongly with the cell surface of antigen-positive cancer cells and in most cases can mediate complement lysis. Based on the distribution of these antigens on various cancers and their demonstrated immunogenicity in KLH conjugate plus QS-21 vaccines, multivalent vaccines against the cell surface carbohydrate antigens of a variety of malignancies are being planned. These malignancies and the antigens considered for inclusion in the multivalent vaccines are listed in Table 1. Phase III multivalent vaccine trials against prostate, breast, melanoma, sarcoma and ovarian cancer may offer an answer to this question and are currently being reported.

Despite these encouraging results, there is still a need to determine the patient populations that will benefit from vaccines, that is, patients with minimal tumour burden versus patients who have been heavily pretreated with significant radiographic disease, and to assess how to analyse the impact of vaccines in these patients. Endpoints vary with the clinical trial design and it is often difficult to standardize how to

interpret whether there is an impact on disease. To date, endpoints using serum biomarkers, for example, PSA used to monitor prostate cancer patients, have not been well-accepted by the Food and Drug Administration of the USA, largely due to the fact that more definitive modalities such as bone or computed axial tomography (CAT) scans are thought to more accurately and objectively reflect the improvement or progression of existing radiographic disease. Biological trials that are evaluated by immunological parameters such as T cell responses as measured by cytokine release following T cell stimulation with antigen, or by antibody induction, respectively, are mainly *in vitro* assays and may not reliably reflect *in vivo* responses in a patient with a rising biomarker but without demonstrable radiographic disease. Therefore, until researchers can develop set guidelines by which we can report the results of biological trials in a uniform manner, there will still be concerns about whether vaccines truly have an impact on cancer growth and metastasis.

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