Carbohydrate Antigens: Synthesis Aspects and Immunological Applications in Cancer

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Abstract: Tumor Associated Carbohydrate Antigens (TACAs) constitute powerful tools as tumor markers and as targets for anticancer immunotherapy. In this review, methods of production of glycopeptide-based vaccines, as well as results of preclinical and clinical studies in cancer patients are discussed.

Key words: Glycosylation, cancer, glycosyltransferase, synthesis, vaccine, immunotherapy, carbohydrate.

I. INTRODUCTION

Malignant transformation is associated with phenotypic changes that influence the cell behavior. Indeed, transformed cells show remarkable differences in some biological processes such as receptor activation, cell adhesion and cell motility. These properties allow tumor cells to proliferate, invade and metastasize throughout the organism [1]. The mechanism of each event is functionally maintained by a combination of defined molecules involved in the transformation and metastasic processes. These molecules, expressed on the surface of cancer cells or shed by these cells, can be detected by the immune system, and they are thus usually called tumor-associated antigens (TAA).

Many of these TAA are constituted by carbohydrates, and are the consequence of deregulations in the glycosylation pathways of the cancer cell. Several studies have established the functional significance of aberrant glycosylation in tumor progression and metastasis. Indeed, glycans are fundamental for the biology of the cell, and in cancer, they participate in tumor cell adhesion, migration, motility, invasiveness, angiogenesis, metastasis and proliferation [2].

II. TUMOR-ASSOCIATED CARBOHYDRATE-RELATED ANTIGENS

1- Expression of Tumor-Associated Carbohydrate Antigens by Cancer Cells

Aberrant glycosylation has been reported in many types of cancers, leading to the accumulation of new glycan structures such as the sialyl-Le^a (sLe^a), sLe^x, Le^y, globo H, Tn, TF, sTn, GM2, GD2, GD3 and fucosyl-GM1 antigens [3, 4]. The structure of these various antigens is detailed in Fig. (**1**). These molecules, usually called tumor-associated carbohydrate antigens (TACA), are expressed on glycolipids or glycoproteins which both contain *N*- and/or *O*-linked glycans. Glycolipids consist of oligosaccharides chains linked to ceramide, forming the gangliosides, the globo- and lacto-series. On the other hand, *N*-glycans are Asn-linked oligosacharides attached to glycoproteins, while *O*-glycans are Ser- or Thr-linked oligosaccharides that predominate on mucin-type glycoproteins (Fig. (**1**)).

In the last two decades, the expression of these antigens by tumor cells has been intensively studied by immunohistochemistry using specific monoclonal antibodies (mAbs) [5] (Table **1**). The Tn, TF and the sTn antigens are broadly expressed in several tumor types including bladder, colorectal, gastrointestinal, prostate, ovarian, breast, pancreas and lung carcinomas [6-10] whereas they are not found in normal tissues [11, 12].

Aberrant expression of blood group antigens (ABO) has also been detected in several cancer tissues [13]. Indeed, an over-expression of the H (O) structure is observed in gastrointestinal, lung, cervical, oral epithelial, urothelial and colon cancers [14, 15]. Likewise, an abnormal expression of sLe^a, sLe^x and Le^y is found in gastric, lung, colon, stomach, pancreas, ovarian and breast cancers [16-19]. Finally, the gangliosides GD2, GD3, GM2 and GM3 are over-expressed in human melanoma and neuroblastoma [15, 20, 21]. The glycolipids fucosyl-GM1 and globo H have been identified in small cell lung cancer cells [22-24] and ovarian, colon and prostate cancers [14, 25], respectively.

2- Mechanisms Involved in TACA Expression

Over the last years, special attention has been paid to the mechanisms which lead to the expression of TACAs. However, due to the variability and heterogeneity of cancer cell models, these mechanisms are still poorly understood (Table **1**). Indeed, although some of these mechanisms have been elucidated, it is likely that the expression of TACAs is not due to a single event, but rather to a complex combination of deregulated processes.

Most efforts have been directed to investigate why small truncated *O*-glycans, like Tn, TF or sTn, are expressed in cancer. The expression of such determinants can be the result of a deregulation of glycosyltransferases (*e.g.* changes in enzyme activity and/or in substrate specificity) or a shift of

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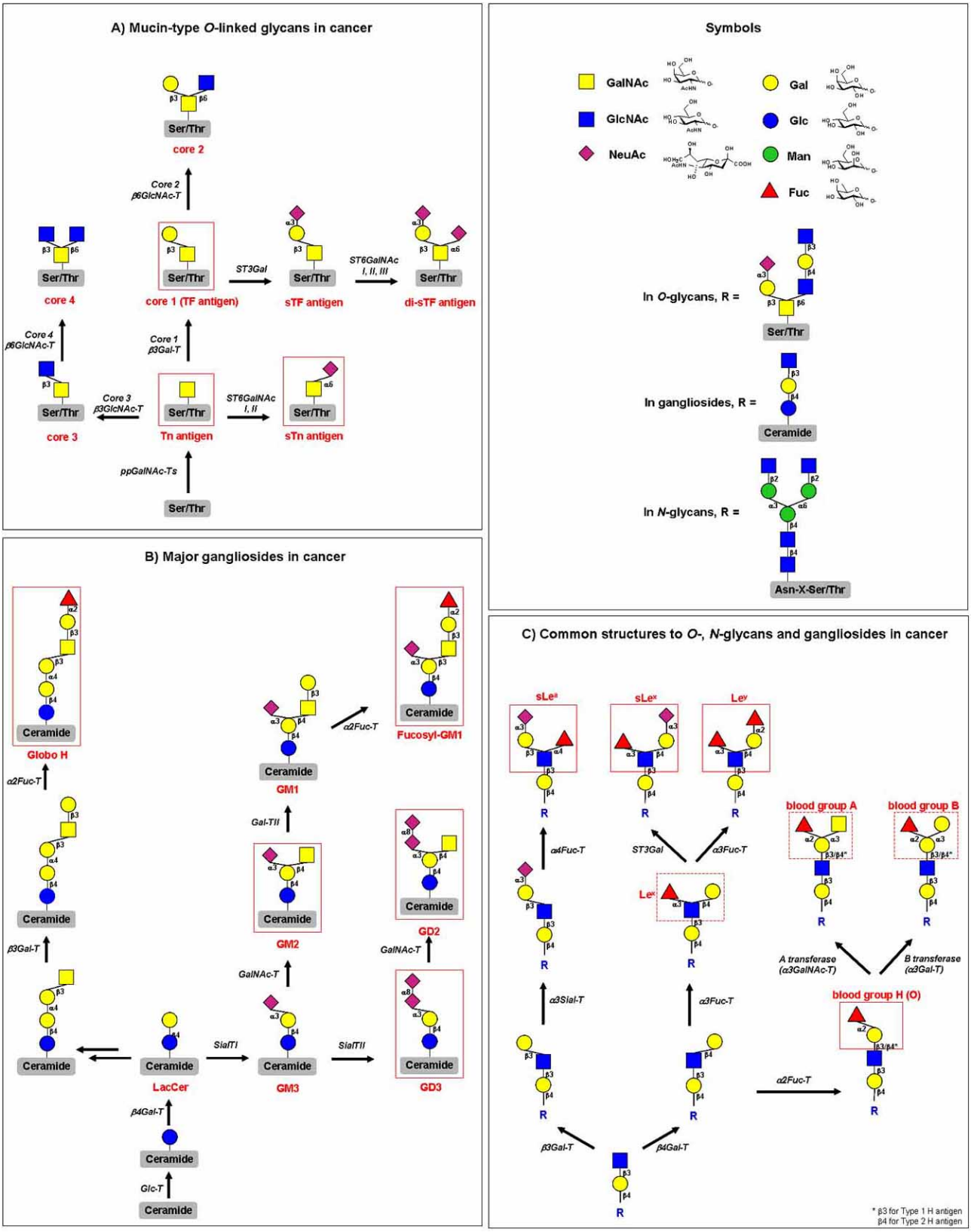

Fig. (1). Structures and biosynthetic pathways of TACAs.

A) Biosynthesis of mucin-type *O*-linked glycans. *O*-glycosylation begins with the addition of GalNAc to a Ser or Thr in a protein. Then, activated sugars are individually added to this structure to generate different cores, which are subsequently glycosylated generating more

(Fig. 1. Contd….)

complex structures. In the recent past years, other cores have been identified (core 5 to 8), whose precursor structure is the Tn antigen. Aberrant glycosylation in cancer leads to the accumulation of Tn, sTn, TF, sTF and di-sTF antigens, which are in red boxes.

B) Biosynthesis of major gangliosides in cancer. Ceramide is glycosylated by the transfer of individual activated sugars by a Glc-T and then the subsequent enzymes catalyze the transfer or each activated sugar to form other structures. The ganglioside structures associated to cancer, such as GM2, GD2, GD3, fucosyl-GM1 and globo-H, are in red boxes.

C) Complex structures associated to cancer (sLe^a, sLe^x, Le^y, blood group H) may be present either in *O*-, *N*-glycans, and/or glycolipids, and are also marked in red boxes (continuous red line).

Glycosyltransferases, which are indicated next to the arrows, are named following the usual nomenclature: XGlyc-T, where "X" corresponds to the resulting carbohydrate linkage, "Glyc" is the type of the transferred carbohydrate, and "T" means transferase.

Table 1. Expression and Biological Effect of TACAs in Cancer

TACA	Expression in cancer	Mechanism of expression	Prognostic and clinical outcome correlations
Tn **TF** **sTn**	Bladder Gastric Colon Prostate Ovary Lung Breast Prostate Pancreas	**Tn** - Absence of core 1 β3Gal-T activity in human colon, LSC and human lymphoblastoid T Jurkat cell lines - Aberrant expression of polypeptide acceptor substrates: MCF7 breast cancer cells **TF** - Decrease of core 3 β3GlcNAc-T activity in colon cancer **sTn** - Absence of core 1 β3Gal-T activity in colon cancer - Increase of ST6GalNAc activity/core 2 β6GlcNAc-T in rat colon cancer LMCR cell line	**Tn** - Decreased survival in colon patients - Indicator of high malignancy of hepatic cancer **sTn** - Unfavorable prognosis in colon and gastric cancer patients - Correlates with the degree of malignancy and metastasis
H blood group	Gastric Lung Cervical Oral epithelial Urothelial Colon	- Reduced activity of A/B glycosyltransferases	- Associated with the grade of metastasis and malignancy - Decreased patient survival
sLe^a **sLe^x** **Le^y**	Lung Colon Stomach Pancreas Breast Gastric Ovarian	**sLe^a** - Increase of α3Sial-T activity in colon human tissues - Increase of α4Fuc-T activity in gastric human tissues **sLe^x** - Incomplete synthesis of sialyl-6-sulfo-Le^x	- Associated with progression of the disease and poor prognosis
GD2 **GD3** **GM2**	Melanoma Neuroblastoma	**GD2** - Up-regulation of GD2 synthase gene in lung cancer cells **GD3** - Increase of α8Sial-T expression in melanoma cell lines	
Globo H	Ovary Colon Prostate		
Fucosyl GM1	Lung (small cell)		

acceptor polypeptides, such as aberrant expression of apomucin genes. For instance, the presence of shorter *O*-glycans in breast cancer cells has been associated with an increase of the enzymatic activity of ST3Gal I (α3sial-T, responsible for transferring sialic acid in α2,3 linkage to the core 1 substrate) [26, 27], and/or with an absence or decrease of core 2 β6GlcNAc-T activity (processing core 1 to core 2) [27] (Fig. (**1A**) and Table **1**). Moreover, an absence of core 1 β3Gal-T activity was found in the human colon cancer LSC cell line, which favors the expression of the Tn and sTn antigens [28]. On the other hand, in LMCR rat colon cancer cells, which have high core 1 β3Gal-T activity levels, sTn expression is controlled by the ratio of ST6GalNAc activity, synthesizing sTn, and core 2 β6GlcNAc-T activity, further processing core 1 to core 2 [29]. Similarly, the increased expression of the TF antigen in colon cancer may be due to a decreased activity of core 3 β3GlcNAc-T [30]. In the human lymphoblastoid T Jurkat cell, the over-expression of the Tn antigen is explained by the lack of core 1 β3Gal-T activity, due to the presence of a mutated chaperone named Cosmc which normally prevents this glycosyltransferase from being targeted to proteasomes [31] (Fig. (**1A**)).

The activity of some glycosyltransferases, such as ppGalNAc-Ts and the enzymes synthesizing *O*-glycan core 1, 2 and 3 have been shown to be differently influenced by the peptide moieties of substrates [32-34]. In another example, we have recently shown that MUC6, which is aberrantly expressed in breast cancer, carries the Tn antigen in MCF7 breast cancer cells. This could be explained by the fact that this mucin is not a good acceptor substrate to the core 1 β3Gal-T, preventing further extension of *O*-glycans and favoring Tn expression [35].

In more complex glycans, the expression of TACAs is mostly controlled by the expression and/or the activity levels of glycosyltransferases synthesizing the novel glycan structure. For instance, an up-regulation of the GD2 synthase gene (β4GalNAc-T) is correlated to a high expression of GD2 in lung cancer cells [36]. On the other hand, melanoma cell lines express very high levels of α8Sial-T, resulting in accumulation of GD3 [37] (Fig. (**1B**)). Likewise, a reduced activity of A transferase (α3GalNAc-T, processing the H (O) to A blood group antigens) leads to a decreased expression of the A antigen, and consequently, to an increase in H blood group antigen levels [38] (Fig. (**1C**)). The over-expression of sLe^a is also related to an increase of α3Sial-T and α4Fuc-T activities in colon and gastric cancer tissues, respectively [39, 40] (Fig. (**1C**)). Another hypothesis has recently been proposed, whereby these antigens are produced by incomplete synthesis of the sugar chains, mainly from sialyl-6-sulfo-Le^x, produced in normal cells [41, 42].

3- Biological and Clinical Effects of TACA Expression in Cancer

Most TACAs contribute to the adhesion and/or invasiveness of cancer cells, and consequently, they participate in the metastasis process. Thus, the expression of several TACAs has been correlated to an unfavorable clinical outcome of cancer patients (Table **1**). High levels of Tn, sTn, TF and $Le^{a/x}$ antigens are associated with poor prognosis and decreased survival of cancer patients [8, 9, 15, 41, 43] and also correlate with metastatic potential of cancer cells [44]. Nevertheless, the mechanisms underlying these phenomena are not completely understood. Their elucidation at cellular and molecular levels is crucial for the development of new efficient anti-cancer therapies.

Extensive data have been assembled about TACA-mediated metastasis. The TF determinant participates in cancer cell adhesion to the endothelium through the binding to galectin-3, a β-galactose-specific lectin [45, 46]. Blocking the interaction between galectin-3 and TF significantly inhibits rolling and stable heterotypic adhesion of MDA-MB-435 breast carcinoma cells to endothelial cells, as well as homotypic tumor cell aggregation [47]. Likewise, the sLe^a and sLe^x determinants serve as ligands for endothelial E-selectin [48, 49] contributing to the adhesion to the endothelium and thus, to the metastasis of cancer cells [44]. In addition, these antigens promote cell proliferation since the inhibition of the glycosyltransferases which synthesize them (α3Fuc-T for Le^x and α4Fuc-T for Le^a) leads to the inhibition of cell proliferation in human colon cancer cells Colo-205 [50] and HT29-LMM [51]. An increase in cell migration and adhesion of human breast cancer cell lines stably transfected with hST6GalNAcI (responsible for sTn synthesis) has also been reported [52, 53].

Gangliosides, and specially GD3, may also promote tumor cell motility and growth, possibly through angiogenesis [54]. Indeed, when GD3 expression was inhibited by stable transfection of the antisense vector against the α8Sial-T gene (also known as GD3-synthase), the growth rate of these tumor cells in nude mice was remarkably reduced [54]. These transfected tumor cells showed greatly reduced cell migration, invasion and metastasis [55]. Moreover, these cells formed small, minimally vascularized tumors, which were correlated with a decrease in vascular endothelial growth factor production [54], indicating an important role for GD3 in tumor angiogenesis.

The overexpression of the blood group H antigen is associated with the grade of metastasis or malignancy and, thus, with lower survival of patients. Moreover, rat colon carcinoma cells (REG) transfected with blood group H α2Fuc-T cDNA are more aggressive and more tumorigenic [56] and exhibited increased resistance to apoptosis [57].

4- TACAs as Tools for Diagnosis and Immunotherapy

Due to their restricted expression to cancer tissues, TACAs constitute powerful tools as tumor markers, for the clinical diagnosis and the follow-up of cancers [58]. Indeed, some epithelial carcinomas, neuroblastomas or melanomas which express high level of TACAs may shed them into the bloodstream [59]. As a result, many attempts to develop assays based on the level of these antigens in cancer patients sera have been carried out.

In most cases, epithelial tumor progression and metastasis were found to be associated with a high serum concentration of certain TACAs. For instance, sLe^x and sLe^a serum levels in gastrointestinal, pancreatic [58], prostate [60] and colorectal cancer patients [61], were shown to correlate with tumor burden and bad prognosis [62]. Tn and sTn levels may also have prognostic value for breast, ovarian, pancreas, gas-

tric and biliary tract cancer patients [38, 63-65]. In particular, high sTn serum levels could be of poor prognostic in colorectal [66] and in gastric cancer patients [67].

Carbohydrate antigens expressed at the cell surface of human cancer cells are also potent targets for passive immunotherapy with mAbs. Indeed, mAbs have shown great promises for the treatment of cancer and several are now available to the market [68]. Although none of them are directed to carbohydrate structures so far, some clinical and preclinical studies with mAbs against gangliosides are very encouraging for a further development in humans [69, 70].

TACAs also constitute very interesting targets for the development of anti-cancer vaccines. The main goal of this strategy is to induce antibodies that selectively eliminate tumor cells through lysis. However, in order to be efficient, these vaccines have to break the immunotolerance to TACAs which are usually self-antigens. Moreover, most TACAs are generally poor immunogens, and they require an appropriate immunogenic carrier to achieve an optimal response. Therefore, an effective cancer vaccine depends not only on the choice of the TACA (which has to be expressed only by cancer cells) but also on a favorable molecular context for eliciting a therapeutically efficient immunological response. The various strategies used for the development of such vaccines are discussed in detail in chapter IV.

III. PREPARATION OF TUMOR CARBOHYDRATE ANTIGENS

1- Introduction: Synthesis Challenges

The development of effective vaccines depends on the accessibility of novel glycoconjugates. Indeed, purification of TACAs from cancer cells leads to very heterogeneous products due to the microheterogeneity associated with their glycosylation. As a result, it is very difficult to isolate the expected compound, particularly when large amounts of material are required. On the other hand, obtaining glycoconjugates through synthesis has been more difficult than for other biomolecules, such as proteins and nucleic acids which can be prepared by automated-solid phase methods. The difficulties to automated carbohydrate synthesis are mainly due to the complexity of their structure: a monosaccharide unit contains several hydroxyl groups of similar chemical reactivity and the formation of the glycosidic linkage connecting two sugar units must be stereospecific.

Fortunately, during the last decade, progress has been made in the synthesis of complex carbohydrates through chemical and chemoenzymatic methods [71-75]. Generally speaking, there are three different approaches for glycoconjugate synthesis (Fig. (**2**)). One involves a sequential assembly on solid-phase with glycosylated aminoacids as building blocks (Fig. (**2B**)). The second one is a convergent synthesis using either chemoselective ligation or enzymes (Fig. (**2C**)). The third strategy is based on the further elongation of glycan chains, mostly by enzymatic methods (Fig. (**2A**)). All three strategies have been extensively exploited, either alone or combined in order to achieve the production of complex glycoconjugates.

Among the different glycoconjugates chemically synthesized for immunotherapeutic purposes, two categories must be distinguished. Hemi-synthetic vaccines are composed of synthetic TACAs conjugated to a protein (usually the keyhole limpet haemocyanin, KLH) through a linker arm. Fully synthetic vaccines also involve synthetic TACAs but associated to an appropriate synthetic peptide or lipid.

2- Chemical Methods

Regarding the synthesis of glycopeptides carrying short *O*-linked TACAs, the building block approach is the most commonly employed strategy (Fig. (**2B**)). Protected glycosyl-aminoacid buildings blocks are prepared and then incorporated into the peptide chain formation. One of the major challenges of this approach is to obtain the appropriate *O*-glycosyl-aminoacid with α-selectivity in the *O*-Ser/Thr linkage. To this end, different reaction conditions have been established, not only for the Tn antigen building block (α-*O*-GalNAc-Ser/Thr) [76, 77], but also for more complex *O*-glycans [77, 78].

In order to overcome the stereochemical difficulties, a new strategy, called the cassette approach, has been established. A GalNAc precursor α-*O*-linked to a serine or threonine residue with an identified acceptor site is joined to a target saccharide having a donor function at its reducing end. This strategy has been successfully used to prepare five building blocks containing core 1, core 2, core 3 or core 6 *O*-linked structures [79], as well as more complex structures [80-83].

Non-natural glycosylated aminoacids are of special interest in the design of new anti-cancer vaccines. Indeed, aminoacids carrying long aliphatic side chains create a distance between the α-carbon of the aminoacid and the carbohydrate antigen and thus facilitates glycopeptide synthesis. The synthesis of such glycosyl-aminoacids has been performed mainly *via* an ozonolysis-Wittig-asymmetric hydrogenation sequence [84-86], by olefin cross-metathesis [87] or by the direct glycosylation of hydroxy-norLeucine [88, 89]. This strategy has been used to prepare a multiantigenic cancer vaccine displaying globo H, Le^y, and Tn, which was shown to be immunogenic in mice [90].

Furthermore, the use of unnatural analogues should increase the half-life of the vaccine. For instance, *C*- or *S*-glycosidic bonds, in which the interglycosidic oxygen is replaced by a methylene or a thiol group respectively, are more stable toward acids, bases and enzymatic hydrolysis. On this basis, different analogues of Tn [91-94], sTn [95, 96] and gangliosides [97] have been prepared.

Despite all the progress in the chemical synthesis of oligosacharides, it remains a slow process because of the need for iterative coupling, deprotection and purification steps. To overcome these drawbacks, the Programmable One-Pot Approach strategy has been developed [98-100]. This new chemoselective strategy is based on the reactivity of different thioglycosides. Donors and donors-acceptors (i.e. thioglycosides with only one hydroxyl exposed) were evaluated to assess quantitatively their relative reactivities. These trends were tabulated to create a database, search engine and computer program (OptiMer one-pot synthesis program). Such information is then used to guide the reactivity-based one-pot synthesis of an oligosaccharide without protecting group

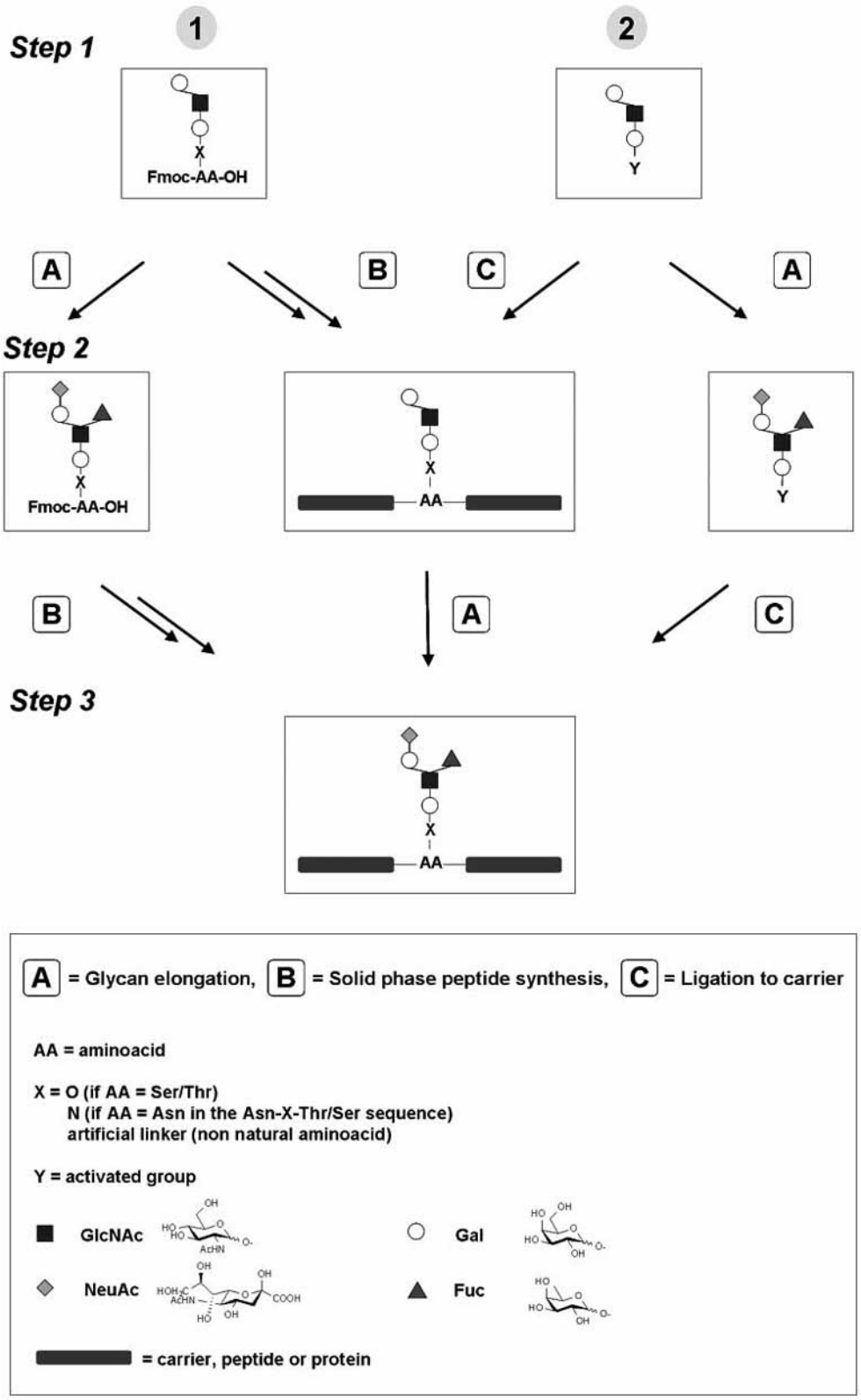

Fig. (2). Principal strategies for glycoconjugate synthesis.

Glycosylated aminoacids (structure 1) or functionalized glycans (structure 2) can be subjected to different synthesis strategies such as elongation of glycan chains by enzymatic methods (A), sequential assembly on solid-phase with glycosylated aminoacids as building blocks (B), or convergent synthesis using either chemoselective ligation or enzymes (C) in order to obtain complex glycoconjugates.

manipulation. By this strategy, globo H [101], Le^y [102] and fucosyl-GM1 [103] were successfully synthesized in one step with short reaction times and very good yields.

Other automated methods of synthesis have also been investigated [104-107]. The Seeberger's group developed the glycosyl phosphate building blocks [108] for the assembly of complex oligosaccharides in solution [109] or on solid phase [110]. This methodology was applied to the automated construction of the Le^x, obtained in 12 hours with a 12.6% yield [108].

In order to obtain efficient glycoconjugates for immunization purposes, TACAs have to be covalently linked to a peptide or a protein. Usually, the glycosylated building block is incorporated in the peptide scaffold by conventional solid-phase methodology using Fmoc-chemistry (Fig. (**2B**)). The *O*-glycosylated aminoacid can be introduced with fully protected carbohydrate [111] or with unprotected carbohydrate functions [112, 113]. Using this methodology, linear glycopeptides bearing hexa-Tn motif [114] and Multiple Antigenic Glycopeptides carrying Tn (MAG:Tn) [115] have been synthesized. The latter is a fully synthetic immunogen based on a dendrimeric lysine core with four arms that does not require a protein carrier (Fig. (**3**)). Multivalent *N*-glycopeptides containing 1 to 4 trisaccharide chains have also been efficiently synthesized by using the unprotected glycosylated Fmoc-Asn as a key building block [116].

Another example of synthetic vaccine is based on regioselectively addressable functionalized templates (RAFTs). Such cyclic templates carrying the Tn antigen, have been recently prepared using a chemoselective ligation *via* an oxime bond [117, 118].

As tumor cells bear heterogeneity in type and distribution of antigens expressed on their surface, vaccines displaying several different carbohydrate antigens on a single carrier protein, have been developed [119]. A multivalent construct was prepared by solution phase synthesis of the glycopeptide moiety [85] and linked to KLH by a sulfhydryl function [120]. Other strategies for the conjugation of carbohydrate moieties to a protein carrier are based on reductive amination (Le^y, Globo H, GM3) [121], or on new coupling reagents [122, 123].

3- *In Vitro* Enzymatic Methods

Although much progress has been made in the development of effective chemical methods, the multistep syntheses of carbohydrate-based conjugates are usually too cumbersome for large-scale production. A powerful alternative to the traditional organic chemistry is the utilization of enzymes which are involved in the glycan metabolism *in vivo,* that is, glycosidases or glycosyltransferases. Indeed, many of these enzymes are now easily available and their routine use in the synthesis of various oligosaccharides is becoming accepted.

Glycosidases have long been known to catalyze not only the hydrolysis of glycosidic bonds but also the stereopecific formation of glycosidic linkages. The capacity of glycosidases to use inexpensive donors, their broad specificity for the acceptor and their wide availability have made these enzymes very attractive tools, but their synthetic applications have been hampered by low yield reactions. However, recent improvements have provided very efficient and promising route to the preparation of *O*-glycosides. For instance, oversaturated βGal donor solutions [124] result in high yield preparation of a TF antigen derivative (60%). Another example is the transfer of GM3 from a water-soluble polymer to ceramide catalyzed by a ceramide glycanase [125]. Remarkably, Withers and co-workers achieved 90% yield transglycosylation by genetic engineering of retaining β-glycosidases. The mutant enzyme, called "glycosynthase", can catalyze the transfer of a sugar from an α-glycosyl fluoride substrate while being unable to hydrolyse the resulting product [126, 127].

In addition to glycosidases, glycosyltransferases have been extensively used to elongate the saccharidic chain. Although narrow acceptor specificity and requirement of expensive donor substrates have initially restricted their use for large scale preparations, they now benefit from the recent progresses in molecular cloning which make them more accessible. Illustrations of this strategy are the use of recombinant sialyltransferases to prepare GM3 [125, 128] or sTn and sTF antigens [129-131]. Very recently, we showed that a recombinant ppGalNAc-T1 allows the large-scale preparation of Tn-mucin glycoproteins [132]. Different groups have also combined series of glycosyltransferases in order to obtain complex oligosaccharidic structures [133-136].

Enzymatic strategies were also successfully applied to solid-phase syntheses which offer unique advantages such as easy purifications throughout the process and potential automation [134, 137-139].

Using an elegant approach, glycosyltransferases were used to remodel the RNAase B glycoprotein carbohydrate profile to give a homogenous sLe^x glycoconjugate [140]. High mannose branched structures were enzymatically removed from the natural protein, leaving only the innermost GlcNAc residues on the protein backbone. By using a series of specific glycosyltransferases, the core was then elongated by different monosaccharides, resulting in the synthesis of large glycoproteins with complex but defined carbohydrate chains.

The combined use of glycosidases and glycosyltransferases also constitutes a promising way for the large scale synthesis of complex glycoconjugates. The synthesis of sTF-glycopeptides was already achieved by regio- and stereoselective enzymatic glycosylation cascade employing both glycosidase and glycosyltransferases [141]. The high specificity of some of these enzymes is such that one-pot multienzyme strategies have been successfully used to prepare different sTF antigens [142, 143].

4- Bioengineering Strategies

Of main importance in the design of a vaccine candidate, is the preparation of glycoconjugates capable of mimicking the saccharidic antigens expressed by cancer cells. To this end, considerable efforts have been invested in the development of *in vivo* production methodologies, generally called bioengineering.

Over the recent past years, the production of cancer-associated *O*-glycoproteins by bioengineering has been extensively evaluated. The use of human tumor cell lines trans-

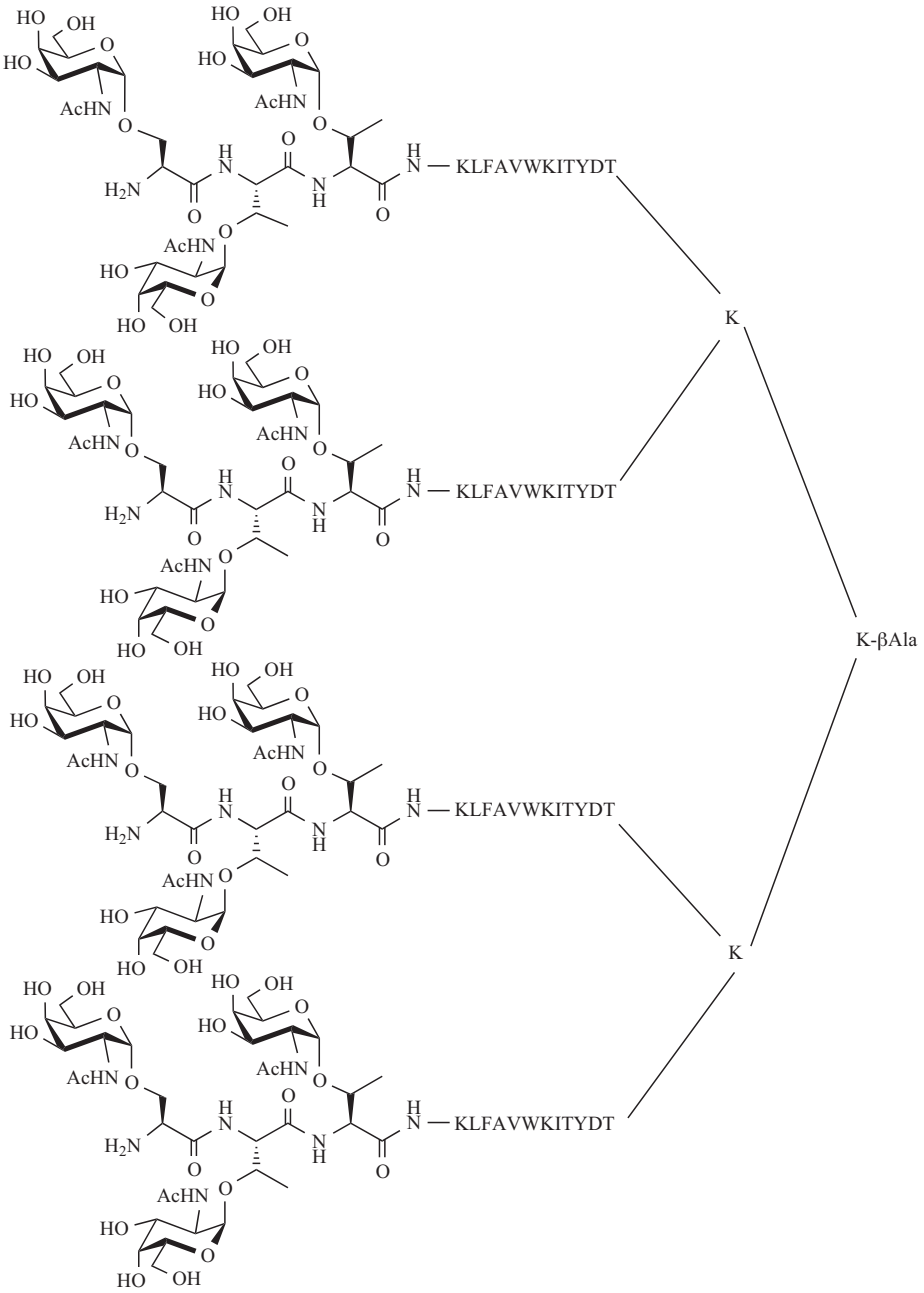

Fig. (3). Multiple antigenic glycopeptide (MAG).

The MAG is a dendrimeric glycopeptide based on a lysine core with four arms, each arm being linked to a peptide backbone (a $CD4^+$ T-cell epitope derived from the poliovirus) with a cluster of three Tn at the *N*-terminal end of the peptide.

fected by mucin-encoding plasmids has been shown to be a valid approach, since the *O*-glycan profile of these recombinant mucins is comparable to the one found in endogenous mucins [144]. This strategy allowed the production of recombinant MUC1 glycoproteins carrying core 1- and core 2-based structures [144-146], similar to that of endogenous MUC1 from breast cancer cells [144, 147]. Also, cell lines can be engineered to modify the *O*-glycosylation profile by under- or over-expressing specific glycosyltransferases. Following such strategies, the number of *O*-glycosylated sites on MUC1 produced by CHO-K1 cells was increased after co-transfection with wild-type ppGalNAc-T4 [146].

However, high amounts of recombinant glycoprotein(s) will be required for immunotherapy applications. To overcome this potential limit, a perfusion process for large-scale

production of a MUC1 glycoprotein has been optimized [148]. Using a Cellferm-pro system and MUC1-CHO K1 transfected cells in protein-free medium, a high production yield of glycosylated MUC1 was achieved (100 mg/day versus 3 mg/day of the initial batch process) [148].

In order to avoid recombinant enzyme production and purification as well as the use of expensive materials (e.g. CMP, activated sugars) which limit application to large-scale synthesis of complex carbohydrates, other strategies have been developed in the recent past years. Novel production systems involving the genetic manipulation of biosynthetic pathways in microorganisms are being exploited [100]. Recently, the large-scale *in vivo* synthesis of the carbohydrate moieties of gangliosides GM2, GM1 and GD3 has been reported [149, 150]. For GM2 oligosaccharide, a genetically modified *E. coli* strain which over-expresses the genes of *Neisseria meningitidis* for CMP-NeuAc synthase, α3Sial-T, UDP-GlcNAc-C4 epimerase, and β4GalNAc-T was used. When this strain was cultured with glycerol, lactose and sialic acid, a maximal yield of 1 g/L of the oligosaccharide was achieved [150]. Similar results were obtained for the carbohydrate moieties of GM1 [150] and GD3 [149] by using differently engineered *E. coli* strains.

Sialyllactose (NeuAc-α3Gal-β4Glc), the carbohydrate moiety of GM3, has also been produced in high yield either using living bacteria [151] or by bacterial coupling [152]. In the latter case, two recombinant *E. coli* strains that overexpressed the *N. gonorrohoeae* genes of CMP-NeuAc synthetase, CTP-synthetase and α3sial-T were used. When coupled with a *Corynebacterium ammoniagenes* strain, which converts orotic acid to UTP, 33 g/L of the oligosaccharide were obtained [152].

The preparative scale synthesis of the disaccharide sTn through the coupling of recombinant bacterial strains, has also been recently reported [153]. In this case, two recombinant *E. coli* strains overexpressing the genes of CMP-NeuAc synthetase and CTP synthetase were used, combined with a recombinant strain of *C. ammoniagenes* that contributed to the formation of UTP from orotic acid. When another recombinant *E. coli* strain overexpressing the α6Sial-T from *Photobacterium damsela* was added, the sTn carbohydrate structure was obtained at 45 g/L [153].

IV. CANCER VACCINES BASED ON CARBOHYDRATE ANTIGENS

In the last two decades, several cancer vaccine strategies based on TACAs have been developed, and some of these have been evaluated in clinical trials. The main aim is to elicit effective humoral and/or cellular immune responses against cell surface antigens capable of eliminating tumor cells. An effective cancer vaccine should be highly specific of tumor cells in order not to elicit autoimmune reactions, and capable of producing a prolonged activity without toxicity.

Except for melanoma patients, for whom naturally occurring antibodies to the ganglioside GM2 have been correlated with improved survival [154], the choice of antigens for the development of cancer vaccines has been problematic because natural antibodies are not generally detected. Moreover, the immune system can develop tolerance to tumor associated antigens, since some TACAs, albeit at very low levels, may be expressed by some normal tissues.

Various approaches for anticancer passive or active immunotherapy have been carried out. These include administration of anti-TAA mAbs, killed tumor cells, plasmidic DNA, dendritic cells or tumor associated antigens conjugated to a carrier [155, 156]. Regarding vaccines based on TACAs, different types of strategies have been evaluated in the last decade. The first pre-clinical assays were carried out with carbohydrate moieties, either from natural sources or chemically synthesized, conjugated to lipids, peptides or carrier proteins. The latter strategy is by far the predominant approach used at the moment. Fully synthetic vaccines are also of interest since they are well-chemically defined molecules. Pre-clinical and clinical studies of these various molecules have been carried out and are discussed in detail below.

1- Development of Anti-Cancer Immunity in Patients

TACAs have attracted much attention since cancer patients were shown to produce high levels of antibodies specific for some TACAs, whereas such antibodies were not detected in normal subjects. Furthermore, in some cases, the levels of anti-TACA antibodies present in the blood of cancer patients have been correlated with a longer survival and/or a better prognosis. For instance, levels of serum IgM antibodies recognizing the GM2 ganglioside were correlated with survival of stage III melanoma patients [154, 157]. These results suggest that these antibodies may suppress melanoma growth in patients, and strongly motivate the development of anti-melanoma vaccines based on such gangliosides [158]. Similarly, anti-Tn [159] and anti-TF [160] antibodies have been detected in cancer patients. Altogether, these results indicate that the immune system of cancer patients is capable of developing a specific immune response against these structures.

2- TACAs as Targets for Anti-Tumoral Immune Responses

The different components of the immune system are able to participate in the control and elimination of tumor cells. TACA-specific antibodies can mediate killing of tumor cells by complement-dependent cytotoxicity (CDC) as well as by antibody-dependent cellular cytotoxicity (ADCC) performed by NK cells or macrophages. These two mechanisms are strongly dependent on the antibody class and on the carbohydrate target type. Indeed, targeting gangliosides would preferentially allow killing through CDC, whereas this mechanism is poorly efficient for TACAs expressed on mucins (TF, Tn and sTn) [161, 162]. It has been suggested that GD3 ganglioside may constitute a direct target for NK cells [163]. However, GD3 presented by CD1 MHC molecules would rather represent a target for NKT cells that can modulate immune responses to tumors through cytokine release [164]. Although still poorly explored, the possibility of raising direct cellular cytotoxicity mediated by $CD8^+$ T cells specific for TACAs is an expanding and exciting new field of investigation for the development of TACA-based immunotherapy.

2.1- Recognition of TACAs by T Cells

The capacity of T cells to control tumors has been widely documented, and the importance of both anti-tumoral CD4 (cytokinic) and CD8 (cytotoxic) T cell responses has been evidenced in many models as well as in clinical settings. In this context, much focus has been put on the mean to elicit peptide-specific MHC-restricted T cell-responses. However, over the last twelve years, molecular evidences of the capacity of glycosylated peptides (such as chicken ovalbumin and hen-egg lysozyme peptides substituted with *N*-GlcNAc and galabiose, respectively) to bind MHC class II molecules and to elicit carbohydrate specific $CD4^+$ T cell responses have been provided [165, 166]. Likewise, an *O*-β-GlcNAc substituted peptide from the nucleoprotein of Sendai virus has been shown to bind MHC class I molecules and to induce GlcNAc-specific cytotoxic T lymphocytes (CTL) [167]. In addition, the resolution of the crystal structure of glycopeptides bound to MHC class I molecules [168, 169] has established the molecular basis of the glycopeptide/MHC interaction and recognition of the carbohydrate moiety by the T cell receptor. The characterization of naturally MHC bound-glycopeptidic forms of antigens has definitively validated the *in vivo* relevance of the MHC presentation of peptides post-translationally modified with carbohydrates [170, 171]. Moreover, the role of glycopeptide-specific T cell responses has been highlighted in pathological situations, such as type II collagen associated rheumatoid arthritis [172] or bee venom phospholipase A2 allergic responses [173].

However, the role of TACA-specific T cells in anti-tumor response is still poorly documented as most studies have been performed with carbohydrates linked to non-tumoral antigens. For instance, the introduction of the Tn antigen within an hemoglobin-derived peptide was shown to induce Tn-specific $CD4^+$ T cell responses [174, 175]. However, T cell responses can be specific for the sole carbohydrate moiety, as suggested by Abdel-Motal and coll., who demonstrated that a vesicular stomatitis virus-derived peptide substituted with galabiose was able to induce CTL that recognize target cells carrying the carbohydrate moiety as part of a glycolipid [176]. In this study, carbohydrate-specific CTL induced by MHC class I/glycopeptide were able to kill carbohydrate-bearing target cells independently of any MHC presentation. In a recent study, Xu *et al*. have also documented the induction of a carbohydrate-specific CTL response by the TF antigen linked to a Sendai virus-derived peptide. These cells were able to kill, although with poor efficiency, B16 melanoma cells transfected with MUC1, independently of the peptide backbone carrying the TF antigen, but in a MHC class I restriction manner [177]. A TF antigen specific-MHC class II restricted T hybridoma, recognizing a MUC1 peptide glycosylated in two independent sites, has also been described, indicating a certain degree of permissiveness regarding the carrier peptide sequence for the recognition of the carbohydrate moiety [178]. Independently of its role on T cell recognition, some TACAs are able to modulate the affinity of the peptide for the MHC, as shown by the increased MHC affinity of a MUC1-derived Tn glycopeptide due to the anchoring role of the carbohydrate moiety into the binding groove of the MHC class I molecule [179].

Whether or not T cell immune responses can be specific for the carbohydrate moiety independently of the peptidic backbone bound to the MHC, still needs to be clearly established. More generally, the relevancy of carbohydrate specific T cell responses in the context of tumor immunity needs to be further documented in murine tumor-experimental models. In this context, aberrantly glycosylated mucins could represent good candidates for immune response, although much care should be taken since mucins, such as MUC1 mucin, have been shown to display immuno-modulating properties on dendritic cells [180, 181].

2.2- Induction of TACA Specific Antibodies and Anti-Tumor Potency

Hemi-Synthetic Vaccines

Hemi-synthetic glycoconjugates have been intensively studied, mostly by the Livingston's and Longenecker's groups [3]. These studies have shown in particular that keyhole limpet haemocyanin (KLH) is the most effective immunogenic carrier for these carbohydrates, as compared with a variety of other proteins [182, 183]. Moreover, conjugating the carbohydrate with the protein carrier KLH is significantly more effective than simply mixing the carbohydrate and KLH [182, 184]. The co-administration of an effective adjuvant to the KLH glycoconjugate (the most used are QS21 and Detox) results in a potent helper T cell type 1 response [90, 182]. Vaccination of mice based on such protocols has proven to be effective in eliciting TACA-specific antibody response against Tn, sTn, TF, Le^y, fucosyl-GM1, GD3 and globo H antigens [183-188]. Furthermore, in some cases, the resulting antibodies recognize the native forms of TACAs produced by human cancer cells [183, 188] and prolong survival of vaccinated mice [187].

Mucins also constitute potential targets for immunotherapy [189, 190]. In particular, MUC1 is undergoing several clinical trials as anti-cancer vaccine [191, 192]. Surprisingly, most of the studies were focused on non-glycosylated backbones (protein or peptide), and very few studies have been published on glycosylated mucins [3, 4]. The immunogenicity of Tn presented on MUC1 or MUC2 mucin peptides conjugated to KLH and of Tn-KLH glycoconjugates have been compared. Interestingly, a 32 mer MUC1 peptide glycosylated at three sites per tandem repeat produced significantly higher anti-Tn antibody titers than even a Tn-KLH conjugate exposing clusters of three Tn (Tn(c)-KLH), and also produced higher antibody titers against the MUC1 peptide than the unglycosylated MUC1-KLH [184]. The importance of both the carbohydrate moiety and of mucin TAA as vaccine targets has led to detailed investigations of their respective role in the context of anti-tumor immunotherapy. Indeed, structural and immunological studies have shown that the carbohydrate structures on mucins may be essential for the definition of the tumor-associated structures [193, 194]. Altogether, these results strongly support the use of mucins with tumor-relevant glycoforms for anti-cancer strategies. On this basis, we have recently developed a glycosylated mucin which holds considerable promise for developing effective anti-cancer vaccines [132].

The use of molecules carrying more than one carbohydrate structure is of special interest, since different carbohy-

drate antigens could be expressed on cancer cells [25, 195]. Moreover, vaccination with multiple antigens may prevent tumor variants from escaping the immune response as a result of tumor antigen loss or variation. Finally, a vaccine that induces a broader immune response will certainly be more efficient in targeting a major set of tumor cells and is likely to induce more efficient immune responses. Immunization of mice with a mix of different TAAs conjugated to KLH (GD3-KLH, Le^y-KLH, MUC1-KLH and MUC2-KLH) plus QS21 was shown to induce antibodies titers against each antigen comparable to the ones obtained when administered each antigen alone in separate groups of mice [196]. Thus, immunogenicity of the individual antigens is not affected by mixing the four conjugates together. Also, Ragupathi and coworkers produced a vaccine construct bearing multiple tumor antigens (globo H, Le^y, and Tn conjugated to KLH) that was as efficient as the mixture of the individual antigens and it stimulated a polyspecific immune response [90].

Totally Synthetic Vaccines

Although very successful, the use of glycoconjugate hemi-synthetic vaccines has major limitations [197]. The main limit concerns the ambiguity of the protein carrier in both composition and structure, which is an obstacle for reproducible preparations. Moreover, protein carriers such as KLH are exogenous non-tumor proteins that represent strong immunogens. Together with its high molecular weight excess over the carbohydrate antigen, glycoconjugates induce a low level of the desired antibodies compared to the total amount of antibodies produced. Finally, KLH can express carbohydrate structures associated to cancer, for instance Tn and TF antigens [198], and may raise another set of antibodies. For these reasons, fully synthetic vaccines are of great interest in anti-cancer immunotherapy. Moreover, they constitute highly homogenous and pure chemically defined structures. Totally synthetic carbohydrate molecules have been tested in pre-clinical studies and constitute potential cancer vaccines to be evaluated in clinical trials.

Some of the synthetic vaccines tested in mice are based on tripalmitoyl-S-glyceryl-cysteinylserine (PAM), which is a potent immune activator acting through TLR2 [199]. PAM is derived from the immunologically active *N*-terminal sequence of an *E. coli* lipoprotein that has been widely used to augment the immunogenicity of peptides and carbohydrates [200]. When glycolipopeptides containing the Le^y-serine or the Tn-serine epitopes conjugated to the PAM moiety were administrated to mice together with QS-21, they induced both IgG and IgM specific antibodies [201, 202]. Interestingly, such compound containing a cluster of three contiguous Le^y serine epitopes was found to be superior to the mono Le^y serine construct in eliciting anti-tumor cell antibodies [201]. Mice immunized with Tn(c)-PAM, also developed anti-Tn IgM and IgG titers, although less efficiently than with Tn(c)-KLH [184]. However, these glycoconjugates have never been tested so far for their capacity to promote tumor rejection**.**

Other examples of fully synthetic vaccines are based on glycopeptidic structures. We have developed linear and dendrimeric glycopeptides in which the TACA is associated with a peptide containing a $CD4^+$ T-cell epitope to allow the B and T cell collaboration required to increase the level and the affinity of the antibody response. Such glycopeptides based on the Tn antigen associated with a murine T cell epitope (poliovirus peptide, PV) successfully induced in mice the production of IgG antibodies that recognized the Tn antigen on human tumor cells. Different linear glycopeptides based on clusters of three (Tn3) and six (Tn6) Tn have also been synthesized, and were shown to induce high levels of anti-Tn antibodies, but with a higher efficiency for the Tn6 cluster-based glycopeptide [114].

As the carbohydrate density on the immunogen was a critical parameter for the level and the fine specificity of the Tn-antibody response, we also designed dendrimeric glycopeptides (MAG, Fig. (**3**)). The MAG is based on a dendrimeric lysine core with four arms, each arm being linked to a peptide backbone containing a $CD4^+$ T-cell epitope with a TACA at the *N*-terminal end of the peptide [112]. Different MAGs with various Tn densities were synthesized and tested. A MAG based on a Tn3 cluster (MAG:Tn3-PV) was the best immunogen since the induced immune response in prophylactic and therapeutic vaccination protocols promoted the survival of mice bearing a murine tumor expressing Tn [115]. The use of Tn as a cluster did not alter the T cell dependency of the anti-TACA antibody response, since the response was abrogated in mice depleted of $CD4^+$, but not $CD8^+$ T cells [115]. Moreover, this MAG induced a higher anti-Tn immune response than the Tn3-KLH conjugate [203].

A second generation of MAGs was developed based on promiscuous HLA-restricted T-cell epitopes in order to be used in humans. Two epitopes were chosen, a tetanus toxin (TT) derived peptide [204] and a non-natural engineered T helper epitope, the Pan-HLA-DR-binding Epitope (PADRE) [205], both capable of binding a wide range of HLA class II molecules. Linear glycopeptides based on TT and PADRE associated with a Tn3-cluster showed a good immunogenic potential in HLA-DR1 and DR4 transgenic mice [206]. The immunogenicity of MAG:Tn3-TT and MAG:Tn3-PADRE was further evaluated in two monkey species (macaque and green monkey). When administered in an adjuvant setting based either on alum plus CpG oligonucleotide or with alum alone, these MAGs were able to induce strong anti-Tn IgG antibodies capable of specifically recognizing Tn-expressing human tumor cells [203]. Furthermore, in the presence of human NK cells, these antibodies mediated ADCC against tumor cells, demonstrating their potential for anti-tumor treatment.

In conclusion, the pre-clinical immunological evaluation of MAGs shows that it represents a safe and highly efficient immunogen to induce anti-TACA antibodies and it is a potent alternative strategy to the traditional carbohydrate-protein conjugates which are developed for vaccine and therapeutic purposes. The proof of concept for MAG in mice and non-human primates as an immunotherapeutic tool for epithelial cancers expressing the Tn antigen has opened the way to a clinical trial, that is currently under organization by the Pasteur Institute.

In direct line with the MAG strategy, we developed RAFTs as a new scaffold for the design of anti-cancer vac-

cines. RAFTs are topological templates consisting of a backbone-cyclized decapeptide of two proline-glycine motifs that displays two independent functional faces *via* lysinyl side chains [117]. One face makes possible the linkage of at least four glycotopic structures that can display diverse and clustered TACAs expressed on tumor cells [207]. The second face is dedicated to T-cell epitopes, enlisting the assistance of T cells for antibody production. Thus, RAFTs can be developed as a polyvalent carrier of different TACAs assembled on the same scaffold to mimic the heterogeneity of TACA expressed at the surface of tumor cells. RAFTs based on Tn antigen were shown to elicit anti-Tn antibodies in mice that are able to recognize Tn-positive human tumor cells [118].

3- Clinical Trials

Based on the antibody titers and cell-surface reactivity of the sera produced in mice immunized with some of these vaccine candidates, several clinical trials have been performed and are still in progress. Pioneering work by Springer and co-workers showed that immunization with desialylated red blood cells expressing the Tn and TF antigens increased survival of breast cancer patients and prevented recurrences [208]. The most significant and recent results of following clinical trials are summarized in Table **2**. In general, after vaccination with hemi-synthetic vaccines based on gangliosides, globo H, Le^y, sTn, TF or Tn antigens coupled to KLH, patients developed specific IgM and IgG antibodies against the tumoral carbohydrate. These antibodies were capable of recognizing tumor cells [209] and mediating ADCC [210-215] or tumor lysis by CDC [162, 185, 216-218]. However, in some cases, the produced antibodies did not recognize the natural epitopes expressed by tumor cells [215, 219], or the antibody titers did not correlate with a good clinical response [217, 220].

In order to increase the immunogenicity of KLH conjugates, various strategies have been evaluated. Several attempts were made to immunize patients with chemically modified gangliosides (GD3 lactones, GD3 amide, GD3 gangliosidol) but only IgM antibodies were induced by these immunogens [221]. Cluster-based KLH conjugates were also tested. Trimeric clusters consisting of Tn or TF saccharide molecules covalently attached to the chains of three serines or threonines, elicited high IgM antibody titers. However, the sera from these patients hardly recognized cancer cells [219, 222].

As in mice, a mix of different glycoconjugates coupled to KLH elicited antibodies capable of recognizing each of the antigens [3, 223], even though they did not generate complement lysis of tumor cells [224]. However, in one of these clinical trials, lower antibody titers were obtained with the mix as compared with antibody responses obtained in a monovalent trial [3, 223].

The only two carbohydrate-KLH glycoconjugates that reached phase III clinical trials so far seem to have failed to meet endpoints. GM2-KLH vaccine administrated with QS21 adjuvant (called GMK by Progenics Pharmaceuticals Inc.) was evaluated in melanoma patients, but failed to meet the disease-free survival endpoint [225]. The second vaccine (sTn-KLH, named Theratope® by Biomira Inc.) was tested in breast cancer patients. Despite the encouraging phase I and II trial results, in 2003 Biomira announced that the Phase III trial for women with metastatic breast cancer failed to meet the two pre-determined statistical endpoints of time to disease progression and overall survival, although a survival advantage was observed in a subset of patients (www.biomira.com).

Regarding the use of fully synthetic vaccines in human trials, only one report is found in the literature. Tn clusters attached to the PAM moiety were used to immunized prostate cancer patients, but only IgM antibodies were elicited [219].

CONCLUSIONS AND FUTURE PROSPECTS

Despite the success obtained with some vaccines in preclinical models, no cancer vaccine has produced so far any significant improvement in clinically relevant endpoints when evaluated in clinical trials. Moreover, in some cases, the results obtained in mice, were not reproduced in humans. These failures may be attributed to the heterogeneity of cancer cells in patients, while in animal models, cancer cell originate from a single clone that may over-express a particular antigen. Moreover, most of these phase I and II studies have been carried out in patients with late stage disease who have already experienced unsuccessful treatments, and presented a relatively large tumor burden.

In spite of the fact that the only two carbohydrate based vaccines (GM2-KLH plus QS21 and sTn-KLH plus Detox) that have reached phase III clinical trials failed to meet endpoints, other cell-based products are available in the market. One of them, called Melacine (Corixa Corp.) is an allogeneic melanoma tumor cell lysate combined with the adjuvant Detox that has been already approved in Canada for melanoma treatment [226]. In addition, OncoVAX (Intracel), which consists of autologous tumor cells with BCG as adjuvant, has been approved in the Netherlands for colorectal cancer [227].

To make cancer vaccines an effective therapeutic tool, there is a need of combining cancer vaccines with other interventions, such as effective adjuvants and traditional treatments. Indeed, they constitute an ideal application to prevent recurrences after the first line of treatment (surgery, radiotherapy, chemotherapy). Various adjuvants have been tested and proven to be effective not only in animal models but also in humans. Indeed, the majority of clinical trials were performed using Detox and QS21 as adjuvants. However, only two adjuvants are approved for clinical use: aluminum-based salts (alum) and a squalene-oil-water emulsion (MF59) [228]. Other examples of adjuvants constitute the use of cytokines, bacterial products (LPS from gram negative bacteria, monophosphoryl lipid A, MPL from Salmonella) or unmethylated CpG oligonucleotides. Interestingly, CpG markedly enhanced the immunogenicity of two vaccines against hepatitis B virus [229] and melanoma [230]. Nevertheless, no CpG has been used in vaccination with TACAs in human clinical trials so far.

Improving efficacy of hemi- or fully-synthetic TACA conjugate vaccines will certainly also require breaking peripheral tolerance due to regulatory T cells in cancer patients. In this context, the combination of the PAM and the MAG

Table 2. Clinical Trials Carried Out Using TACA-Conjugates

Vaccine	Type of Cancer (Stage)	Immune response elicited/Therapeutic effect	Reference
Tn-KLH	Prostate	IgM and IgG which slightly recognize LSC cells (5 of 15 patients) Decrease of PSA progression	[219]
Tn(c)-PAM	Prostate	Weak IgM antibodies which do not recognize LSC cells	[219]
TF-KLH	Ovary (metastatic)	IgM and IgG which recognize human tumor cells, CDC	[235]
TF-KLH	Prostate	IgM (mostly) which do not recognize human tumor cells Decrease of PSA progression	[236]
sTn-KLH	Colorectum metastatic (Dukes' B, C or D)	IgM + IgG which do not recognize native TACA	[215] www.biomira.com
sTn-KLH	Breast, colorectum (D)	Correlation between Ab level and improved survival	[213]
sTn-KLH	Ovarian	IgM + IgG. Increased pre-ASI CA-125 serum levels in ovarian cancer patients were predictors of poor survival	[213]
sTn-KLH	Breast (metastatic)	Improved survival only when chemotherapy + hormonotherapy Failed to meet TDP and OS endpoints	www.biomira.com
Le^y-KLH	Ovarian (I-IV)	Mostly IgM which recognize human tumor cells, CDC	[216]
GD3-KLH GD3-Lactone-KLH	Melanoma (III, IV)	IgM + IgG	[237, 238]
GD3-Lactone-KLH + BEC2 anti-idiotypic mAb	Melanoma (III, IV)	IgM + IgG Ab response does not correlate with clinical outcome	[220]
GM2-KLH	Melanoma (III)	Not better than interferon-α Failed to meet DFS endpoints	[225, 239]
GD2-KLH GD2-Lactone-KLH	Melanoma (III, IV)	IgM + IgG which recognize native TACA, CDC	[240]
Fuc-GM1-KLH	Lung (small cell)	IgM + IgG, which recognize native TACA, CDC	[209, 241]
GloboH-KLH	Prostate	IgM (mostly) which recognize native structures, CDC Decrease of PSA progression	[222]
GloboH-KLH	Breast	IgM (mostly) which recognize native structures, CDC	[218]
GM2-KLH + GD2-KLH	Melanoma (III, IV)	IgM + IgG	[223]
(MUC2-Tn)-KLH + GloboH-KLH	Prostate	IgM + IgG, CDC	[224]
(MUC1-Tn)-KLH + GM2-KLH + Globo H-KLH + Tn(c)-KLH + TF(c)-KLH + Le^y-KLH conjugates	Prostate	Ab titers lower than when immunizing with monovalent vaccine	[3]

CDC: Complement-Dependent Cytotoxicity, TDP: Time to Disease Progression, OS: Overall Survival, PSA: Prostate Specific Antigen, DFS: Disease Free Survival, Ab: Antibody.

strategies could be fruitful to stimulate the innate and the adaptive immune system. Indeed, the characterization of an increasing number of pathogen-associated molecular patterns (PAMPs) and of their receptors, such as TLR, as well as the molecular pathways subsequently activated in mammal cells, has opened up new perspectives for designing adjuvants for vaccines. TLR ligands, such as PAM, U/C ORN and CpG ODN targeting TLR2, 7 and 9, respectively, are small molecules that are easy to produce by chemical synthesis methods. From the immunological point of view, TLR triggering can positively act on the adaptive immune response by appropriately activating antigen-presenting cells [231]. In addition, this activation pathway has recently been shown to control regulatory T cells activity, either through IL-6 release rendering effector T cell refractory to suppression [232], or by directly acting on regulatory T cells functions [233]. Therefore, the aim would be to develop a vaccine scaffold with immunological effects on the innate and adaptive systems capable of stimulating TACA-specific immunity with a single synthetic molecule. The linkage of TLR ligands to the TACA vaccine would trigger more efficiently TACA-specific immune responses, which is essential for immunotherapeutic purposes.

Although TACAs have been exclusively tested in therapy of advanced cancer, they constitute, as highly purified ho-

mogenous antigens, ideal candidates for prophylactic vaccination of individuals who are at high risk of developing cancer. Indeed, a proportion of human tumors are of hereditary origin [234]. Moreover, many of the potential problems that limit the therapeutic effects of cancer vaccines would not need to be considered in the setting of cancer prevention, since the primed immune system would destroy the tumor before it becomes heterogeneous and capable of evading the immune response [228]. Some types of cancer may be ideal to test these prophylactic vaccines, such as breast cancer, (especially those with mutations in the genes encoding BRCA1 or BRCA2), colon cancer (15% of colon cancer corresponds to familial adenomatous polyposis syndrome), or pancreatic cancer (mainly patients with hereditary pancreatitis). Patients exhibiting some of these characteristics have an increased risk of developing these types of cancer [228] and could strongly benefit from such preventive approaches.

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