Glycopeptide- and carbohydrate-based synthetic vaccines for the immunotherapy of cancer

R. Rao Koganty, Mark A. Reddish and B. Michael Longenecker

On cancer cells, MUC-1 mucin displays distinct carbohydrate structures, such as Thomsen–Friedenreich (TF) and sialyl-Tn, the presence of which is attributed to reduced glycosylation activity. The core peptide is increasingly exposed and is recognized by the immune system. Vaccines based on both the exposed core protein, which contains major histocompatibility complex unrestricted epitopes, and carbohydrate structures are targets for the immunotherapy of cancers of epithelial origin. A vaccine formulated using synthetic sialyl-Tn has proven to be highly target-specific in human trials, and the induction of high anti-STn antibody titers correlated with prolonged survival of breast cancer patients. Peptides and glycopeptides formulated as liposome-based vaccines have been effective in animal models.

Traditional vaccines are mixtures of adjuvants and cell wall fragments or whole inactivated bacteria or virus that are used prophylactically to prevent infections. The approach to vaccine design is simple. The surface antigens of the bacteria in the form of whole cell wall are used to stimulate and program the immune system's memory to protect and defend the host against the pathogen. The mammalian host has so little in common with the infecting bacterium/virus that the whole organism can function as a vaccine as long as its capacity to reproduce and propagate is inhibited.

This oversimplified concept has so far been unsuccessful in cancer therapy, as clinical experiments using vaccine preparations based on whole tumor cell wall have met with limited success. The cancer cell, being a product of our body, has much in common with the body's normal cells, in spite of a few cryptic structures displayed on the cell surface. Processing and selection of epitopes from a cell membrane-based mixture of antigens, for presentation by the major histocompatibility complex (MHC), is not a clearly understood phenomenon and may lead to autoimmune responses. Vaccines with epitopic and structural definition are necessary in dealing with human carcinoma-associated antigens to prevent autoimmune responses, since such specificity may accurately direct the immune system to the target.

An ever-increasing array of functions are attributed to complex carbohydrate structures displayed on a normal cell surface. Large carbohydrate structures dominated by high mannose and *N*-acetyllactosamine content[1,2] appear to mask core peptide epitopes in the vicinity. In cancer cells, changes in detectable glycosylation patterns form the basis for research into diagnosis and immunotherapy of cancers. Altered patterns of glycosylation, which result in the appearance of several distinctive carbohydrate structures on cancer cell surfaces, produce highly tissue-specific glycoforms. Consequently, it becomes harder to design a unique cancer-specific antigen to function as a vaccine that can direct the immune system to detect and destroy cancer cells. An ideal cancer vaccine may incorporate many structural features that are scattered over the entire population of cancer cells. So far, glycosylation defects seem to be the most consistent changes that are displayed by cancer cells. Cell surface glycolipids and glycoproteins, particularly epithelial

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mucins, are among the widely studied cancer-associated molecules.

MUC-1 mucin is a high molecular weight glycoprotein with a carbohydrate content of up to 70% of its molecular weight. Although MUC-1 is expressed by many normal secretory epithelial cells at the apical cell surface, cancer cells of the same origin have increased expression of the mucin with significant changes in glycosylation patterns, while the core sequence remains the same in both normal and cancer cells. The extracellular domain of the peptide core is formed by a repeating (in tandem) 20 amino acid sequence dominated by the presence of serine and threonine residues, which carry large *O*-linked carbohydrate chains[3]. The tandem repeat is characterized by the presence of an immunogenic epitope to which cellular immune responses (CTL) have been described in breast cancer patients[4,5] and multiparous women[6]. It appears that MUC-1 mucin becomes an 'autoantigen' as a result of abnormal glycosylation, which results in the exposure of core peptide epitopes and loss of tolerance. The well-known Thomsen–Friedenreich family of carbohydrate structures, such as Tn and TF, have been found on the MUC-1 mucin together with their sialylated analogs. The mucin core peptide is highly polymorphic because the number of tandem repeats varies considerably between individuals and the glycosylation pattern is highly tissue specific.

There are five potential glycosylation sites per tandem repeat, which account for 25% of the tandem repeat sequence (Figure 1). In normal cells, because most of these sites are extensively glycosylated with branched carbohydrate side chains, the flexibility and reach of the long carbohydrate structures may completely obscure the core protein sequence from immune recognition[1]. However, on cancer cells, where the glycosylation structures are characteristically truncated, several cryptic structures (Figure 2) have been identified on the MUC-1 mucin.

The effects of glycosylation and size of the carbohydrate side chain structures on the folding of the protein core have been the subject of some recent investigations[2,7,8]. Large oligosaccharides may induce additional tertiary structural features to the protein core, and their presence in a series, as is commonly seen in mucins, appears to impose a characteristic core orientation in order to accommodate their clustered presence (Figure 3). However, conformational analysis using small carbohydrate structures has revealed no significant changes in the local conformation of the MUC-1 mucin tandem repeat sequence[7]. A pattern of underglycosylation relaxes the core protein to normal linearity and exposes

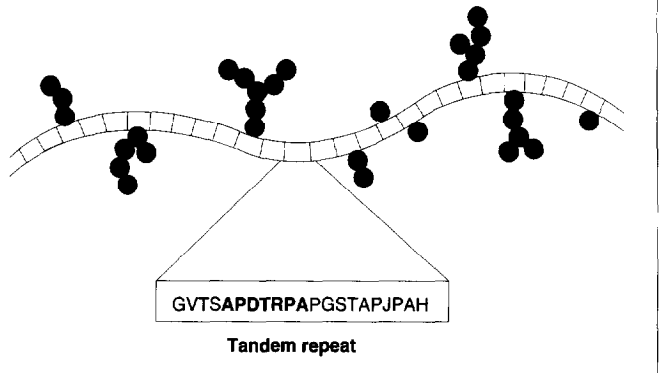

Figure 1. *Tumor-associated MUC-1 protein core showing reduced glycosylation and exposed epitope (bold) in the tandem repeat.*

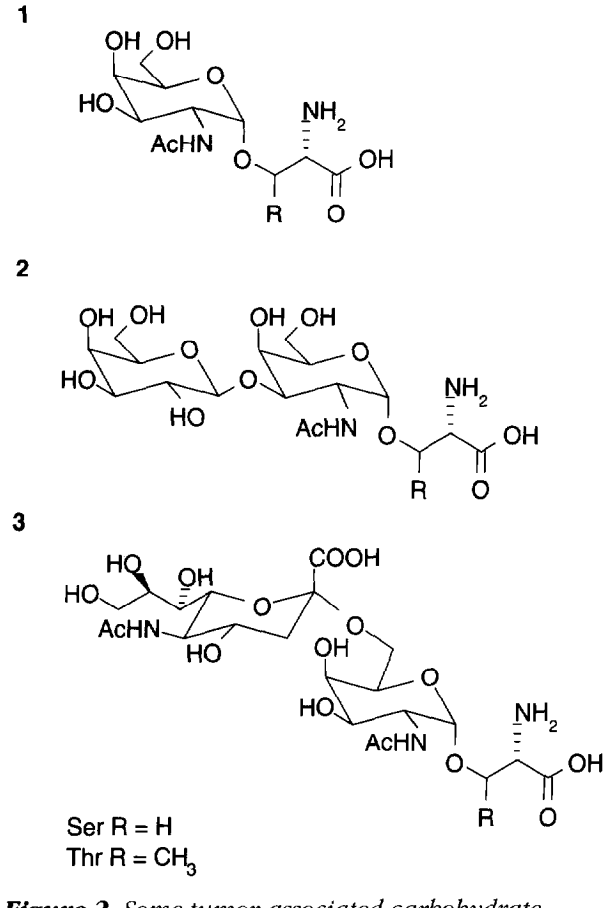

Figure 2. *Some tumor-associated carbohydrate structures that are extensively expressed on MUC-1 mucin: Tn (**1**), Thomsen–Friedenreich (**2**) and sialyl-Tn (**3**).*

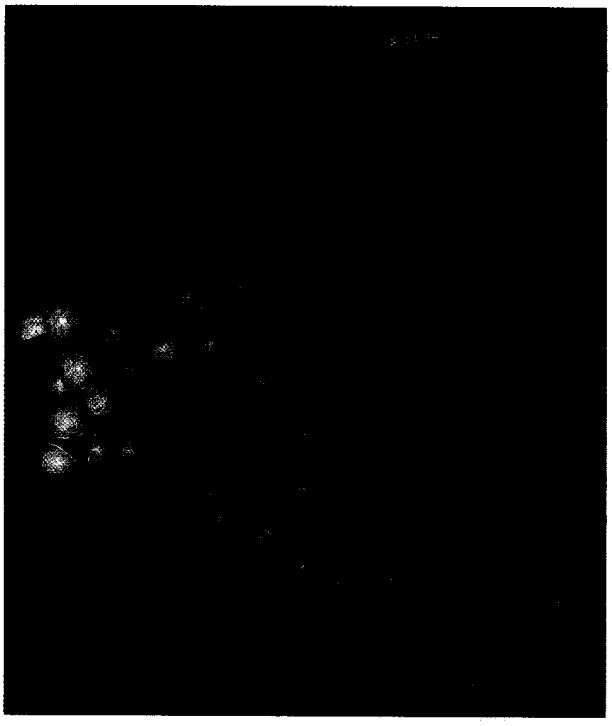

***Figure 3**. Three sialyl-Tn structures (radially extending from the Ser-Ser-Ser core at the center) forcing the core protein to assume a spiral shape.*

Box 1. Carbohydrates and proteins: a functional combination

Statistical diversity in the arrangements of amino acids in protein sequences adds distinctiveness to every glycosylation site, in spite of the fact that only three amino acids are glycosylated (i.e. asparagine as an *N*-glycosylation site, and serine and threonine as *O*-glycosylation sites). On the other hand, the linking of carbohydrate units seems to follow a restricted pattern in spite of the multitude of permutations and combinations[9] of all possible linking sites, in addition to the axial/equatorial configurations of such linkages. In the formation of a glycosidic bond, a simple rule that only the anomeric carbon of the incoming sugar is linked to a nonanomeric carbon of another is adhered to, without exception, by glycosyltransferases that are involved in the construction of all protein and lipid-based carbohydrate structures. Several such restrictions, particularly in protein glycosylations, underscore the importance of sequence and location-specific variations in the functions of the same carbohydrate structure[1] that may be found on different proteins. One such important function may be to 'camouflage' and protect an epitope expressed on a core protein (e.g. of a cell surface mucin) from immune recognition leading to cancer cell destruction.

possible core epitopes for immune recognition (see Box 1). The cryptic carbohydrate structures, as well as the exposed core epitopes, are potential targets for immunotherapy for a variety of common epithelial cancers such as lung, breast, ovary and colon cancer.

Synthetic target molecules

Cell-surface carbohydrate structures, particularly in the form of glycopeptides that are segments of tumor-associated mucins, have excellent potential to function as cancer antigens. Immune responses, both humoral and cell mediated, generated against these antigens have been effective in protection against cancers that display similar structures. For example, TF is a disaccharide (**2**, Figure 2) expressed on epiglycanin, a mucin derived from murine breast adenocarcinoma cell line TA3Ha. Mice immunized with a TF vaccine prevented tumor growth when challenged with TA3Ha cells[10]. High titers of hapten-specific IgM and IgG antibody titers have been generated in ovarian cancer patients in a Phase I clinical trial[11]. Sialyl-Tn (**3**, Figure 2), expressed on cancer-associated MUC-1, is well known as a prognostic indicator[12,13], and has proven to be an effective target for therapy of cancers. Similar results have been obtained with a synthetic peptide (GVTSA**PDTRP**APGSTA) containing a known mucin core epitope (indicated in bold letters) conjugated to keyhole limpet hemocyanin (KLH) and administered with DETOX-B SE adjuvant[14]. MUC-1 glycopeptides and peptides with sequence variations (Table 1) have been tested in animals to determine the extent and type of immune response, i.e. humoral or cell mediated. Consequently, there has been a need for efficient synthetic methods for large-scale production of carbohydrate- and glycopeptide-based vaccine formulations for the immunotherapy of cancers.

Chemical synthesis of carbohydrate structures requires the assembly of various strategically protected monosaccharides. Production of large quantities of cancer-associated structures in the form of *O*-glycosylated serine and threonine requires an efficient process, either enzymatic, chemical or a combination of both, that uses readily available cheap raw materials. Recently, we have reported new glycosyl donors based on commercially available *N*-acetylgalactosamine for the synthesis of serine and threonine-based *O*-linked carbohydrate structures[15,16]. The novel glycosyl donors are key synthetic intermediates that create an α-linkage, exclusively, in spite of the presence of the deterrent 2-acetamido group in the

pyranose ring. This process allows for the synthesis of cancer-associated carbohydrate structures (Figure 2) in the form of glycosylated amino acids which can be used for the commercial manufacture of glycopeptides (Table 1) with multiple site-specific carbohydrate structures. Sialyl-Tn vaccine is made by first synthesizing the carbohydrate on a linker arm and then chemically linking it to KLH (Figure 4).

Table 1. Some glycopeptides and lipopeptides that have been tested in animals to determine the extent and type of immune response (see text)

Name	Sequence
BP.24 TAP (20, 21Tn)	TAPPAHGVTSAPDTRPAPGS(Tn)T(Tn)APP[a]
BP.24 TAP (20STn, 21Tn)	TAPPAHGVTSAPDTRPAPGS(STn)T(Tn)APP[b]
BP.24 (TAP (20STn, 21STn)	TAPPAHGVTSAPDTRPAPGS(STn)T(STn)APP
BP.24 TAP $(Pal)_2$	TAPPAHGVTSAPDTRPAPGSTAPP·K(Pal)K(Pal)G[c]
BP.16 GVT	GVTSAPDTRPAPGSTA

[a,b] Tn and STn are carbohydrate structures as shown in Figure 2

[c] K(Pal) is a lysine with a palmitoyl chain attached to the 4-amino group [$HNCH(CO)(CH_2)_4NH$-$CO(CH_2)_{14}CH_3$]

New-generation vaccine delivery systems

Vaccines for cancers must be tailored to address specific targets at the molecular level, unlike conventional vaccines, which take a global approach to the target – usually a pathogen. As a prerequisite, the chemical synthesis of a structurally well-defined target in the form of a peptide, a carbohydrate or a glycopeptide is required. An immunological equivalence between the synthetic structure and the natural epitopes on tumor tissue or a cell line must be established through cross-reactivity of a monoclonal antibody to the synthetic structure. In order to determine the therapeutic utility, the immune sera or antigen-specific T cells generated against the synthetic structure must recognize the tumor target. When these preliminary criteria are met, the synthetic structure becomes a vaccine candidate for testing in a mouse immunotherapeutic model.

Totally synthetic vaccines, which are either a carbohydrate or a peptide/glycopeptide, are advancing rapidly through the developmental stages of synthesis, production, formulation and analysis of immune responses. These small molecules are poorly immunogenic and must depend on carrier delivery systems capable of eliciting nonspecific immune stimulation. The carbohydrate structure is synthesized with a linker arm at the reducing end, which is later used to chemically link (Figure 4) the structure to an immunogenic protein such as KLH. Peptides and glycopeptides can be chemically conjugated to KLH either through the carboxy or the amino function. A bacterial cell wall-derived adjuvant, such as monophosphoryl lipid A (MPLA), is usually added to these KLH conjugates to make them more immunogenic. Protein conjugate vaccines are highly immunogenic in humans[17–21], generating nonspecific anti-protein immune responses along with the desired anti-epitopic response. Sialyl-Tn–KLH (STn–KLH) vaccine has generated excellent epitope-specific IgM and IgG titers[20,21]. The responses that are specific to sialyl-Tn are measured by titers against ovine submaxillary mucin (OSM), a mucin similar to MUC-1 in STn expression. Immune suppression is commonly associated with cancer as a result of the release of large quantities of mucins into the serum. Before immunotherapy, the tumor model animals are pretreated with a low intravenous dose of the cytotoxic drug cyclophosphamide to reduce the suppressor cell population. A low dose of cyclophosphamide is known to have immunomodulatory properties and enhances the immune response in humans[22]. BP.16GVT (Table 1) conjugated to KLH has elicited immune responses to the peptide, and the antibody generated showed a protective influence against tumor challenge with MUC-1 transfected 410.4 cells in animal models[14]. That the tandem repeat contains a Th1 epitope is confirmed by the strong delayed-type hypersensitivity reactions generated by mice immunized with the conjugate antigen and foot pad challenged with human serum albumin conjugated BP.16GVT.

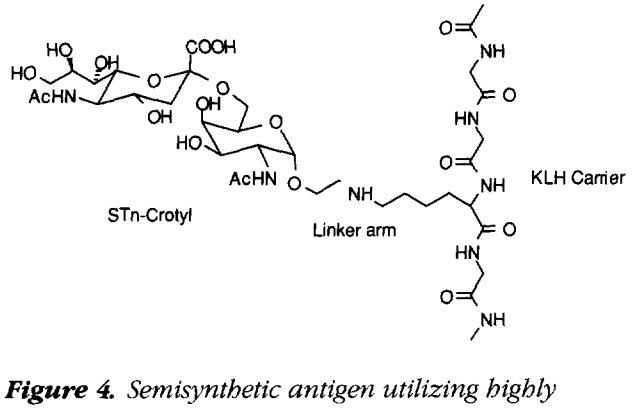

Figure 4. *Semisynthetic antigen utilizing highly immunogenic keyhole limpet hemocyanin (KLH) to carry synthetic sialyl-Tn structures attached to the lysines through a linker arm.*

Structural definition is necessary for cancer vaccines if all essential functions of a vaccine can be attributed to a single well-defined chemical structure. A synthetic molecule with a built-in property of 'assembling' into a macromolecular structure, or a 'particle' that is recognized and processed by the immune system, may be a vaccine of the future offering both structural definition and target specificity. A self-assembling antigen may be multifunctional in that it is composed of an epitope to which an immune response is desired and an adjuvant that can elicit a cascade of useful cytokines such as gamma-interferon (IFN-γ) and tumor necrosis factor. This concept, although experimental at this stage, is based on the current chemical research on molecular self-assembly. Synthetic glycolipids and phospholipids such as phosphatidylcholine, a major component of natural lipid membranes, fit the definition of self-assembling molecules.

The term 'liposome' (Figure 5) is used for artificially formulated globular bodies composed of phospholipids with strongly hydrophilic 'heads', which assemble into uni- or multilamellar vesicles filled with aqueous phase. Clinical experiments have been conducted with soluble drugs incorporated into the aqueous space enclosed by liposomes or within the lipid bilayer. These lipid vesicles may be effectively used to deliver soluble cancer-associated antigens to macrophages and other antigen presenting cells through various routes of inoculation. The strong lipophilic property of liposomes enables them to be adsorbed in large numbers onto the surface of macrophages. It has been shown *in vitro*, using cultured peritoneal macrophages, that up to 8,500 small liposomes may be adsorbed onto the surface of a single macrophage in about 4 h, assuming that a maximum of 75% of the membrane surface is available for adsorption[23]. Although the process of adsorption is reversible, it provides a fast and effective mechanism for the antigen to be taken up, processed and presented by macrophages. This process can augment the immune response by involving macrophages to enlist Th1 helper mechanisms.

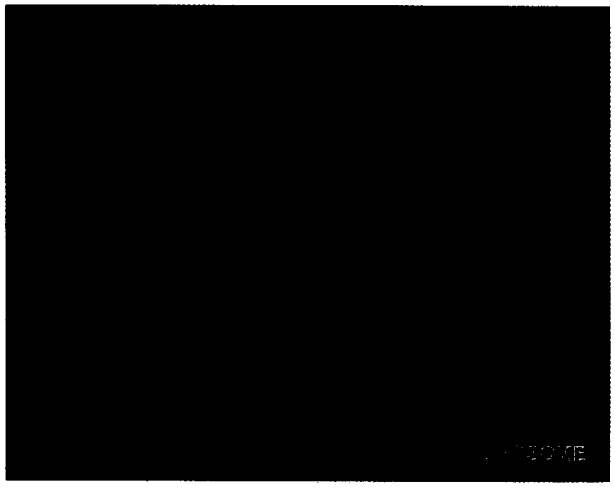

Figure 5. *Pictorial cross-section of a bilamellar liposome-based vaccine incorporating lipoantigen (dark red globes) evenly distributed throughout the lipid bilayer.*

Recent evidence suggests that a structurally well-defined molecule can indeed function as a vaccine if it fulfills the characteristics that are required for an effective vaccine. Tripalmitoyl-*S*-glycerylcysteinylserine (or P_3CS)[24] is a lipopeptide derived from *Escherichia coli* with strong immunogenic and adjuvant characteristics. When the peptide part of P_3CS is extended with a glycopeptide through two consecutive serines carrying α-*O*-linked *N*-acetylgalactosamine (see Box 2), the molecule behaves very much like an effective vaccine[24]. This large molecule with lipid chains and a hydrophilic head probably assembles into micellar structures that are highly immunogenic without an added adjuvant. Such integrated liposomal formulations can be very stable with a better shelf life than aqueous solutions of antigens enclosed in the lamellar pockets of liposomes.

An inherent advantage of using integrated liposomes is the ease of formulation as uniform particles with the antigen distributed evenly about the liposome surface (Figure 5). This concept may be useful in administering multiepitopic vaccines. The expression of carbohydrate structures that are different in size and content may influence the peptide conformations differently[7,28–30]. Consequently, the same core peptide epitope carrying Tn, TF and STn separately may not be recognized by the same core-specific antibody. Multiepitopic vaccines as one liposomal formulation using several lipid-based structures is not only feasible, but may also be an effective alternative in dealing with metastatic cancers (see Box 3).

Animal models for tumor immunology

A consistent need in drug development is an animal model to simulate the disease and the therapy in humans. In the area of human tumor immunology, the animal models chosen should mimic the disease being studied, and the tumor cell lines used must express the antigen(s) of interest. The antigen may be expressed in murine cells by an engineered

Box 2. Prognostic indicators

Tn and TF are basic foundations on which most *O*-linked carbohydrate structures are built, and yet their presence is widely regarded as cryptic and cancer associated. Termination of further biosynthesis from Tn and TF or abnormal glycosylations that create 'cryptotopes' represents the failure of the natural glycosylation pathways and triggers autoimmune responses that have been reported in all humans, while early cancer patients show more pronounced responses that are sustained throughout the progression of the disease. Although Tn is a precursor of TF, the ratio of their expression varies considerably and is tissue specific, and their quantitative expression is often correlated to the aggressiveness of carcinomas[25]. More recently, expression of a Tn successor, α-sialyl(2–6)-Tn, is regarded as an indicator of poor prognosis and cancer aggression[12,13] in patients with colorectal and ovarian cancers, as well as in breast[26] and gastric[27] cancers.

Box 3. Living with cancer

Preventing metastatic spread of cancer cells through the circulation may be a practical way to manage cancers. Presurgical immunization of cancer patients to generate an immune response to destroy tumor cells that spill into the circulation during surgery may reduce the risk of metastasis and recurrence. Vaccination protocols to induce humoral immune responses to cancer-associated carbohydrate structures to prevent bloodborne metastasis, followed by cellular responses to a specific peptide antigen to attack pre-existent solid tumors or micrometastasis, may be clinically effective. Multiepitopic vaccines using relevant antigenic structures may be evaluated using the tools described here, and the results may be used in designing clinical protocols for human trials.

human gene transfected into the murine tumor cell. In order to study the potential value of vaccine immunotherapy in human breast cancer, we have developed murine models ($CB6F_1$ mice) in which the transfected murine mammary tumor grows expressing the human MUC-1 mucin. This allows for the study of human MUC-1 synthetic peptide vaccine formulations in the murine system. Cells derived from epithelial tumors of the breast are highly metastatic. Although breast cancer starts as a single focus in soft tissues, the disease becomes lethal when it spreads from the primary site to multiple vital organ systems. Hence it is necessary to include analysis of metastatic processes in an animal model.

The murine breast adenocarcinoma cell line 410.4 has been transfected with a full-size construct of the human MUC-1 gene[31], and the cells maintained in selective media (Genetisin) to ensure consistent expression. Antigen expression was monitored by fluorescein-activated cell sorting (FACS) analysis after staining the cells with the anti-MUC-1 monoclonal antibody B27.29[32]. The transfected cells were sorted using flow cytometry (FACS) to enrich for either high expression (GZHi) or for low levels of expression (GZLo) of MUC-1 mucin. [Gabrielle Zimmerman (GZ), a graduate student with B.M. Longenecker, cultured the transfected cells and classified them into high and low MUC-1 expressor groups and sorted them by flow cytometry.]

Limiting dilution was used to establish subclones of the sorted populations. These clonal lines were then analyzed for long-term stability of MUC-1 gene expression under non-selective conditions in the animal host. Based on stable antigen expression at 8 weeks, the GZHi subclone A5.3 was grown into a working cell bank for the study of tumor biology in various models.

Two types of tumor challenge are commonly used for tumor immunology. In the first, subcutaneous challenge results in the generation of a solid tumor under the skin that can be easily measured bidimensionally using calipers. In practice, the large standard errors observed when estimating the mean tumor volume for a group limit statistical significance, even with large groups of animals in each experiment. Kaplan–Meier statistical survival analysis can also be performed on subcutaneously challenged animals. The second type of tumor model is an artificial metastasis model in which suspensions of tumor cells are injected intravenously. This results in organ-specific establishment of a large number of small tumor foci in the lung and is lethal at 35–45 days. The tumors are quantitated by intravenous injection of [^{125}I]iododeoxyuridine, which is incorporated into the rapidly dividing tumor cells. After 2 h, the lungs are surgically excised, washed and counted for radioactivity. Normal age-matched mice are used to determine the tissue background levels. The experimental and control group lungs are weighed and the number of visible tumor foci are counted as additional measures of tumor burden.

Prevaccination tumor challenge

One of the commonly used protocols in tumor immunology is vaccination, followed by subcutaneous or intravenous tumor challenge. It is important to determine the effects of nonspecific and antigen-specific immune responses in this

model, since nonspecific responses may significantly influence the rate of tumor implantation. Antigen-specific immunity is memory oriented and long lasting, while nonspecific responses are short lived. When tumor challenge was given only 3 days after the last treatment, at a time of high cytokine production and macrophage activation, a significant nonspecific protective effect was seen for the adjuvant/vehicle control groups. As the tumor challenge is delayed to 8 days and 14 days after treatment, the nonspecific protective effects observed in the adjuvant control group at day 3 are no longer significant. In contrast, the results obtained with two different antigen-specific vaccine formulations of MUC-1 peptides show consistent results throughout the time course experiment. The statistical significance in differentiating the treatment groups from the controls is notably increased when the 14-day interval is used.

The subcutaneous route of tumor challenge results in the development of a measurable subcutaneous solid tumor in the soft tissue, which is a functional model for primary human breast tumor. Histological examination of lung tissues (Figure 6) from an animal subcutaneously injected with GZHiA5.3 on the lower flank 35 days earlier, indicates that the primary tumor has metastasized to the lungs and is seen infiltrating the endothelial lining of the microvasculature. Subcutaneous challenge with the GZHiA5.3 MUC-1$^+$ tumor cell line results in a highly aggressive metastatic disease that is lethal at 6–7 weeks after challenge. This model is an appropriate representation of the human disease and can be used for the screening of vaccine formulations for effectiveness in treating the human cancer. Immunohistochemical staining of tumor tissues with monoclonal antibody B27.29 demonstrates that the MUC-1 encoded antigen is still expressed by the tumor.

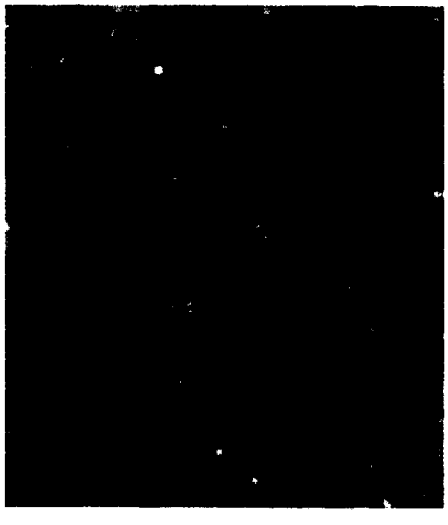

Figure 6. *Hematoxylin and eosin-stained section of lung, 35 days after subcutaneous tumor challenge on the lower flank, showing micrometastatic disease within the capillary venules of the lung along with evidence of modest inflammatory cell infiltrate. The capillary venule appears to be compromised (center right) with tumor cells extravasating to the extracapillary space.*

Active specific immunotherapy of tumor-bearing hosts

Prevaccination models are clearly less relevant to the active specific immunotherapy (ASI) of cancer patients than ASI models. In an ASI model, the tumor challenge (subcutaneous or intravenous) is given before the beginning of the immunotherapy. The longer the duration between tumor challenge and immunotherapy, the higher the tumor burden of the test animals. When effective vaccine formulations are not statistically distinguishable in efficacy in the prevaccination models, it is necessary to use the ASI model for evaluation. In Figure 7, the results of an ASI time course are presented in which two vaccines are compared for treatments starting at day 3 or day 9 after tumor challenge. It is obvious that only the liposomal, synthetic MUC-1 formulation generated responses that led to a significant reduction in tumor burden in the day 9 group. An examination of excised lungs after intravenous tumor challenge demonstrates significant protection by the vaccine based on MUC-1 mucin, which offers hope for the immunotherapy of breast adenocarcinomas in humans.

In order to determine the antigen specificity and the activities that are associated with protective immunity, the responses in age-matched animals have been investigated. Vaccine formulations that induce strong peptide-specific T-cell proliferative responses and Th1-type cytokine profiles are protective in all the animals models investigated. Antigen-specific proliferation and IFN-γ production from CD4$^+$ T cells appear to correlate strongly with protective immunity, particularly in the solid tumor models (Figure 8). In contrast, vaccine formulations that primarily induce Th_2-type cytokine responses and antigen-specific antibody production did not show efficacy in the solid tumor models.

Human cancer therapy

In clinical trials, patients with metastatic breast cancer underwent immunotherapy with THERATOPE® STn–KLH vaccine following a low pretreatment dose of an immunomodulatory cyclophosphamide.

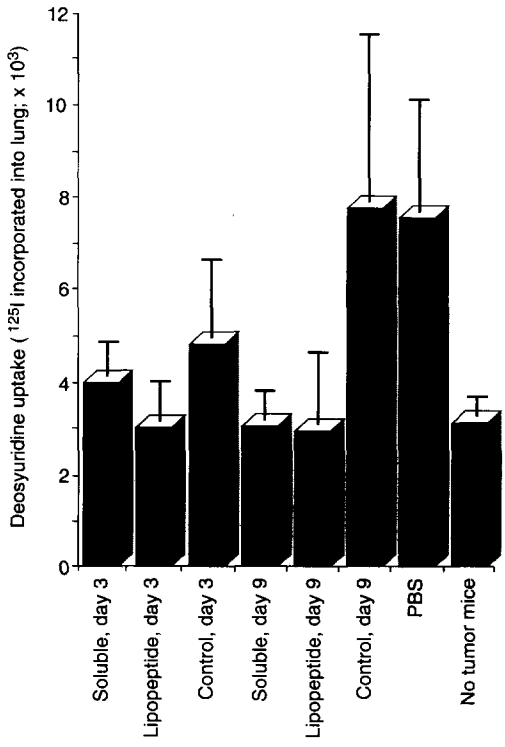

Figure 7. *Lung tumor burden data obtained in the evaluation of two liposomal formulations in a tumor bearing mouse model. Vaccine treatments begin either on day 3 (boosted on day 17) or on day 9 (boosted on day 23) after intravenous challenge with 5 × 10^5 cells. Formulations represent a large liposome (2–5 μm) made by entrapping a soluble peptide antigen with monophosphoryl lipid A (MPLA; Ribi, Hamilton, MT, USA), or a small integrated liposome (< 0.2 μm) using a lipopeptide and MPLA. Liposomal vehicle controls do show some protective effects relative to the phosphate buffered saline (PBS) control when treatments begin on day 3, a time when established tumor volume is low. Both peptide delivery vehicles generated significant tumor burden reductions relative to both controls in the 9 day tumor burden analysis. Data presented are the mean (with standard errors,* n=*10 per group) of lung incorporated ^{125}I obtained by 2 h* in vivo *[^{125}I]deoxyuridine labeling.*

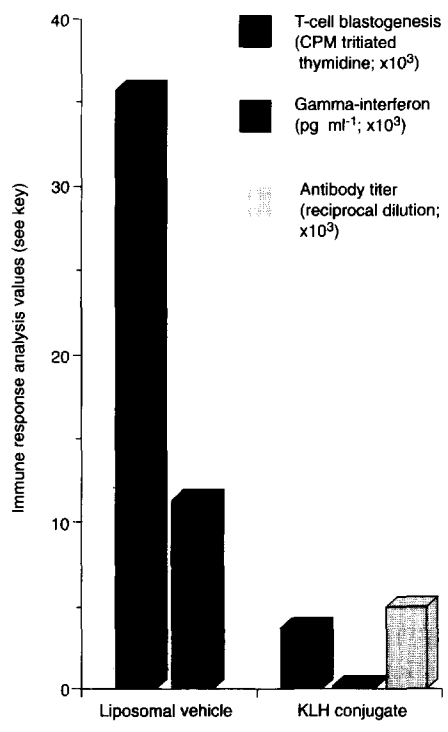

Figure 8. *Immune response analysis of CB6F_1 mice includes antigen-specific T cell blastogenesis and gamma-interferon (IFN-γ) production by draining lymph node T- cells along with serum antibody titrations (21 day IgG titers). Vaccine constructs based on a liposomal delivery system typically show strong T-cell proliferation and Th1-type cytokine production profiles with an absence of peptide-specific antibodies (titers <1/80). In contrast, the peptide keyhole limpet hemocyanin (KLH) conjugate vaccines in DETOX™-B SE induced modest T cell proliferative and cytokine responses but typically vigorous antibody production (mean titers >1/5,120). Subjects were primed and boosted with 5 μg doses subcutaneously in the inguinal draining flank at two sites on days 0 and 14. Inguinal node T cells were obtained by nylon wool passage on day 21 and incubated with purified splenic antigen presenting cells (mitomycin treated) from nonvaccinated CB6F_1. Peptide pulsed and control cultures were for 5 days prior to supernatant harvest for cytokine analysis and tritiated thymidine was added with fresh media to determine blastogenesis. Supernatants were analyzed for IFN-γ and IL-4 (data not shown) by capture ELISA techniques using reference standards (Genzyme, Boston, MA, USA). Antibody titer (by peptide solid-phase ELISA) is reported as the last serial dilution generating an optical density greater than two times that observed for the preimmune serum from each subject.*

Patients who were pretreated with single low-dose intravenous cyclophosphamide generated stronger immune responses than did those who received an oral dose or no cyclophosphamide. The anti-STn and anti-OSM IgG titers were higher in the former group compared with those who received oral doses. The patients with high titers also have significantly longer survival (with a median survival of

23.3 months *vs* an actual median of 12.6 months) compared to the patients who received oral or no cyclophosphamide (Figure 9)[21]. The anti-STn antibody titer showed an inverse correlation with growth in measurable tumors. Antibody responses against a highly tumor-specific target such as STn may lead to better prognosis through ancillary mechanisms such as formation of immune complexes with immunosuppressive mucins[19,33] in addition to antibody-dependent cellular cytotoxicity.

Conclusions

The results from Phase II clinical trials using THERATOPE® vaccine, formulated with structurally defined synthetic cancer-associated sialyl-Tn, have indicated that target-specific immune responses, both humoral and cell mediated, can be generated. These responses have translated into a statistically significant increase in the survival of cancer patients. The safety of targeting cryptic structures that are expressed on cancer cells for immunotherapy using THERATOPE® and synthetic peptide-based liposomal vaccines has been sufficiently established. Although there is more to be accomplished at this stage, the statistical significance of these results suggests that the comprehensive developments and concepts described above may lead to therapy of cancer.

Factors such as the ability to modulate the humoral and cell-mediated immune responses, the induction of cytokines such as IFN-γ and target specificity may be incorporated into synthetic vaccines, as has been shown. Such accomplishments are necessary in order to further enhance the therapeutic utility of the immune responses against cancer cells whose characteristic features are only subtly different from those of the normal. In a hostile environment that relentlessly brings about gene mutations, enlistment of the body's immune system to eliminate the cancer cell through modulatory vaccines of high target specificity is a viable and safe approach. Such cancer vaccines, besides being safer, less expensive and simpler to use compared to chemotherapeutic drugs, are similar in many respects to the traditional vaccines that have received the widest ever public acceptance for a therapeutic agent.

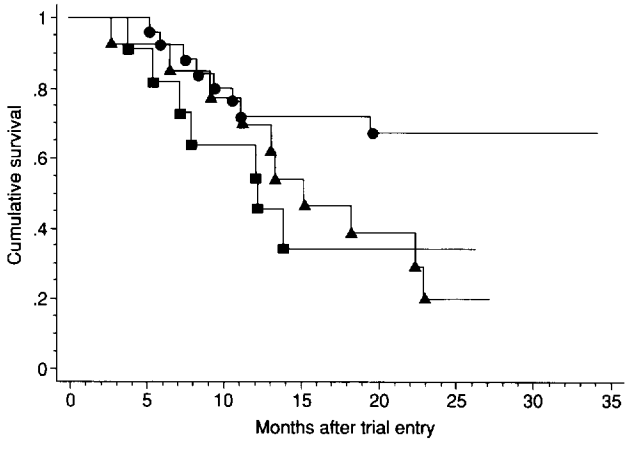

Figure 9. *Cumulative survival plots (Kaplan–Meier) of breast cancer patients who were treated with STn–KLH (keyhole limpet hemocyanin) vaccine. The survival plots compare patients who received intravenous (circles), oral (triangles) or no (squares) cyclophosphamide. The log rank (Mantel–Cox) test indicates a significance of* P = *0.024.*

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