NOVEL STRATEGY FOR CARBOHYDRATE-BASED THERAPEUTIC VACCINES

FIELD OF THE INVENTION

This invention relates to the field of medical treatments and therapeutic compositions for use therein. More specifically, it relates to methods and compositions for treatment and prophylaxis of cancer in human patients.

BACKGROUND OF THE INVENTION

Despite the very extensive research efforts and expenditures over recent years, cancer remains one of the most life threatening diseases in the world. Cancer therapy remains very difficult. Scientists have long been exploring the possibility of developing vaccines for the treatment and prevention of cancer in human patients. Although this approach has been viewed as the therapy of the future, progress has been modest and the incidence of clinical failure has been very high.

Creating cancer vaccines is problematic, due largely to the fact that patients fail to mount an effective immune response to cancerous cells, because cancer cells fail to produce immunogenic markers that sufficiently distinguish them from normal cells. Although the patterns of cell surface carbohydrate antigens of cancer cells differ from those of normal cells, the individual structures of their antigens are identical.

BRIEF REFERENCE TO THE PRIOR ART

Despite the structural identity of individual antigens found on normal cells and cancer cells, attempts have been made to exploit cancer cell carbohydrate antigens as potential cancer vaccines. The observation that specific antigens are overexpressed by certain tumor types has enabled the development of simple monovalent antigen vaccines against various tumor types. It has also

been observed that, in animals and humans, provided that the carbohydrate antigens are conjugated to a protein carrier, the resulting conjugate vaccines can be used to raise antibodies that are specific for each carbohydrate antigen.

However, the antibodies so induced are usually of low titer and poor endurance (mostly IgM). Despite this drawback, they are used, on the basis that, after surgical or chemical treatment of cancer, the antibody levels will remain sufficiently high, during a short convalescence period, to dispose of any remaining cancer cells.

Clinical trials using some of these carbohydrate antigen-protein conjugate vaccines have demonstrated that they can increase remission times in some patients. However, their use as therapeutic agents is far from satisfactory.

α2-8 Polysialic acid is expressed in a number of important human cancers, including small cell lung cancer, neuroblastoma and Wilms' tumor. It is strongly expressed in neonatal tissue, but is not prevalently expressed in normal human tissue following the neonatal period (1 month). Normal cells express a variety of different sialylated surface antigens.

It is known (U.S. Patent 5,811,102 Jennings et al.) how to prepare vaccine compositions based on chemically modified meningococcal polysaccharides, and that they are useful for immunizing mammals against Neisseria meningitidis and E. coli K1, microorganisms which are leading causes of meningitis in humans. A modified B polysaccharide of N. meningitidis is prepared chemically, from the polysaccharide isolated from N. meningitidis. The modified polysaccharide has sialic acid residue N-acetyl groups (C_2) replaced by a saturated or unsaturated C_{3-5} acyl group. This modified polysaccharide is conjugated to an immunologically suitable protein to produce a conjugate of enhanced immunogenicity. A mammal may be immunized with the vaccine composition, to induce a specific immune response in the animal suitable to provide active protection from N. meningitidis infection. Alternatively, blood may

be collected and the gamma globulin fraction may be separated from the immune serum, to provide a fraction for administration to a suitable subject to provide passive protection against or to treat on-going infection caused by these microorganisms.

Many different surface antigens containing modified sialic acid residues have been expressed in normal cells, using N-propionylated and N-levulinoyl-D-mannosamine as precursors. In one report, N-propionylated-D-mannose was introduced into a cancer cell line (hepatoma).

It is an object of the present invention to provide novel compositions capable of being used as anti-cancer vaccines.

It is a further object of the invention to provide a process for enhancing the specific immunogenicity of mammalian cancer cells, and exploiting this enhanced immunogenicity in a vaccination approach to the management of cancer in human patients.

SUMMARY OF THE INVENTION

In the present invention, from one aspect, the sialic acid component of a sialic acid unit-containing cell surface marker characteristic of cancerous mammalian cells is modified so that cells normally expressing such a marker express instead a modified sialic acid unit-containing cell surface marker which is strongly immunogenic. For example, the present invention enables, in a portion of patient cells which regularly express α2-8 polysialic acid (i.e. various types of cancer cells), the expression of a highly immunogenic surface antigen namely, modified α2-8 sialic acid. Antibodies specific for the modified antigen, which can be induced using a conjugate of a suitable portion of the modified sialic acid unit-containing marker (such as α2-8 polysialic acid) and a protein, can then be used to eliminate cells such as the aforementioned cancer cells which express α2-8 polysialic acid. Vaccines can be prepared utilizing conjugates of the modified sialic

acid-containing marker (such as modified α2-8 polysialic acid), or utilizing antibodies produced in response to exposure of a suitable subject to the modified sialic acid-containing marker, for managing cancer conditions which involve cancer cells characterized, at least in part, by expression of modified sialic acid unit containing marker.

The binding of appropriate antibodies which antibodies are prepared by standard antibody raising techniques using the modified polysialic acids described herein, to cancer cells expressing the corresponding modified polysialic acid, in the presence of complement, will result in at least partial destruction of the cancer cells. Appropriate antibodies can be induced in the patient using a modified polysialic acid-protein conjugates such as an N-acylated-α2-8 polysialic acid-protein conjugate, or alternatively the antibody can be raised outside the patient's body, by known techniques such as hybridoma incubation, and administered passively to the patient following antigen modification.

Thus, according to one aspect of the present invention, there is provided a process of enhancing the specific immunogenicity of viable, proliferating mammalian cancer cells to levels sufficient to allow the effective recognition and destruction of such cells by an immuno-response in vivo, which comprises providing to said cells a chemically modified precursor of a suitable sialic acid unit-containing cell surface marker capable of rendering said cancer cells immunologically distinctive from related, normal cells; causing biochemical incorporation of said modified precursor into the sialic acid unit-containing cell surface marker during intracellular synthetic processes; and eventual surface expression of said sialic acid unit-containing surface marker incorporating said modified precursor in a form capable of eliciting said level of immune response.

A further aspect of the invention comprises immunogenic mammalian cancer cells, said cells having surface markers incorporating modified sialic acid units capable of initiating an immune response in the mammalian system containing them which is sufficiently strong to effectively combat the proliferation

and even the viability of such cells.

A further aspect of the invention provides a conjugate of a modified α2-8 polysialic acid incorporating N-acylated D-mannosamine units and a protein and the use of the conjugate in the preparation of a vaccine for managing cancer conditions in mammalian patients.

Another aspect of the invention provides use of chemically modified sialic acid precursors, such as N-propionylated mannosamine, in creating mammalian cancer cells expressing sialic acid unit-containing surface markers capable of eliciting an immune response, conjugates of a modified α2-8 polysialic acid incorporating N-acylated D-mannosamine units and a protein and the use of these conjugates, to combat said mammalian cancer cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation of results obtained from experiments described in Example 3;

Figure 2 is a graphical representation of results obtained from experiments described in Example 3;

Figures 3A and 3B are graphical representations of results obtained from experiments described in Example 4;

Figures 4A, B and C are graphical representations of results obtained from experiments described in Example 5.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is preferred, according to the invention to choose as the sialic acid-unit containing cell surface marker for modification contiguous sialic acid residues.

This is desirable as contiguous sialic acid residues are large enough to stimulate an effective immune response. Preferably, between 2 and 100 contiguous sialic acid residues are used. More preferably, between 5 and 60 contiguous sialic acid residues are used. Even more preferably, between 15 and 25 contiguous sialic acid residues are used. As used herein, the term "polysialic acid" refers to two or more contiguous sialic acid residues.

One example of an appropriate polysialic acid modification for use in the present invention is N-acylation of α2-8 polysialic acid. This can be accomplished by feeding the cells an N-acylated precursor of α2-8 polysialic acid, for example N-propionylated-D-mannosamine, which in the natural intracellular biochemical synthesis of α2-8 polysialic acid becomes chemically incorporated therein and thus results in the production of N-propionylated α2-8 polysialic acid. Such N-acylated α2-8 polysialic acids are strongly immunogenic, in contrast to the naturally expressed α2-8 polysialic acid.

Antibodies specific for the modified antigens can be induced by normal antibody raising techniques using a corresponding chemically modified polysialic acid-protein conjugate, such as an N-acylated α2-8 polysialic acid-protein conjugate. N-acylated α2-8 polysialic acids for antibody production and vaccine preparation may be produced from commercially available poly-2,8-N-acetylneuraminic acid using methods disclosed herein. Alternatively, though less preferably, they may be purified by standard means from cells expressing them. Examples of cells expressing N-acylated α2-8 polysialic acid include cells such as RMA tumor cells which normally produce 2-8 poly-N-acetylneuraminic acid, when induced to use an N-propionated precursor such as N-propionated mannosamine. The choice of immunologically suitable protein for conjugation, and techniques for binding the polysaccharide to the protein to form the conjugate for use as the vaccine are matters within the skill of the art. Tetanus toxoid TT is a specific, suitable example of such a protein.

It is believed that two distinct classes of antibodies recognizing

polysialic acids exist. Thus, as cancer cells may express polysialic acids of varying lengths (ranging from 2 contiguous sialic acid residues to at least 60, 100 and likely as many as 200 contiguous sialic acid residues), it may be desirable to select the length of polysialic acid used to induce an immune response.

A first class of antibodies recognizes "short" polysialic acids, having at least 2 and no more than 10 to 12 sialic acid residues. A second class of antibodies recognizes "long" polysialic acids, having at least 10 or 12 sialic acid residues. Antibodies recognizing "short" polysialic acids can be distinguished from those recognizing "long" polysialic acids by routine experimentation, in light of the disclosure contained herein and of the prior art, and particularly, *Pon, et al.* J. Exp. Med., Vol. 185(11), pages 1929-1938, 1997.

In some instances it will be desirable to induce an immune response using a conjugate of a modified polysialic acid and a protein where the length of the polysialic acid is selected to be either short or long. When it is desired to induce an immune response to a short polysialic acid, preferably between 2 and 12 contiguous sialic acid residues are used in the conjugate. Even more preferably, between 4 and 10 contiguous sialic acid residues are used. Yet more preferably, between 6 and 8 contiguous sialic acid residues are used.

When it is desired to induce an immune response to a long polysialic acid, preferably between 10 and 200 contiguous sialic acid residues are used in the conjugate. More preferably, between 12 and 100 contiguous sialic acid residues are used. Yet more preferably, between 14 and 60 contiguous sialic acid residues are used.

One specific example of a suitable polysialic acid unit-containing cell surface marker for modification is α2-8 polysialic acid, since this marker is well recognized as a characteristic of certain cancer cells, and its biochemical synthesis is quite well understood. A variety of different N-acyl modifications can be made to the polysialic acid for use in the present invention, by appropriate chemical

modification of the mannosamine precursor. One can introduce the appropriate chemical modifying group by simple chemical reaction of the mannosamine with the organic acid carrying the appropriate acyl group, i.e. an acid (or acid equivalent such acid halide, anhydride, etc.) of formula R-COOH where R represents a C_{1-6} alkyl group, straight or branch chained, a C_{1-6} alkenyl group, straight or branch chained, a C_{1-6} ketonic group or the like. Specific preferred examples include butyric acid, acrylic acid, propionic acid and levulinic acid.

The invention is further described, for illustrative purposes, in the following specific examples.

Example 1 - Synthesis of N-propionylated Group B meningiococcal polysaccharide - tetanus toxoid (NPrGBMP-TT) conjugate vaccine (ref.):

A) Synthesis of NPRGBMP

Colominic acid (500 mg) was N-deacetylated in 2N NaOH (15ml) containing $NaBH_4$ (50 mg) for 7 hours at 105°C. The solution was neutralized with 5N HCl to ca. pH 8.0 and then dialyzed against distilled water overnight. The resulting product was sized by passing a Bio-Gel A.5 column to collect the fraction of MW ~ 10-11 kD. After lyophilization, deacetylated GBMP was obtained (370 mg. ^1H NMR of the product indicated that the deacetylation was complete.

To the deacetylated GBMP (300 mg) solution in 0.1N NaOH (10 ml) was added propionyl anhydride (1 ml) in 5 portions while 2N NaOH was added to maintain the pH at 8-9. Four hours later, the reaction was adjusted to pH 11-12 and stirred for 1 h. The reaction mixture was dialyzed against distilled water and then lyophilized to give NPrGBMP (310 mg). ^1H NMR of the product indicated that the propionylation was complete.

B) Synthesis of NPrGBMP-TT conjugate

NPrGBMP (150 mg) was activated by oxidation of the non-reducing terminus using $NaIO_4$ (100 mg) in 0.1N NaOAc-HOAc solution at pH 6.5 overnight. The mixture was dialyzed against distilled water and then lyophilized.

Oxidized NPrGBMP (20 mg), freshly prepared TT monomer (8 mg) and $NaBCNH_3$ (10 mg) were dissolved in 0.1 N $NaHCO_3$ (1ml) and the mixture was incubated at room temperature for 3 days. The product was separated by gel filtration chromatography on a Bio-Gel A.5 column (2x45 cm). The first peak contained the expected glycoconjugate. It was then dialyzed and lyophilized to give NPrGBMP-TT conjugate (8.1-8.2 mg). Sialic acid content of the conjugate was determined using the resorcinol method and the protein content was measured using BCA protein microanalysis. The final conjugate was found to contain about 20% (wt/wt) of sialic acid, which is equivalent to 4 sialic acid chains per TT molecule.

In similar manner, GBMP-TT conjugate, as control conjugate vaccine, was also prepared. (R.A. Pon, M. Lussier, Q.-L. Yang, H.J. Jennings, *J. Exp. Med.*, 1997, 185, 1929).

EXAMPLE 2 - Synthesis of the biosynthetic precursor of sialic acid - N-propionyl mannosamine (NPrMan)

To a solution of D-mannosamine hydrogen chloride (10 g) in 0.1N NaOH (40 ml) was added propionyl anhydride (5 ml) in portions while 2N NaOH was added to the solution to maintain the pH at 6.5-7.0. Four hours later, the reaction mixture was purified by chromatography on a Silica-Gel column (10x20 cm) using ethyl acetate and methanol (2:1 - 1:1) as eluent. The first portion contained various partially O-acetylated N-propionyl mannosamines and some N-propionyl mannosamine (NPrMan). The second pure portion was the expected product of NPrMan (6.4 g) as a mixture of both α-(60%) and β-anomers (40%). ^1H NMR(D_2O):5.09 (s, α H-1), 5.01 (s, β H-1), 4.44 (d, J 3.5 Hz, β H-2), 4.30 (d, J 4.0

Hz, α H-2), 4.03 (dd, J 3.5, 9.8 Hz, α H-3), 3.88-3.76 (m), 3.60 (t, J 9.5 Hz, α H-4), 3.50 (t, J 9.8 Hz, β H-4), 3.39 (m, α H-5), 2.35-2.28 (m, CH_3CH_2CO), 1.12-1.04 (m, CH_3CH_2CO).

EXAMPLE 3 - Surface expression of N-Propionylated Polysialic acid

Tumor cells (RMA) were incubated with the precursor N-propionylated mannosamine (2 mg/ml) for various time periods indicated in Figure 1 in RPMI plus 8% FBS. After each incubation, cells were harvested and incubated with either 13D9 ("mAb13D9", anti-N-propionyl polysialic acid) or 735 ("mAb735", anti-N-acetyl polysialic acid) monoclonal antibodies. Subsequently, cells were stained with FITC anti-mouse IgG2a antibody and fixed with 1% formaldehyde before analysis by Flow cytometry. Figure 1 clearly indicates that the binding of 13D9 antibody on tumor cells increases with the duration of incubation of the precursor N-propionylated mannosamine in the previous culture. Interestingly, the binding of 735 antibody (which recognizes N-acetyl polysaccharide) is down-regulated with the incorporation of N-propionylated mannosamine. Figure 2 shows the measurement of the mean fluorescent intensity of tumor cells binding to 13D9 or 735 antibodies. Clearly, the expression of N-propionyl groups on the surface polysialic acid of tumor cells is associated with a reduction in their expression of N-acetyl groups. This data suggests that the precursor N-propionylated mannosamine is metabolized efficiently by the tumor cells.

EXAMPLE 4- Susceptibility to cell death due to expression of N-Propionylated Polysialic acid

It was determined whether the expression of N-propionylated sialic acid makes cells more susceptible to killing by the antibody 13D9 (anti-N-propionyl polysialic acid). After feeding tumor cells with the precursor for various time intervals (indicated in Figure 3), cells were harvested and incubated with either 13D9 or 735 monoclonal antibodies for 4 hours in the presence of rabbit

complement (low toxicity grade). Cytotoxicity towards tumor cells was measured by MTT method that measures the viability of cells in culture (Figure 3).

Figure 3A depicts results obtained from incubating RMA cells with NPrMan (4 mg/ml) and harvesting aliquots at specified time intervals. The harvested aliquots were washed with PBS and with either: complement and no antibody, complement and mAb13D9, or complement and mAb735.

Figure 3B depicts results obtained from incubating RBL-2H3 cells with specified concentrations of NPrMan. At daily intervals, cells were harvested, washed with PBS and incubated with mAb13D9 and rabbit complement, and then subjected to the cytotoxicity assay.

In the presence of complement alone, low level cytotoxicity was noted (Figure 3A). The addition of monoclonal antibody 735 resulted in a strong cytotoxicity indicating that the expression of sialic acid makes the cells vulnerable to killing depending on the presence of antibody. This cytotoxicity, mediated by the antibody 735, persisted at various time points tested and only at late time point was a reduction in cytotoxicity mediated by 735 observed. Taken together with Figure 2, this indicates that even if the expression of N-acetyl groups is low on the surface of tumor cells, the level of expression is still sufficient to result in tumor cell killing. The addition of the antibody 13D9 during the incubation resulted in an increase in killing of tumor cells in a time dependent manner. This correlates with the increase in the expression of N-propionyl groups on sialic acid on the tumor cells (Figure 2). Before incubation of cells with the precursor, a background killing of 25% was observed and this killing went up to about 80% with the incorporation of N-propionyl groups on the surface of tumor cells. Similar results have also obtained with polyclonal antisera against N-propionyl polysialic acid. Similar results were also obtained with another tumor cell line.

Taken together these results indicate that tumor cells can be made to metabolize and incorporate a precursor N-propionyl mannosamine which gets

expressed on the surface polysialic acid residues of the tumor cells. This expression of N-propionyl polysialic acid makes the tumor cells vulnerable to immune attack depending on the presence of antibody against N-propionyl polysialic acid.

Example 5 - Control of Tumor Growth *In Vivo*

Mice were inoculated with RMA cells (10^6 cells/mouse) and 5 days after inoculation the mice were treated daily (by i.p. injection near the tumor site) with mAb 13D9 (200 µg/mouse) and precursor NPrMan (5 mg/mouse) for a period of 8 days. Tumor growth was routinely monitored by measurement of tumor size. In combination with NPrMan, mAb 13D9 had a greater effect on tumor size than mAb 13D9 alone, although mAb 13D9 alone was also able to reduce tumor size when compared with a control group of mice (Figs. 4A, 4B and 4C).

Figure 4A depicts results from individual C57BL/6 mice which were injected subcutaneously with $1x10^5$ RMA tumor cells in the shaven area of the rear flank. After 5 days, groups of 5 or 6 mice were injected daily, with mAb 13D9 (200 µg, intraperitoneally) and NPrMan (5 mg, intraperitoneally). Figures 4B and 4C depict the results of similar experiments wherein mice were injected with (B) mAb 13D9 alone (200 µg, intraperitoneally) or (C) with PBS. Tumor growth was monitored routinely by measuring the tumor size. A control IgG2a mAb did not inhibit tumor growth.

Example 6 - Control of Metastatic Cancer Cells

The experiments in mice were carried out as described in Example 5 using RMA cells except that in this case the spleens of the mice were analyzed for the presence of metastatic cells. On day 25, spleens were excised and cell suspensions prepared in medium RMPI = 8% FEBS. One fifth of the aliquots from the individual mice were used to initiate serial two fold dilution in 24 well plates in 1 mL of RPMI 8% FEBS. Cultures were fed regularly and monitored over a period

of one month to score positive wells containing tumors. Spleen samples that had tumor cells were scored positive and the samples that had no tumor cells at all dilutions were scored negative. Following cell cultures of the spleen cells, the metastatisized tumor cells were easily distinguished from the normal spleen cells, by microscopic examination. Table I shows that there were no tumor cells in the spleen of the mice treated with a combination of mAb 13D9 and NPrMan, indicating that all transported metastatisized tumor cells were polysialyated and therefore were completely eliminated from the mice. The data also revealed that mAb 13D9 alone could also partially reduce the metastasis of tumor cells in comparison with a control group of mice (Table 1).

Thus, the metastasis of tumor cells can be controlled by modification of surface polysialic acid glycoconjugates to their N-propionylated analogs and then applying immunotherapy based on antibodies specific for the modified antigen. These antibodies could be either passively administered as described herein, or induced *in situ* by direct immunization using an appropriate N-propionated (NPr) polysialic acid - protein conjugate vaccine.

Thus, it will be appreciated that there have been provided novel compositions capable of being used as anti-cancer vaccines and, further, a process for enhancing the specific immunogenicity of mammalian cancer cells, and exploiting this enhanced immunogenicity in a vaccination approach to the management of cancer in human patients.

Table 1

Table 1 - Antibodies against NPr polysialic acid control tumor metastasis *in vivo*.

Group	Tumors in Spleen*	Percentage metastasis
13D9+precursor	0/5	0.0%
13D9	2/6	33.3%
-	4/5	80.0%

*Number per mouse

WHAT IS CLAIMED IS:

1. A process of enhancing the specific immunogenicity of viable, proliferating mammalian cancer cells to levels sufficient to allow the effective recognition and destruction of such cells by an immuno-response *in vivo*, which comprises providing to said cells a chemically modified precursor of a suitable sialic acid unit-containing cell surface marker capable of rendering said cancer cells immunologically distinctive from related, normal cells; causing biochemical incorporation of said modified precursor into the sialic acid unit-containing cell surface marker during intracellular synthetic processes; and eventual surface expression of said sialic acid unit-containing surface marker incorporating said modified precursor in a form capable of eliciting said level of immune response.

2. The process of claim 1 wherein the sialic acid unit-containing cell surface marker comprises contiguous sialic acid residues.

3. The process of claim 2 wherein the sialic acid unit-containing cell surface marker comprises between 2 and 100 contiguous sialic acid residues.

4. The process of claim 2 wherein the sialic acid unit-containing cell surface marker comprises between 5 and 60 contiguous sialic acid residues.

5. The process of claim 2 wherein the sialic acid-containing cell surface marker comprises between 15 and 25 contiguous sialic acid residues.

6. The process of claim 2 wherein the sialic acid unit-containing cell surface marker comprises α2-8 polysialic acid.

7. The process of either of claim 1 or claim 6 wherein the chemically modified precursor is an N-acylated precursor.

8. The process of claim 7 wherein the chemically modified precursor is

an N-acylated D-mannosamine.

9. The process of claim 8 wherein the precursor is N-propionylated-D-mannosamine.

10. Immunogenic mammalian cancer cells, said cells having cell surface markers incorporating modified sialic acid units capable of initiating an immune response in a mammalian system containing them which is sufficiently strong to effectively combat the proliferation of such cells.

11. A conjugate of a modified polysialic acid incorporating N-acylated D-mannosamine units and a protein.

12. The conjugate of claim 11 wherein said polysialic acid is a short polysialic acid.

13. The conjugate of claim 12 wherein the short polysialic acid has between 2 and 12 contiguous sialic acid residues.

14. The conjugate of claim 13 wherein the short polysialic acid has between 4 and 10 contiguous sialic acid residues.

15. The conjugate of claim 14, wherein the short polysialic acid has between 6 and 8 contiguous sialic acid residues.

16. The conjugate of claim 11 wherein said polysialic acid comprises a long polysialic acid.

17. The conjugate of claim 16 wherein the long polysialic acid comprises between 10 and 200 contiguous sialic acid residues.

18. The conjugate of claim 17 wherein the long polysialic acid comprises

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between 12 and 100 contiguous sialic acid residues.

19. The conjugate of claim 18 wherein the long polysialic acid comprises between 14 and 60 contiguous sialic acid residues.

20. The conjugate of any one of claims 11, 12 or 16 wherein said polysialic acid comprises an α2-8 polysialic acid.

21. Use of the conjugate of any one of claim 11, 12 or 16 in the preparation of vaccine for managing cancer conditions in mammalian patients.

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