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(54) Title: METHOD FOR THE PREPARATION OF SPECIFIC ANTIBODIES AGAINST SACCHARIDIC ANTIGENS

(57) Abstract: The present invention relates to new compounds useful for the stimulation of the production of antibodies. Said compounds comprises a saccharidic tumor antigen and a polymeric scaffold. The present invention also encompasses conjugated compounds usefull in ELISA assay for the selection of antibodies against saccharidic antigens.



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Method for the preparation of specific antibodies against saccharidic antigens

The present invention finds application in the field of immunodiagnosis and immunotherapy and, more in particular, it relates to compounds useful in the preparation of antibodies directed against Tumor Associated Carbohydrate Antigens.

Background

Carbohydrates are known to exert an important role in several biological and physiological pathways. It has been found, in fact, that they may contribute to the protein stability or resistance to degradation, to the regulation of intracellular signalling and targeting of glycoproteins to membranes or cell organelles, and to have implications in immunological recognition [see, as a general reference, Varki, A.; *Glycobiology* **1993**, 3, 97–130, Bertozzi, C.L., Kiessling, L.L.; *Science* **2001**, 291, 2357-64, Angeloni, S.; *Glycobiology* **2005**, 15 (1), 31-41)].

In addition to that, several studies and research projects have discovered that carbohydrates and derivatives thereof, for instance glycans, take also part in some neoplastic modifications of cells. In fact, [as reported by Hakomori, S.; Chapter 4 in Montreuil, J., Vliegenthart, J.F.G., Schachter, H. (editors) *Glycoproteins and disease*. Elsevier, Amsterdam, 1996 or by Kobata A.; *Cancer cells and metastasis*. Chapter 3 in Montreuil, J., Vliegenthart, J.F.G., Schachter, H. (editors) *Glycoproteins and disease*. Elsevier, Amsterdam, 1996 or by Kumamoto et al in *Biochemical and Biophysical Research Communications* **1998**, 247, 514-7 or by Hakomori, S.; *PNAS* **2002**, 99 (16), 10231-33], it has been observed that glycans, known to be present on cell membranes, usually exhibit structural modifications, for instance a high degree of branching in cancer cell membranes over normal cell membranes.

Likewise, besides any structural modification, it has also been observed an altered composition in membrane carbohydrates with respect to normal cells.

As an additional example of a saccharidic moiety playing a role in neoplastic processes there is, for instance, sialic acid (see The Merck Index; XIII Ed.; No. 8558), a 9-carbon atom amino sugar being a member of neuraminic acid related compounds. There has been observed, in fact, that the high metastatic activity of certain tumoral forms may be well correlated with an increased concentration of sialic acid in cell membranes, thus

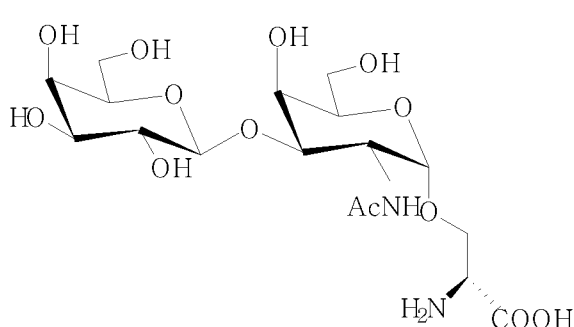
yielding to a reduced adhesive capability of the extra-cellular matrix cells in metastatic phenomena (see, for a reference, Varki, A. et al *Essentials of glycobiology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York **1999**).

Additionally, for instance as reported by Sames, D. et al in *Nature* **1997**, 389, 587-91 or by Dwek, M.V. et al. (*Clinica Chimica Acta* **1998**, 271, 191-202) or by Burchell et al (*Glycobiology* **1999**, 9 (12), 1307-11) or by Taylor-Papadimitriou et al. (*Biochim. Biophys. Acta* **1999**, 1455, 301-13) or by Hanisch et al (*Glycobiology* **2000**, 10 (5), 439-49) or by Schuman, J. et al (*Glycoconjugate Journal* **2000**, 17, 835-48) within complex saccharidic compounds, a highly glycosilated glycoprotein like altered MUC1, which shows a high number of O-glycosidic residues and that provides a protective layer on epithelial surfaces and it is involved in cell-cell interactions, signalling, and metastasis (see, for a reference Parry S, Silverman HS et al., *Biochem. Biophys. Res. Commun.* 2001; 11; 283(3): 715-20), seems to contribute to the metastatic potential of the neoplastic cells.

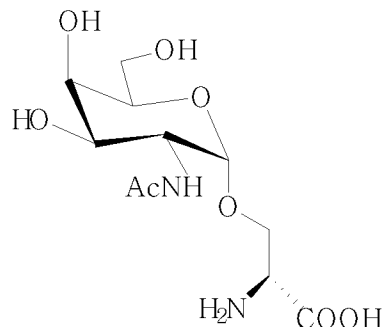
Though numerous molecular alterations have been found to be associated with each single developing or progressing phase of a tumor, the exact relationship among them is not yet completely understood.

The phenotypic alterations in saccharides found to characterize a neoplasia, are generally known as Tumor Associated Carbohydrate Antigens (hereinafter shortly referred to as TACAs).

The epitopes Gal- β -1 $\rightarrow$ 3-GalNAc- α -O.Ser/Thr and GalNAc- α -O.Ser/Thr (see the chemical structures below), commonly referred to as TF and Tn respectively, are among the most widespread known TACAs.



TF antigen (Gal- β -1 $\rightarrow$ 3-GalNAc- α -O.Ser/Thr)



Tn antigen (GalNAc- α -O.Ser/Thr)

As from the above reported formulae, the Tn immunodeterminant group is a GalNAc residue α linked to the hydroxyl group of serine or threonine, which appears in the amino-terminal region of glycoproteins. In its turn, Gal- β -1 $\rightarrow$ 3-GalNAc- α -O.Ser/Thr (TF antigen) is then formed by the action of the enzyme β -galactosyltransferase, which adds a galactose residue, taken from a Gal-nucleotide, to a Tn residue.

The occurrence of these antigens in normal tissues has been investigated in order to verify their specificity toward pathological tissues (see, for example, Cao, *Histochem.Cell Biol.*1996, 106, 197-207).

Moreover, TF and Tn antigens were found to be over-expressed in immunoreactive form, to a significant extent, in about 90 % of carcinoma tissues (see, for a reference, Springer, G.F.; *Science* **1984**, 224, 1198-206) and their relative proportion in human carcinomas frequently correlates with the carcinoma's aggressiveness itself.

The results of several studies also suggested that at the early stages of the metastatic process, TF and Tn antigens both play a key role in adhesion phenomena between the tumoral cells and the adjacent normal cells (see, for a reference, Kischikawa et al, in *Jpn. J. Cancer Res.* 1999, 90, 326-32).

The isolation and structural identification of carbohydrate antigens specifically over-expressed in cancer cells, and thus of TF and Tn, represents a first step for the development of a strategy for the therapy and diagnosis of neoplastic diseases: the carbohydrate-based immunotherapy and immunodiagnosis (see, for instance, Allen in *J. Am. Chem. Soc.*, 2001, 123, 1890-7).

It is well known in the art that immunotherapy involves the stimulation of the natural body defenses, i.e. immunosystem, against harmful agents such as microorganisms, pollutants, chemicals, foods and the like. Therefore, it has been envisaged that cancer cells, characterized by an altered expression of TACAs on their membrane, might represent the target of the human or animal immunosystem.

There was found that the stimulation of the immunosystem through suitable tumoral antigens such as, for example Tn or TF antigens, could be a promising therapeutic tool against tumor.

Toyokuni et al. (see, for a reference, *Bioorg. Med. Chem.* 1994, 2, 119-32) describes the preparation of totally synthetic carbohydrate vaccines by the use of monomeric, dimeric

and trimeric Tn antigens further conjugated with Ovine Serum Albumin, Starbust® dendrimer and tripalmitoyl-S-glycerylcysteinylserine (P₃CS).

Lo-Man et al. (see, for example, J. Immunol. 2001, 166, 2849-54) discloses the development of a fully synthetic immunogen called multiple antigenic glycopeptide and based on a dendrimeric lysine core with four arms.

Kuduk et al. (see, for a reference, J. Am. Chem. Soc. 1998, 120, 12474-12485) describes the use of KLH, BSA and a lipopeptide (pam) for the clusterization of Tn and TF antigens, while Dziadek et al. (Angew. Chem. Int. Ed. 2005, 44, 7624-7630) discloses a similar approach for the preparation of compounds comprising TACAs and BSA and MUC1 peptides.

Kagan et al. (see, for a reference, Cancer Immunol. Immunother. 2005, 54, 424-430) describes the tests made on several Tn conjugates: Tn monosaccharide, Tn(c) prepared on a triple threonine backbone and Tn prepared on a partially or fully glycosylated MUC1 backbone.

Among the major limitations of the above reported conjugated compounds, however, is the fact that when used as vaccines, they lead to the production of poor quantities of the desired antibodies and, also, that an aspecific mixture of the same is finally recovered (for example, see Grigalevicius et al, Bioconjugate Chemistry, 2005, 16, 1149-1159).

We have now found novel conjugated compounds of TF and Tn antigens that may be used as vaccines in the therapy against tumors. The conjugated compounds of the invention, in addition, allow to overcome the above drawbacks of the TACAs conjugated compounds of the prior art.

Description of the invention

It is therefore a first object of the present invention a compound comprising a plurality of Tn or TF antigens conjugated to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through any suitable linker.

In the present description, and unless otherwise provided, with the term polymeric scaffold we intend any of the above scaffolds like cBSA, alginate or dextran having polymeric structure.

Among the polymeric scaffold within the scope of the present invention, there is Bovine Serum Albumin (BSA), a highly immunogenic protein, that can be further modified through the introduction of alkylamino groups at the aspartic acid or glutammic acid residues, thus obtaining cationized Bovine Serum Albumin (cBSA). According to the present invention, the molecular weight of cBSA is approximately comprised between about 65 to about 77 KDa.

For a general reference to cBSA and its preparation see, for instance, Domen et al. in J. Immunol. 1987, 139, (10), 3195-8.

Alginate is a mixture of polysaccharidic chains of different molecular weight (see, as an example, The Merck Index, XIII Ed., No. 239).

Typically, according to the present invention, the molecular weight of alginate may range from about 130,000 to about 170,000 and, more preferably, from about 140,000 to about 160,000.

Finally, for a general reference to dextran see, for instance, The Merck Index, XIII Ed., No. 2965.

The preferred conjugated compounds of the present invention comprise cBSA and alginate and, even more preferably, they comprise cBSA.

As formerly reported, the above polymeric scaffolds are conjugated either directly, that is through a covalent bond, or by means of a suitable linker, to a plurality of Tn or TF antigens.

Suitable examples of linkers may comprise either straight or branched, synthetic or naturally occurring amino acids and derivatives thereof including, for instance, suitable peptides or carbohydrates, other similar linkers being within the knowledge of the skilled person.

More preferably, the said amino acids or peptides may comprise from 2 to about 10 carbon atoms.

According to a preferred embodiment of the invention, however, the conjugated compounds comprise the above antigens directly linked to the selected scaffold.

As far as both Tn and TF antigens are concerned, whose structural formula have been above reported, they are linked to the rest of the moiety (e.g. scaffold) through carboxamido bonds, that is to say through any of their amino or carboxy groups.

With the term “a plurality of Tn or TF antigens” we intend, unless otherwise provided, that more than one of the said antigens are linked, directly or through a suitable linker, to the polymeric scaffold.

Typically, the number of antigens linked to the polymeric support, within the
5 conjugated compounds of the invention, may vary to a great extent according to the number of monomeric units forming the polymeric scaffold itself and bearing suitable reactive functional groups (e.g. amino, carboxy) as well as from the operative experimental conditions being adopted in the synthesis of the conjugated compound.

In particular, in the case of the conjugated compounds of the invention comprising
10 cBSA, from about 5 to about 30 moieties of Tn or of TF antigens, for instance from about 10 to about 20 moieties of Tn or of TF antigens, may be linked to each cBSA molecule.

Likewise, in the case of the conjugated compounds comprising alginate, from about 200
15 to about 250 moieties of Tn or of TF antigens, for instance from about 220 to about 230 moieties of Tn or of TF antigens, may be linked to each alginate molecule.

According to a preferred embodiment, the present invention refers to a conjugated compound comprising cBSA directly linked to a plurality of Tn antigens.

According to another preferred embodiment, the invention relates to a conjugated compound comprising alginate directly linked to a plurality of Tn antigens.

20 According to an additional object of the invention, herewith provided there is a process for the preparation of the above reported conjugated compounds, said process comprising the steps of:

- 1) dissolving any one of the polymeric scaffolds in a suitable buffered aqueous solution;
- 2) adding to the solution of step (1) a condensing agent and a suitable amount of the
25 selected Tn or TF antigen, each of which is optionally connected to a suitable linker, in the presence of a condensing agent; and
- 3) mixing and stirring the resulting solution from step (2) for a suitable period of time and recovering the obtained product.

The above process, and any variant thereof, is carried out as per step (1) in suitable
30 aqueous buffers, such as 2-morpholino-ethansulphonic acid (MES); suitable concentration is, for example 25 mM, while suitable pH is comprised between about 4.5 to about 7, preferably being 6.5.

As far as step (2) is concerned, the amount of selected antigen is the one that allows the antigen and the scaffold to react in the expected molar ratio, so as to obtain the desired conjugated compound of the invention.

The above reaction is carried out in the presence of any suitable condensing agent conventionally adopted in the formation of carboxamido bonds. Preferably, said
5 condensing agent is N-Hydroxysuccinimide (NHS), either alone or in combination with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

The above mentioned condensing agents are preferably used in a slight stoichiometric excess.

10 From the above, it is clear to the skilled person that the linker, whenever present, may be already bonded (e.g. connected) to the selected antigen before the same undergoes coupling with the scaffold or, alternatively, it may be bonded (e.g. connected) to the scaffold, at first, so that the resulting intermediate compound is then coupled with the selected antigen.

15 In this respect, both of these optional variants are within the scope of the present invention.

In addition, as the antigen and the scaffolds are coupled, either per se or by means of any suitable linker, through the formation of carboxamido bonds, e.g. $-\text{CONH}-$ or $-\text{NHCO}-$, it is clear to the skilled person that any other reactive group optionally
20 present in any one of the antigen, the scaffold or even the linker itself, and which may be susceptible to react under the above operative conditions so as to give rise to unwanted side products, need to be properly protected/deprotected according to well known methods.

According to the present invention, the recovery of the final product in step (3) is
25 performed by conventional methods including, for example, dialysis and freeze-drying.

Within the present process, and as per any variant thereof, either the reactants as well as any of the starting materials including the selected antigen and the scaffold, each of which being optionally coupled to a linker, and the linker per se, are all known compounds or they can be prepared according to conventional methods known in the
30 art.

The determination of the substitution degree of the compounds of the present invention, that is the number of Tn or TF antigens residues linked to each scaffold, is carried out according to known methods.

As an example, the determination of the substitution degree of the compounds of the present invention comprising cBSA is carried out via enzymatic reaction with the use of the enzyme α -N-Acetylgalactosaminidase from *Acremonium sp.* according to the experimental conditions indicated by the supplier of the enzyme [Seikagaku (Tokyo, Japan)]. More in detail, for example, as for Tn antigen, the hydrolysis is performed by incubating 0.25 ml of the substrate mixture containing Tn antigen with 0.05 ml of enzyme (having an activity of about 13 U/ml) in citrate buffer for 2 hours at pH 4.5 and at 37°C. The reaction is then quenched by the addition of 0.2 M borate buffer at pH 9.8. The quantification of the resulting sugar residue, i.e. N-Acetylgalactosamine (GalNac) from Tn antigen, is made by capillary electrophoresis as further described in the Materials and Method section. In turn, the substitution degree of the compounds of the present invention comprising alginate or dextran is determined by automated elementary analysis carried out on a lyophilized sample of the conjugated compound.

The results of the determination of the substitution degree (see Examples 3 and 4 of the Experimental Section), i.e. the number of Tn or TF antigens residues linked to each scaffold, are then expressed as a percentage. Therefore, for example, a 15% substitution degree of alginate means that 15 over 100 repetitive monosaccharide units of alginate are linked to one molecule of Tn or TF antigen.

As formerly reported, the conjugated compounds of the invention may find application in therapy and prophylaxis and, more in particular, they may be used as vaccines against tumors.

It is therefore a further object of the invention, a pharmaceutical composition comprising a conjugated compound wherein a plurality of Tn or TF antigens is linked to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through any suitable linker, together with pharmaceutically acceptable excipients including, solvents, carriers, buffers, stabilizers and the like, as per the general pharmaceutical practice.

In general, any suitable excipient may be properly included in the pharmaceutical compositions of the invention, the preparation of which may be accomplished according to general methods well known to the skilled person in the art.

It is therefore a further object of the present invention a compound comprising a plurality of Tn or TF antigens conjugated to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through a suitable linker, for use in therapy or prophylaxis.

Additionally, a further object of the invention is represented by a method for the treatment or the prophylaxis of tumors, which method comprises the administration to a mammal of a suitable amount of a conjugated compound of the invention wherein a plurality of TF or Tn antigens is linked to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through any suitable linker, or of any pharmaceutical composition thereof.

For the intended purpose, suitable amounts of the conjugated compounds of the invention are those typically employed for therapeutic or prophylactic purposes comprising the administration of antigens and derivatives thereof and they may be determined according to a selected suitable therapeutic protocol.

Accordingly, it is an additional object of the invention, the use of a compound comprising a plurality of Tn or TF antigens conjugated to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through any suitable linker, in the preparation of a medicament or vaccine for the treatment or prevention of tumors.

According to an additional embodiment, the conjugated compounds of the invention may also be used for the preparation of specific antibodies against Tn or TF antigens. Typically, the aforementioned antibodies may be prepared by means of well known methods employed in the production of monoclonal antibodies (hereinafter referred to as mAbs).

For the purpose of the present invention, experiments with animals involved normal mice, which were used solely to produce mAbs. They have been housed in animal facilities according to the standards for animal care established by national regulations and maintained in accordance with individual institutional guidelines under approved

authority protocols. They also have been regularly checked by veterinarians and handled by qualified animal care takers within an authorized animal facility.

For a general reference to the preparation of monoclonal antibodies see, for instance, Köhler, G., Milstein, C.; *Nature* 1975, 256, 495-7.

5 As an example, the claimed method for the preparation of the given antibodies may thus comprise the following steps:

- 1) BALB/c mice immunization;
- 2) sacrifice of the animals and spleen excision;
- 3) immortalization antibody-producing cells by somatic cell hybridization (fusion);
- 10 4) selection of the hybrid cells in HAT culture medium;
- 5) screening by ELISA assay to obtain the most specific clones; and
- 6) subcloning.

Immunization, as per step (1), is performed by the injection of the conjugated compound of the present invention. Preferably, the conjugated compound is the one
15 wherein a plurality of Tn antigens is directly linked to cBSA.

Steps 2), 3), 4) and 6) are performed according to known methods.

In particular, according to the present invention, as far as step (5) is concerned, the Enzyme Linked Immuno Sorbent Assay (better known as ELISA) comprises the use of a solid phase onto which the selected Tn or TF antigens, corresponding to that used for
20 immunization when conjugated with the scaffolds of the invention, is properly immobilized.

More in particular, the polymeric scaffold is a biotinylated polymeric scaffold, so that it can be linked/immobilized onto the solid support through a streptavidin/biotin linkage.

ELISA is a well-know assay for the detection and quantification of substances, for
25 example proteins, antibodies and hormones. The first step of ELISA requires the immobilization of specific antigens onto an ELISA plate; then, a sample containing the antibodies to be selected according to the present invention is added to the solid phase and incubation is allowed so that a complex between the immobilized antigens and the complementary antibodies may form. Washing is then carried out in order to remove
30 unbound materials. The further step includes the addition of a secondary antibody directed to the formed complex and linked to a suitable enzyme [the most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP)].

Finally, as an example, the subsequent addition of a suitable chromogen will result in a variation of colour intensity depending upon the quantity of antibodies bound to the immobilized antigen and, as far as this latter variation is assessed, to the quantity of antibodies present in the sample.

- 5 For a general reference to the ELISA technique, as per the present invention, see the following Experimental Section.

We have observed, in particular, that the immobilization of the selected antigen onto the solid phase, for ELISA purpose, may be conveniently carried out by immobilizing the biotinylated conjugated compound of the invention. In this respect, the immobilization
10 of the antigen-biotinylated alginate conjugate proved to be particularly selective in detecting antibodies originating from the inoculation of any of the conjugated compounds of the invention themselves.

In other words, when immunization and antibodies preparation follow proper inoculation to mice of the selected conjugated compound of the invention, for instance
15 of the conjugated between Tn and cBSA, the ELISA test may be conveniently performed by adding the sample containing the antibodies thus produced to the solid phase comprising the biotinylated conjugated compounds of the invention, preferably the biotinylated alginate conjugate with that same TACA, that is to say, Tn antigen.

From the above, it is clear to the skilled person that analogous consideration may apply
20 when referring to TF antigens and conjugated compounds thereof.

Therefore, it is another object of the present invention a compound comprising a plurality of Tn or TF antigens conjugated to a biotinylated polymeric scaffold selected from the group consisting of biotinylated alginate, biotinylated cationized bovine serum albumin (biotinylated cBSA) or biotinylated dextran, either directly or through any
25 suitable linker.

Preferably, the biotinylated polymeric scaffold is biotinylated alginate.

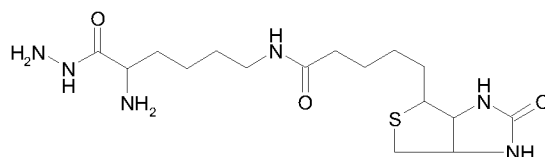
In this respect, cBSA, alginate and dextran are defined as formerly reported.

Likewise, the number of Tn or TF antigens linked to the biotinylated scaffolds of the present invention is as defined above.

- 30 Biotin (see The Merck Index, XIII Ed., No. 1231) is a naturally occurring growth factor known to play a role in carboxylation reactions with proteins or polypeptides.

As biotin selectively binds to streptavidin, which is especially used for the *in vitro* preparation of solid beds, biotin may be usefully linked to molecules to be immobilized onto said solid phases in separation as well as in purification techniques.

Among biotin derivatives, N-(+)-biotinyl-L-lysine hydrazide may be usefully employed in the biotinylation of polymeric scaffolds. In particular, it is a commercially available compound, having the chemical formula shown below:



N-ε-(+)-biotinyl-L-lysine hydrazide

The linkage of biotin to polymers, i.e. cBSA, alginate or dextran, occurs randomly through any of the carboxy groups of these polymeric compounds.

As for the preparation of the conjugated compounds of the present invention wherein the polymeric scaffold is a biotinylated polymeric scaffold, said process includes the steps of:

- 1) dissolving any one of the polymeric scaffolds in a suitable buffered aqueous solution;
- 2) adding to the solution of step (1) a suitable amount of biotin or of a biotinylating agent, in the presence of a suitable condensing agent;
- 3) adding to the solution obtained from step (2) a suitable condensing agent and a suitable amount of the selected Tn or TF antigen, each of which is optionally connected to a suitable linker; and
- 4) mixing and stirring the resulting solution from step (3) for a suitable period of time and recovering the obtained product.

As for the above process, a suitable amount of biotin or a biotinylating agent is within the knowledge of the skilled person so as to get the desired biotinylated polymer.

However, for a specific reference to the operative conditions being employed in the process of the invention, and details thereof, see the following Experimental Section.

A preferred condensing agent is, for instance, NHS used alone or in combination with EDC.

It is therefore an additional object of the invention the use of a conjugated compound comprising Tn or TF antigens linked to biotinylated alginate, biotinylated cBSA or biotinylated dextran, properly immobilized onto a solid phase, in an ELISA test for the detection of antibodies produced upon immunization techniques wherein Tn or TF are those same Tn or TF antigens respectively conjugated with cBSA, alginate or dextran.

According to an embodiment of the invention, particularly preferred is the use of a conjugated compound comprising Tn or TF antigens linked to biotinylated alginate, properly immobilized onto a solid phase, in the detection through ELISA test of antibodies produced upon immunization techniques and comprising those same Tn or TF antigens conjugated with cBSA, alginate or dextran.

Typically, unless otherwise provided, proper immobilization onto the solid phase of the Tn or TF antigen conjugated with the biotinylated polymer may comprise coating a microtiter ELISA plate wells with streptavidin, filling the wells with the selected conjugate between the biotinylated polymer and Tn or TF and leave incubating for a suitable period time.

The above operative conditions are well known in the art and details thereof are reported in the Experimental Section.

The antibodies thus produced and selected according to the present invention may also be used for therapeutic purposes.

Additionally, as they selectively target specific tumor sites or tissues, they may be properly functionalized, according to known methods, with diagnostically or therapeutically active compounds.

In fact, as they selectively attack and bind to tumoral cells expressing on their surface Tn and/or TF antigens, they may also act as carriers to bring therapeutically active moieties on the tumoral sites or tissues or to bring at those sites or tissues diagnostic moieties for enabling the visualization.

Typically, functionalization with therapeutically or diagnostically active compounds may comprise reacting the said antibodies with given antitumoral drugs or detectable labels known in the art.

From the above, it is clear to the skilled person that the antibody and the therapeutically or diagnostically active moiety may be linked to each other either directly or, alternatively, through any suitable linker or spacer known in the art.

These linkers may include, but are not restricted to, substituted or unsubstituted alkyl chains, polyethylene glycol derivatives, amino acid spacers, sugars or aliphatic or aromatic spacers commonly known in the art.

Suitable examples of diagnostic moieties according to the present invention include, for instance, paramagnetic metal chelates suitable for magnetic resonance imaging (MRI), radiolabels suitable for X-ray imaging, ultrasound microspheres or liposomes suitable for ultrasound detection or optical imaging dyes.

Among the paramagnetic chelates, the preferred metals elements are those having atomic number ranging between 20 and 31, 39, 42, 43, 44, 49 and between 57 and 83.

More preferred are paramagnetic metal ions selected from the following: Fe(2+), Fe(3+), Cu(2+), Ni(2+), Rh(2+), Co(2+), Cr(3+), Gd(3+), Eu(3+), Dy(3+), Tb(3+), Pm(3+), Nd(3+), Tm(3+), Ce(3+), Y(3+), Ho(3+), Er(3+), La(3+), Yb(3+), Mn(3+), Mn(2+) wherein Gd(3+) is the most preferred. The organic chelator is a molecule having one or more polar groups that act as a ligand for, and complex with, a paramagnetic metal. Suitable chelators are known in the art and include acids with methylene phosphonic acid groups, methylene carbohydroxamine acid groups, carboxyethylidene groups, or carboxymethylene groups. Examples of chelators include, but are not limited to, diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetraacetic acid (DOTA), ethylenediaminetetraacetic acid (EDTA), and 1,4,8,11-tetraazacyclotetradecane-1,4,8, 11 -tetraacetic acid (TETA). Additional chelating ligands are ethylenebis-(2-hydroxy-phenylglycine) (EHPG), and derivatives thereof, including 5-Cl-EHPG, 5Br-EHPG, 5-Me-EHPG, 5t-Bu-EHPG, and 5sec-Bu-EHPG; benzodiethylenetriamine pentaacetic acid (benzo-DTPA) and derivatives thereof, including dibenzo-DTPA, phenyl-DTPA, diphenyl-DTPA, benzyl-DTPA, and dibenzyl DTPA; bis-2 (hydroxybenzyl)-ethylene-diaminediacetic acid (HBED) and derivatives thereof, the class of macrocyclic compounds which contain at least 3 carbon atoms, more preferably at least 6, and at least two heteroatoms (O and/or N), which macrocyclic compounds can consist of one ring, or two or three rings joined together at the hetero ring elements, e.g., benzo-DOTA, dibenzo-DOTA, and benzo-NOTA, where NOTA is 1,4,7-triazacyclononane N,N',N"-triacetic acid, benzo-TETA, benzo-DOTMA, where DOTMA is 1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetra(methyl tetraacetic acid), and benzo-TETMA, where TETMA is 1,4,8,11-

- tetraazacyclotetradecane-1,4,8,11-(methyl tetraacetic acid); derivatives of 1,3-propylenediaminetetraacetic acid (PDTA) and triethylenetetraaminehexaacetic acid (TTHA); derivatives of 1,5,10-N,N',N''-tris(2,3-dihydroxybenzoyl)-tricatecholate (LICAM) and 1,3,5-N,N',N''-tris(2,3-dihydroxybenzoyl) aminomethylbenzene (MECAM), (4S)-4-(4-ethoxybenzyl)-3,6,9-triscarboxylatomethyl)-3,6,9-triazaundecandioic acid-disodium (EOB-DTPA), N,N-bis[2-[bis(carboxymethyl)amino]ethyl]-L-glutamic acid (DTPA-GLU), N,N-bis[2-[bis(carboxymethyl)amino]ethyl]-L-lysine (DTPA-LYS), the DTPA mono- or bis-amide derivatives, such as N,N-bis[2-[carboxymethyl[(methylcarbamoyl)methyl]-amino]ethyl] glycine (DTPA-BMA), 1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid (DO3A), 10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid (HPDO3A), 4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan-13-oic acid (BOPTA), 2-methyl-1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid (MCTA), ($\alpha,\alpha',\alpha'',\alpha'''$)-tetramethyl-1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid (DOTMA), 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA) and 6-[bis(carboxymethyl)amino]-tetrahydro-6-methyl-1H-1,4-diazepine-1,4(5H)-diacetic acid (AAZTA) and derivatives thereof, as per the international patent application WO 03/008390 incorporated herein by reference.
- Examples of representative chelators and chelating groups contemplated by the present invention are described in WO 98/18496, WO 86/06605, WO 91/03200, WO 95/28179, WO 96/23526, WO 97/36619, PCT/US98/01473, PCT/US98/20182, and U.S. 4,899,755, all of which are hereby incorporated by reference.
- The above compounds, in particular those intended for diagnostic applications, and, even more particularly, those to be used for MRI, may all be prepared according to conventional methods known in the art.

Description of the Figures

- Fig. 1** illustrates the monoclonal antibodies production technique.

Fig. 2 shows the ELISA result of mouse “#1” and mouse “#2” serum after Tn-cBSA immunization tested on biotinylated alginate-Tn conjugated compound (Alg-Tn); alginate (Alg) and Tn antigen (Tn) are the used references.

5 **Fig. 3** shows an example of anti Tn hybridoma selection. In the graph the adsorbance values of clone supernatants tested on biotinylated alginate-Tn conjugated compound are reported.

The following Examples will now disclose the present invention in more detail; other similar applications, even if not explicitly encompassed, are deemed to be within the scope of the present application.

Experimental Section

Materials and methods

N- Hydroxysuccinimide (NHS), N-Acetylgalactosamine, 2-aminobenzoic acid (2-AA), sodium cyanoborohydride and biotin were from Sigma Chemical Co. (St. Louis., MO),
5 Nε-(+)-biotinyl-L-lysinehydrazide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2-morpholinoethanesulphonic acid (MES) monohydrate were from Fluka (Buchs, Switzerland), α-N-Acetylgalactosaminidase from *Acremonium sp.* was from Seikagaku (Tokyo, Japan), PBS (Phosphate-buffered saline; Cambrex Bio Science, Verviers, Belgium), Tween 20 (Sigma-Aldrich, St. Louis, MO), Ovalbumin (Sigma-
10 Aldrich, St. Louis, MO) ABTS peroxidase substrate (Sigma-Aldrich, St. Louis, MO), anti mouse IgG-Peroxidase conjugated (Amersham Biosciences, Freiburg, Germany), Streptavidin (Sigma-Aldrich, St. Louis, MO).

Capillary electrophoresis

15 High-performance capillary electrophoresis system was from Hewlett-Packard (Agilent, Waldbronn, Germany), Model HP3DCE, with HP Chemstation software.

Derivatization of GalNAc (standard or arising from hydrolysis of BSA-Tn) with 2-AA was carried out by mixing an aqueous solution of the sugar with the derivatizing solution (0.25 M 2-AA, 0.159 M sodium cyanoborohydride in 99% methanol and 1%
20 acetic acid). The solution was heated at 60°C for 5 hours. Before capillary electrophoresis analysis, the samples were diluted five times in water.

The analysis conditions are reported in Table A. Before each run, a capillary wash run with sodium hydroxide (2 min, 985 mbar) and with working buffer (4 min, 985 mbar) was performed.

HPLC-UV

Column	Synergy Polar (4.6 x 250 mm; 5 µm) from Agilent
Column temperature	RT or 45 °C (see text for details)
Mobile phase	Eluent A: CH ₃ OH Eluent B: 5 mM CH ₃ COONH ₄ pH 3.5
Elution profile	0-8': 3%A, 97%D; 13': 40%A, 60%B, 20':90%A,10%B;38': 90%A, 10%B; 45': 3%A, 97%B
Flow rate	1 mL/min
Injection volume	10 µL
Detection with PDA detector	Run time: 30 min
	Scan wavelength: 190-800 nm
	Channel A: 195 nm

Table 1- HPLC-UV experimental conditions

- 5 *Instruments:* HPLC-UV Merck-Hitachi equipped with a L-6200. Pump, an AS-200A Autosampler and a L-4500A Diode Array Detector. Data acquisition and analysis was performed by D-700 Chromatography Data Station software (Merck- Hitachi).

HPLC-MS

Column	Synergi Polar – RP 80A (2.0 x 250 mm; 4 µm) from Phenomenex
Column temperature	45 °C
Mobile phase	Eluent A: CH ₃ OH Eluent B: 5 mM CH ₃ COONH ₄ pH 3.5
Elution profile	0-8': 3%A, 97%D; 13': 40%A, 60%B, 20':90%A,10%B;38': 90%A, 10%B; 45': 3%A, 97%B
Flow rate	200 µL/min
Injection volume	10 µL
Detection with PDA detector	Run time: 45 min
	Scan wavelength: 200-500 nm
	Channel A: 195 nm
Detection with MS detector	Run time: 45 min
	ESI voltage: 5 kV
	Polarity: positive
	Capillary temperature: 250 °C
	Sheath gas nature and flow: N ₂ at 60 unit
	Auxiliary gas nature and flow: not used
	Capillary voltage: 46 V
	Tube lens offset: 55 V
	Mass range: 50-2000

Table 2- HPLC-MS experimental conditions

- 5 *Instrument:* HPLC SURVEYOR Thermo Finnigan system equipped with MS Pump, Autosampler and PDA Detector, coupled with a LCQ DECA XP Plus Thermo Finnigan mass spectrometer with Electrospray Ionisation (ESI). The Xcalibur software was used for managing system and data.

10 Example 1Preparation of cBSA-Tn conjugated compound

Tn antigen (1 mg) and cBSA (2 mg) were dissolved in a solution containing MES buffer (0.2 M), pH 6; EDC and NHS were added in a slight stoichiometric excess, and the

mixture was stirred for 2 hours. The solution was then dialyzed and freeze-dried and the title compound was recovered accordingly.

By working in analogous way and by using any suitable antigen and any polymeric scaffold of the invention, the following compounds can be obtained:

- 5 -alginate-Tn conjugated compound;
- dextran-Tn conjugated compound;
- cBSA-TF conjugated compound;
- alginate-TF conjugated compound;
- dextran-TF conjugated compound.

10

Example 2

Preparation of biotinylated alginate-Tn conjugated compound

1) Biotinylation of alginate

Alginate isolated from *Laminaria hyperborean* was provided by Protan A/S (Norway) and was purified by precipitation in isopropanol. 30 mg (0.15 mmol-repetitive unit) of alginate were dissolved in 10 mL of MES buffer (0.2 M) at pH 6. Then, EDC and NHS are added in a slight stoichiometric excess; N-ε-(+)-biotinyl-L-lysinehydrazide was then added (3.2 mg) and the solution was stirred for 6-8 hours, then dialyzed and freeze-dried.

20 By working in an analogous way and by using any suitable polymeric scaffold of the invention and any proper biotinylating moiety, biotinylated-cBSA and biotinylated-dextran can be obtained.

2) Derivatization of biotinylated alginate with Tn

14 mg (0.071 mmol) of biotinylated alginate were dissolved in 5.0 mL of 0.2 M MES buffer, pH 6.0. After solubilization, EDC (33.9 mg) and NHS (4.7 mg) were added and, after 10 min, 0.6 mg (1.95×10^{-3} mmol, 0.027 eq) of β-GalNAc-α-O-Ser. The solution was stirred for 8 h, dialyzed and freeze-dried and the title compound was recovered accordingly.

By working in an analogous way by starting with any biotinylated scaffolds as per step (1), those same biotinylated scaffolds can be also derivatised/conjugated with TF antigen.

30

Example 3Determination of the degree of substitution of the conjugated compound of Example 1

The experimental conditions applied are those indicated by the supplier of the enzyme as stated in the above specification.

- 5 The molar substitution degree of the compound of previous Example 1 resulted to be 3%.

Example 4Determination of the degree of substitution of the conjugated compound of Example 2

- 10 The determination of the degree of substitution of the conjugated compounds of the present invention, comprising alginate or dextran as the polymeric scaffold, is carried out by elemental analysis.

The molar substitution degree of the compound of previous Example 2 resulted to be 15%.

15

Example 5Production and selection of anti-Tn and anti-TF antibodies

Anti-Tn and anti-TF antibodies were produced according to the preferred methods disclosed in the description of the invention and here below reported:

- 20 1) Two 8-week-old female BALB/c mice were immunized by 3 intraperitoneal injections of 200 µg of Tn-cBSA properly emulsified in Ribi's (MPL® + TDM Adjuvant System; Sigma); so as to observe the immune response. Three additional intravenous injections of 20 µg of the selected conjugate compound properly diluted in PBS followed;
- 25 2) animals were sacrificed and spleen excised;
- 3) antibody-producing cells were immortalized by somatic cell hybridization (fusion);
- 4) hybrid cells were selected in HAT culture medium;
- 5) ELISA test was performed to obtain the most specific clones; and
- 30 6) subcloning was performed.

Steps 2), 3), 4) and 6) of the above disclosed method were performed according to known methods (see the references in the former description of the invention).

Step 5) was performed according to the method disclosed in the following Example 6.

Example 6

Selection of antibodies

- 5 ELISA test according to the method disclosed in the description of the invention was carried out as follows:
- the microtiter plate wells were coated with 10 µg/ml streptavidin in carbonate buffer 100 mM pH 9,6 over night at 4 °C;
 - after a PBS wash the wells were filled with 100 µg/ml of Tn- biotinylated alginate
 - 10 conjugate and incubated for 1h at 37°C;
 - washing with PBS was performed three times at room temperature;
 - blocking with a PBS Ovalbumin , 1% solution for 1h at 37°C;
 - washing with PBS was performed three times at room temperature;
 - mouse serum hybridoma supernatant was added to the wells and incubated for 1h at
 - 15 37°C;
 - washing with a PBS Tween 0,02% solution for three times;
 - anti-mouse IgG-HRP conjugated secondary antibody was added and incubated for 1h at room temperature;
 - washing with a PBS Tween 0,02% solution for three times at room temperature;
 - 20 - 0,5 mg/ml ABTS peroxidase substrate was added;
 - colorimetric quantification of bound antigen on ELISA plate at 405 nm.

CLAIMS:

1. A compound comprising a plurality of Tn or TF antigens conjugated to a polymeric scaffold selected from the group consisting of cationized bovine serum albumin (cBSA), alginate or dextran, either directly or through any suitable linker.
5
2. A compound according to claim 1, wherein the polymeric scaffold is cBSA.
3. A compound according to claim 1, wherein the antigen is directly linked to the polymeric scaffold.
10
4. A compound according to claim 1, wherein the antigens are conjugated to the polymeric scaffold through any suitable linker.
5. A compound according to claim 4, wherein the linker is selected from the group consisting of either straight or branched, synthetic or naturally occurring, amino acids and derivatives thereof, suitable peptides and carbohydrates.
15
6. A compound according to claim 5, wherein said amino acid or carbohydrate linker comprises from 2 to 10 carbon atoms.
20
7. A compound according to claim 1, wherein a plurality of Tn antigen is directly linked to cBSA.
8. A compound according to any one of claims from 1 to 7 for use as a medicament.
25
9. Use according to claim 8 in the preparation of a vaccine against tumors.
10. A pharmaceutical composition comprising an effective amount of any one of the compounds of claims from 1 to 7 together with a suitable solvent and with one or more pharmaceutically acceptable excipients.
30

11. A method for the treatment of tumors, comprising the administration to a mammal of an effective amount of a compound according to claims from 1 to 7 or of any pharmaceutical composition thereof.

5 **12.** A process for the preparation of a compound of any one of claims from 1 to 7, which process comprises the steps of:

1) dissolving any one of the polymeric scaffolds in a suitable buffered aqueous solution;

2) adding to the solution of step (1) a condensing agent and a suitable amount of Tn or TF antigens, each of which is optionally connected to a suitable linker in the presence of a condensing agent; and

3) mixing and stirring the resulting solution from step (2) for a suitable period of time and recovering the obtained product.

15 **13.** A process for the preparation of anti-Tn antibodies or of anti-TF antibodies, comprising the steps of:

1) BALB/c mice immunization

2) sacrifice of the animals and spleen excision

3) immortalizing antibody-producing cells by somatic cell hybridization (fusion)

20 4) selection of the hybrid cells in HAT culture medium

5) screening by ELISA assay to obtain the most specific clones

6) subcloning;

wherein step (1) comprises injecting a compound according to any one of claims 1 to 7, wherein the antigen is Tn or TF, respectively.

25 **14.** A compound comprising a plurality of Tn or TF antigens conjugated to biotinylated alginate, either directly or through any suitable linker.

15. A compound according to claim 14, wherein biotinylated alginate is directly linked to Tn or TF antigens.

30

16. A process for the preparation of any one of the conjugated compounds of claim 14 or 15 comprising the steps of:

- 1) dissolving alginate in a buffered solution;
- 2) adding to the previously obtained solution a suitable amount of biotin or a biotinylating agent;
- 3) adding to the solution obtained from step (2) a suitable amount of Tn or TF antigens, each of which is optionally connected to a suitable linker selected from the group consisting of either straight or branched, synthetic or naturally occurring, amino acids and derivatives thereof, suitable peptides and carbohydrates; and
- 4) mixing and stirring the resulting solution for a suitable period of time and recovering the obtained product.

17. Use of a conjugated compound according to claim 14 or 15 for the selection of anti-Tn or anti-TF antigens.

18. An ELISA test for the selection of anti-Tn or anti-TF antibodies comprising the steps of:

- 1) coating microtiter well plates with streptavidin;
- 2) immobilizing onto the solid phase obtained from step 1) a conjugated compound of any one of claims 14 or 15.

19. Antibodies produced according to claim 13 and/or selected according to claim 18.

20. Antibodies according to claim 19 further functionalised with a therapeutic or diagnostic moiety.

5

FIG. 1/3

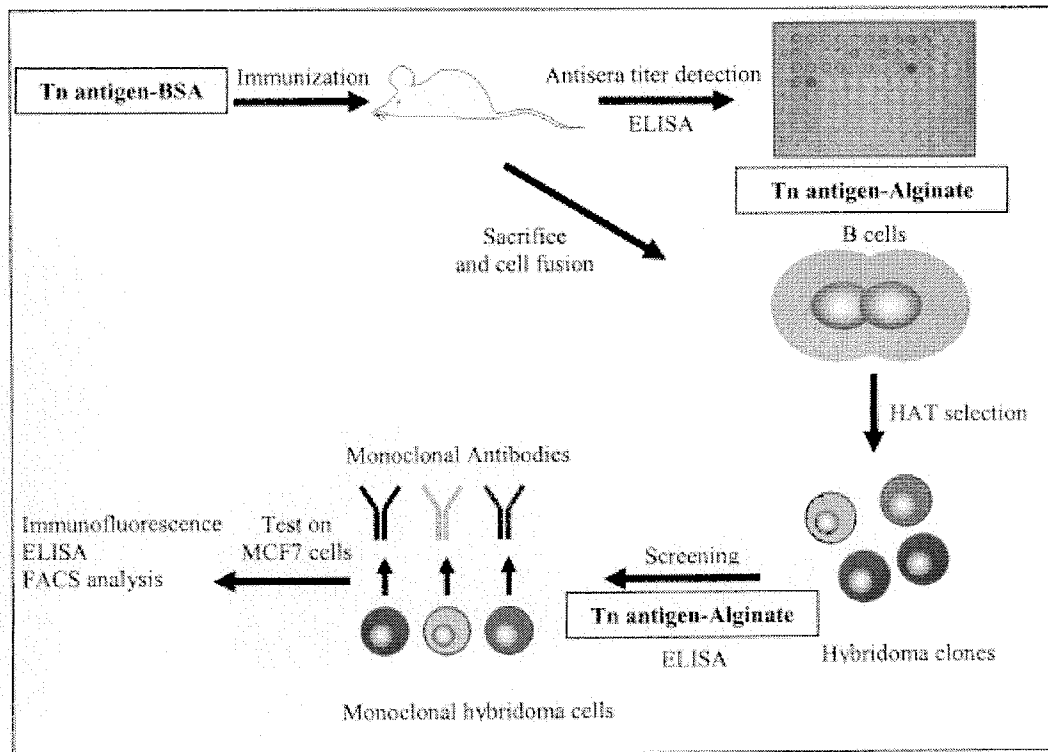


FIG. 1

10

15

FIG. 2/3

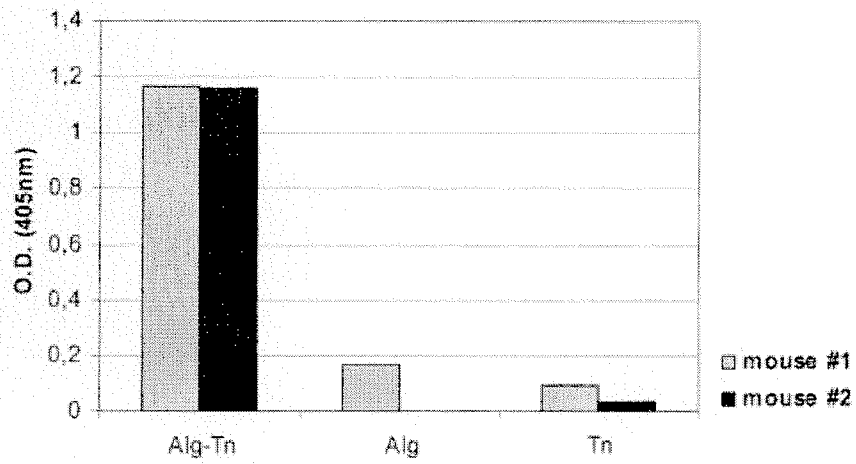


FIG. 2

FIG. 3/3

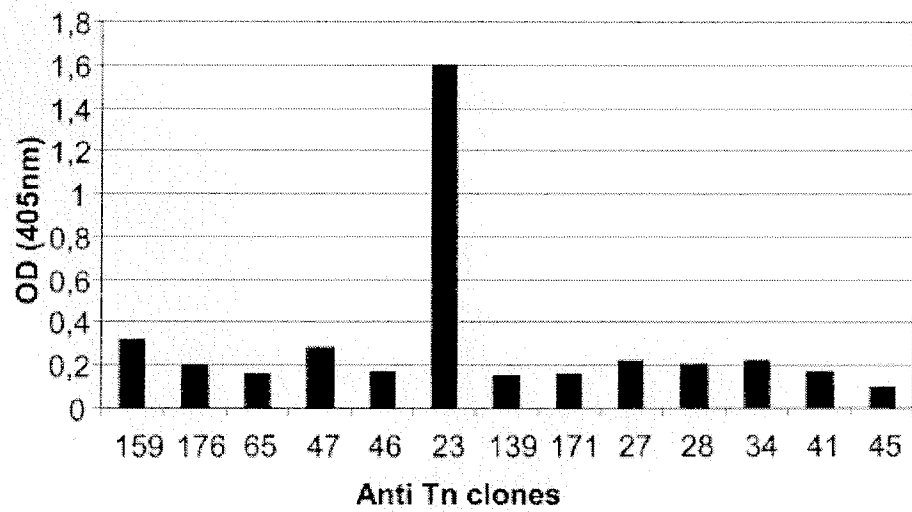


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/056081

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/385 C07K16/30 G01N33/543 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. KUDUK ET AL.: "Synthetic and immunological studies on clustered modes of mucin-related Tn and TF O-linked antigens: The preparation of a glycopeptide-based vaccine for clinical trials against prostate cancer." JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 120, 1998, pages 12474-12485, XP002956518 U.S.A. cited in the application abstract	1-13, 19, 20
Y	----- -/--	14-18

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

21 August 2007

Date of mailing of the international search report

06/09/2007

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Nooij, Frans

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2007/056081

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	T. TOYOKUNI ET AL.: "Synthetic carbohydrate vaccines: Synthesis and immunogenicity of Tn antigen conjugates." BIOORGANIC & MEDICINAL CHEMISTRY, vol. 2, no. 11, 1994, pages 1119-1132, XP000938458 U.K. cited in the application abstract page 1123, right-hand column, last paragraph - page 1125, left-hand column, paragraph 1	1-13, 19, 20
A	----- E. KAGAN ET AL.: "Comparison of antigen constructs and carrier molecules for augmenting the immunogenicity of the monosaccharide epithelial cancer antigen Tn." CANCER IMMUNOLOGY, IMMUNOTHERAPY, vol. 54, no. 5, May 2005 (2005-05), pages 424-430, XP019333116 Germany cited in the application abstract	1-13, 19, 20
A	----- P. DOMEN ET AL.: "Cationization of protein antigens. III. Abrogation of oral tolerance." THE JOURNAL OF IMMUNOLOGY, vol. 139, no. 10, 15 November 1987 (1987-11-15), pages 3195-3198, XP002447216 U.S.A. cited in the application abstract page 3198, left-hand column, last paragraph	1-7, 12, 13
A	----- H. KUNZ ET AL.: "Synthesis of glycopeptides with the TN and T antigen structures, and their coupling to bovine serum albumin." CARBOHYDRATE RESEARCH, vol. 202, 1990, pages 207-223, XP002447217 abstract	1-7
A	----- K. DAHLENBORG ET AL.: "Human monoclonal antibodies specific for the tumour associated Thomsen-Friedenreich antigen." INTERNATIONAL JOURNAL OF CANCER, vol. 70, no. 1, 6 January 1997 (1997-01-06), pages 63-71, XP002447218 U.S.A. abstract	13, 19
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/056081

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99/58978 A (THE UNIVERSITY OF NOTTINGHAM) 18 November 1999 (1999-11-18) examples 5,8 -----	14-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/056081

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 11 (partially)
because they relate to subject matter not required to be searched by this Authority, namely:
Although present claim 11 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2007/056081

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
W0 9958978	A	18-11-1999	AT 314651 T	15-01-2006
			AU 3838899 A	29-11-1999
			DE 69929185 T2	31-08-2006
			DK 1078264 T3	15-05-2006
			EP 1078264 A2	28-02-2001
			ES 2257048 T3	16-07-2006
			PT 1078264 T	31-05-2006