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(71) Applicant(s)  
**Biomembrane Institute**

(72) Inventor(s)  
**Levery, Steven; Stroud, Mark; Hakomori, Sen-itiroh**

(74) Agent / Attorney  
**Davies Collison Cave, Level 15 1 Nicholson Street, MELBOURNE, VIC, 3000**

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(74) Agent: NAKAMURA, Dean; Roylance, Abrams, Berdo & Goodman, L.L.P., 1300 19th Street, N.W., Suite 600, Washington, DC 20036 (US).

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(71) Applicant: BIOMEMBRANE INSTITUTE [US/US]; 720 Broadway, Seattle, WA 98122 (US).

(72) Inventors: LEVERY, Steven; Biomembrane Institute, 720 Broadway, Seattle, WA 98122 (US). HAKOMORI, Sen-Itiroh; Biomembrane Institute, 720 Broadway, Seattle, WA 98122 (US). STROUD, Mark; Biomembrane Institute, 720 Broadway, Seattle, WA 98122 (US).



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(54) Title: EXTENDED TYPE 1 CHAIN GLYCOSPHINGOLIPIDS AS TUMOR-ASSOCIATED ANTIGENS

(57) Abstract: A variety of compounds are provided which are useful as immunogens and as tumor markers. The present invention discloses methods relating to the detection of cancer. Extended forms of the lacto-series type 1 chain are shown to be present in various cancer tissues. The present invention also provides a cell line and the monoclonal antibody produced therefrom. Such an antibody has a number of uses, including in diagnostic or therapeutic methods.

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**Description**

**EXTENDED TYPE 1 CHAIN GLYCOSPHINGOLIPIDS AS  
TUMOR-ASSOCIATED ANTIGENS**

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**Technical Field**

The present invention relates generally to new human tumor-associated antigens. This invention is more particularly related to isolated carbohydrates of extended type 1 chain glycosphingolipids and their uses, e.g., as immunogens and as tumor markers.

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**Background of the Invention**

Despite enormous investments of financial and human resources, cancer remains one of the major causes of death. Current cancer therapies cure only about 50% of the patients who develop a malignant tumor. In most human malignancies, metastasis is the major cause of death.

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Metastasis is the formation of a secondary tumor colony at a distant site. In most human malignancies, distant metastases are often too small to be detected at the time the primary tumor is treated. Furthermore, widespread initiation of metastatic colonies usually occurs before clinical symptoms of metastatic disease are evident. The size and age variation in metastases, their dispersed anatomical location, and their heterogeneous composition are all factors that hinder surgical removal and limit the concentration of anticancer drugs that can be delivered to the metastatic colonies. Therefore, detection of malignancies prior to dissemination of the tumor cells from the primary site is needed to enhance the effectiveness of current cancer therapies.

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Aberrant glycosylation has been observed to be a common feature for most cancer types. Most of the carbohydrate antigens used for the diagnosis of human cancers carry polylactosamine structures, i.e., they contain  $\text{Gal}\beta 1\rightarrow 3/4\text{GlcNAc}$ . Polylactosamines are usually classified into two categories according to their polylactosamine unit structure. The polylactosamine having the  $\text{Gal}\beta 1\rightarrow 3\text{GlcNAc}$  structure is called the type 1 chain, and that having the  $\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}$  structure is referred to as the type 2 chain. The most common tumor-associated antigens found in major human cancers have the lacto-series type 2 chain

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structure, which usually has been sialylated and/or fucosylated. Type 1 chain antigens are abundant in normal cells and tissues, and also are cancer-associated. For example, 2→3 sialylated Le<sup>a</sup> antigen (the CA 19-9 antigen defined by the N19-9 antibody) is a cancer-associated type 1 chain-antigen. However, cancer diagnostic methods based on the 5 detection of these known antigens have been hampered by high false positive and/or high false negative incidences.

Due to the difficulties in the current approaches to the diagnosis of cancer, there is a need in the art for improved compositions and methods. The present invention fills this need, and further provides other related advantages.

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### Summary of the Invention

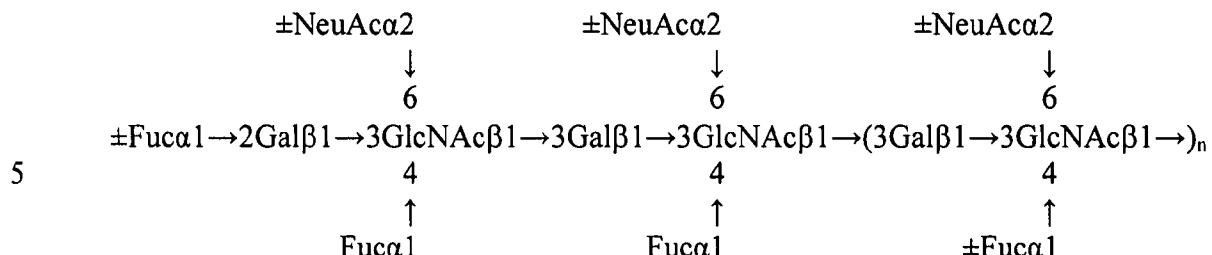
Briefly stated, the present invention provides isolated compounds. In one aspect, the present invention provides an isolated compound having the formula:

15 Gal $\beta$ 1→3GlcNAc $\beta$ 1→3Gal $\beta$ 1→3GlcNAc $\beta$ 1→(3Gal $\beta$ 1→3GlcNAc $\beta$ 1→)<sub>n</sub>3Gal $\beta$ 1→4Glc

wherein n is 0 or an integer of 1 or more, there are at least two fucosyl residues, Gal represents galactose, Glc represents glucose, GlcNAc represents N-acetylglucosamine, wherein said at least two fucosyl residues are linked to the GlcNAc residues via an  $\alpha$ 1→4 linkage and/or to the terminal Gal residue via an  $\alpha$ 1→2 linkage and further comprising one or more N-acetylneurameric acid (sialic acid) residues linked to the terminal Gal residue via an  $\alpha$ 2→3 linkage and/or to one or more of the subterminal GlcNAc residues via an  $\alpha$ 2→6 linkage.

20 In a further aspect, the present invention provides the above-described isolated 25 compound having the formula:

- 3 -

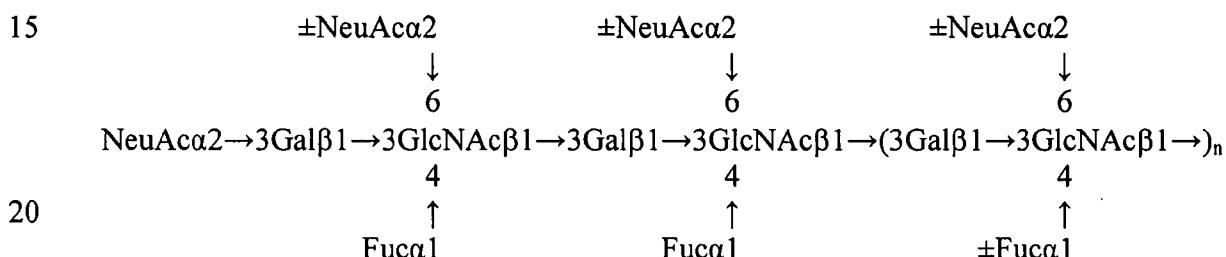


3Gal $\beta$ 1 $\rightarrow$ 4Glc

10

wherein Fuc represents fucose and NeuAc represents N-acetylneuraminic acid.

In another aspect the invention provides the first-described compound having the formula:



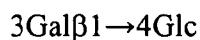
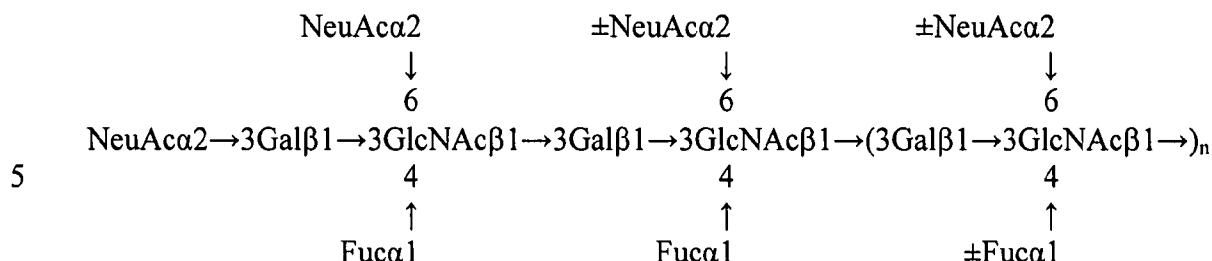
3Gal $\beta$ 1 $\rightarrow$ 4Glc

25

wherein Fuc represents fucose and NeuAc represents N-acetylneurameric acid.

In another aspect the invention provides the first described compound having the formula:

- 4 -

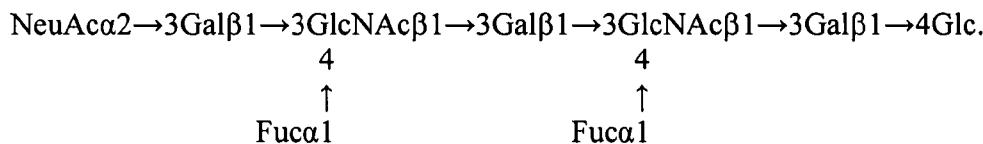


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wherein Fuc represents fucose and NeuAc represents N-acetylneuraminic acid.

Within an even further aspect, the present invention provides an isolated compound comprising an epitope having the formula:

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In yet other aspects, any of the compounds of the present invention may be used as an immunogen for the production of polyclonal or monoclonal antibodies.

In another aspect of the present invention methods for screening for cancer are provided. The methods comprise (a) isolating a biological sample from a warm-blooded animal; and (b) testing the sample for the presence or amount of a compound.

#### Brief Description of the Drawing

Figure 1 is the <sup>1</sup>H-NMR spectrum of extended sialyl Le<sup>a</sup> from chemical shift at 4.20 ppm to 5.60 ppm covering sugar I(Glc), II(Gal), III(GlcNAc), IV(Gal), V(GlcNAc) and

30

VI(Gal as well as fucose linked to III GlcNAc identified as F<sub>III</sub> and fucose linked to V GlcNAc as indicated by F<sub>IV</sub>). In this spectrum, all anomeric proton spectrums of F<sub>V</sub> and F<sub>III</sub> are indicated as F<sub>V</sub>-1 and F<sub>III</sub>-1. In addition, spectrum C5 proton of fucoses are indicated by multiple coupling as indicated by F<sub>III</sub>-5 and F<sub>V</sub>-5. Spectrum marked as Cis is 5 a Cis double bond of sphingosine and R-5 and R-4 indicate spectrum of sphingosine.

### Detailed Description of the Invention

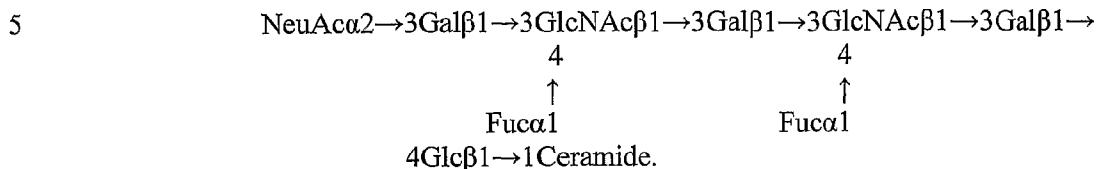
10 The present invention is generally directed towards compounds and methods relating to the detection of cancers. More specifically, the disclosure of the present invention shows that lacto-series type 1 chain occurs in extended forms in cancer tissues.

15 As noted above, type 1 chain lactosamine (Gal $\beta$ 1 $\rightarrow$ 3GlcNAc) is known to be abundant in normal cells and tissues. Although polylactosamine antigens having an extended type 2 chain (i.e., Gal $\beta$ 1 $\rightarrow$ 4GlcNAc core structure is repeated) have been detected, those with an extended type 1 chain have not been detected. Thus, lacto-series type 1 chain has traditionally been considered not to occur in extended form.

20 As disclosed within the present invention, extended forms of lacto-series type I chain (i.e., Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$ 1 $\rightarrow$ [3Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$  $\rightarrow$ ]<sub>n</sub>3Gal $\beta$ 1 $\rightarrow$ R, with or without sialyl and/or fucosyl residues) are present in cancer tissues. Two representative extended forms of 25 lacto-series type 1 chain were isolated by subjecting a glycolipid fraction (extracted from tumor cells) to preparative column and thin layer chromatography.

25 A slow-migrating sialyl-Lewis<sup>a</sup> (sLe<sup>a</sup>) active glycosphingolipid (GSL) was purified to homogeneity from the monosialyl ganglioside fraction of the colonic adenocarcinoma cell line Colo205. This compound was purified by HPLC and preparative HPTLC in two different solvent systems and stained strongly by TLC immunostaining using the  $\alpha$ -sLe<sup>a</sup> monoclonal antibody (MAb) NKH-1. Mild acid hydrolysis (1% acetic acid, 100°C for 1 hour) yielded a faster migrating component that co-migrated with a dimeric-Le<sup>a</sup> standard

GSL and stained strongly by the  $\alpha$ -dimeric Le<sup>a</sup> MAb ST-421. The structure was confirmed by <sup>1</sup>H-NMR spectroscopy as sialyl-dimeric Le<sup>a</sup> (see structure below).



10 In addition to the particular glycolipids depicted above, the  $\text{Le}^a$ - $\text{Le}^a$  and  $\text{Le}^b$ -  
 $\text{Le}^a$  epitopes may be present as extended type 1 chains with additional  
 $[\text{3Gal}\beta 1\rightarrow\text{3GlcNAc}\beta 1\rightarrow]_n$  units. Furthermore, the  $\text{Le}^a$ - $\text{Le}^a$  and  $\text{Le}^b$ - $\text{Le}^a$  epitopes may be  
carried by glycoproteins, e.g., high molecular weight mucin-like sera glycoproteins.

Given the teachings provided herein, it would be evident to those of ordinary skill in the art that other extended forms of lacto-series type 1 chain compounds may be isolated from biological starting materials, such as cancer tissue, or synthesized chemically (and/or enzymatically) following structural identification. Briefly, the structure of carbohydrates bound to either lipids or proteins may be determined based on degradation, mass spectrometry, including electron-impact direct-probe (EI) and fast atom bombardment (FAB), and methylation analysis (techniques described below and, for example, in Nudelman et al., *J. Biol. Chem.* **261**:5487-5495, 1986). Degradation analysis may be accomplished chemically and/or enzymatically, e.g., by glycosidases. The carbohydrate sequence suggested by degradation analysis may be determined by methylation analysis (e.g., Hakomori, *J. Biochem.* **55**:205-208, 1964) followed by chemical ionization mass spectrometry of permethylated sugars (e.g., Stellner et al., *Arch. Biochem. Biophys.* **155**:464-472, 1974; Levery et al., *Meth. Enzymol.* **138**:13-25, 1987). Alternatively, or in conjunction with these techniques, EI mass spectrometry may be performed on permethylated glycans or after the appropriate degradation of intact glycans (e.g., Kannagi et al., *J. Biol. Chem.* **259**:8444-8451, 1984; Nudelman et al., *J. Biol. Chem.* **263**:13942-13951, 1988). Homogeneity of the carbohydrate sequence may be demonstrated based on various chemical and physical criteria, including proton NMR spectroscopy of intact or methylated

glycans and FAB mass spectrometry. Once a carbohydrate structure has been determined, the carbohydrate or derivatives thereof or non-carbohydrate functional equivalents thereof may be synthesized using techniques well known to those of ordinary skill in the art.

The compounds of the present invention may be used as immunogens for the production of polyclonal and monoclonal antibodies (MAbs). Polyclonal antibodies may be produced by standard methodologies. For example, briefly, polyclonal antibodies may be produced by immunization of an animal with a compound of the present invention and subsequent collection of its sera. It is generally preferred to follow the initial immunization with one or more boosters prior to sera collection. MAbs may be generally produced by the 5 method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol. 6:511-519, 10 1976). Briefly, the lymph nodes and/or spleens of an animal immunized with a compound of the present invention are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin and, like the myeloma cells, has the potential for indefinite cell division. An alternative to the 15 production of MAbs via hybridomas is the creation of MAb expression libraries using bacteriophage and bacteria (e.g., Sastry et al., Proc. Natl. Acad. Sci. USA 86:5728, 1989; Huse et al., Science 246:1275, 1989). Selection of antibodies exhibiting a desired specificity may be performed in a variety of ways well known to those of ordinary skill in the art.

It may be desirable to combine a compound of the present invention with a carrier in 20 order to increase their immunogenicity. Suitable carriers include inactivated bacteria, keyhole limpet hemocyanin, thyroglobulin, bovine serum albumin and derivatives thereof. For example, all or a portion of the carbohydrate residues of the GSLs  $Le^a$ - $Le^a$  or  $Le^b$ - $Le^a$  25 may be combined with a carrier. A compound of the present invention may be combined with a carrier by a variety of means, including adsorption and covalent attachment.

A representative example of the use of a compound of the present invention as an 30 immunogen is the immunization of mice with  $Le^b$ - $Le^a$  antigen. In brief,  $Le^b$ - $Le^a$  isolated from Colo205 cells was combined with a suspension of acid-treated Salmonella minnesota, injected via tail vein into BALB/c mice, and the injection repeated three times with 10-day intervals. Following the final injection, splenocytes of immunized mice were harvested and fused with myeloma cells. A hybridoma, IMH2, which showed preferential reactivity with the immunogen, was established and deposited with ATCC (American Type Culture

Collection, 10801 University Boulevard, Manassas, Virginia 20110 USA) as ATCC No. HB 11026. The hybridoma produces a MAb 1MH2 with an IgG3 isotype.

Methods for the detection of extended forms of type 1 chain antigen, such as  $\text{Le}^a$ - $\text{Le}^a$  and/or  $\text{Le}^b$ - $\text{Le}^a$  antigens, may be used to screen for cancers. For example, the GSL  $\text{Le}^b$ - $\text{Le}^a$  and the GSL  $\text{Le}^a$ - $\text{Le}^a$  detected by TLC immunostaining with MAb 1MH2 and MAb NCC-ST-421 (established according to Watanabe et al., *Jpn. J. Cancer Res (Gann)* **76**:43-52, 1985), respectively, of neutral glycolipid fractions prepared from various tumor samples. Such samples include tissue from colonic cancer, breast cancer, Hodgkin's disease, gallbladder cancer and embryonal rhabdomyosarcoma. The GSL  $\text{Le}^a$ - $\text{Le}^a$ , for example, was not detected in glycolipid fractions from normal tissue from spleen, liver, kidney, placenta and lung. Given the teachings provided herein, it would be evident to those of ordinary skill in the art that a variety of means for detecting tumor-associated extended type 1 antigens (including the use of binding partners specific for tumor-associated extended type 1 antigens; such as GSL  $\text{Le}^a$ - $\text{Le}^a$  and  $\text{Le}^b$ - $\text{Le}^a$ ) could be employed within the methods of the present invention. For example, antibodies specific for  $\text{Le}^a$ - $\text{Le}^a$  or  $\text{Le}^b$ - $\text{Le}^a$  epitopes may be produced as described above, and the presence of immunocomplexes may be tested following contact (e.g., incubation) of such antibodies with a biological sample under conditions and for a time sufficient to permit the formation of immunocomplexes.

Detection of the presence of immunocomplexes formed between an antigen described above and an antibody specific for the antigen may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). Suitable immunoassays include the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Patent 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide et al., in Kirkham and Hunter, eds., *Radioimmunoassay Methods* E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Patent 4,452,901); immunoprecipitation of labeled ligand (Brown et al., *J. Biol. Chem.* **255**:4980-4983, 1980); enzyme-linked immunosorbent assays as described by, for example, Raines and Ross (*J. Biol. Chem.* **257**:5154-5160, 1982); immunocytochemical techniques, including the use of fluorochromes (Brooks et al., *Clin. Exp. Immunol.* **39**:477,

1980); and neutralization of activity (Bowen-Pope et al., Proc. Natl. Acad. Sci. USA 81:2396-2400, 1984). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S Patent Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

5 For detection purposes, the antibodies may either be labeled or unlabeled. When unlabeled, the antibodies find use in agglutination assays. In addition, unlabeled antibodies can be used in combination with labeled molecules that are reactive with immunocomplexes, or in combination with labeled antibodies (second antibodies) that are reactive with the antibody directed against the compound, such as antibodies specific for immunoglobulin.  
10 Alternatively, the antibodies can be directly labeled. Where they are labeled, the reporter group can include radioisotopes, fluorophores, enzymes, luminescers, or dye particles. These and other labels are well known in the art and are described, for example, in the following U.S. patents: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

15 In one preferred embodiment for detecting immunocomplexes, a reporter group is bound to the antibody. The step of detecting immunocomplexes involves removing substantially any unbound antibody and then detecting the presence of the reporter group. Unbound antibody is antibody which has not bound to the antigen.

20 In another preferred embodiment, a reporter group is bound to a second antibody capable of binding to the antibodies specific for the antigen. The step of detecting immunocomplexes involves (a) removing substantially any unbound antibody (i.e., antibody not bound to the antigen), (b) adding the second antibody, (c) removing substantially any unbound second antibody and then (d) detecting the presence of the reporter group. For example, where the antibody specific for the antigen is derived from a mouse, the second antibody is an anti-murine antibody.

25 In a third preferred embodiment for detecting immunocomplexes, a reporter group is bound to a molecule capable of binding to the immunocomplexes. The step of detecting involves (a) adding the molecule, (b) removing substantially any unbound molecule, and then (c) detecting the presence of the reporter group. An example of a molecule capable of binding to the immunocomplexes is protein A.

30 An alternative to the use of labeled antibodies, labeled second antibodies or labeled molecules reactive with immunocomplexes generally, is an immunoassay employing a labeled antigen. In such an assay ("indirect" or "competitive"), an antigen present in a

sample will compete with labeled antigen for the antibodies.

It will be evident to those of ordinary skill in the art that a variety of methods for detecting immunocomplexes may be employed within the present invention. Reporter groups suitable for use in any of the methods include radioisotopes, fluorophores, enzymes, luminescers, and dye particles. Further, it will be appreciated that binding partners (other than antibodies) specific for tumor-associated extended type 1 antigens of the present invention may be used to test for such antigens and that complexes formed between such binding partners and antigens may be detected by techniques analogous to those described above for immunocomplexes.

10 The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

15

### **EXAMPLE 1**

#### **HPTLC IMMUNOSTAINING AND IMMUNOASSAY WITH MAB NCC-ST-421 OF NEUTRAL GLYCOLIPIDS PREPARED FROM TUMORS AND NORMAL TISSUES**

20 A. **Monoclonal Antibodies and Immunoassays**

MAb ST-421 was established as previously described (Watanabe et al., Jpn. J. Cancer Res. (Gann) 76:43-52, 1985). MAb MNH-1, which defines type 1 chain N-acetyllactosamine ( $\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow \text{R}$ ), was prepared in the laboratory of the inventors; MAb 1B2, which defines type 2 chain N-acetyllactosamine ( $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow \text{R}$ ), was established as previously described (Young et al., J. Biol. Chem. 256:10967-10972, 1981). Anti- $\text{Le}^a$  MAb was obtained from Chembimed Ltd. (Edmonton, Alberta, Canada). Anti- $\text{Le}^y$  MAb AH6 was established as previously described (Abe et al., J. Biol. Chem. 258:11793-11797, 1983), and did not show any cross-reactivity with  $\text{Le}^b$ . Anti- $\text{Le}^b$  MAb was purchased from Chembimed Ltd. (Edmonton, Alberta, Canada), and showed cross-reactivity with type 1 chain H. Another anti- $\text{Le}^b$  MAb was

5 purchased from Monocarb (Lund, Sweden), and showed reactivity with  $\text{Le}^b$ , type 1 chain H, and  $\text{Le}^y$ . HPTLC immunostaining was performed using Whatman HPTLC plates (HP-KF) by a modified version (Kannagi et al., *J. Biol. Chem.* **257**:4438-4442, 1982; Kannagi et al., *J. Biol. Chem.* **257**:14865-14874, 1982) of the method originally described by Magnani et al. (Magnani et al, *Anal. Biochem.* **109**:399-402, 1980).

### B. Glycolipid Preparation

All glycolipid samples used were either isolated or synthesized enzymatically. VI<sup>3</sup>NeuAcnLc<sub>6</sub>, IV<sup>3</sup>NeuAcIII<sup>4</sup>FucLc<sub>4</sub>, VI<sup>2</sup>FucnLc<sub>6</sub>, and IV<sup>2</sup>FucLc<sub>4</sub> were isolated from 10 human placenta, liver adenocarcinoma, human type O erythrocytes, and porcine intestine, respectively, after extraction with IHW (55:25:20) followed by Folch partition, DEAE-Sephadex chromatography, and HPTLC on an Iatrobeads 6RS-8010 column (Magnani et al., *J. Biol. Chem.* **257**:14365-14369, 1982; Watanabe et al., *J. Biol. Chem.* **254**:8223-8229, 1979; Hakomori et al., *J. Immunol.* **98**:31-38, 1967; Stellner et al., *Biochemistry* **12**:656-15 661, 1973). nLc<sub>6</sub> and III<sup>4</sup>FucLc<sub>4</sub> were prepared by desialylation of VI<sup>3</sup>NeuAcnLc<sub>6</sub> and IV<sup>3</sup>NeuAcIII<sup>4</sup>FucLc<sub>4</sub>, respectively, by heating the samples at 100°C for 1 hr in 1% acetic acid. IV<sup>3</sup>GlcNAcnLc<sub>4</sub>, IV<sub>3</sub>Galβ1→3-GlcNAcnLc<sub>4</sub>, IV<sup>3</sup>Galβ1→3[Fuc1→4]GlcNAcnLc<sub>4</sub> and IV<sup>3</sup>Galβ1→3[Fuc1→4]GlcNAcIII<sup>3</sup>FucnLc<sub>4</sub>(Le<sup>a</sup>-Le<sup>x</sup>) were prepared by enzymatic 20 synthesis. IV<sup>3</sup>Galβ1→3GlcNAcIII<sup>3</sup>FucnLc<sub>4</sub> was prepared by α-fucosidase treatment of IV<sup>3</sup>Galβ1→3[Fuc1→4]GlcNAcIII<sup>3</sup>FucnLc<sub>4</sub>; i.e., 100 µg of the glycolipid was incubated with 0.2 M citrate buffer (pH 4.5) containing 0.05 units bovine kidney α-L-fucosidase (Sigma Chemical Co., St. Louis, Mo.) for 2 hr at 37°C. IV<sup>2</sup>III<sup>4</sup>Fuc<sub>2</sub>Lc<sub>4</sub>, V<sup>3</sup>III<sup>3</sup>Fuc<sub>2</sub>nLc<sub>6</sub>, and VI<sup>2</sup>V<sup>3</sup>Fuc<sub>2</sub>nLc<sub>6</sub> were prepared biosynthetically by α1→3 fucosylation of IV<sup>2</sup>FucLc<sub>4</sub>, nLc<sub>6</sub>, and VI<sup>2</sup>FucnLc<sub>6</sub> (respectively) as substrates, using α1→3/4 fucosyltransferase from 25 Colo2O5. α1→3/4 fucosyltransferase was solubilized from Colo2O5 cells by homogenization in two volumes of 50 mM Hepes buffer (pH 7.0), 0.5 M sucrose, 1 mM

EDTA, and 1% Triton CF-54 in a Potter-Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 100,000 x g for 1 hr, and the supernatant was concentrated to the original volume of cells by dialysis. The enzyme preparation was stored at -80°C until needed.

Enzymatic  $\alpha$ 1→3/4 fucosylation was performed in a reaction mixture containing 1 mg 5 glycosphingolipid (GSL) substrate, 1 mg deoxytaurocholate, 10  $\mu$ mol MnCl<sub>2</sub>, 25  $\mu$ mol Hepes buffer (pH 7.0), 5  $\mu$ mol CDP-choline, 6  $\mu$ mol GDP-fucose, and 500  $\mu$ l enzyme preparation in a total volume of 1 ml. The reaction mixture was incubated at 37°C for 16 hr, then lyophilized, extracted with isopropanol-hexane-water (IHW) (55:25:20) by sonication, and centrifuged. The supernate was subjected to HPLC on an Iatrobeads 6RS-8010 column 10 using gradient elution of IHW from 55:40:5 to 55:25:20 over 200 min. Two ml fractions were collected and tubes containing the final product were pooled according to HPTLC migration in chloroform-methanol-water 50:40:10. GSL bands were visualized by orcinol spray reagent.

Each GSL with defined structure was characterized by reactivity with specific 15 MAb(s), i.e., Le<sup>b</sup>/Le<sup>a</sup> antigen reacted with anti-Le<sup>b</sup> MAbs but not with anti-Le<sup>y</sup> MAb AH6; Le<sup>y</sup>/Le<sup>x</sup> reacted with AH6 but not with anti-Le<sup>b</sup> nor anti-Le<sup>x</sup> MAbs; Le<sup>a</sup>/Le<sup>a</sup> and Le<sup>a</sup>/Le<sup>x</sup> reacted with anti-Le<sup>a</sup> MAb as well as with MAb ST-421.

### C. TLC Immunostaining

20 TLC immunostaining of neutral glycolipid fractions prepared from various tumor samples showed the presence of a positive band migrating slower than -Le<sup>a</sup>- active ceramide pentasaccharide, and cross-reacting with anti-Le<sup>a</sup> MAb. This band was strongly stained by MAb NCC-ST-421, and was seen in the majority of tumors so far examined. Examples 25 from colonic cancer, breast cancer, Hodgkin's disease, gallbladder cancer, and embryonal rhabdomyosarcoma.

**EXAMPLE 2****ISOLATION OF DIMERIC  $\text{Le}^{\text{a}}$  ANTIGEN AND  $\text{Le}^{\text{b}}\text{-}\text{Le}^{\text{a}}$  ANTIGEN**5    **A. Preparation of Tumor Tissue**

Colo205 cells (ATCC) (Semple et al., *Cancer Res.* 38:1345-1355, 1978) were grown in RPMI 1640 medium containing 10% fetal calf serum. Cells were harvested and passed approximately every 7 days. Cells harvested were trypsinized, centrifuged, washed twice with phosphate-buffered saline (pH 7.4) and counted using a hemocytometer.  $4 \times 10^6$  cells 10 were injected subcutaneously into each of 6 athymic (nude) mice. Tumors (approximately 2 ml each) were excised after 2 weeks and stored frozen at  $-80^{\circ}\text{C}$  until needed.

15    **B. Isolation of the Slow-Migrating,  $\text{Le}^{\text{a}}$ -Active Component (Dimeric  $\text{Le}^{\text{a}}$ ) from Colo205 Tumor**

Approximately 200 g of tumors were extracted with isopropanol-hexane-water (IHW) (55:25:20) followed by Folch partition, DEAE-sephadex chromatography and HPLC on an Iatrobeads 6RS-8010 column. Gradient elution of the upper-phase neutral fraction was performed in IHW from 55:40:5 to 55:25:20 over 200 minutes. Two-ml fractions were collected and pooled according to HPTLC migration in chloroform-methanol-water 20 (50:40:10). The slow-migrating  $\text{Le}^{\text{a}}$ -active fraction (revealed by TLC immunostaining) was further purified by preparative TLC on Merck HPTLC plates (Silica Gel 60, Merck, Darmstadt, Germany) and used for structural characterization.

25    **C. Isolation of  $\text{Le}^{\text{b}}\text{-}\text{Le}^{\text{a}}$  Antigen**

A positive band (by immunostaining with MAb NCC-ST-421 according to Example 1) which migrates just below dimeric  $\text{Le}^{\text{a}}$  antigen was purified using the methods described in section B above.

## EXAMPLE 3

CHARACTERIZATION OF DIMERIC Le<sup>a</sup> AND Le<sup>b</sup> - Le<sup>a</sup> ANTIGENSA. Enzymatic Degradation

5 Enzymatic degradation of 1 mg dimeric Le<sup>a</sup> was performed by sequential hydrolysis with 0.5 units of  $\alpha$ -fucosidase (bovine kidney), 0.5 units of  $\beta$ -galactosidase (jackbean), and 0.5 units of  $\beta$ -N-acetylglucosaminidase (bovine epididymis) (Sigma Chemical Co., St. Louis, Mo.). All reactions were carried out in 0.2 M sodium citrate (pH 4.5) for 4 hr at 37°C in a water bath with shaking. Purification of each degradative product was performed by 10 preparative HPTLC.

B. In vitro Cytotoxicity of IMH21. Cell Lines

15 Colo205 was originally obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 IU/ml penicillin, and 10  $\mu$ g/ml streptomycin. Human epidermoid carcinoma A431 cell line (MacLeod et al., *J. Cell. Physiol.* 127:175-182, 1986) was originally donated by Dr. Carol MacLeod (Gildred Cancer Facility, UCSD School of Medicine, San Diego, CA). This cell line expresses Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup>, Le<sup>y</sup>, and ALe<sup>b</sup> on the EGF receptor (Gooi et al., *Biosci. Reports* 5:83-94, 1985). A431 cells were cultured in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal calf serum, 1 mM glutamine, 110 mg/l sodium pyruvate, 100 IU/ml penicillin, and 10  $\mu$ g/ml streptomycin. 20 Cells (about  $5 \times 10^5$ /ml) were seeded and harvested at confluence by EDTA treatment followed by washing with PBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup>. These were used as target cells in in vitro cytotoxicity assay, or used for testing tumorigenicity in nude mice by 25 subcutaneous inoculation of  $5 \times 10^6$  cells. Human erythroleukemia K562 cells (Lozzio et al.; *Blood* 45:321-334, 1975) were used as controls for natural killer (NK) activity of lymphocytes used in the assay system.

2. **Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC)**

For the ADCC assay, human peripheral blood leukocytes (HPBL) (used as effector cells) were obtained from buffy coat fraction of blood from healthy volunteer donors.

5 Briefly, mononuclear cells were separated by centrifugation through Ficoll-Hypaque gradient solution at 2000 rpm for 20 min (Mishell et al., in Mishell, B.B and Shiigi, S.M. (eds.), Selected Methods in Cellular Immunology, pp. 3-27, W.H. Freeman & Co., San Francisco, CA, 1980). Mouse splenocytes and mouse peritoneal macrophages (effector cells) were prepared as previously described by Mishell et al., with some modification as  
10 follows. Target cells ( $5 \times 10^6$ ) were labeled by incubation with 100  $\mu$ l of  $^{51}\text{Cr}$  for 90 min at 37°C. After washing (3x) and incubation (1 hr at 37°C), cells ( $1 \times 10^6$ /ml) were suspended in RPMI-1640 supplemented with 25 mM HEPES buffer and 3% bovine serum albumin. Twenty  $\mu$ l of labeled cells, 100  $\mu$ l of IMH2 or ST-421, and 100  $\mu$ l of effector cell suspension were mixed into Microtiter U-bottom plates (Corning, NY). Non-specific mouse  
15 Ig (Sigma, St. Louis, MO) was used as a negative control. After 4 hr incubation, the plates were centrifuged (500 x g, 2 min) with a hanging plate-holder assembled in a centrifuge, and radioactivity in 100  $\mu$ l supernatant in each well was measured with a gamma counter. Each experimental group was tested in triplicate. Percent specific lysis was calculated according to the formula  $([A-B] \times 100)/C$ , where A=cpm in lysed experimental cells; B=cpm in  
20 unlysed target cells; C=cpm in total target cells. Spontaneous release never exceeded 15% of maximally releasable labeled radioactivity.

25 For CDC,  $^{51}\text{Cr}$ -release assay was performed using a procedure similar to that for ADCC, except that 100  $\mu$ l of diluted human serum was added as a complement source instead of effector cells. The serum was inactivated at 56°C for 30 min and used as a control. Percent specific lysis was calculated as described above.

Since Colo2O5 cells have been characterized as expressing extended type 1 chain  $\text{Le}^a/\text{Le}^a$  and  $\text{Le}^b/\text{Le}^a$  antigens, which react strongly with MAbs ST-421 and IMH2, respectively, cytotoxic effect of IMH2 against Colo2O5 was evaluated and compared to that of ST-421. Both MAbs showed striking ADCC killing of Colo2O5 cells. This killing was correlated with effector:target cell (E:T) ratio and with MAb concentration. The cytotoxic  
30

effect was maximal at an E:T ratio of 100:1-200:1, and at a MAb concentration of 35-70 µg/ml. Control mouse IgG and other non-specific MAbs showed no cytotoxic effect regardless of E:T ratio or MAb concentration. When the same cytotoxicity test was performed with mouse splenocytes, the corresponding values were only 7% and 17% lysis (E:T ratio 200:1, MAb concentration 30 µg/ml). The MAbs showed a weak cytotoxic effect against A431 cells (Table). Comparison of maximum IMH2-dependent lysis of Colo 205, A431, and K562 cells is shown in the Table. High lysis values (e.g., 65% and 94% lysis of Colo205 cells with IMH2 and ST-421, respectively) were only pronounced in the presence of HPBL; values were much less with mouse splenocytes, as observed previously with ST-421 (Watanabe et al., *Cancer Res.* 51:2199-2204, 1991). CDC mediated by IMH2 and ST-421 was similarly correlated with complement concentration and with MAb concentration.

**TABLE** MAb-dependent cytotoxic effect on Colo205, A431, and K562 cells by MAbs ST-421 and IMH2

15

| Target Cell       | Antibody/<br>Reactivity <sup>b</sup> | Percent lysis <sup>a</sup> |                    |                     |
|-------------------|--------------------------------------|----------------------------|--------------------|---------------------|
|                   |                                      | eff. cell +<br>MAb+        | eff. Cell+<br>MAb- | eff. cell -<br>MAb+ |
| Colo205           | ST-421 +                             | 94.5                       | 2.7                | 0.8                 |
|                   | IMH2 +                               | 65.0                       | 2.7                | 0.7                 |
| A431              | ST-421 ±                             | 14.4                       | 10.9               | 1.1                 |
|                   | IMH2 ±                               | 7.6                        | 9.2                | 0.6                 |
| K562 <sup>c</sup> | ST-421 -                             | 48.2                       | 36.2               | 0.5                 |
|                   | IMH2 -                               | 44.8                       | 36.2               | 0.3                 |

a. Percent lysis at E:T (effector:target cell) ratio of 100:1 with IMH2 (35 µg/ml) and ST-421 (x100 diluted ascites).

b. Determined by flow cytometry. +, positive; ±, weakly positive; -, negative.

c. The high cytotoxic effect of K562 cells is also observed in the absence of MAb, and is considered to reflect natural killer cell activity.

25

**C. In vivo Tumor Suppression**

Colo205 and A431 cells used for in vivo experiments were grown in vitro, washed 2x with medium, and reconstituted at the desired cell density in PBS. Cells ( $5 \times 10^6$ /100  $\mu$ l) were subcutaneously injected into the backs of 5- to 7-week-old athymic BALB/c mice, and 5 intraperitoneal administration of MAb was started immediately after injection. Purified IMH2 (1.1 mg/ml) or ST-421 in ascites fluid with corresponding concentration of IgG (1.1-1.2 ng/ml) at a dosage of 0.2 ml/animal were intraperitoneally injected 1x/day for 2 weeks. Width and length of tumors were measured by the same observer 3x/week. Tumor weight 10 was estimated as (width<sup>2</sup> x length)/2. Control animals received ascites protein produced by mouse myeloma cell line NS1 in BALB/c mice. Seven mice per group were used for each experiment, and experiments were run in duplicate. Mean values of tumor weight based on the duplicate experiments were plotted.

Both MAbs IMH2 and ST-421 showed striking inhibition of Colo205 tumor growth in nude mice. In contrast, both MAbs showed minimal inhibitory effect on A431 tumor 15 growth. Thus, high expression of the defined antigen appears to be essential for susceptibility to antibody-dependent inhibition of tumor growth in vivo.

**D. Reactivity of IMH2 With Various Tumors and Normal Tissues**

Various tumors and adjacent normal tissues were obtained from surgical specimens 20 fixed with formalin and paraffin-embedded. In addition, normal tissues and some tumor tissues from brain, thymus, lung, liver, stomach, colon, kidney, adrenal gland, spleen, pancreas, uterus (with endometrium), and skin were obtained by fresh necropsy from accident victims. Both surgical and necropsy specimens were provided through the courtesy 25 of the Department of Pathology, Swedish Medical Center, Seattle, WA, and Ms. Debbie Bennett of The Biomembrane Institute. Samples were sectioned (3  $\mu$ M thickness), deparaffinized with zylene, dehydrated in ethanol, treated with primary MAb, subsequently treated with biotinylated secondary MAb and peroxidase-conjugated avidin, and stained with 3',3'-diaminobenzidine. Endogenous peroxidase activity was blocked by treatment of sections with 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min. Some sections were incubated with mouse IgG as a 30 negative control. Biotinylated goat anti-mouse IgM, avidin, and biotin were from Vectastain

(Burlingame, CA).

MAb IMH2 reacted strongly and with high incidence with tumors from colon, rectum, liver, pancreas, and endometrium (Table I). In contrast, it showed no reactivity with normal mucosae of distal colon and rectum, including crypt regions and goblet cells. It reacted with 5 lung adenocarcinoma, but not with large cell or small cell carcinoma. One out of 5 cases of squamous cell carcinoma showed strong positive reactivity. MAb IMH2 did not react with tissues of normal brain, lung, spleen, skin, or with various blood cells including granulocytes.

Observed locations of normal tissues with strong staining were as follows: Hassall's 10 bodies and epithelial reticular cells of thymus (thymocytes were negative); mucous epithelium and secretory glands of gastric mucosa (lamina propria, serosa, and muscle layer were negative); both medulla and cortex of adrenal glands. Locations of normal tissues with moderate to weak positive staining were: epithelial cells of proximal and distal convolutions of kidney (other parts were negative); cells in Langerhans' islets in pancreas (other parts of 15 pancreas were negative); cecal mucosa; urothelium. Very weak staining was observed for hepatocytes (other parts of liver, infralobular connective tissue, central vein, bile duct, and Kupffer's cells were negative). These results are summarized in Table I.

**TABLE I.** Immunohistological staining by MAb IMH2 of normal tissues and carcinomas.

|               | Tissue       | Staining | Localization/comments           |
|---------------|--------------|----------|---------------------------------|
| <u>Normal</u> |              |          |                                 |
|               | brain        | -        |                                 |
| 25            | lung         | -        | including broncheolar epithelia |
|               | spleen       | -        |                                 |
|               | rectum       | -        | including crypt area            |
|               | colon        | -        | -11/12, ± 1/12                  |
|               | cecum        | +        |                                 |
| 30            | skin         | -        |                                 |
|               | granulocytes | -        |                                 |
|               | lymphocytes  | -        |                                 |

|    |                    |     |                                                                        |
|----|--------------------|-----|------------------------------------------------------------------------|
|    | pancreas           | +   | + in islets of Langerhans; others-                                     |
|    | liver              | ±   | faintly + /± hepatocytes; others --                                    |
|    | thymus             | ++  | ++ in Hassal's bodies, epithelial and reticular cells; - in thymocytes |
| 5  | stomach            | +++ | mucosa, glandular cells (see text)                                     |
|    | kidney             | +   | weakly + in tubular epithelia (see text)                               |
|    | adrenal glands     | +++ |                                                                        |
|    | uterus/endometrium | -/+ | - or weakly + in endometrium; -                                        |
| 10 |                    |     | 9/15, ± 2/15, + 4/15 (total positive cases 4/15 = 27%)                 |

#### Carcinomas

|    |                     |        |                                                                  |
|----|---------------------|--------|------------------------------------------------------------------|
|    | colon/rectum        | ++/+++ | ++++ 4, ++ 6, + 4, ± 1, - 1 (total positive cases 14/16 = 88%)   |
|    | liver (primary)     | ++     | 2/3                                                              |
| 15 | pancreas            | +++    | 2/2                                                              |
|    | lung adenocarcinoma | ++     | 2/4                                                              |
|    | squamous            | +      | 1/5                                                              |
|    | large cell          | -      | 0/3                                                              |
|    | small cell          | -      | 0/5                                                              |
| 20 | endometrium         | -/+++  | +++ 4, ++ 11, + 6/24, ±/-3<br>(total positive cases 21/24 = 88%) |

25      **E. Reactivity of IMH2 With Normal and Malignant Colonic and Bladder Tissues From Patients With Known Lewis and Secretor Status**

Expression of  $Le^b$  and  $Le^y$  determinants is correlated with secretor status of the individual (Sakamoto et al., Molec. Immun. **21**:1093-1098, 1984; Ørnloft et al., J. Urol. **138**:171-176, 1987), whereas expression of Lewis antigens in some tumors is unrelated to host Lewis status (Ørnloft et al., Lab. Invest. **58**:576-583, 1988; Ørnloft et al., Blood **77**:1389-1396, 1991). Therefore, reactivity of MAb IMH2 with normal and malignant

colonic and bladder tissues from patients with known Lewis and secretor status was studied. Results are summarized in Tables II and III. IMH2 was reactive with rectal tumors but not with normal rectal tissue, and this reactivity was unrelated to secretor status. Conversely, IMH2 was reactive with normal cecum but less so with the single cecal tumor sample studied. These results suggest that the trend of IMH2 epitope expression in normal and malignant colonic tissues is similar to the well-established expression pattern of ABH antigens. Genuine Lewis-negative ( $Le^{a-b-}$ ) individuals (Ørntoft et al., *Lab. Invest.* **58**:576-583, 1988), expressed IMH2 epitope in both normal and malignant colonic tissues (Tables II and III).

IMH2 epitope is expressed in normal urothelium, but its expression is diminished to varying degrees in bladder tumors. There seems to be a correlation with grade of atypia, i.e., IMH2 epitope expression is lowest in highly invasive tumors. Again, this trend is similar to that of ABH antigen expression in normal and malignant bladder tissues. However, in contrast to colonic tissues, IMH2 epitope expression in bladder tissues from blood group A individuals is correlated with secretor status. Genuine Lewis-negative ( $Le^{a-b-}$ ) individuals expressed IMH2 epitope in both normal and malignant bladder tissues.

**TABLE II.** Immunohistological staining by MAb IMH2 of normal and malignant colonic tissues: Relationship with host Lewis status.

|    |                                | Normal |                  | Malignant |       |
|----|--------------------------------|--------|------------------|-----------|-------|
|    |                                | rectum | cecum            |           |       |
|    |                                |        |                  | rectum    | cecum |
| 20 | A Le <sup>a-b+</sup>           | 0/5    | 1/1              | 3/4       | 1/1   |
|    | A Le <sup>a+b-</sup>           | 0/4    | ND               | 2/2       | ND    |
|    | O Le <sup>a-b+</sup>           | 0/2    | ND               | 2/3       | ND    |
|    | O Le <sup>a+b-</sup>           | 0/2    | ND               | 1/1       | ND    |
| 25 | genuine Le <sup>a-b-</sup>     | 0/1    | 1/1 <sup>a</sup> | 1/1       | 0/1   |
|    | non-genuine Le <sup>a-b-</sup> | 0/2    | ND               | 1/1       | 0/1   |

Figures indicate positive specimens divided by total specimens examined. ND = not determined. For Le<sup>a-b-</sup> individuals (genuine and non-genuine), phenotypic status was determined by  $\alpha 1 \rightarrow 4$  fucosyltransferase activity in saliva, and erythrocyte reactivity with anti-Le<sup>a</sup> and -Le<sup>b</sup> MAbs. Definitions of phenotypes may be found in Holmes et al., *Arch. Biochem. Biophys.* 274:14-25, 1989, and Ørntoft et al., *Lab. Invest.* 58:576-583, 1988.

<sup>a</sup> Non-secretor.

10 **TABLE III.** Immunohistological staining by MAb IMH2 of normal and malignant bladder tissues: Relationship with host Lewis status.

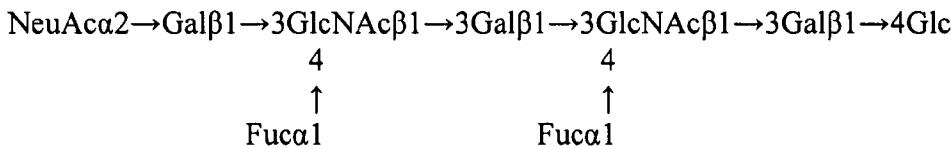
|    |                                | Bladder carcinoma |             |          |
|----|--------------------------------|-------------------|-------------|----------|
|    |                                | normal            | noninvasive | invasive |
| 15 | A Le <sup>a-b+</sup>           | 4/4               | 1/1         | 1/2      |
|    | A Le <sup>a+b-</sup>           | 0/2               | 1/2         | 1/3      |
| 20 | O Le <sup>a-b+</sup>           | 1/1               | 1/1         | 1/2      |
|    | O Le <sup>a+b-</sup>           | 2/2               | 1/1         | 0/1      |
|    | genuine Le <sup>a-b-</sup>     | 2/2               | ND          | 0/1      |
|    | non-genuine Le <sup>a-b-</sup> | ND                | ND          | ND       |

25 Main footnote as for Table II.

**F. Isolation of Extended sialyl-Le<sup>a</sup> (or S Le<sup>a</sup> - Le<sup>a</sup>)**

30 Examination of monosialo-ganglioside fraction of Colo 205 cells led to isolation or resulted in isolation of one major ganglioside by a high performance thin layer chromatography technique. The major band was extracted and characterized. The structure was identified as:

- 22 -



5

This structure was verified by  $^1\text{H}$ -NMR spectroscopy.

Extended sialyl-  $\text{Le}^a$  on the  $\text{SLe}^a - \text{Le}^a$  structure was verified by enzymatic degradation with sialidase to yield the same compound as  $\text{Le}^a\text{-Le}^a$  as verified by thin layer chromatography as well as immunostaining with monoclonal antibody ST-241. The 10 original sialyl  $\text{Le}^a\text{-Le}^a$  or extended  $\text{Le}^a$  do not show any reactivity with MAb ST-421. However, this compound showed reactivity with MAb directed to sialyl-  $\text{Le}^a$  such as N-19- 9, NKH-1 and NKH-2.

15 The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" and 20 "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

- 23 -

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated compound having the formula:

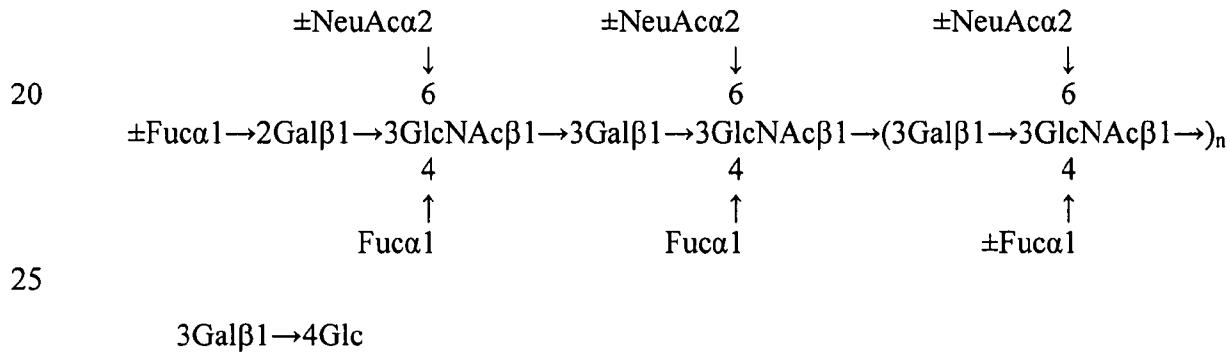
5  $\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow (\text{3Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow)_n 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}$

wherein n is 0 or an integer of 1 or more, and there are at least two fucosyl residues, Gal represents galactose, GlcNAc represents N-acetylglucosamine, and Glc represents glucose, wherein said at least two fucosyl residues are linked to the GlcNAc residues via an  $\alpha 1 \rightarrow 4$  linkage and/or to the terminal Gal residue via an  $\alpha 1 \rightarrow 2$  linkage and further comprising one 10 or more N-acetylneuraminic acid (sialic acid) residues linked to the terminal Gal residue via an  $\alpha 2 \rightarrow 3$  linkage and/or to one or more of the subterminal GlcNAc residues via an  $\alpha 2 \rightarrow 6$  linkage.

2. The isolated compound of claim 1, wherein there are at least two sialyl residues.

15

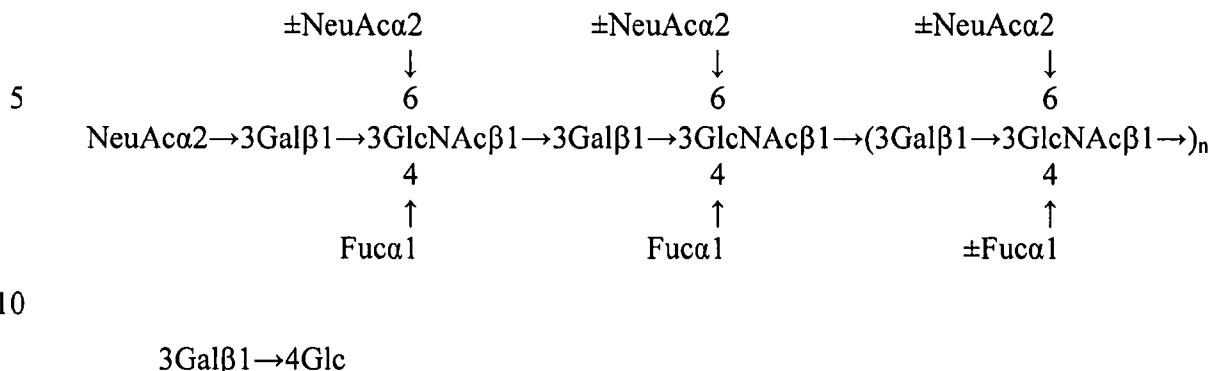
3. The isolated compound of claim 1, having the formula:



wherein Fuc represents fucose and NeuAc represents N-acetylneuramic acid.

- 24 -

4. The isolated compound of claim 1 having the formula:

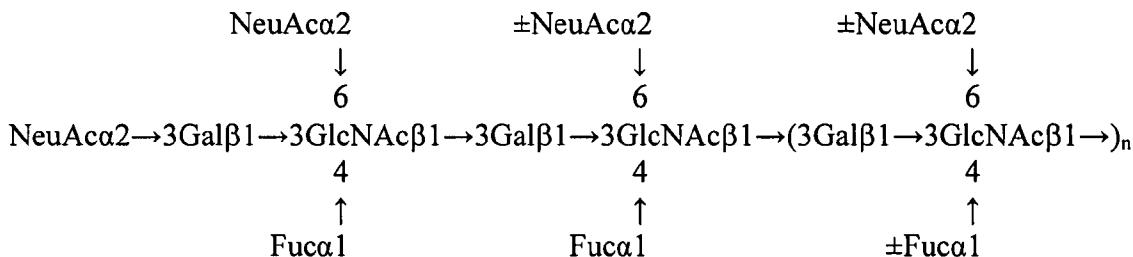


wherein Fuc represents fucose and NeuAc represents N-acetylneuraminic acid.

5. The isolated compound of claim 1 having the formula:

15

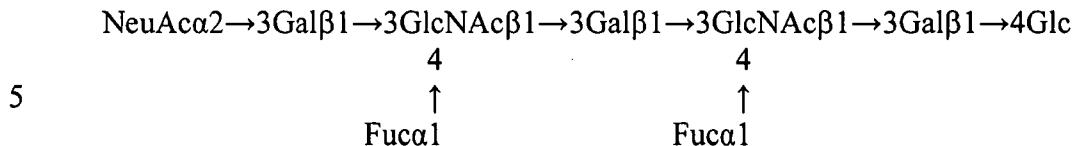
20



3Gal $\beta$ 1 $\rightarrow$ 4Glc

25 wherein Fuc represents fucose and NeuAc represents N-acetylneuraminic acid.

6. An isolated compound having the formula:



wherein NeuAc represents N-acetylneuraminic acid, and Fuc represents fucose.

10 7. An isolated compound substantially as hereinbefore described and/or exemplified.

DATED this 23rd day of September, 2005

## Biomembrane Institute

15 By DAVIES COLLISON CAVE

## Patent Attorneys for the Applicant

220 Colozu<sup>n</sup> mucoganglioside

VI      V      IV      III      II      I  
 NeuAc $\alpha$ 2 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$ 1 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 3GlcNAc $\beta$ 1 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4Glc

A

4

4

Fuc $\alpha$ 1  
 F<sub>VI</sub>

F<sub>III</sub>

F<sub>V</sub>  
 F<sub>I</sub>  
 F<sub>III</sub><sup>-1</sup>

F<sub>III</sub><sup>-1</sup>

Cis

R-5

R-4

F<sub>III</sub><sup>-5</sup>

F<sub>II</sub><sup>-1</sup>, F<sub>II</sub><sup>-1</sup>

F<sub>II</sub><sup>-1</sup>

I<sup>-1</sup>

5.60      5.40      5.20      5.00      4.80      4.60      4.40      4.20

ppm