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The Characterization of Cross-reactive Antibodies to Thomsen-Friedenreich α/β and Related Glycan-conjugates with Polyacrylamide Carriers in Patients with Gastrointestinal Cancer

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Abstract

The level of IgG and IgM antibodies (Abs) to the tumor-associated Thomsen-Friedenreich antigen (TF, Galβ1-3GalNAcα) in the serum of patients with gastrointestinal cancer is reduced and the elevated anti-TF IgG level is positively associated with survival of patients with gastric cancer as shown earlier using ELISA with the TF-polyacrylamide (TF-pAA, an amide-type conjugate). The reactivity of Abs to the standard conjugate TF-PAA is low. To characterize the specificity of Abs, they were affinity-isolated from sera of patients by using different TF-sorbents. These were: 1) IgG populations that differed in the reactivity and cross-reactivity to TF, TFβ (Galβ1-3GalNAcβ), GA1 and $Gb5_{tri}$ (the Gb5 trisaccharide, Galβ1-3GalNAcβ1-3Gal) conjugates. However, all the populations showed a cross-reactivity to the pAA-carrier. 2) The pAA-carrier-independent cross-reactive IgG Abs to TF, TFβ, GA1 and $Gb5_{tri}$ glycans, where TFβ and its cross-reactive TF were minimal ligands to Abs. 3) The pAA-non-reactive IgM Abs whose profile of reactivity was similar to that of population 2 but their specificity to TFβ was lower. In the most samples the Abs were more specific to TFβ than TF conjugates. The terminal Galβ residue was essential for antibody binding. IC_{50} of glycoconjugates was in the range of from 3×10^{-8} to 5×10^{-6} M. GA1-PAA-reactive Abs bound the GA1 glycolipid and weakly bound GM1. No or weak binding of the IgG antibodies to the unrelated antigens used in the determination of polyreactivity was observed. Thus, the antibody populations varied in reactivity and cross-reactivity to TF, TFβ, GA1 and $Gb5_{tri}$. The cross-reactivity of Abs to the pAA-carrier with unsubstituted amide groups may be explained by its spatial similarity to these glycans. The determination of antibody populations using TFβ, GA1 or $Gb5_{tri}$ conjugates instead of TF-pAA may be more informative for diagnostic purposes and monitoring of patients with cancer.

Keywords: Cross-reactive antibodies; GA1; Asialo-GM1; Terminal Gb5 trisaccharide; TF-αβ-polyacrylamide; Gastrointestinal cancer survivors

Abbreviations: TF: Thomsen-Friedenreich Antigen; TFβ: β Anomer of Thomsen-Friedenreich Antigen; ELISA: Enzyme-Linked Immunosorbent Assay; pAA: Polyacrylamide Carrier (Amide Type); PAA: Standard Polyacrylamide Carrier (Ethanolamide Type); sp: Spacer; SSEA-3: Stage-Specific Embryonic Antigen-3; OSM: Ovine Submaxillary Mucin; AOSM: Asialo-OSM; AG: Asialo-Glycophorin; MPG: Macroporous Glass; MAb: Monoclonal Antibody; RPA to GA1: Monospecific Rabbit Polyclonal IgG Antiserum to GA1; BSA: Bovine Serum Albumin; NGS: Normal Goat Serum

Introduction

The expression of tumor-associated carbohydrate antigens (TACA), namely TF (Galβ1-3GalNAcα) and its precursor Tn (GalNAcα) in human malignant tumors, as well as their association with metastases has been described in numerous papers [1,2]. TF and Tn are exposed in tumor cells as epitopes of mucins while they are usually concealed in normal mucosa [1,3]. In humans, carbohydrate antibodies are constantly produced, being inherent in the innate and adaptive immunity. The TF and Tn antibodies are predominately induced by the intestinal flora [4]. The research undertaken by Dr. George Springer marked the beginning of a study of TF and Tn antigens as well as naturally occurring TF and Tn antibodies in cancer [5]. In recent years, only little has been done to systematically evaluate the significance of spontaneously occurring tumor-associated autoantibodies [6]. These antibodies draw attention as diagnostic and prognostic biomarkers in cancer. The immunotolerance of the host to many tumor-associated antigens is a well-known phenomenon. The decreased level of TF antibodies may be indicative of human breast carcinoma [5,7]. The specificity and cross-reactivity of spontaneously occurring antibodies to the tumor-associated TF and structurally related antigens deserve a thorough investigation to elucidate the role of antibodies in targeting antigens.

In immunoassays, synthetic glycoconjugate-models have certain advantages over natural antigens containing usually different determinants. Polyacrylamide (PAA)-glycoconjugates are homogenous antigens with a single reiterative epitope that enables the detection of epitope-specific antibodies [8]. We have performed earlier numerous immunoassays with a 10% TF-pAA (an amide-type conjugate) in donors and patients with cancer. The decreased level of anti-TF IgM and IgG in the serum of patients including patients with the disease in its early stage was observed that is in accordance with results obtained by other researchers who used erythrocyte-derived TF-antigen [7]. However, in some patients, on the contrary, the high level of antibodies to TF and Tn was demonstrated [9-11]. The low level of anti-TF IgG is

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associated with a lower differentiated carcinoma and advanced gastric cancer [12]. The authors have undertaken a long-term follow-up of cancer patients to determine changes in the postoperative level of TF and Tn antibodies, as well as to elucidate the association of this level with the progression of cancer, and survival. A significant postoperative elevation of TF and Tn antibody levels was observed during follow-up [13]. The higher level of anti-TF IgG in primary patients is associated with a benefit in survival [14]. These results prompted us to elucidate the carbohydrate specificity of antibodies and their reactivity to natural glycoconjugates. A TF-similar sequence Galβ1-3GalNAcβ (TFβ) is an external fragment of GA1 and Gb5 glycolipids. In the inhibition assay of anti-TF-positive sera, the cross-reactivity of IgG antibodies to TF-pAA and TFβ-pAA was demonstrated [15]. In our earlier papers, the TF-pAA conjugate was designated as a generally accepted TF-PAA. The aim of the present study was to characterize the specificity of antibodies in cancer survivors having a high level of antibodies to TF-pAA.

Materials and Methods

Patients and serum probes

The investigation was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Research Ethics Committee, Estonia. The informed consent was obtained from patients under examination. A follow-up study of ninety-four patients with gastric and colorectal cancers in stages I-IV with a diagnosis verified by the pTNM system [16] was undertaken earlier. The median age of gastric cancer patients, 59 years (range 28 to 74 years); and of colorectal cancer patients, 60 years (range 41 to 75 years). The patients who received chemo- or X-ray-therapy were excluded from the study. Venous blood serum probes were stored at -50 °C before use. The IgG antibodies from the serum of long-term survivors were affinity-isolated and characterized (Table 1).

Conjugates, saccharides, affinity sorbents and antibodies

The synthetic glycoconjugates and affinity sorbents were obtained from Lectinity, Russia. The following glycoconjugates were used: TF, Galβ1-3GalNAcα; TFβ ($T_{ββ}$), Galβ1-3GalNAcβ; $Gb5_{tri}$ (the terminal trisaccharide of Gb5), Galβ1-3GalNAcβ1-3Gal; GA1 (asialo-GM1), Galβ1-3GalNAcβ1-4Galβ1-4Glcβ; GA2 (asialo-GM2), GalNAcβ1-4Galβ1-4Glcβ; PF_{di} (the terminal disaccharide of the para-Forssman glycolipid, X3b), GalNAcβ1-3GalNAcβ; GalNAcβ; Tn, GalNAcα; $T_{αα}$, Galα1-3GalNAcα; A_{di} (A-disaccharide), GalNAcα1-3Galβ; B_{di} (αGal disaccharide), Galα1-3Galβ. A density of TF epitope in glycoconjugates was 10, 20 or 80 %mol. (TF_{max}), a density of TFβ epitope was 10 %mol., while other glycoconjugates had a density 20 %mol. The soluble glycoconjugates with the N-substituted poly [N-(2-hydroxyethyl)acrylamide] (the ethanolamide-type conjugate, 30 kDa) were designated as glycan-PAA. TF, TFβ and GA1 were conjugates with either PAA or N-unsubstituted polyacrylamide, the amide-type conjugate that was designated as glycan-pAA. The nonconjugated TF-spacer (TF-sp, TF-$O(CH_2)_3NH_2$); Tn-spacer, Tn-$O(CH_2)_3NHCOCF_3$ and free $Gb5_{tri}$ (trisaccharide GLY126, core type 4, ELICITYL, France) were used in the inhibition assay. Tris(hydroxymethyl)aminomethane-PAA (20% Tris-PAA) was used as a negative control and a 20% Glucitol-PAA or a 10% Glucitol-pAA was used as a TF-unrelated conjugate. The binding of antibodies to the pAA-carrier consisting of a 10 %mol. of N-substituted 2-hydroxyethylamide instead of glycan, and a 90 %mol. of the N-unsubstituted amide was investigated as well. Ovine submaxillary mucin (OSM) was purchased from IsoSep AB, Sweden. Asialo-OSM (AOSM) was produced by hydrolysis as described in [17]. Asialo-glycophorin (AG) was purchased from Sigma-Aldrich. Ganglioside GM1a (a sodium salt) and GA1 were purchased from Alexis Biochemicals. Affinity sorbents were TF, Tn, B_{di}, A_{di}, PF_{di} and GalNAcβ ligands conjugated to PAA and covalently attached to the macroporous glass (MPG) or Sepharose FF. The TF-spacer-Sepharose or TF-$O(CH_2)_3NHCO(CH_2)_5NH$-Sepharose (TF-sp-long-Sehparose) without the PAA linkage were also used. Human IgM monoclonal antibody (MAb) TF1 (immunogen AG) was a gift of Dr. Bo Jansson. The monospecific rabbit polyclonal IgG antiserum to bovine GA1 (RPA to GA1) was purchased from LifeSpan BioSciences.

The indirect and competitive ELISA

Assays were performed as described earlier [18]. The antibody level was estimated in the indirect ELISA as a ratio of $A_{test}/A_{control}$, where A_{test} is the absorbance with glycoconjugate and $A_{control,}$ with Tris-PAA. The antibody-isotype reactivity to glycoconjugates was determined using alkaline phosphatase labelled secondary antibodies, *i.e.* goat anti-human IgG or rabbit anti-human IgM or IgA diluted in a 0.05 M Tris HCl/0.2 M NaCl/0.02% sodium azide (TBS), pH 7.5, with a 0.05% Tween-20 and a 0.25% bovine serum albumin (BSA).

The cross-reactivity of antibodies was evaluated by the competitive assay. The inhibition of antibody binding to the adsorbed glycoconjugate by the soluble glycoconjugate or saccharide was evaluated in percentages by using the subtracted value of absorbance with the glycoconjugate and Tris-PAA. The human sera diluted with TBS/0.05% Tween-20/5 mM EDTA/0.25% BSA or purified antibodies diluted in the same buffer without BSA were incubated with the solution of glycoconjugates or saccharides at different concentrations for 2 h at 26°C. The mixture was applied to the wells coated with glycoconjugates and the plate was incubated (2 h, 26°C). The sera or antibodies incubated with the buffer at an appropriate dilution were used as control. After washing, the goat anti-human IgG-phosphatase conjugate and, further, the solution of the p-nitrophenylphosphate disodium salt (NPP) were added as described [18].

The indirect ELISA with GM1 and GA1 glycolipids was carried out as follows. 50 µl of the glycolipid solution in methanol (100 µg/

Code	Diagnosis	Postoperative survival in months	Age	Sex	Blood group	Isotype
KO	Colon cancer, III stage, $pT_3N_1M_0G_3$	> 112	41	F	A	IgG
AM	Gastric cancer, II stage, $pT_3N_0M_0G_3$	> 190	61	F	B	IgG
DM	Gastric cancer, III stage, $pT_3N_1M_0G_2$	> 73	67	M	O	IgG
KT	Colon cancer, III stage, $pT_3N_1M_0G_2$	> 148	54	F	B	IgG
RA	Rectal cancer, III stage, $pT_4N_1M_0G_2$	< 9	40	F	O	IgM

Table 1: The characterization of patients selected for the isolation of antibodies.

ml, test) or methanol (control) were applied to the Nunc-Immuno plate (MaxiSorp). The plate was dried at room temperature and kept overnight at 4°C. The wells were blocked for 2 h at 26°C with 200 µl of TBS/1% BSA containing 0.1% normal goat serum (NGS). The plate was washed with TBS/0.1% BSA and the antibodies diluted in this buffer were applied. The plate was kept for 2 h at 4°C and all further operations were performed at 4°C. The plate was washed with TBS/0.1% BSA and the goat anti-human IgG-alkaline phosphatase conjugate in TBS/0.1% BSA/0.1% NGS was applied. After incubation (1.5 h, 4°C) and washing with cold TBS, the empty plate was kept for 1 h at 26C. The solution of NPP (1 mg/ml) in 0.1 M glycine-buffer, pH 10.3, was added and the plate was incubated for 2 to 4 h at 26°C. The absorbance (A) at 405 nm was measured using a Labsystem Multiscan MCC/340 (Finland).

The competitive ELISA using glycolipids was performed at 26°C. 50 µl of GM1 or GA1 at a concentration of 2 mg/ml in chloroform/methanol (1:2) or solvent (the control) was injected in a glass test tube containing 250 µl of deionised water. The emulsion was evaporated in vacuum at stirring by vortex up to 100 µl and 10 µl of isopropanol was added. 200 µl of 0.05 M Tris HCl, pH 7.5, saturated with n-butanol at 26°C was added and the solution was incubated for 2 min at 80 °C with stirring in the closed test tube. Butanol was added to prevent the formation of micelles [19]. The solution was kept overnight at 4°C. The antibodies were mixed with the solution of glycolipids or the control solution and after incubation for 2 h at 26°C the mixture was transferred to the plate coated with glycoconjugates. Further operations with labeled secondary antibodies and NPP were performed as described [18].

The dot blot immunoassay

0.5 µl of the solution of glycolipids in methanol (2 mg/ml) was put on the rings of the polyvinyl difluoride membrane (PVDF) and dried. The degassed TBS/2% BSA/0.1% NGS was added to the wells of the immunoplate (200 µl/well) and the rings put on the buffer. The plate was kept in vacuum for 2 h at room temperature to remove bubbles. The soaked rings were washed with TBS/0.1% BSA and the isolated TF antibodies or irrelevant isolated human anti-Tn and A_{di} antibodies in the same buffer (100 µl/well) were added. The plate was incubated overnight at 4°C and all further operations were also performed in the cold. The rings were washed with TBS/0.1% BSA and the goat anti-human IgG-alkaline phosphatase conjugate in TBS/0.1% BSA/0.1% NGS or the rabbit anti-human IgM-alkaline phosphatase conjugate in TBS/0.1% BSA/0.1% normal rabbit serum was applied. The rings were incubated for 1.5 h and washed with TBS. Then the rings were transferred to the wells with the solution of a 5-bromo-4-chloro-indolylphosphate disodium salt (BCIP, 0.2 mg/ml) and nitroblue tetrazolium (NBT, 0.4 mg/ml), (Sigma-Aldrich) in 0.1 M Tris HCl/0.1 M NaCl/5mM $MgCl_2$/0.02% sodium azide, pH 9.5. The rings were kept for 0.5 to 1 h at 4°C and washed.

The isolation of antibodies

The antibodies were purified from the serum of patients with gastrointestinal cancer by using TF-, Tn-, GalNAcβ-, PF_{di}-, B_{di}- and A_{di}-PAA or TF-sp without a PAA covalently bound to MPG or Sepharose FF [18]. All serum probes were taken in an excess amount as the serum passing through the column contained the remaining antibody reactivity to glycoconjugates. The admixture of IgA and IgM was separated from the affinity-purified IgG by immunoadsorbtion on the goat-anti-human IgA (α-chain specific)- and/or goat-anti-human IgM (µ-chain specific)-agarose (Sigma-Aldrich). The affinity-purified IgM antibodies were separated from IgG by using Protein-G agarose (Pierce). The concentration of antibodies in the purified samples was determined by ELISA [18]. The admixture of αGal antibodies was present in some IgG samples affinity-purified on the TF-sp-Sepharose. The admixture was separated by immunoadsorbtion on the B_{di}-PAA-Sepharose.

Results and Discussion

The cross-reactive antibodies to the pAA-carrier and TF, TFβ, GA1 and $Gb5_{tri}$ conjugates. The populations IgG-I.

In general, the antibodies of some serum probes and isolated antibodies were highly reactive and cross-reactive to pAA conjugates. However, low or no reactivity and cross-reactivity to PAA conjugates except individual PAA-conjugated TF, Tn, TFβ, GA1 or $Gb5_{tri}$ glycans was observed.

The higher reactivity was mainly associated with the binding to pAA itself and cross-reactive glycans TF, TFβ, GA1 and $Gb5_{tri}$ as was confirmed in the competitive ELISA of isolated antibodies. The antibodies were highly cross-reactive to TF-pAA and pAA (Figure 1, strip TF). Moreover, being reactive to the former, the serum probes tested also revealed reactivity to the latter with a good correlation

No	Glycoconjugates	r	P	n
1	10% TFβ-PAA *vs* 20% $Gb5_{tri}$-PAA	0.88	< 0.01	21
2	10% TFβ-PAA *vs* 20% GA1-PAA	0.47	0.03	23
3	20% GA1-PAA *vs* 20% $Gb5_{tri}$-PAA	0.51	0.02	21
4	10% TF-pAA *vs* 20% GA1-PAA	0.46	0.04	21
5	pAA vs 20% GA1-PAA	0.37	0.08	18
6	10% TF-pAA *vs* pAA	0.85	< 0.01	18
7	10% TF-pAA *vs* pAA	0.96	<0.001	15

Table 2: The correlation between A_{405} values of the indirect ELISA. The determination of serum IgG (Nos 1-6) and IgM (No 7) antibodies to glycoconjugates.

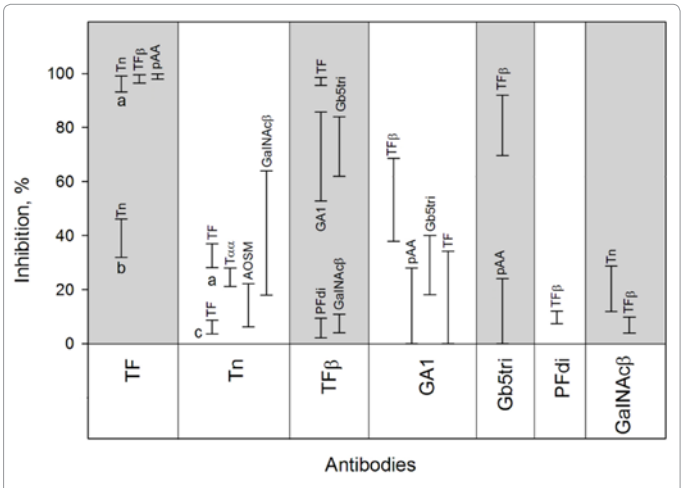

Figure 1: The cross-reactivity of IgG antibodies to structurally related glycoconjugates as established in the competitive ELISA using serum probes. The glycoconjugates adsorbed onto the plates are shown under the horizontal zero axis. In the figure, the glycoconjugates-inhibitors are written over intervals of inhibition. The interval designates the extreme values of inhibition for the four sera used. The binding activity of antibodies was calculated as (A_{test} minus $A_{control}$). A_{test} is the absorbance with the PAA glycoconjugate; $A_{control}$ is the absorbance with Tris-PAA. **a** - TF-pAA /Tn-PAA reactive sera; **b** - TF-pAA reactive/Tn-PAA nonreactive sera; **c** - Tn-PAA reactive/TF-pAA nonreactive sera. TF is a 10% TF-pAA, TFβ is a 10% TFβ-pAA. The other glycoconjugates are conjugates with PAA.

(Table 2). Moderate to high antibody cross-reactivity to TF-pAA and Tn-PAA was observed in serum probes reactive to TF-pAA only (Figure 1, strips TF and Tn, **a**, **b** *vs*. non-reactive sera **c**). Probes of type **b** (Figure 1, strip TF) were used in the isolation of samples 1, 2 and 6 while type **a** was used for samples 3, 4 and 5. In spite of using the TF-PAA sorbent in the isolation of samples 1, 3 and 6 the antibody samples demonstrated a lower reactivity to TF- or TFβ-PAA conjugates than that to pAA and its conjugates. Affinity-isolated on TF-sorbents, the IgG antibodies of types **a** and **b** were weakly or not reactive to Tn-PAA (Table 3). The Tn-monoreactive IgG antibodies were purified on the Tn-PAA sorbent from eight mixed serum probes (type **c**) in which TF-pAA reactive antibodies were absent. These antibodies showed the binding to the free saccharide Tn-sp in the inhibition assay [15]. However, the antibodies showed even a higher reactivity to TF-pAA or pAA than to Tn-PAA when they were isolated on the Tn-PAA sorbent from the pAA-reactive serum ((Table 3, sample 4). These antibodies did not bind Tn-sp and exhibited a similar pattern of reactivity to TF- and TFβ-pAA/PAA conjugates as samples 1 and 3 isolated on the TF-PAA sorbent (Table 3). These observations concern serum probes with reactivity to pAA and its conjugates. Worth mentioning is that seven samples of glycan-monoreactive IgG antibodies were isolated from pAA-nonreactive sera by using different glycan-PAA sorbents and none of them showed reactivity to the pAA carrier [15,18,20]. But the antibody reactivity to pAA in sera should be taken into account in the isolation of antibodies because this reactivity in the product may predominate over antibody reactivity to ligands used in affinity chromatography.

In the indirect and competitive ELISA, the IgG antibodies of sample 1 and 3 showed no or low binding to10% TFβ-PAA, the 10% or 20% TF-PAA, the 20% Tn-PAA, AG, and a free saccharides TF-sp and $Gb5_{tri}$. In the low or missing reactivity to TFβ-PAA and AG, samples 1 and 3 were similar to human anti-TF IgG population isolated on the

Conjugate	Sample and key ligand [a]							MAb TF1 IgM	RPA GA1 IgG
	1 TF[b] IgG (KO)	2 TF[c] IgG (KO)	3 TF[b] IgG (AM)	4 Tn[b] IgG (DM)	5 TF[c] IgG (DM)	6 TF[b] IgG (KT)	7 TF[c] IgM (RA)		
10% TF-pAA	100	100	100	399	100	100	100	100	100
10% TFβ-pAA	677	499	207	446	88	173	242	6	47
pAA	218	297	190	350	430	171	0	0	12
TF_{max}-pAA	0	0	NT	0	NT	27	10	139	NT
10% TF-PAA	2	4	0	0	9	6	74	28	NT
10% TFβ-PAA	10	1381	2	12	380	9	157	3	11
20% TF-PAA	8	88	0	0	56	57	413	197	NT
20% GA1-PAA	31	83	4	122	18	14	91	1	600
20% GA2-PAA	0	0	0	0	0	0	0	NT	0
20% Tn-PAA	0	7	11	100	0	0	0	NT	NT
20% $Gb5_{tri}$-PAA	0	843	6	0	71	109	214	27	54
AG	0	175	5	NT	0	189	86	222	NT

[a] The key ligand is a glycan that was used in the isolation of antibodies. The reactivity of antibodies to key-ligand conjugates was taken as 100%.
[b] Antibodies isolated on PAA sorbents.
[c] Antibodies isolated on the TF-sp-sorbents.
NT – not tested.

Table 3: The reactivity of antibodies to glycoconjugates (A_{test} minus $A_{control}$ in %, indirect ELISA).

Inhibitors	Samples				
	1 (IgG) [a]	2 (IgG) [b]	3 (IgG) [a]	5 (IgG) [b]	7 (IgM) [b]
10% TFβ	0.04	0.12	0.03	0.31	3.03
pAA	0.04	UD	0.04	0.09	UD
10% TF	0.63	5.10	0.88	UD	NT
20% TF	1.79	0.90	2.25	NT	UD
20% Tn	2.32	UD	2.84	UD	NT
20% GA1-PAA	2.05	0.59	UD	UD	1.24
20% $Gb5_{tri}$-PAA	UD	1.44	UD	0.56	1.18
Free TF-sp	UD	82	UD	UD	UD
Free $Gb5_{tri}$	UD	46	NT	UD	UD
GA1 glycolipid	UD	58	NT	NT	NT

[a] Antibodies isolated on the TF-PAA-sorbent. Inhibitors are pAA carrier or pAA conjugated glycans except GA1-PAA. The 10% TFβ-pAA was adsorbed onto the plate.
[b] Antibodies isolated on the TF-sp-sorbents. Inhibitors are the pAA carrier or PAA conjugated glycans. The 10% TFβ-PAA was adsorbed onto the plate.
NT - not tested. UD - undeterminable.

Table 4: The IC_{50} values (μM) of ligands to antibodies isolated on TF-sorbents.

TF-PAA sorbent and described by other authors [21] but reactivity to TF-PAA was low. However, the antibody reactivity to TF-PAA and AG was demonstrated in sample 6. The antibodies of samples 1 and 3 were more specific to a 10% TFβ-pAA and pAA than a 10% TF-pAA (Table 4). The affinity of a 20% TF-pAA to antibodies was lower than that of a 10% TF-pAA. Besides, antibodies did not bind TF_{max}-pAA. This may be due to the partial steric hindrance of antibody binding to the pAA-carrier in conjugates containing a higher percentage of TF. The values of the affinity of a 20% Tn-pAA and a 20% TF-pAA were close. These results show that the high IgG reactivity to pAA glycoconjugates is due to the binding of IgG to the pAA-carrier mainly. However, the pAA-reactive IgG antibodies isolated on PAA sorbents (samples 1 and 4) were found to be selectively cross-reactive to GA1-PAA. GA1-PAA showed a potent dose-dependent inhibition of antibody binding to either TFβ-pAA or pAA, moreover, the affinity to antibodies was close in values (Figure 2A, 2.05 *vs* 1.48 μM). No or faint inhibition was observed with other PAA-conjugates including GA2-PAA with a similar structure. The antibodies of samples 1 and 4 bound GA1 glycolipid in dot blot immunoassay and ELISA (see below). The antiserum RPA to the GA1 glycolipid showed a lower reactivity to pAA than TF- or TFβ-pAA (Table 3). Thus, these pAA-reactive populations bound the GA1 ligand. These antibodies were isolated from sera cross-reactive to pAA vs GA1 but antibodies of sample 3 isolated from the non-cross-reactive serum were reactive mainly to the pAA-carrier and its conjugates (Table 3). The lack of the correlation between the levels of antibodies to pAA and GA1-PAA in a number of serum probes tested may be due to the testing of mixed pAA/GA1-cross-reactive and non-cross-reactive probes. But the correlation existed when these probes were tested by the level of antibodies to TF-pAA and GA1-PAA (Table 2). The correlation between the levels of serum IgG antibodies (Table 2) as well as the levels of antibodies to pAA-conjugated TF- *vs* TFβ and TFβ *vs* GA1 observed earlier [15] appeared to be due to the cross-reactivity to both pAA and ligands. The good correlation between the levels of antibodies to TF-pAA and pAA was rather associated with the reactivity of antibodies to the common pAA-carrier (Table 2). The IgG of sample 5 isolated on the TF-sp-long-Sepharose without PAA differed in reactivity from the IgG of sample 4 isolated from the serum of the same individual (DM). The IgGs of both samples were pAA-reactive but the antibodies of sample 5 bound predominately TFβ- and $Gb5_{tri}$-PAA, and were less specific to GA1-PAA whereas antibodies of sample 4 bound GA1-PAA (Table 3, Figure 2B). This points to the isolation of different populations of IgG.

We hypothesized that the spatial structure of pAA mimics that of

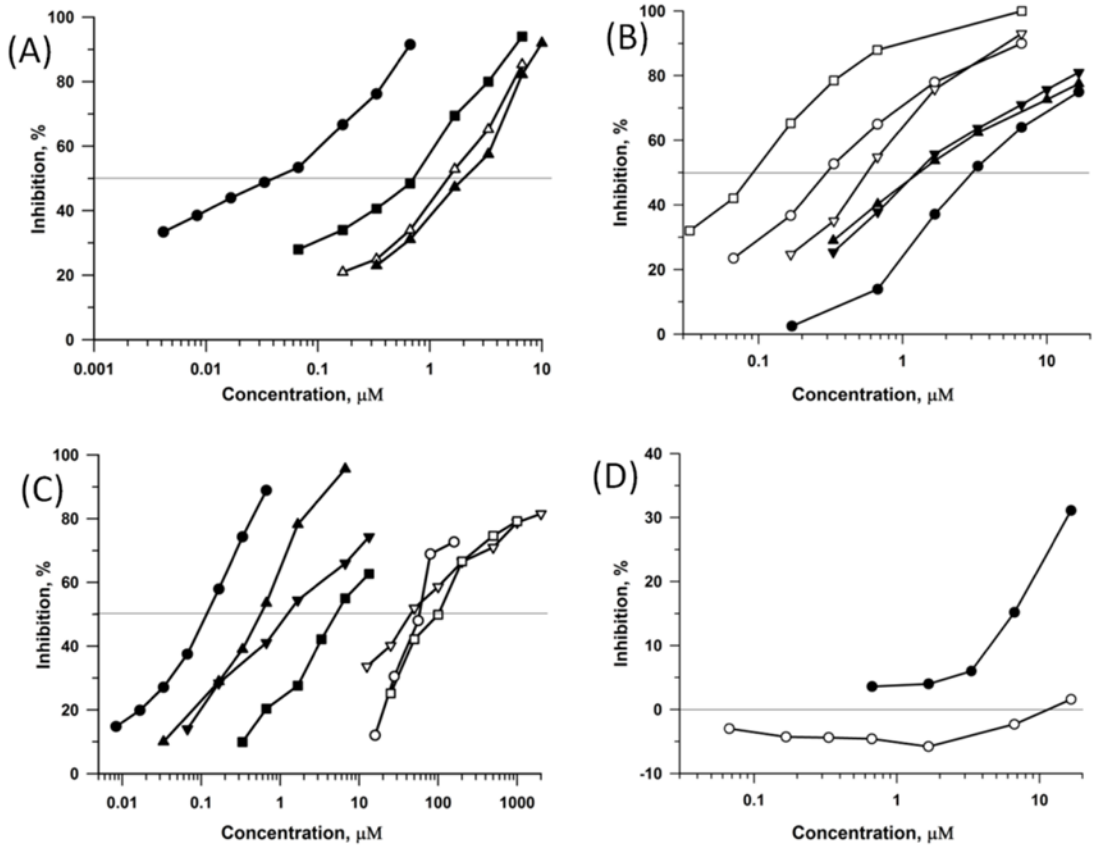

Figure 2: The cross-reactivity of isolated antibodies as established in the competitive ELISA. (**A**) Sample 1. The inhibition of the binding of IgG to the adsorbed 10% TFβ-pAA conjugate by soluble conjugates: 10% TFβ-pAA (•), 10% TF-pAA (■) and 20% GA1-PAA (▲, IC_{50} = 2.05 μM). The inhibition of the antibody binding to the pAA-carrier by the soluble 20% GA1-PAA (△, IC_{50} = 1.48 M). (**B**) The inhibition of the binding of IgG (sample 5) to the adsorbed 10% TFβ-PAA conjugate by soluble conjugates: 10% TFβ-PAA (○), pAA (□) and 20% $Gb5_{tri}$-PAA (▽). The inhibition of the binding of IgM (sample 7) to the adsorbed 10% TFβ-PAA conjugate by soluble conjugates: 10% TFβ-PAA (•), 20% GA1-PAA (▲) and 20% $Gb5_{tri}$-PAA (▼). (**C**) The inhibition of the binding of IgG (sample 2) to the adsorbed 10% TFβ-PAA conjugate by soluble conjugates: 10% TFβ-PAA (•), 10% TF-PAA (■), 20% GA1-PAA (▲) and 20% $Gb5_{tri}$-PAA (▼), free TF-sp (□) and free $Gb5_{tri}$ (▽), and GA1 glycolipid (○). (**D**) The effect of the soluble pAA carrier on the IgG antibodies of sample 2: the inhibition of the antibody binding to the adsorbed 10% TFβ-pAA (•) and 10% TFβ-PAA (○) conjugates.

the determinants of TF, TFβ, GA1 and $Gb5_{tri}$. The unsubstituted amide groups in pAA and the acetamide group in GalNAc of glycans might be involved in the binding and cross-reactivity of antibodies. But the terminated Galβ residue in glycans is essential for antibody binding because all samples did not bind GA2- and PF_{di}-PAA.

Thus, pAA-cross-reactive populations IgG-I (samples 1, 3, 4, 5 and 6) isolated from different individuals were either reactive and cross-reactive to TF-, TFβ-, GA1- or $Gb5_{tri}$-PAA or not. The antibodies of sample 1 recognized the whole GA1 because antibodies bound weakly TFβ (the external moiety of GA1) and did not bind GA2 (the internal moiety). Antibodies of samples 1 and 4 recognized the 1-4 linkage in GA1 as they did not bind the $Gb5_{tri}$ ligand having the 1-3 linkage (Figure 3). The antibodies of samples 5 and 6, on the contrary, bound $Gb5_{tri}$-PAA and only weakly bound GA1-PAA.

The pAA-non-cross-reactive antibodies to TF, TFβ, GA1 and $Gb5_{tri}$ ligands. The population IgG-II and IgM antibodies

To elucidate the role of the pAA-carrier in the antibody cross-reactivity to TF and related ligands, IgG antibodies (sample 2) were isolated on the TF-sp-Sepharose without PAA. The spacer (sp) contained no amide groups either. A serum probe with a high IgG reactivity to pAA was taken from the same individual (KO) whose serum was used in the isolation of sample 1 on the TF-PAA-sorbent. Unlike samples 1, 3 and 4, sample 2 showed a high reactivity to TFβ-PAA and $Gb5_{tri}$-PAA, moreover, the main specificity of antibodies was directed to TFβ-PAA (Tables 3, 4 and Figure 2C). The antibody cross-reactivity to the PAA-conjugated TF, TFβ, GA1 and $Gb5_{tri}$ but not to GA2 was observed in sample 2 (Figure 2C). The isolated IgG antibodies showed reactivity to pAA and pAA-conjugated TF and TFβ (Table 3, sample 2). However, pAA inhibited antibodies only partly, i.e. inhibited antibody binding to TFβ-pAA but not to TFβ-PAA (Figure 2D). This is indicative of the presence of different antibody populations: pAA-reactive (minor) and non-reactive (major). The reactivity of pAA-non-reactive population was associated with carbohydrate ligands because free saccharides TF-sp and $Gb5_{tri}$ as well as GA1 glycolipid inhibited antibodies (Table 4, Figure 2C). Although antibodies bound AG in the indirect ELISA, no or faint cross-reactivity to TFβ/TF conjugates and AG was observed.

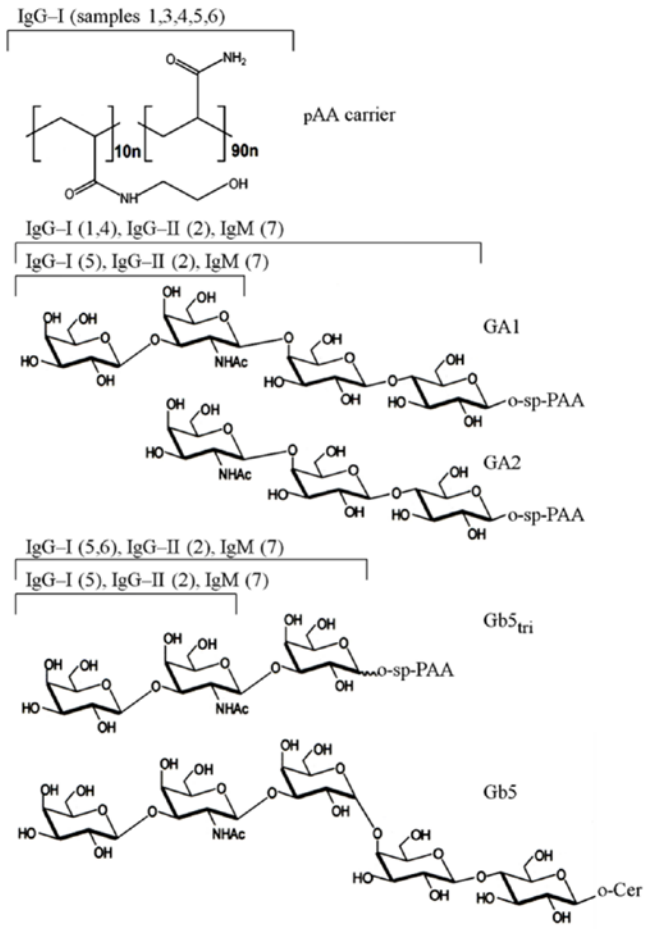

Figure 3: A schematic presentation of pAA and glycotopes, and antibodies reactive to them.

The IgM antibodies of sample 7 were isolated on the TF-sp-Sepharose from the pAA-reactive serum (RA) in which their reactivity to TF and TFβ conjugates preponderated over that of IgG (the attempt to isolate anti-TF IgG failed). They did not react to pAA and bears some resemblance to antibodies of sample 2 (Table 3). The IgM antibodies were cross-reactive to $Gb5_{tri}$-PAA and GA1-PAA, and more specific to these conjugates than to TFβ-PAA (Figure 2B, Table 4). AG (200 μg/ml) inhibited the antibody binding to TF- or TFβ-PAA in the range of 21-43%. Like antibodies of sample 2 and human monoclonal TF1 IgM antibody, IgM of sample 7 was more reactive to the 20% TF-PAA than to the 10% TF-PAA (Table 3).

Thus, the pAA-non-cross-reactive IgG-II of sample 2 and IgM (sample 7) demonstrated the cross-reactivity to TF, TFβ and ligands with the terminated TFβ residue GA1 and $Gb5_{tri}$ (Figure 3). The disaccharide TFβ or its cross-reactive TF are minimal ligands for antibodies because no or faint reactivity to monosaccharides GalNAcβ or Tn was observed.

All isolated IgG antibodies showed an insignificant binding to GalNAcβ-PAA and a slight or no binding to PF_{di}-PAA in the indirect and competitive ELISA. Also, isolated on GalNAcβ- or PF_{di}-PAA-Sepharose, IgG antibodies of other serum probes did not bind TF-, TFβ-pAA and pAA. IgG antibodies (samples 1-6) did not bind ferritin either and only slightly bound casein and DNA that are antigens used in the determination of antibody polyreactivity.

The pAA-reactive and non-reactive antibodies that bound GA1-PAA (samples 1, 2, 4 and 7) also bound GA1 glycosphingolipid and only faintly bound GM1 in a dot blot immunoassay and indirect or competitive ELISA (Figure 2C). Human anti-Tn or anti-A_{di} IgG isolated on Tn or A_{di} sorbents, which were used as irrelevant antibodies, did not show any binding to the GA1glycolipid.

In the present paper and in the paper [15] we demonstrated that anti-TF IgG of numerous serum probes and isolated antibodies of patients with gastrointestinal cancer are less specific to TF (TFα) than TFβ epitope presented in glycolipids. Despite the isolation of antibodies on TF-sorbents, a higher IgG reactivity and specificity to TFβ than to TF, irrespective of the conjugate used, was demonstrated (Tables 3, 4). This is not surprising because five human monoclonal IgM and IgA antibodies induced by the TF antigen AG were more inclined to bind a synthetic TFβ form [22]. But one of them, MAb TF1, still showed a higher reactivity to TF conjugates (Table 3).

Literature data on the antibody level to the neutral glycolipids and its relation to clinical manifestations in cancer are scanty. High titres of antibodies to GA1 were found to be associated with gynaecologic

cancer [23]. We tested different glycoconjugates and found a 10% TF-pAA to be a more suitable conjugate in oncodiagnostics [9]. The level of antibodies in sera determined in ELISA using this conjugate was significantly higher than by using a 10 or 20% TF-PAA. The survival of patients with gastric cancer was positively associated with the higher level of IgG but not IgM antibodies to TF-pAA [14]. As a rule, in patients with a high level of serum IgG antibodies to TF-pAA/pAA the low level of IgM antibodies to these conjugates was observed but no correlation was found. Natural anti-carbohydrate immunoglobulins are mostly antibodies of IgM-class. The high level of anti-TF IgG and/or its postoperative elevation observed in some patients with cancer may be a sign of an adaptive immune response which is indicative of the switching of antibody to the IgG-class.

Two populations of IgM antibodies in normal human plasma are characterized: monoreactive with high affinity to GA1 and cross-reactive to glycoconjugates carrying terminal Gal beta1-3GalNAc with low affinity [24,25]. Approximately, 10% of normal human sera contain a low titer of specific anti-GA1 IgG antibodies. High titers of IgG antibodies reacting only with GA1 are detected rarely in sera of patients with neurological disorders [26]. Human neuropathy-associated IgG and IgM antibodies were also differentiated in the reactivity to the whole ganglioside GM1 and terminal TFβ disaccharide common in GM1, GA1 and GD1b [27]. We did not observe any clinical manifestations of neuropathy during follow-up in eight cancer patients with a high serum level of IgG to GA1 and TFβ conjugates.

The natural glycoconjugates as supposed targets of antibodies

The whole GA1 and glycolipids with TFβ-terminated residues may be considered targets of isolated antibodies. Gb5 (stage-specific embryonic antigen 3, SSEA-3) is a precursor of the Globo H antigen and both are frequently expressed in human breast cancer specimens [28]. As shown earlier by using the carbohydrate microarray, the polyclonal IgG and IgM antibodies of the serum from breast cancer patients bind either a synthetic hexasaccharide Globo H or pentasaccharide SSEA-3 without a significant difference but the binding to the terminated tetrasaccharide moiety of Globo H was significantly lower [29]. We suggest that the serum anti-SSEA-3/Globo H antibodies described by Huang et al. [29] should be low specific to the terminated trisaccharide $Gb5_{tri}$ as well, i.e. the $Gb5_{tri}$-reactive populations described in the present study seems to be not related to SSEA-3 antibodies.

Human polyclonal anti-TF and -Tn antibodies may react with different human carcinoma cell lines [30]. The purified TF-pAA-reactive IgG antibodies were previously found to be highly reactive to the mucinous fraction isolated from human breast cancer tissues. The fraction void of glycolipids showed a dose-dependent inhibition of antibodies [17]. A substance reactive to antibodies needs to be identified. As found later, antibodies bound only rare mucinous probes and an insufficient specificity of antibodies is an obstacle to their use for the determination of TF antigens in tumor-derived probes. As was shown by Sriram et al. [31], the immunohistochemical staining of human colorectal adenocarcinoma tissues using anti-GA1 IgY is positive in 84% of tissue sections. The authors explain it by a prevalence of the TF-beta (terminated residue of GA1) conformation in mucinous antigens. However, in our opinion, the cross-reactivity of antibodies to TF, TFβ and related ligands, including MAbs, makes the accurate recognition of ligands difficult (see also the reactivity of RPA, antiserum to GA1, Table 3).

Conclusion

The previously examined anti-TF IgG antibodies, whose level is decreased in patients with gastric cancer, while their higher level is positively associated with better survival, differ in specificity. The specificity and cross-reactivity of antibodies affinity-isolated from the serum of long-term survivors was evaluated in immunoassays with homogeneous glycoconjugates and saccharides. Being mainly reactive to TFβ or pAA, the antibodies varied in the cross-reactivity to TF, GA1 and $Gb5_{tri}$ glycans. As there was a high correlation between the levels of serum antibodies to TF-pAA and pAA, the decreased level of anti-TF IgG and IgM in patients and survival may be related to the presence of pAA-cross-reactive antibodies. What glycoconjugate should be used in immunoassays to elucidate more informative antibody populations for diagnostic and monitoring purposes needs further study.

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References

1. Springer GF (1997) Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J Mol Med 75: 594-602.
2. Yu LG (2007) The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression. Glycoconj J 24: 411-420.
3. Campbell BJ, Finnie IA, Hounsell EF, Rhodes JM (1995) Direct demonstration of increased expression of Thomsen-Friedenreich (TF) antigen in colonic adenocarcinoma and ulcerative colitis mucin and its concealment in normal mucin. J Clin Invest 95: 571-576.
4. Springer GF, Tegtmeyer H (1981) Origin of anti-Thomsen-Friedenreich (T) and Tn agglutinins in man and in white Leghorn chicks. Br J Haemat 47: 453-460.
5. Springer GF (1984) T and Tn, General carcinoma autoantigens. Science 224: 1198-1206.
6. Kobold S, Lütkens T, Cao Y, Bokemeyer C, Atanackovic D (2010) Autoantibodies against tumor-related antigens: incidence and biologic significance. Hum Immunol 71: 643-651.
7. Desai PR, Ujjainwala LH, Carlstedt SC, Springer GF (1995) Anti-Thomsen-Friedenreich (T) antibody-based ELISA and its application to human breast carcinoma detection. J Immunol Meth 188: 175-185.
8. Bovin NV (1998) Polyacrylamide-based glycoconjugates as tools in glycobiology. Glycoconj J 15: 431-446.
9. Smorodin EP, Jansson B, Milyukhina L, Paaski G, Bovin NV, et al. (1997) Enzyme-linked immunosorbent assay of IgM antibodies to Thomsen-Friedenreich (TF) hapten in oncodiagnostics: comparison of data obtained with four TF-glycoconjugates. Bioorg Khim 23: 795-799.
10. Smorodin JP, Kurtenkov OA, Miljukhina LM, Sergeyev BL, Hint EK, et al. (1997) Thomsen-Friedenreich antigen-specific IgM antibodies: diagnostic significance for gastric and breast cancer. Exp Oncol 19: 338-342.
11. Smorodin EP, Kurtenkov OA, Sergeyev BL, Lilleorg AL, Chuzmarov VI (2001) Antibodies to tumor-associated carbohydrate epitopes in sera of cancer patients and blood donors. Exp Oncol 23: 109-113.
12. Smorodin EP, Kurtenkov OA, Sergeyev BL, Lipping AA, Chuzmarov VI, et al. (2002) The relation of serum anti-TF, Tn and alpha-Gal IgG antibody levels to cancer progression and histopathological grading. Exp Oncol. 24: 270-273.

13. Smorodin EP, Kurtenkov OA, Sergeyev BL, Kodar KE, Chuzmarov VI, et al. (2008) Postoperative change of anti-Thomsen-Friedenreich and Tn IgG level: the follow-up study of gastrointestinal cancer patients. World J Gastroenterol 14: 4352-4358.

14. Kurtenkov O, Klaamas K, Mensdorff-Pouilly S, Miljukhina L, Shljapnikova L, et al. (2007) Humoral immune response to MUC1 and to the Thomsen-Friedenreich (TF) glycotope in patients with gastric cancer: Relation to survival. Acta Oncol 46: 316-323.

15. Smorodin EP, Kurtenkov OA, Sergeyev BL, Pazynina GV, Bovin NV (2004) Specificity of human anti-carbohydrate IgG antibodies as probed with polyacrylamide-based glycoconjugates. Glycoconj J 20: 83-89.

16. Sobin LH, Wittekind CH (2002) TNM Classification of Malignant Tumours. 6th ed. New York: Wiley Press.

17. Smorodin EP, Kurtenkov OA, Sergeyev BL (2000) The application of human natural polyclonal IgG-antibodies to Thomsen-Friedenreich epitope (TFE) for evaluation of TFE-expression in cancer-associated mucins. Exp Oncol. 22: 44-47.

18. Smorodin EP, Kurtenkov OA, Sergeyev BL, Branovets JS, Izotova JG, et al. (2011) Specificity of serum anti-A(di) IgG antibodies from patients with gastrointestinal cancer. J Immunoassay Immunochem 32: 170-190.

19. Rauvala H (1979) Monomer-micelle transition of the ganglioside GM1 and the hydrolysis by Clostridium perfringens neuraminidase. Eur J Biochem 97: 555-564.

20. Smorodin EP, Kurtenkov OA, Shevchuk IN, Tanner RH (2005) The isolation and characterization of human natural αGal-specific IgG antibodies applicable to the detection of αGal-glycosphingolipids. J Immunoassay Immunochem 26: 145-156.

21. Butschak G, Karsten U (2002) Isolation and characterization of Thomsen-Friedenreich-specific antibodies from human serum. Tumor Biol 23: 113-122.

22. Dahlenborg K, Hultman L, Carlsson R, Jansson B (1997). Human monoclonal antibodies specific for the tumour associated Thomsen-Friedenreich antigen. Int J Cancer 70: 63-71.

23. Witkin SS, Bongiovanni AM, Birnbaum S, Caputo T, Ledger WJ (1985) Antibodies to the neutral glycolipid asialo ganglio-N-tetraosylceramide: association with gynecologic cancers. Am J Obstet Gynecol 151: 679-681.

24. Mizutamari RK, Wiegandt H, Nores GA (1994) Characterization of anti-ganglioside antibodies present in normal human plasma. J Neuroimmunol 50: 215-220.

25. Lopez PH, Lardone RD, Irazoqui FJ, Maccioni M, Nores GA (2002) The origin of anti-GM1 antibodies in neuropathies: the “binding site drift” hypothesis. Neurochem Res 27: 687-695.

26. Lopez PH, Comín R, Villa AM, Di Egidio M, Saizar RD, et al. (2006) A new type of anti-ganglioside antibodies present in neurological patients. Biochim Biophys Acta 1762: 357-361.

27. Townson K, Boffey J, Nicholl D, Veitch J, Bundle D, et al. (2007) Solid phase immunoadsorption for therapeutic and analytical studies on neuropathy-associated anti-GM1 antibodies. Glycobiology 17: 294-303.

28. Chang WW, Lee CH, Lee P, Lin J, Hsu CW, et al. (2008) Expression of Globo H and SSEA3 in breast cancer stem cells and the involvement of fucosyl transferases 1 and 2 in Globo H synthesis. Proc Natl Acad Sci U S A 105: 11667-11672.

29. Huang CY, Thayer DA, Chang AY, Best MD, Hoffmann J, et al. (2006) Carbohydrate microarray for profiling the antibodies interacting with Globo H tumor antigen. Proc Natl Acad Sci U S A 103: 15-20.

30. Avichezer D, Springer GF, Schechter B, Arnon R (1997) Immunoreactivities of polyclonal and monoclonal anti-T and anti-Tn antibodies with human carcinoma cells, grown in vitro and in a xenograft model. Int J Cancer 72: 119-127.

31. Sriram V, Jebaraj CE, Yogeeswaran G (1999) Chicken egg yolk anti-asialoGM1 immunoglobulin (IgY): an inexpensive glycohistochemical probe for localization of T-antigen in human colorectal adenocarcinomas. Indian J Exp Biol 37: 639-649.

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