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Application of chemically desialylated and degalactosylated human glycophorin for induction and characterization of anti-Tn monoclonal antibodies

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Human erythrocyte glycophorin was desialylated by mild acid hydrolysis and degalactosylated by Smith degradation. Two monoclonal antibodies (Tn5 and Tn56) obtained by immunization of mice with this 'artificial' Tn antigen were characterized and compared in some experiments with two antibodies (BRIC111 and LM225) obtained in other laboratories by immunization with Tn erythrocytes. The specific binding of the antibodies to glycophorins desialylated and degalactosylated on the nitrocellulose blot and to asialo-agalactoglycophorin-coated ELISA plates, and reactions with authentic Tn antigen served for identification of their anti-Tn specificity. The antibodies were further characterized in inhibition assay with various glycoproteins. The antibody Tn5 (similar to BRIC111) was shown to be specific for human erythrocyte Tn antigen, whereas Tn56 reacted strongly with different glycoproteins carrying O-linked GalNAcα- residues, and was strongly bound to the murine adenocarcinoma cell line Ta3-Ha. The antibodies Tn5, Tn56 and BRIC111 were similarly inhibited by ovine submaxillary mucin (OSM) and asialoOSM, but the antibody LM225 showed a distinct preference in reaction with OSM (sialosyl-Tn antigen). The results show that Tn antigen, obtained by chemical modifications of human glycophorin, enables the preparation and characterization of anti-Tn monoclonal antibodies, without using rare Tn erythrocytes.

Keywords: Glycophorin, Smith degradation, Tn, sialosyl-Tn, monoclonal antibodies.

Abbreviations: HuGph, human erythrocyte glycophorin; HoGph, horse erythrocyte glycophorin; OSM, ovine submaxillary mucin; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline (0.01 M Na_2HPO_4/0.15 M NaCl, pH 7.2); BSA, bovine serum albumin; TBS, 0.05 M Tris-HCl/0.15 M NaCl, pH 7.2; TGr, transformation grade.

The erythrocytes of some rare individuals show Tn type polyagglutination, which can be defined by some lectins [1]. Tn erythrocytes have glycophorins with defective O-linked oligosaccharide chains (GalNAcα- and NeuAcα2-6GalNAcα-) [2, 3], due to the lack of 3-β-D-galactosyltransferase activity [4, 5]. This defect is an acquired long lasting syndrome resulting from somatic mutation in precursor cells [6], and usually only a fraction of erythrocytes and other cells (descendants of the mutated cells) of a given individual shows the Tn characteristics.

Due to frequently occurring defective glycosylation in tumour cells, the Tn antigen has been found to be a marker of some types of cancer [7–12]. This finding stimulated an interest in obtaining anti-Tn monoclonal antibodies, which have been raised by immunization of mice with Tn erythrocytes [12–15], Tn-positive cancer cells or cancer-derived Tn antigens [7, 16, 17], synthetic Tn antigen (GalNAc-Ser linked to protein carrier) [11], or enzymatically degalactosylated human erythrocyte asialo-glycophorin [15].

In this report we describe the use of chemically desialylated and degalactosylated human glycophorin for induction of anti-Tn monoclonal antibodies. Two new anti-Tn antibodies obtained were characterized by immunoblotting and microtitre plate ELISA, with the use of the asialo-agalactoglycophorin as the target antigen.

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Materials and methods

Glycophorins and other glycoproteins

Human glycophorin (HuGph) was obtained by the phenol extraction of membranes of outdated human OM erythrocytes [18] obtained from the District Blood Transfusion Centre in Wrocław. The unfractionated mixture of glycophorins A (predominant component), B and C was used. Sialic acid was removed by hydrolysis of glycophorin in 0.025 M sulfuric acid for 4 h at 60 °C, which was followed by dialysis and lyophilization. The galactose residues were removed from asialoHuGph by one step Smith degradation. The 0.5% asialoHuGph solution in 0.05 M sodium acetate, pH 4.5, containing 0.05 M sodium periodate, was incubated for 18 h at 4 °C in darkness and dialysed against 0.15 M NaCl and then against distilled water. The sample was mixed with an equal volume of 0.3 M $NaBH_4$ in 0.2 M sodium borate buffer, pH 8.0, and, after 3 h at 20 °C, the excess $NaBH_4$ was decomposed with acetic acid. The sample was dialysed, hydrolysed in 0.025 M sulfuric acid for 1 h at 80 °C, neutralized with NaOH, dialysed again and lyophilized. The colorimetric determination of sialic acid [19] and neutral sugars [20] in the preparations obtained revealed the following values: HuGph, 18.4% *N*-acetylneuraminic acid, 13.8% neutral sugars; asialoHuGph, 1.7% *N*-acetylneuraminic acid, 13.3% neutral sugars; asialo-agalactoHuGph, 0.0% *N*-acetylneuraminic acid, 3.3% neutral sugars. Horse glycophorin (HoGph) and its asialo- and asialo-agalacto-derivatives were obtained by the same methods from horse erythrocyte membranes.

Ovine submaxillary mucin (OSM), which has predominantly NeuAcα2-6GalNAcα- chains, was isolated from the water extract of ovine submaxillary glands by fractionation with hexadecyltrimethylammonium bromide and ethanol [21]. OSM was desialylated by mild acid hydrolysis (0.025 M sulfuric acid, 1 h at 80 °C).

The blood group glycoproteins from ovarian cyst fluids of A and B patients were isolated by phenol extraction, ammonium sulfate precipitation and ethanol precipitation, as described by Morgan [22]. The A and B glycoproteins obtained showed a strong and specific inhibition (< 1 μg/ml) of agglutination of A and B erythrocytes by anti-A and anti-B human sera, respectively.

Monoclonal antibodies (mAbs)

Antibodies Tn5 and Tn56 were raised by immunization of mice with asialo-agalactoHuGph. Adult BALB/c mice were injected subcutaneously with 100 μg antigen in complete Freund adjuvant, then three times intraperitoneally at weekly intervals with 50 μg antigen, and then once intravenously with 50 μg antigen. Three days after the last injection, the spleen cells of immunized animals were fused with SP2/O-Ag14 hybrid plasmacytoma cells [23]. The hybridoma cells were grown on tissue culture plates under standard conditions. The supernatants from growing clones were screened by haemagglutination of untreated and desialylated erythrocytes and by binding to ELISA plates coated with HuGph, asialoHuGph and asialo-agalactoHuGph. The antibodies reacting with the last antigen and showing negative reactions with native or desialylated HuGph were considered as anti-Tn. The selected clones were subcloned three times by the limiting dilution procedure. Two clones, Tn5 and Tn56, were expanded *in vitro* and then grown intraperitoneally in BALB/c mice and the ascites fluids were collected and used in experiments. The Tn5 and Tn56 antibodies were typed as IgG1 and IgM, respectively, using double immunodiffusion in agarose gel and monospecific anti-mouse Ig type sera (Bionetics, USA).

Two other anti-Tn antibodies, BRIC111 (IgG1) and LM225/463.21 (IgM), were supplied to the Second International Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens (Lund, Sweden, 1990) by Dr D. J. Anstee (Bristol, UK) and Dr R. H. Frazer (Carluke, Lanarkshire, UK), respectively. Both antibodies were obtained by immunization of BALB/c mice with Tn erythrocytes and were used as supernatants from *in vitro* culture. The mAb LM225/463.21 for simplicity is referred to as LM225.

Tn erythrocytes

The frozen Tn erythrocytes of group B were kindly supplied by Dr. G. Kuśnierz-Alejska, Institute of Haematology, Warsaw.

Cell lines and flow cytometry analysis

The human urothelial cell lines represented transformation grade (TGr) II (nontumourigenic, noninvasive cells: HCV29, Hu609, Hu1734) and III (tumourigenic and invasive cells: HCV29T, Hu609T, Hu456, Hu549, Hu1703He) [24]. These cells and mammary adenocarcinoma cell lines, human T47D and murine Ta3-Ha, were obtained from the Fibiger Institute (Copenhagen, Denmark). The cells were maintained in Fib41B growth medium under routine conditions.

The cell suspensions for binding experiments were prepared with the use of 0.25% EDTA in PBS and, after washing, the cells were suspended in PBS–1% BSA. The cells, untreated or treated with neuraminidase from *Vibrio cholerae* (BDH, England), were incubated with the serially diluted mAbs Tn5 and Tn56 for 1 h on ice. After washing, the cells were treated with FITC-conjugated rabbit anti-mouse Ig antibodies (Dako A/S, Denmark) diluted 1:50 for 30 min on ice and washed again (the washing and dilution of the antibodies were done with PBS–1% BSA containing 0.01% sodium azide). The cells were analysed by flow cytometry in the FACStar (Becton-Dickinson, USA) cytofluorimeter: 5000 cells were acquired for each data file. Data were processed with the FACStar Plus Research Software Version 1.0 06/87.

Microtitre plate enzyme-linked immunosorbent assay (ELISA)

The ELISA plates (Nunc, Maxi Sorp, Austria) were coated overnight at 4 °C with HuGph, asialoHuGph or asialo-agalactoHuGph in 0.05 M sodium carbonate buffer, pH 9.6 (1 μg 50 μl^{-1} per well). The binding of serially diluted anti-Tn mAbs was determined with the use of alkaline phosphatase-conjugated goat antibodies against mouse Ig (Dakopatts, Denmark), or against mouse IgM (Sigma, USA) and Sigma 104 Phosphatase Substrate Tablets. The details of the procedure were as described previously [25], except that the volumes of all reagents were reduced to 50 μl per well. To measure the inhibition of binding, the serially diluted inhibitor samples were mixed with an equal volume of anti-Tn antibody at constant dilution (Tn5 1:20,000, Tn56 1:2,000, BRIC111 1:100, and LM225 1:5) and, after 1 h at 20 °C, the binding of these samples to asialo-agalactoHuGph-coated plates was determined as described above.

Immunoblotting

The erythrocyte membrane proteins (40 μg protein per lane) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel [26], and were electrophoretically transferred to nitrocellulose BA85 (Schleicher & Schuell, Germany) [27]. The blots were quenched with 5% human serum albumin for 1 h at 37 °C, and were used directly or after the following modifications. To desialylate glycophorins bound to nitrocellulose, the blots were incubated overnight at 20 °C with neuraminidase from *Vibrio cholerae* (Serva, Germany) at an enzyme concentration of 10^{-4} U ml^{-1}. The subsequent degalactosylation was performed by successive incubations of the neuraminidase-pretreated and washed blots with 0.05 M sodium periodate for 18 h at 4 °C, in 0.15 M $NaBH_4$ in 0.1 M sodium borate buffer, pH 8.0, for 2–3 h at 20 °C, and in 0.025 M sulfuric acid for 1 h at 80 °C, with severalfold washing of the blot with 0.15 M NaCl after each incubation. The nitrocellulose blots (untreated or modified) were incubated overnight at 4 °C with the mAbs Tn5 and Tn56, diluted 1:500 with TBS. After washing, the blots were overlaid for 1 h at 20 °C with horseradish peroxidase-conjugated goat antibodies against mouse Ig (Sigma, USA) and finally with the substrate, 4-chloro-1-naphthol (Sigma) solution.

Results

Haemagglutination

The new mAbs Tn5 and Tn56 were tested with untreated and desialylated normal erythrocytes of all blood group A, B, O and M, N phenotypes. The mAb Tn5 did not agglutinate any erythrocyte sample, whereas Tn56 showed weak agglutination (2+, up to 50-fold dilution of the ascites fluid) of A erythrocytes only. However, both antibodies showed strong agglutination of Tn erythrocytes, the agglutination titres of Tn5 and Tn56 were 1 600 and 160 000, respectively.

Inhibition of the antibodies measured by ELISA

The mAbs Tn5 and Tn56, as well as two Workshop mAbs BRIC111 and LM225, bound to asialo-agalactoHuGph-coated ELISA plates, and did not bind either to HuGph or to asialoHuGph (Fig. 1). The binding to asialo-agalactoHuGph enabled us to test the inhibition of the antibodies with various glycoproteins (Table 1). As expected, the antibodies were not inhibited by HuGph or asialoHuGph but were strongly inhibited by asialo-agalactoHuGph. It confirmed the recognition of O-linked GalNAcα- residues by these antibodies. Horse erythrocyte glycophorin (HoGph), which has O-linked chains of the same core structure as HuGph, but a different amino acid sequence and distribution of O-linked oligosaccharides [28], and other glycoproteins were used to check the dependence of reactivity of the antibodies on the localization of *N*-acetylgalactosamine residues in the antigen. As with the HuGph preparations, the antibodies were not inhibited by HoGph in the native or desialylated form. However, asialo-agalactoHoGph showed some activity, but much weaker and more differentiated than asialo-agalactoHuGph (Table 1). The mAb Tn5 seemed to be specific for asialo-agalactoHuGph, since it was inhibited very weakly not only by asialo-agalactoHoGph but also by OSM, asialo-OSM and *N*-acetylgalactosamine, and was not inhibited by blood group A glycoprotein. With respect to the specificity, the mAb Tn5 was similar to BRIC111, but the latter antibody showed relatively higher cross-reactivity with OSM, asialoOSM and GalNAc than Tn5. The mAb

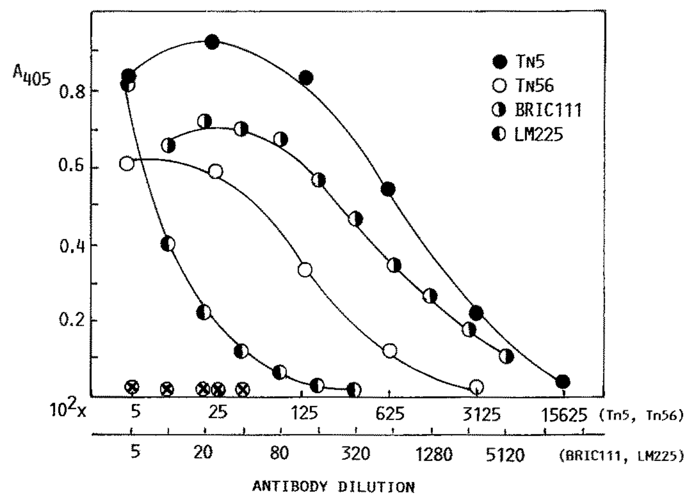

Figure 1. Binding of serially diluted mAbs Tn5, Tn56, BRIC111 and LM225 to ELISA plates coated with asialo-agalactoHuGph. The crossed circles demonstrate the lack of binding of these antibodies to the plates coated with HuGph or asialoHuGph.

Table 1. Inhibition of anti-Tn antibodies by various glycoproteins, measured by ELISA.

Inhibitor	*Monoclonal antibodies*			
	Tn5	*Tn56*	*BRIC111*	*LM225*
		μg ml^{-1} [a]		
Human glycophorin				
untreated	>2500 (0%)[b]	>2500 (0%)	>2500 (0%)	>2500 (0%)
asialo	>2500 (0%)	>2500 (0%)	>2500 (0%)	>2500 (0%)
asialo-agalacto	0.7	2.8	2.1	5.0
Horse glycophorin				
untreated	>2500 (0%)	>2500 (0%)	>2500 (0%)	>2500 (0%)
asialo	>2500 (0%)	>2500 (0%)	>2500 (0%)	>2500 (0%)
asialo-agalacto	2000	45	>2500 (42%)	>2500 (0%)
OSM				
untreated	>2500 (18%)	1.5	250	0.8
asialo	>2500 (36%)	1.5	250	100
Cyst fluid glycoprotein				
blood group A	>2500 (0%)	>2500 (46%)	n.t.[c]	n.t.
blood group B	n.t.	>2500 (41%)	n.t.	n.t.
N-Acetylgalactosamine	28,000	>40,000 (12%)	5000	n.t.

[a] Concentration of inhibitor required for 50% inhibition of antibody binding to asialo-agalactoHuGph-coated ELISA plate.
[b] Preceding the number by > denotes that at this concentration no, or lower than 50%, inhibition (indicated in parentheses) was obtained.
[c] n.t., Not tested.

Tn56 showed the broadest specificity, since it was inhibited by OSM and asialoOSM and, almost equally strongly, by asialo-agalactoHuGph, and was more effectively inhibited by asialo-agalactoHoGph than the remaining antibodies. Moreover, Tn56 showed weak reactivity with ovarian cyst fluid glycoproteins of blood group A and B, but was not inhibited by *N*-acetylgalactosamine. The mAb LM225, which did not react with asialo-agalactoHoGph, was inhibited by asialoOSM and most strongly by untreated OSM, which was about six-times and 120-times stronger inhibitor than asialo-agalactoHuGph and asialoOSM, respectively. It indicated that the mAb LM225 reacts preferentially with sialosyl-Tn determinant, whereas the other antibodies tested here did not show any difference in reaction with OSM (sialosyl-Tn) and asialoOSM (Tn).

Immunoblotting

The membrane proteins of untreated erythrocytes and of erythrocytes treated with trypsin or chymotrypsin were fractionated by SDS-PAGE and blotted to nitrocellulose. The glycoproteins were desialylated with neuraminidase and degalactosylated by Smith degradation on the blot. The mAbs Tn5 and Tn56 did not show binding to untreated or desialylated blots, but strongly stained glycophorin bands on desialylated, Smith-degraded blots, giving typical patterns of bands in the membranes derived from untreated and protease-treated erythrocytes (Fig. 2). The immunoblotting showed a characteristic difference between the mAbs Tn5 and Tn56. Tn5 stained the bands of glycophorins A and B only, whereas Tn56 was bound to asialo-agalactoglycophorins A, B and C. Glycophorins A and B show a high degree of homology in amino acid sequence and have identical glycosylated portions comprising the amino acid residues 1–26, and glycophorin C has a different amino acid sequence and distribution of oligosaccharide chains [29]. Therefore, the results of immunoblotting confirmed that Tn5 has the specificity restricted to Tn epitopes present in structurally identical portions of glycophorins A and B, and Tn56 has a broader specificity and reacts with structurally dissimilar glycoproteins carrying O-linked *N*-acetylgalactosamine residues. The mAbs Tn5 and Tn56 also stained glycophorins of Tn erythrocytes (Fig. 2). The Tn glycophorins showed higher microheterogeneity and, higher electrophoretic mobility than normal counterparts.

Binding of the antibodies to cultured cells measured by flow cytometry

The mAbs Tn5 and Tn56 (diluted 1:20 to 1:400) gave negative or weakly positive reactions (up to 15% of positively stained cells) with the human urothelial cell lines

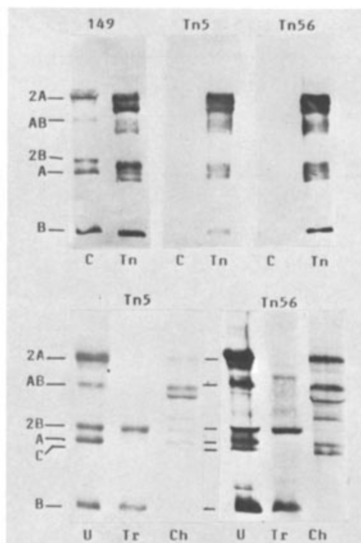

Figure 2. Detection of Tn type glycophorins with the mAbs Tn5 and Tn56 in immunoblotting. Upper part: blots with membrane components of control normal erythrocytes (C) and Tn erythrocytes (Tn) overlayed with the control mAb 149 (reactive with glycophorins A and B, 2nd International Workshop, Lund, 1990) and with anti-Tn mAbs Tn5 and Tn56. Lower part: blots with membrane components of normal erythrocytes. The membranes were obtained from untreated (U), trypsin-treated (Tr), or chymotrypsin-treated (Ch) red blood cells. The blots were submitted to neuraminidase treatment and Smith-degradation procedure (as described in the Materials and methods section) before overlaying with the mAbs Tn5 or Tn56. The positions of glycophorins A, B, C, monomers and dimers, are indicated on the left side.

of TGr II and III (see the Materials and methods section). Treatment of the cells with neuraminidase did not change the results, and no correlation between the binding of the antibodies and TGr of the cells was observed. The results of Radzikowski *et al.* [30], obtained using a computer-connected microscope photometer, indicated that transfection of HCV-29 cells with the v-raf gene increased the binding of the mAb Tn5 to the transfectants. Both mAbs (diluted 1:20–1:400) also showed only weak binding to human mammary carcinoma cells T47D (up to 10% of positively stained cells). However, a big difference between mAbs Tn5 and Tn56 was found in the binding to mouse adenocarcinoma Ta3-Ha cells. The mAb Tn5 (diluted 1:400) gave a weakly positive reaction, similar to that observed with the other cells tested, but the mAb Tn56 reacted strongly with these cells. The optimal binding of the mAb Tn56 to untreated and neuraminidase treated Ta3-Ha cells (60% of positive cells) was detected at mAb dilutions of 1:4 000–1:40 000, and a weak binding was still observed at mAb dilutions of 1:100 000–1:200 000.

Discussion

The results show that asialo-agalactoHuGph obtained by chemical modifications of HuGph has similar antigenic properties to glycophorin present in Tn erythrocytes. This was demonstrated by the strong reactivity of this preparation with the mAbs raised by immunization with Tn erythrocytes and, *vice versa*, by the strong reactivity of authentic Tn type glycophorin with the mAbs Tn5 and Tn56 elicited by immunization with the 'artificial' Tn antigen. Therefore, asialo-agalactoHuGph, which is easy to obtain by chemical methods, is a convenient alternative to rare Tn erythrocytes for raising and testing anti-Tn monoclonal antibodies. Moreover, it was shown that transformation of glycophorins into Tn antigen can also be done in nitrocellulose blots. The latter method can be used more generally for identification of components carrying not only 'cryptic' Tn antigens, but also other 'cryptic' carbohydrate antigens.

The properties of anti-Tn monoclonal antibodies described here and in other papers raises the problem of the definition of a Tn epitope or Tn antigen. Historically, a Tn antigen is asialo-agalactoglycophorin present in human Tn erythrocytes. Later, the term Tn antigen was extended to other glycoproteins which have O-linked, unsubstituted GalNAc- residues and are present mainly in cancer cells. According to the latter definition, anti-Tn antibodies should recognize GalNAcα-Ser/Thr residues, independently of their environment. However, these residues are relatively small and some anti-Tn antibodies are specific for larger epitopes, including not only GalNAcα-Ser/Thr residues but also other adjacent amino acid or carbohydrate components. Such antibodies react most strongly or exclusively with the Tn antigen used for immunization. Examples of this type of antibody are Tn5 and BRIC111 (the latter antibody showed in our hands properties in accord with those described recently by King *et al.* [14]). These antibodies are specific for Tn antigen of human erythrocyte glycophorins A and B, and cross-react only weakly with other Tn antigens. On the other hand, anti-Tn antibodies which show a broad specificity may react not only with any antigen having GalNAcα-Ser/Thr residues (Tn antigen), but they also may cross-react with blood group A antigen [14, 16]. The mAb Tn56 described in this report seems to be a broadly specific reagent reacting strongly with various Tn antigens. Its cross-reactivity with blood group A antigen was negligible and detectable only by a weak agglutination of A erythrocytes. The weak and equal inhibition of the mAb Tn56 by blood group A and B glycoproteins suggested that this inhibition was caused by unsubstituted GalNAcα-Ser/Thr residues present in the preparations used [31], rather than by blood group A determinants. A lack of inhibition of the mAb Tn56 by *N*-acetylgalactosamine also suggested the importance of the linkage of this sugar to Ser/Thr residues in the epitope recognized by this antibody.

In conclusion, the mAb Tn56 is a potentially useful reagent for detecting Tn antigens in cancer cells.

An antigen related to Tn is sialosyl-Tn (NeuAcα2-6GalNAcα-Ser/Thr residues), which is coexpressed in Tn erythrocytes [3] and is also a marker of cancer cells [12, 32, 33]. An example of sialosyl-Tn antigen is OSM, which was used for raising specific anti-sialosyl-Tn monoclonal antibodies [32]. Most anti-Tn monoclonal antibodies tested so far react also with sialosyl-Tn [14, 32], which indicates that the substitution of *N*-acetylgalactosamine at C-6 has no or little influence on its recognition by these antibodies. The mAbs Tn5, Tn56 and BRIC111 were also found to belong to this category, since they were similarly inhibited by OSM and asialoOSM. However, the mAb LM225 was much more strongly inhibited by OSM than by asialoOSM, indicating the preference of its reactivity with sialosyl-Tn.

In conclusion, the four antibodies described in this report showed distinct differences in fine specificity. However, a common property defining them as anti-Tn antibodies was the dependence of their reactivity on the presence in the antigen of O-linked GalNAcα-residues, which cannot be substituted at C-3 by Gal, but can carry α(2-6)-linked sialosyl residues.

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