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Correlative Fine Specificity of Several Thomsen-Friedenreich Disaccharide-Binding Proteins with an Effect on Tumor Cell Proliferation[1]

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Epithelial cancer cells show increased cell surface expression of mucin antigens with aberrant *O*-glycosylation, notably type I core (Galβ1-3GalNAcα), termed Thomsen-Friedenreich disaccharide (TFD), a chemically well-defined carbohydrate antigen with a proven link to malignancy. Several TFD-binding proteins influence the proliferation of cells to which they bind. We studied the fine specificity of TFD-binding proteins and its relationship with epithelial tumor cell proliferation. Competitive binding assays against asialoglycophorin showed that *Agaricus bisporus* lectin (ABL) and human anti-TFD monoclonal antibody (mAb) TF1 were inhibited only by TFD and its α-derivatives. Peanut agglutinin (PNA), mAb TF2, and mAb TF5 were also inhibited by other carbohydrates such as lacto-N-biose (Galβ1-3GlcNAc), lactose, and (Meα or β) Gal, indicating lower recognition of the axial C-4 hydroxyl group position of GalNAc from TFD, and the major relevance of the terminal Gal on interaction of these three TFD-binding proteins. In the direct glycolipid-binding assay, ABL bound mostly to α-anomeric TFD-bearing glycolipids, whereas PNA interacted mainly with β-linked TFD. Of the three anti-TFD mAbs analyzed, all bound N5b (terminal β-TFD), but only TF2 interacted with N6 (terminal α-TFD). These findings indicate that TFD-binding proteins that stimulate the proliferation of epithelial tumor cell lines recognize mainly a terminal β-Gal region of β-linked TFD, whereas ABL, which inhibits the proliferation of these tumor cells, binds mainly to subterminal GalNAc of α-anomeric TFD.

Key words: carbohydrate-binding proteins, cell proliferation, Thomsen-Friedenreich disaccharide.

Interest in the post-translational modification of serine and threonine hydroxyl groups by glycosylation has been recently revived because the resulting *O*-linked oligosaccharide chains tend to cluster over short stretches of peptides and hence display multivalent carbohydrate antigenic or functional determinants for antibody and non-immune carbohydrate-binding protein recognition (*1–3*). Aberrant *O*-glycosylation of cell surface mucin antigens occurs on epithelial cancer cells, exposing cryptic regions as a type I core structure (α-anomeric Galβ1-3GalNAc) called Thomsen-Friedenreich disaccharide (TFD) (*4, 5*), one of the few chemically well-defined carbohydrate antigens with a documented link to malignancy (*6–8*). Low levels of natural human anti-TFD antibodies have been correlated with tumor progression and aggressiveness (*9–11*). These observations provide the basis for the use of TFD-bearing molecules for active specific immunotherapy in patients with epithelial tumors (*12–14*). Interaction among *O*-glycans and human anti-TFD monoclonal antibodies (mAbs), TF2 and TF5, stimulates the proliferation of epithelial tumor cell lines (*15, 16*). TFD-binding lectins found in certain foods, such as peanut agglutinin (PNA) and lectin from the common edible mushroom *Agaricus bisporus* (ABL), respectively, stimulate or inhibit without cytotoxicity the proliferation of human colon cancer cells (*17–20*).

We studied the fine TFD-binding specificity of these proteins, using competitive and direct-binding assays, in order to clarify the relationship between carbohydrate-binding specificity and the effect on epithelial tumor cell proliferation.

MATERIALS AND METHODS

Materials—Reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Glycolipids N5a, N5b, N6, N7,

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Abbreviations: ABL, *Agaricus bisporus* lectin; ASG, asialoglycophorin; Bzl, benzyl; CELA, competitive enzyme-lectin assay; ID50, concentration required for 50% inhibition; lacto-N-biose, Galβ1-3GlcNAc; lactose, Galβ1-4Glc; mAb, monoclonal antibody; Me, methyl; PNA, peanut agglutinin; pNP, *p*-nitrophenyl; TFD, Thomsen-Friedenreich disaccharide.

A5b, and A6 were obtained from *Calliphora vicina* as reported previously (*21, 22*). GA1 was prepared by mild acid hydrolysis of GM1 from cow brain (*23*). Human anti-TFD monoclonal antibodies TF1 (IgM, κ), TF2 (IgA, κ), and TF5 (IgM, λ) were generated after in vitro immunization or antigen specific isolation of normal peripheral blood B cells using asialoglycophorin (ASG) (*15*). ABL was purified and conjugated to HRP as described previously (*24*).

Lectin Affinity Constants and Antibody Titer Measurement—Polystyrene microtitration plates (Corning) were coated with ASG in phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.2, 150 mM NaCl) for 1 h at 37°C, saturated with PBS–0.05% Tween 20 (PBS-T) for 1 h at 37°C, incubated with a range of concentrations of conjugated lectins or antibodies for 2 h at 23°C, and then washed six times with PBS-T. mAbs were detected with anti-human IgA or IgM antibodies conjugated to peroxidase (1/1,000) followed by incubation for 2 h at room temperature. The color reaction was developed with 2 mg/ml *o*-phenylenediamine and 0.02% H_2O_2 in 0.1 M sodium citrate, pH 5.0, for 30 min. Reactions were stopped by adding 2.5 M sulfuric acid, and absorbance values were read at 450 nm with a microplate reader. Lectin data were fitted with LIGAND Soft v. 3.1 (*25, 26*) for measurement of affinity constants (K_a). The ELISA titer was defined as the highest dilution yielding an absorbance value of ≥0.1 more than that of normal serum (*27*). All assays were performed in triplicate.

Competitive Assays—Competitive enzyme-lectin assays (CELA) and competitive ELISA (CELISA) were performed as described previously (*24*). Briefly, polystyrene microtitration plates were coated with 5 μg/ml ASG in PBS for 1 h at 37°C and then saturated with PBS-T for 1 h at 37°C. Carbohydrates were preincubated with conjugated lectin or human anti-TFD mAb for 1 h at room temperature, and then added to wells. Plates were incubated for 2 h at room temperature and then washed six times with PBS-T. mAbs were detected and the color reaction was developed as described above.

HPTLC Staining—Glycolipids were separated on HPTLC silica gel 60 (Merck) in running solvent chloroform–methanol–aqueous 0.2% $CaCl_2$ (45:45:10) using a tank to obtain highly reproducible chromatograms (*28*). The plates were air-dried for 15 min, coated by dipping in a 0.5% solution of polyisobutylmethacrylate (Plexigum P 28; Rohm & Haas, Darmstadt, Germany) in hexane:chloroform (9:1) for 1 min, air-dried again for 10 min, and then incubated with 0.08 μg/ml ABL-HRP or 15 μg/ml PNA-HRP or human anti-TFD mAbs (512 antibody titer) in PBS-T overnight at 4°C. mAbs were detected with anti–human IgA or IgM antibodies conjugated to peroxidase (1/1,000) followed by incubation for 2 h at 4°C. After five washes with PBS-T in 5 min, the color reaction was developed with 0.5 mg/ml 4-chloro-1-naphtol and 0.02% H_2O_2 in methanol–PBS (1:29) for 30 min. Reactions were stopped by washing with distilled water (*29*).

Molecular Modeling—Minimum energy conformations of carbohydrates were modeled using molecular mechanic calculations with MM2 force field. Three-dimensional structures were constructed using CPK models (Harvard Apparatus, South Natick, MA).

TABLE I. **Affinity constants for ASG-lectin interactions.**

Lectin	K_a (M^{-1}) × 10^{-8}
ABL	16 (±2.2)
PNA	1.2 (±0.18)

Values are calculated for 23°C. Data were fitted using LIGAND Soft v. 3.1 (*25*). Parentheses indicate standard deviation (n = 3).

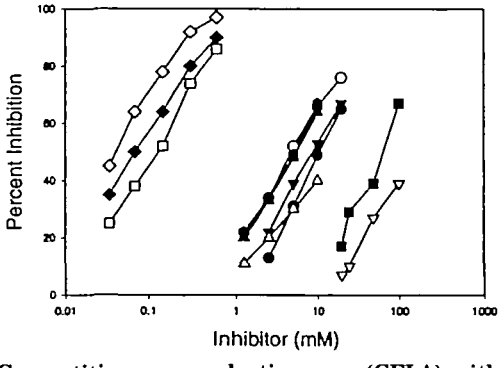


Fig. 1. **Competitive enzyme-lectin assay (CELA) with carbohydrates as inhibitors of the PNA–ASG interaction.** Wells coated with ASG were assayed against PNA-HRP, with previous incubation of the lectin with the following carbohydrates: Gal (solid circles), MeαGal (open circles), MeβGal (solid inverted triangles), GalNAc (open inverted triangles), GlcNAc (solid squares), TFD (open squares), BzlαTFD (solid diamonds), pNPαTFD (open diamonds), lacto-N-biose (solid triangles), Galβ1-6GlcNAc (open triangles), and lactose (solid hexagons). Washing and the color reaction were performed as described under "MATERIALS AND METHODS."

RESULTS

Table I shows the affinity constants for interaction between ASG and the lectin from *A. bisporus* or peanut. The affinity constant for the ABL-ASG interaction is ~13-fold higher than that for the PNA-ASG interaction.

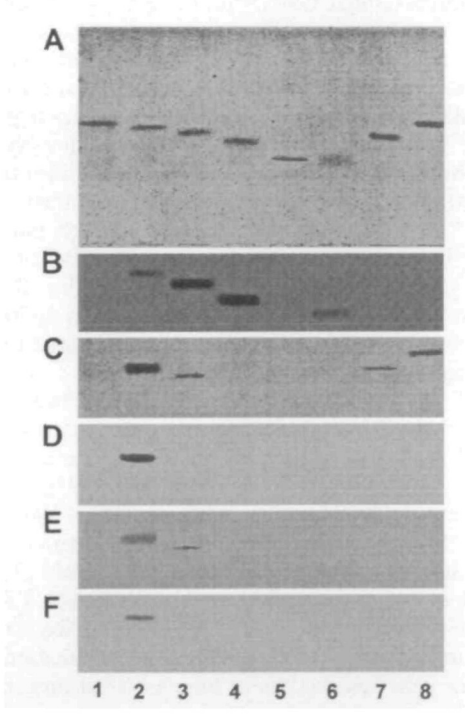
The optimal concentration of conjugated lectin or human anti-TFD mAb showing an optical density of 1.0 against ASG was determined in preliminary experiments for the competitive assay. The inhibitory ability of carbohydrates on PNA binding is shown in Fig. 1. Table II summarizes the TFD-related carbohydrate concentrations required for 50% inhibition (ID50) in CELA or CELISA for lectins and various anti-TFD mAbs (TF1, TF2, and TF5). Of the five

TABLE II. **Inhibition of interactions between ASG and TFD-binding proteins with TFD-related carbohydrates.**

Carbohydrate	Concentration (mM) required for 50% inhibition (ID50)				
	Lectin		Human anti-TFD mAb		
	ABL	PNA	TF1	TF2	TF5
Gal	>100	10	>100	50	5.0
MeαGal	>100	5.0	>100	60	1.2
MeβGal	>100	10	>100	25	6.0
GalNAc	>100	>100	>100	>100	>100
GlcNAc	>100	75	>100	>100	>100
TFD	5.0	0.15	1.2	0.2	2.0
BzlαTFD	2.5	0.07	1.0	0.03	1.4
pNPαTFD	0.5	0.04	0.5	0.13	1.4
Lacto-*N*-biose	>10	5.0	>10	4.0	0.4
Galβ1-6GlcNAc	>10	>10	>10	>10	>10
Lactose	>100	5.0	>100	2.8	2.5

carbohydrate-binding proteins analyzed, four showed that TFD or its α-derivatives (BzlαTFD and pNPαTFD) were the major sugar inhibitors. The exception was the ASG-mAb TF5 interaction, for which lacto-N-biose (Galβ1-3GlcNAc) was the most powerful inhibitor, while TFD exhibited a similar ID50 to Gal. The interaction of ASG with ABL or mAb TF1 was inhibited only by TFD and its α-derivatives; in both cases, pNPαTFD was a stronger inhibitor than BzlαTFD or free TFD. The PNA-ASG interaction was inhibited by (α-derivatives) TFD as well as TFD-related carbohydrates lacto-N-biose, lactose, Gal, MeαGal, MeβGal, and GlcNAc. For the mAb TF2-ASG interaction, BzlαTFD was a more potent inhibitor than pNPαTFD or free TFD. mAb TF2 showed a TFD-related carbohydrate ID50 profile similar to that of PNA, and its interaction was inhibited by lacto-N-biose, lactose, Gal, MeαGal, and Meβ‴ Gal; however, compared to PNA, a higher concentration of monosaccharides was required for 50% inhibition.

To obtain additional information on the fine specificity of these carbohydrate-binding proteins, we performed direct binding analysis of glycolipids. Figure 2 shows the glycolipid relative mobility (A) and interaction ability of several carbohydrate-binding proteins (B–F) by chromatographic separation on HPTLC-silica gel plates and HPTLC-binding staining, respectively. Of the glycolipids assayed, N5a, A5b, GM1, and GA1 did not interact with ABL, whereas N5b, N6, N7, and A6 showed significant lectin binding. PNA bound to N5b, N6, GM1, and GA1, but did not interact with N5a, N7, A5b, or A6. Of the human anti-TFD mAbs assayed, all recognized N5b, and TF2 also the bound to N6 glycolipid. No other glycolipid-mAb interaction was observed.



1- N5a: GalNAcα1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
2- N5b: Galβ1-3GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
3- N6: Galβ1-3GalNAcα1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
4- N7: GlcNAcβ1-3Galβ1-3GalNAcα1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
5- A5b: GlcAβ1-3Galβ1-3GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
6- A6: GlcAβ1-3 Galβ1-3GalNAcα1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ-Cer
7- GM1: Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ-Cer
8- GA1: Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer

Fig. 2. **Direct glycolipid-binding protein assaying by HPTLC separation.** The glycolipids: N5a (1), N5b (2), N6 (3), N7 (4), A5b (5), A6 (6), GM1 (7), and GA1 (8) were visualized either chemically (A) using orcinol-sulfuric acid spray reagent or as to the binding ability of the proteins: ABL-HRP (B), PNA-HRP (C), mAb TF1 (D), TF2 (E), and TF5 (F). The mAbs were detected using anti-human Ig conjugated to peroxidase. After washing, the color reaction was developed with 0.5 mg/ml 4-chloro-1-naphtol and 0.02% H_2O_2 in methanol-PBS (1:29) for 30 min. Reactions were stopped by washing with distilled water.

DISCUSSION

Increased cell surface mucin antigens resulting from aberrant O-glycosylation are expressed on epithelial cancer cells. They include TFD, a chemically well-defined carbohydrate antigen with a documented link to malignancy (*6–8*). Many carbohydrate-binding proteins affect the proliferation of cells to which they bind (*30, 31*). We analyzed the fine specificity of several TFD-binding proteins by means of competitive and direct-binding assays, and found that the interaction of ABL with ASG is ~7-fold stronger than that with the human IgA1 subclass (*24*). ASG has 15 *O*-glycan moieties per protein molecule (*32*); in contrast, human IgA1 has four *O*-glycan chains in its heavy chain hinge region (*33*). The different numbers of *O*-glycan chains could explain the higher ABL-ASG affinity. As a consequence of the high affinity constant for the ABL-ASG interaction, competitive effects of weak inhibitors of the ABL-IgA1 interaction such as GalNAc, lactose, and Galβ1-6GlcNAc (*23*) were not observed in the present model, in which only TFD, and its Bzlα and pNPα derivatives inhibited the ABL-ASG interaction. The fact that the introduction of a bulk hydrophobic α-substituent to TFD improves the inhibitory ability of the disaccharide suggests that a hydrophobic interaction adjacent to the carbohydrate-binding site affects the ABL–ASG interaction.

TFD and its α-derivatives were also potent inhibitors of the PNA–ASG interaction, in addition to other TFD-related saccharides. Lacto-*N*-biose significantly inhibited this interaction, showing the lesser importance of the axial C-4 hydroxyl group position of GalNAc from TFD in this case compared to the ABL-ASG interaction. This demonstrates a key difference in the stereochemical requirements of carbohydrate on comparative binding of PNA *vs.* ABL. Lacto-*N*-biose had no inhibitory effect on the ABL interaction with ASG or human IgA1, its affinity constant (*24*) being similar to that of the PNA-ASG interaction. The influence of Gal on the PNA-ASG interaction is also relevant. This is well documented in the CELA, in which Gal, MeαGal, and MeβGal inhibited the PNA interaction. In contrast, pNPα- and pNPβ-GalNAc inhibited the ABL interaction but Gal did not (*23*). These results suggest than PNA recognizes mainly the terminal non-reducing Gal from TFD, in agreement with the results of a crystallographic study (*34*). In the direct glycolipid-binding assay, PNA mainly recognizes terminal β-linked Galβ1-3GalNAc because this lectin interacts mostly with N5b and GA1. PNA binds weakly to terminal α-anomeric TFD in contrast to ABL, which interacts strongly with N6, weakly with N5b, and not at all with GA1. These PNA binding properties could be explained by steric hindrance of neighboring regions to TFD. N6 has ter-

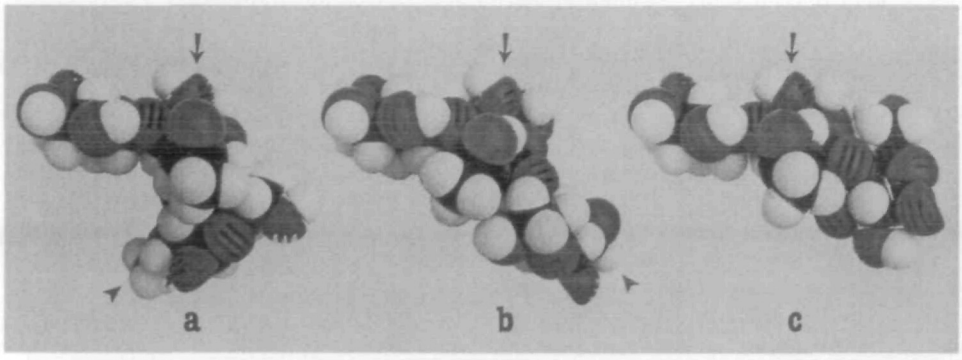


Fig. 3. **CPK models of terminal trisaccharides corresponding to N6 (a), N5b (b), and GA1 (c).** The side view shows the common Galβ1-3GalNAc terminal and the adjacent carbohydrate that carries it. Arrow, axial C-4 hydroxyl group position of GalNAc from terminal TFD. Arrowhead, differential position of the C-2 acetamido residue from the carbohydrate carrier to common TFD.

minal TFD α-linked through the axial C-4 oxygen atom of the adjacent GalNAc, which placies its C-2 acetamido residue in a position that makes binding difficult, as observed on conformational analysis (Fig. 3). In contrast, β-TFD in N5b is carried by GlcNAc through the equatorial C-4 oxygen atom; thus, the remaining GlcNAc is located in a similar plane to its terminal TFD and does not impair TFD binding. Similarly, GA1 does not have a C-2 acetamido residue, and the residual molecule that links TFD has a molecular plane different from that of N6. These data suggest that PNA recognizes mainly the lower plane of TFD (Fig. 3), whereas ABL shows TFD binding through the upper face (*23*). This interpretation is supported by the lower relevance of the axial C-4 hydroxyl group position of GalNAc from TFD (Fig. 3, upper plane) for the PNA interaction than for the ABL interaction. When the C-3 hydroxyl residue of the terminal Gal from TFD is substituted by glucuronic acid, PNA binding is prevented, as seen on comparison of N5b with A5b and N6 with A6. These data are consistent with those previously reported for PNA (*9, 35–37*), *e.g.*, inhibition of its interaction when sialic acid is β-linked to the C-3 oxygen of the terminal Gal of TFD, or when β-GlcNAc is added to the equatorial C-3 hydroxyl residue of the terminal Gal from N6 (N7). Inhibition is less pronounced when sialic acid is β-linked to the equatorial C-3 hydroxyl residue of non-terminal Gal, as in GM1. Thus, the addition of a neutral or anionic carbohydrate β-linked to the C-3 oxygen atom of the terminal Gal of TFD impairs the PNA interaction, confirming the relevance of the terminal β-Gal region of TFD for PNA binding, as observed previously in CELA.

Like PNA, CELISA of human anti-TFD mAbs TF1 and TF5 shows the enhanced inhibitory effect of TFD when a hydrophobic residue is α-added to the disaccharide, but to a lesser degree than observed for ABL, suggesting the presence of low-relevant hydrophobic-binding loci adjacent to the carbohydrate-binding site. The TF1-ASG interaction was inhibited by (α-derivatives) TFD but not by other TFD-related mono- or disaccharides, suggesting a high specificity for TFD with an inhibitory profile similar to that observed for the ABL-ASG interaction. However, in direct-binding assay TF1 binds only N5b, indicating that its carbohydrate-binding specificity is different from that of ABL. ABL is a reversible non-cytotoxic inhibitor of epithelial tumor cell proliferation (*19, 20*), whereas TF1 has no effect on proliferation (*16*).

CELISA shows that Gal has a greater influence than GalNAc on the ASG-TF2 and ASG-TF5 interactions because (Meα or β) Gal is a more powerful inhibitor than GalNAc. The lower significance of the axial C-4 hydroxyl group position of GalNAc from TFD is evidenced by the significant lacto-*N*-biose ID50 observed in the TF2 and TF5 competitive assays. The β-anomeric TFD interaction observed in the glycolipid-binding assay with mAbs is consistent with previous observations that anti-TFD mAbs preferentially bind β-form [Galβ1-GalNAcβ-*O*-2-(2-carbomethoxyethylthioethyl)-bovine serum albumin] relative to α-anomeric synthetic glycoprotein (Galβ1-GalNAcα-*O*-aminophenylethyl-human serum albumin) (*15*). These results indicate great similarity in the carbohydrate recognition properties among PNA, TF2, and TF5, in accordance with their similar stimulatory effects on tumor cell proliferation (*16–18*). The latter effect could be explained by clustering at the cell surface of a few glycoprotein ligands which modulate phosphorylation, similar to the previous finding of Badache *et al.* (*30*) for cerebellar soluble lectin. The situation is different for ABL, which is internalized into endocytotic vesicles; its anti-proliferative effect is a consequence of lectin trafficking to the nuclear periphery (*20, 38*).

We conclude that TFD-binding proteins that stimulate the proliferation of epithelial cell lines recognize mainly the terminal β-Gal region of β-linked TFD. In contrast, ABL, an inhibitor of the proliferation of these tumor cells, binds mostly subterminal GalNAc of α-anomeric TFD. We hypothesize that the selection of hybridomas using the criteria of high N6, low N5b, and no GA1 reactivity would allow the production of human anti-TFD mAbs with fine TFD-binding specificity similar to that of ABL. Such mAbs could have a non-cytotoxic inhibitory effect on the proliferation of epithelial tumor cells and be useful for passive immunization.

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