Novel immunogenicity of Thomsen-Friedenreich disaccharide obtained by a molecular rotation on its carrier linkage

Fernando J. Irazoqui1, Pablo H.H. López, Miguel A. Vides2 and Gustavo A. Nores

Departamento de Química Biológica-CIQUIBIC-CONICET and 1Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina

Received on November 22, 1999; revised on February 10, 2000; accepted on February 11, 2000

The α-anomeric Galβ1-3GalNAc, called Thomsen-Friedenreich disaccharide (TFD), is overexpressed in epithelial cancer cells by aberrant O-glycosylation. TFD is also the main ligand of Agaricus bisporus lectin (ABL), a reversible noncytotoxic inhibitor of proliferation of epithelial cell lines. In order to obtain anti-TFD antibody response with a fine carbohydrate-binding specificity similar to that of ABL, we designed an immunogen of TFD with a molecular rotation on its carrier linkage that exposes more GalNAc than Gal, since ABL recognizes GalNAc more than Gal in TFD. The synthesis was accomplished by C-6 oxidation of Gal from TFD or its α-benzyl derivative (Bzlα-TFD), followed by reductive amination between the C-6 aldehyde yielded and the available amine of protein. Mice immunized with TFD-KLH (keyhole limpet hemocyanin) or Bzlα-TFD-KLH produced antibodies which were then analyzed by ELISA against several target antigens. Both immunogens raised anti-KLH antibody titers; however, TFD-KLH did not raise anti-TFD antibodies showing low TFD immunogenicity. In contrast, Bzlα-TFD-KLH gave much higher anti-TFD antibody response, indicating that benzyl residue helps improve anti-carbohydrate immune response. When IgG and IgM anti-TFD antibodies were analyzed by competitive ELISA using TFD-related carbohydrates as inhibitors, a high specificity to TFD as well as an enhanced binding to GalNAc over Gal were observed. The axial C-4 hydroxyl group of GalNAc interacted with IgG anti-TFD antibody, as evidenced by the lack of inhibitory activity of GlcNAc in contrast to GalNAc. These findings indicate that the anti-TFD antibodies have fine carbohydrate-binding specificity more similar to ABL than to other TFD-binding proteins that stimulate proliferation of epithelial cell lines.

Key words: immunogenicity/Thomsen-Friedenreich disaccharide/tumor-associated antigen

Introduction

Thomsen-Friedenreich disaccharide (TFD) is chemically defined as α-anomeric Galβ1–3GalNAc and described as type I core of O-linked oligosaccharide constituents of mucins (Hounsell et al., 1996). Sugars formed by O-glycosylation are added individually and sequentially (Brockhausen, 1995), and alterations in the early steps of the biosynthetic pathways change the final carbohydrate composition of the glycan, thereby altering its properties and biological functions (Taylor-Papadimitriou and Finn, 1997). Aberrant O-glycosylation has frequently been described as a tumor-associated alteration (Brockhausen et al., 1995; Hakomori, 1996; Lloyd et al., 1996), resulting in expression of novel carbohydrate epitopes such as TFD, a well-defined antigen with a proven link to tumors. Synthesis of the novel immunogen was first shown by Springer (1984), several papers have described over-expression of TFD in various tumor tissues (Carneiro et al., 1994; Campbell et al., 1995; Langkilde, 1996; Kanitakis et al., 1998; Said et al., 1999). Human adenocarcinoma patients have increased levels of serum mucus which are correlated with poor prognosis (Gouvevitch et al., 1995; MacLean et al., 1997), probably due to their immunosuppressive effect (Fung and Longenecker, 1991; Agrawal et al., 1998). Low levels of natural human anti-TFD antibody have been associated with tumor progression and aggressiveness (Chen et al., 1995; Desai et al., 1995; Kurtenkov et al., 1995). These data provide the basis for use of TFD-bearing molecules in active specific immunotherapy of patients with epithelial tumors (MacLean et al., 1992; Graham et al., 1996; Springer, 1997), and for use of monoclonal anti-TFD antibody (anti-TFD mAb) for inhibition of liver metastases (Shigeoka et al., 1999).

We demonstrated recently that Agaricus bisporus lectin (ABL) binds mainly to TFD where GalNAc (through its axial C-4 hydroxyl group and C-2 acetamido residue) has a more significant role in the interaction than terminal β-linked Gal (Irazoqui et al., 1999). ABL is a reversible noncytotoxic inhibitor of proliferation of epithelial cell lines (Yu et al., 1993; Yu et al., 1999), in contrast to TFD-specific lectin, peanut agglutinin (PNA), and human anti-TFD mAbs, which stimulate proliferation of these cells (Ryder et al., 1994a, 1994b; Yu et al., 1997). Both PNA and anti-TFD mAbs recognize primarily the terminal Gal of TFD (Lotan et al., 1975; Dahlenborg et al., 1997; Loris et al., 1998).

The purpose of the present study was to construct an immunogen that gives rise to anti-TFD antibodies with a fine carbohydrate-binding specificity similar to that of ABL. Such immunogen could be an alternative carbohydrate target antigen for active specific immunotherapy of patients with epithelial tumors. Synthesis of the novel immunogen was accomplished...
by molecular rotation of TFD on its carrier conjugation, where it is linked to protein through C-6 of Gal, thereby exposing more GalNAc than Gal.

Results

Synthesis of glycoconjugate

Galactose oxidase was used to yield aldehyde in C-6 of galactose from TFD or its α-benzyl derivative (BzlαTFD; see Figure 1).

Figure 2 shows the carbohydrate C-6 aldehyde after 2,4-dinitrophenylhydrazine (DNPH) reaction and high-performance thin-layer chromatography (HPTLC) separation. This shows the ability of galactose oxidase to yield aldehyde from BzlαTFD because the carbonyl group can react with DNPH, and major mobility in HPTLC separation is observed.

To the mix of carbohydrate C-6 aldehyde with carrier protein, NaCNBH₃ was added as a catalyst for reductive amination. Synthetic glycoconjugates were analyzed by Western blot and enzyme-lectin assay (ELA). Conjugation of TFD and BzlαTFD to bovine serum albumin (BSA) was detected by a slight difference in their electrophoretic mobility relative to BSA (Figure 3A). The presence of carbohydrates linked to BSA was detected by Western blot using a labeled lectin as ABL-HRP (Figure 3B), which showed the linkage carbohydrates as well as ABL binding ability when (Bzlα)TFD was linked through C-6 to BSA or KLH (Figure 4A). In contrast, for PNA, (Bzlα)TFD C-6 linked to protein did not bind to PNA-HRP (Figure 4B).

Immune response

Table I shows antibody titers measured by ELISA when antisera of mice immunized with (Bzlα)TFD-KLH were analyzed against several target antigens. All sera showed high response against carrier protein (KLH) and no reactivity to BSA. Low immunogenicity of TFD-KLH for anti-TFD antibody response was observed, at least by the immunization protocol used here. However, immunization with BzlαTFD-KLH yielded high antibody titers against BzlαTFD-BSA, and a lower but important IgG and IgM anti-TFD antibody response was observed using TFD-BSA as target antigen in ELISA. BzlαTFD-KLH also raised anti-asialoglycophorin (ASG) and anti-crude porcine stomach mucin (MUC) antibody titers.

Fine carbohydrate-binding specificity of anti-TFD antibodies

IgG and IgM anti-TFD antibodies raised by BzlαTFD-KLH immunization were analyzed by competitive ELISA (CELISA) to determine the fine specificity of their carbohydrate interaction. Figure 5 shows the inhibitory activity of TFD-related disaccharides and monosaccharides on anti-TFD antibody interaction. TFD and pNPhαTFD are the major inhibitors for the assayed carbohydrates, and no significant difference between them was
Novel immunogenicity of Thomsen-Friedenreich disaccharide

noted. Their high specificity is reflected by the fact that both are 5000- to 10,000-fold stronger inhibitors than GalNAc (Table II). Of the other disaccharides assayed, lacto-N-biose showed significant inhibition whereas lactose, N-acetyllactosamine and Galβ1–6GlcNAc showed no inhibitory activity on anti-TFD antibody interaction. Analysis of TFD-related monosaccharides showed that GalNAc was recognized by IgG and IgM antibodies, whereas Gal, MeβGal, and MeβGal showed no competitive activity. GlcNAc inhibited IgM but not IgG anti-TFD antibody interaction.

Discussion

Aberrant O-glycosylation of cell surface mucin antigens occurs on epithelial cancer cells, and TFD is a chemically well-defined carbohydrate antigen with a documented link to malignancy (Cao et al., 1995; Yang and Shamsuddin, 1996; Baldus et al., 1998). Many studies have attempted to improve immune response to TFD and related carbohydrates, using

### Table I. Anti-carbohydrate antibody titers measured by ELISA$^a$

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>TFD-KLH</th>
<th>BzlαTFD-KLH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>KLH</td>
<td>&gt;6400</td>
<td>0</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BzlαTFD-BSA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TFD-BSA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASG</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>MUC</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Target antigens (10 µg/ml) were coated on ELISA plates in PBS overnight at 4°C and saturated with PBS-T for 1 h at 37°C. Serially diluted antiserum was incubated with the coated antigen for 2 h at room temperature, then washed six times with PBS-T. Adherent antigen-antibody complex was detected with 1/1000 goat anti-IgG or IgM antibody conjugate with peroxidase in PBS-T, incubated at room temperature for 2 h. After washing six times with PBS-T, color reaction was developed as ELA. The ELISA titer was defined as the highest dilution yielding an absorbance value 20.1 more than that of normal serum (Ragupathi et al., 1998). Value 0 is <50 dilution.

$^a$Median value from five mice.

### Table II. Inhibition of anti-TFD antibody interaction using TFD-related carbohydrates

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Concentration (mM) required for 50% inhibition</th>
<th>Relative inhibitory potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>Gal</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>MeβGal</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>GalNAc</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>&gt;200</td>
<td>100</td>
</tr>
<tr>
<td>Galβ1–3GlcNAc</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Galβ1–3GalNAc</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Galβ1–3GalNAcO-pNPh</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Galβ1–4Glc</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Galβ1–4GlcNAc</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Galβ1–6GlcNAc</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>
oligosaccharide immunogens. These include (1) cells expressing polymorphic epithelial mucins (Lalani et al., 1991); (2) TFD or Tn (GalNAC-O-Ser/Thr) present in human group O erythrocyte membrane (Springer, 1997); (3) Tn or sialylated Tn of ovine submaxillary mucin (Singhal et al., 1991); (4) synthetic TFD attached by a linker to various carrier proteins (MacLean et al., 1992; Adluri et al., 1995; Rittenhouse-Diakun et al., 1998); (5) synthetic clustered sialyl TFD linked to peptide chain (Sames et al., 1997); (6) synthetic Tn-lipopeptide conjugate (Toyokuni and Singhal, 1995). In these previous studies, conjugation of the carbohydrate used as immunogen is through C-1 of GalNAc, whereas in the present study TFD is conjugated through C-6 of Gal. To our knowledge, this is the first example of a disaccharide molecular rotation made to test the immunogenicity of an alternative stereochemical configuration. The molecular rotation of TFD on its carrier linkage was obtained by specific C-6 oxidation of Gal using galactose oxidase (Avigad et al., 1962), following use of NaCNBH3 as catalyst of reductive amination between the obtained aldehyde and the available amine group of protein (Gray, 1974), probably ε-amine of lysine because it is the most reactive amine group (Schwartz and Gray, 1977). The synthetic TFD linkage through C-6 of Gal was chosen because the C-6 hydroxyl group is not relevant to the TFD-ABL interaction (Chen et al., 1995) and because TFD conjugated through C-6 can bind ABL (Figure 3), in contrast to PNA, where the C-6 linkage makes it unavailable to PNA interaction (Figure 4). These data suggest that immune response to TFD using the synthesized immunogen should be more similar to ABL than to PNA based on fine carbohydrate-binding specificity. As a consequence of C-6 conjugation, the GalNAc residue of TFD has a terminal position relative to natural α-anomeric Galβ1–3GalNAc O-linked in glycoproteins (Figure 1C,D). Thus, it appears more likely that the immune response to TFD is directed to GalNAc over Gal, since the major immune response to carbohydrate is usually to terminal structures (Bundle and Young, 1992).

Comparison of ABL binding to BSA or KLH by ELA indicates a major interaction with KLH (Figure 4). This observation is consistent with the presence of O-glycans in KLH (Wirguin et al., 1995). However, immunization of mice with KLH containing synthetic TFD C-6 linkage in addition to natural O-glycans did not raise significant anti-TFD antibody titer, confirming the poor immunogenicity of TFD. In contrast, when BzlxTFD was C-6 attached to KLH and used as immunogen, strong immune response against TFD was obtained. This illustrates the effect of chemical modification, in this case benzyl group, on carbohydrate target immunogenicity to improve anti-TFD antibody titer. Similarly, Gu et al. (1998) observed enhanced cellular and humoral immunity using an hydrophobized polysaccharide.

BzlxTFD-KLH immunogen raises anti-ASG antibody titer, demonstrating recognition of TFD when it is α-linked through C-1 of GalNAc. BzlxTFD-KLH also improves anti-MUC antibody titers, and shows a binding ability more similar to ABL than to PNA, since PNA does not bind to MUC (Figure 4B).

The anti-TFD antibodies were analyzed in terms of fine carbohydrate-binding specificity, using TFD-related carbohydrates as inhibitors in CELISA. High specificity of IgG and IgM antibodies to TFD was evidenced by the fact that TFD and

---

**Fig. 4.** Reactivity of (glyco)proteins with lectins by enzyme-lectin assay (ELA). Polystyrene microtitration plates were coated with various concentrations of BSA (solid circles), KLH (open circles), TFD-KLH (open inverted triangles), BzlxTFD-KLH (solid inverted triangles), asialoglycophosphoryl (solid squares) and crude porcine stomach mucin (open squares) in PBS overnight at 4°C, and incubated with 0.04 μg/ml ABL-HRP (A) or PNA-HRP (B) for 2 h at room temperature and washed six times with PBS-T. Color reaction was developed using 2 mg/ml o-phenylenediamine and 0.02% H2O2 in 100 mM sodium citrate, pH 5.0, at room temperature for 30 min. Reaction was stopped by adding 100 μl of 2.5 M sulfuric acid, and absorbance values were read at 492 nm with a microplate reader.

---

**Fig. 5.** Competitive ELISA (CELISA) using carbohydrates as inhibitors of anti-TFD antibody interaction. Wells coated with TFD-BSA were assayed against mouse IgM (A) and IgG (B) anti-TFD antibody, previous incubation of antibodies with following carbohydrates: Gal (open triangle), Me Gal (solid circles), MeβGal (solid triangles), GlcNAc (open inverted triangle), Gal (solid circles), lactose (solid inverted triangles), TFD (solid diamonds), lacto-N-biose (solid squares), N-acetyllactosamine (asterisks), Galβ1–6GlcNAc (open diamonds), and pNPhT (open squares). Washing, color reaction, and absorbance reading were performed as described in ELA.
pNPhtTFD were the major inhibitors of all assayed carbohydrates. Both disaccharides showed ID$_{50}$ of ~0.02 mM, similar to that reported for inhibition of ABL interaction (Irazoqui et al., 1999). When TFD-related monosaccharides were assayed as competitors, GalNAc was found to have greater inhibitory activity than Gal on TFD-IgG and -IgM antibody interaction. These findings are contrary to those for previously described anti-TFD mAbs (Dahlenborg et al., 1997) and PNA (Lotan et al., 1975; Loris et al., 1998) where Gal was more relevant than GalNAc on TFD interaction.

Our results indicate that C-2 acetamido addition improves binding of Gal, and the axial C-4 hydroxyl group in GalNAc provides a significant binding locus, since GlcNAc does not bind to the IgG antibody. However, the GalNAc ID$_{50}$ obtained here (100 mM) is 4-fold higher than that described for ABL. This may reflect minor importance of the C-4 hydroxyl group of GalNAc from TFD, as evidenced by the fact that lacto-N-biose (Galβ1–3GlcNAc) is a better inhibitor of the competitive assays of these antibodies than for ABL (Irazoqui et al., 1999). This explanation is also supported by the lack of inhibitory activity of lactose and Galβ1–6GlcNAc on TFD-antibody interaction, in contrast to ABL. Binding of ABL is attributed to the C-4 and C-3 hydroxyl groups of reducing Glc from lactose and Galβ1–6GlcNAc respectively, since conformational analysis shows that they occupy a position similar to C-4 hydroxyl group of GalNAc from TFD. This C-4 hydroxyl group position is less relevant for IgM anti-TFD antibody interaction, since GalNAc and GlcNAc do not differ significantly in their inhibitory activity. CELISA also indicates a lower ID$_{50}$ difference between TFD and lacto-N-biose for IgM anti-TFD antibody.

In conclusion, we found that immunization of mice with BzlαTFD conjugated to KLH through C-6 of Gal produces anti-TFD antibodies with enhanced binding to GalNAc over Gal, and consequently a fine carbohydrate-binding specificity more similar to that of ABL than other TFD-binding proteins. The TFD immunogen synthesized as described here could be an effective alternative carbohydrate target antigen for active specific immunotherapy of patients with epithelial tumors. Experiments to test this hypothesis are in progress.

Materials and methods

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO). ABL was purified and conjugated to HRP (ABL-HRP) as described previously (Irazoqui et al., 1997).

Preparation of (Bzlα)TFD C-6 aldehyde and its conjugation to protein

Galactose oxidase (50 U) was immobilized to cyanogen bromide-activated Sepharose 4B gel (100 mg) as described by Cuatrecasas and Anfinsen (1971). One milliliter of 2 mM carbohydrate (TFD or BzlαTFD) in PBS was incubated with immobilized galactose oxidase for 2 h at room temperature with constant stirring. The carbohydrate C-6 aldehyde was recovered by gel separation, detected by DNH reaction (Liggins and Furth, 1997), and separated by HPTLC. Briefly, 50 µl carbohydrate solution was added with an equal volume of methanol and 7 mg/ml DNH (50 µl) in methanol:HCl (80:1) for 2 h at room temperature, then added with 2.5 mM pyridine (50 µl) in methanol. The tubes were dried in nitrogen atmosphere and dissolved in 50 µl methanol for HPTLC analysis. Carbohydrates were separated on HPTLC silica gel 60 (Merck) in the running solvent chloroform-methanol-aqueous 0.2% CaCl$_2$ (60:37:8), using a tank to obtain highly reproducible chromatograms (Nores et al., 1994). After air-drying for 15 min, carbohydrates were visualized chemically using orcinol-sulfuric acid spray reagent, for 5 min at 120°C.

TFD and BzlαTFD C-6 aldehydes were mixed with 3 mg BSA or KLH in PBS for 10 min, then added with 3 mg NaCNBH$_3$. The reaction mixture was incubated with gentle stirring for 4 h at room temperature. The glycoconjugate was diazylated with four changes of PBS, at 4°C. The presence of carbohydrates linked to proteins was analyzed by Western blot for BSA and enzyme-lectin assay (ELA) for KLH.

Immunization of mice

Groups of 5 mice (Rockefeller female, age 6 weeks) were intradermally injected with 100 µg TFD-KLH or BzlαTFD-KLH mixed with 100 µl Freund’s complete adjuvant, at four shaved sites. Three weeks later this process was repeated, but using Freund’s incomplete adjuvant. Three weeks later, mice were boosted by subcutaneous injection with the same sample as the second injection. Mice were bled at day 0 and day 10 after the third immunization.

Competitive ELISA (CELISA)

The three antisera having highest anti-TFD titer were analyzed by CELISA and the results averaged. The optimal antiserum dilution showing 1.0 optical density against TFD-BSA (10 µg/ml) as target antigen was determined in preliminary experiments. All steps of CELISA were developed as ELISA, except that the optimal antiserum dilution was preincubated with several carbohydrates for 1 h at room temperature before adding to wells.

Acknowledgments

We thank I.Orsingher for her language assistance, Dr. J.A.Curtino for helpful discussion and Dr. S.Anderson for editing. This work was supported by grants (to G.A.N.) from CONICOR, SeCyT (UNC), CONICET and FONCYT, Argentina. F.J.I. and P.H.H.L. acknowledge the fellowship assistance from CONICET and CONICOR, respectively.

Abbreviations

ABL, Agaricus bisporus lectin; ASG, asialoglycophorin; DNH, 2,4 dinitrophenylhydrazine; ELA, enzyme-lectin assay; ID50, concentration required for 50% inhibition; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MUC, crude porcine stomach mucin; PNA, peanut agglutinin; TFD, Thomsen-Friedenreich disaccharide.

References

patients with gastric cancer related to ABO (H) blood-group phenotype. Int. J. Cancer, 60, 781–785.


