Carbohydrate Research, 132 (1984) 93–103
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ANTISERA SPECIFICITIES TO β-D-GALACTOPYRANOSIDE CLUSTER LIGANDS*

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(Received February 13th, 1984; accepted for publication, March 12th, 1984)

ABSTRACT

Mono-, di-, and tri-β-D-galactopyranosides of 2-(5-hydrazinocarbonylpentanamido)-2-(hydroxymethyl)-1,3-propanediol [$(Gal)_n$-TA] have been conjugated to bovine serum albumin (BSA), and used to study the binding specificities to the Gal receptors of liver parenchymal cells. In this study, rabbit antisera produced to the $(Gal)_n$-TA-BSA were characterized by using an enzyme-linked, immunosorbent assay under conditions that allow only the antibodies directed to the carbohydrate part of the antigen to react with the solid-phase $(Gal)_n$-TA-BSA antigens. Inhibition assays using $(Gal)_n$-TA-BSA conjugates showed a relative specificity of the antisera for the number of Gal residues on the TA bridging group to the BSA carrier-protein, indicating that antibodies having specificities to oligosaccharide branch points can be produced. Inhibition assays with $(Gal)_n$-TA haptens, Gal, and methyl β-D-Gal indicated that the antibody combining-sites interact mainly with the Gal units; no inhibition was observed with the TA bridging group used as a hapten inhibitor. The spatial distances of the Gal units were apparently important for interaction with the anti-$(Gal)_n$-TA-BSA antibody-combining-sites, as $(Lac)_3$-TA-BSA and $(Lac)_3$-TA exhibited relatively little inhibitory activity.

INTRODUCTION**

Neoglycoproteins have been synthesized and then used to study the role of carbohydrates in many biological interactions[1–3] and to examine the specificities of anti-carbohydrate antibodies[4,5]. Most of these neoglycoproteins have consisted mainly of monosaccharides or unbranched oligosaccharides conjugated to carrier

*This work was supported, in part, by Grant No. CA-30070 awarded by the National Cancer Institute, and by Grant No. AM-9970 from the National Institutes of Health, DHHS.

**Abbreviations used. TA: 2-(5-hydrazinocarbonylpentanamido)-2-(hydroxymethyl)-1,3-propanediol; $(Gal)_n$-TA: *n* β-D-Gal residues glycosidically linked to the TA group; $(Gal)_1$-, $(Gal)_2$-, and $(Gal)_3$-TA: 1, 2, and 3 D-Gal residues linked to the TA group; BSA: Bovine serum albumin; PBS: phosphate-buffered saline; PBSA: 1% BSA in PBS plus 0.1% of Tween-20; ELISA: enzyme-linked, immunosorbent assay; Lac: lactose, β-D-Gal-(1→4)-D-Glc; ONPG: *o*-nitrophenyl β-D-galactopyranoside.

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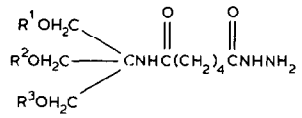

(mono-Gal-TA)$_n$ R^1 = Gal, R^2 = R^3 = H

1

(di-Gal-TA)$_n$ R^1 = R^2 = Gal, R^3 = H

2

(tri-Gal-TA)$_n$ R^1 = R^2 = R^3 = Gal

3

(tri-Lac-TA)$_n$ R^1 = R^2 = R^3 = Lac

4

protein. However, in Nature, many glycoconjugates contain one or more branched oligosaccharides per molecule. In order to study such glycoconjugates, the mono-, di-, and tri-β-D-galactopyranosides (**1–3**) of 2-(5-hydrazinocarbonylpentanamido)-2-(hydroxymethyl)-1,3-propanediol [(Gal)$_n$-TA] were synthesized[6,7]. The (Gal)$_n$-TA glycosides were then coupled to BSA by the acyl azide method[8], to afford neoglycoproteins.

These neoglycoproteins were tested for their inhibitory effect on the binding of asialoglycoproteins to the Gal receptor of rabbit-liver membranes, their interaction with the isolated Gal-binding protein, and uptake by intact, rat hepatocytes[7].

We now describe the preparation of rabbit antisera to the (Gal)$_1$-, (Gal)$_2$- and (Gal)$_3$-TA-BSA conjugates, and the specificities of antibodies to the closely spaced, Gal units

EXPERIMENTAL

Mono- and oligo-saccharides. — Compound **3** was prepared by reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**5**) with 2-(hydroxymethyl)-2-(5-methoxycarbonylpentanamido)-1,3-propanediol (**6**) and mercuric cyanide, and the fully protected tri-D-galactoside was converted into the hydrazide by treatment with hydrazine in absolute ethanol[6,7]. Compounds **1** and **2** were prepared by the same method, but using a lower ratio of compound **5** to **6**, and separating the mono-, di-, and tri-D-galactosides on a column of Sephadex LH-20, followed by conversion into the hydrazides. Gal, Lac, melibiose, gentiobiose, and methyl β-D-Gal were purchased from Sigma Chemical Co., St. Louis, MO.

Antigens and immunizations. — (Gal)$_n$-TA and (Lac)$_3$-TA were conjugated to BSA by the acyl azide method, and the carbohydrate content of the resulting conjugates was analyzed as previously described[7]. Rabbits were immunized with the (Gal)$_n$-TA-BSA conjugates over multiple sites. The first injection consisted of 0.5 mg of the conjugate mixed with Freund's complete adjuvant (CalBiochem-

Behring, San Diego, CA). Subsequent injections were given with antigen mixed in incomplete Freund's adjuvant at approximately 3-week intervals. Antisera were collected 7–10 days after the injections; pre-immune sera were collected for use as controls.

Enzyme-linked, immunosorbent assay (ELISA). — The wells of 96-well ELISA plates (Immulon 2, Cooke Engineering Co., Alexandria, VA) were coated with 200 μL of $(Gal)_n$-TA-BSA in 0.1M $NaHCO_3$, pH 9.0, for 2 h at 22°. The wells were then backcoated with 1% ovalbumin (Sigma Chemical Co.) in 0.01M sodium phosphate buffer, pH 7.3, containing 0.15M NaCl (PBS) for 1 h at 22°. Wells were now washed 3 times with PBS containing 1% of BSA (PBSA) and 0.1% of Tween-20 (Sigma Chemical Co.) after each step, following backcoating. Antisera diluted in PBSA (200 μL) were next added to the wells, and these were incubated for 2 h at 22°. After washing to remove excess antisera, β-D-galactosidase (Sigma Chemical Co.) conjugated goat anti-rabbit IgG (affinity purified; Cappel Laboratories, West Chester, PA), prepared according to the method of Boraker *et al.*[9] and diluted in PBSA, was added and the mixture was incubated overnight at 4°. After washing with PBSA, a 4 mg/mL solution (200 μL) of ONPG (Sigma Chemical Co.) in 0.1M sodium phosphate buffer, pH 7.3, containing 0.1M 2-mercaptoethanol and 5mM $MgCl_2$ was added. After incubation, the optical absorbances of *o*-nitrophenol were read at 405 nm by using a Titertek Multiskan (Flow Laboratories, McClean, VA). In certain experiments, peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) was used instead of the β-D-galactosidase-conjugated second antibody, and the color reaction was developed with *o*-phenylenediamine · 2 HCl in substrate diluent (Abbott Laboratories, N. Chicago, IL). All experimental points were obtained in duplicate, and each assay included pre-immune, secondary antibody, substrate, and other controls, as indicated.

Inhibition assays. — Inhibition assays were performed as just described, except that various concentrations of the inhibitors were incubated with the diluted antisera for 2 h before addition to the wells of the ELISA plates.

Glycoproteins. — Desialized, ovine submaxillary mucin was a gift from Dr. D. Carlson. Ovomucoid (Sigma Chemical Co.) was desialyzed by hydrolysis with 0.05M H_2SO_4 for 1 h at 80°. Hog gastric mucin was purchased from Sigma Chemical Co., and purified by ethanol fractionation[10]. The desialyzed ovomucoid and the hog mucin preparations were processed through two cycles of periodate oxidation and Smith degradation, as described by Feizi *et al.*[11]. In order to ascertain binding of diluted anti-$(Gal)_n$-TA-BSA sera to the glycoproteins, each glycoprotein (1 μg/mL of solution) was adsorbed to the wells of the ELISA plates, and the assay performed as already described.

RESULTS

The degrees of substitution of the conjugates by carbohydrate were 16, 21.5, and 23 for $(Gal)_1$-TA-BSA, $(Gal)_2$-TA-BSA, and $(Gal)_3$-TA-BSA, respectively,

and 40 for $(Lac)_3$-TA-BSA. The molar concentrations in the competitive-binding experiments with solution-phase $(Gal)_n$-TA-BSA conjugates were calculated on the basis of the oligosaccharide cluster substitutions.

The assays were conducted in 1% BSA solution in order to prevent the measurement of anti-BSA antibodies. No inhibition was obtained on using BSA, and complete inhibition could be obtained by using the $(Gal)_n$-TA haptenic groups, indicating that the reactions detected were not due to antibodies directed to the BSA-carrier molecule, but were due only to binding to the $(Gal)_n$-TA portions of the conjugates.

For each of the anti-$(Gal)_n$-TA-BSA sera, the concentrations of coating antigens and dilutions of antisera were initially varied, in order to yield ~1 unit of absorbance at 405 nm in an incubation time of 30–60 min with the ONPG substrate. The conditions determined were: $(Gal)_1$-TA-BSA, 25 ng/mL for antigen coating and 1:60,000 dilution of anti-$(Gal)_1$-TA-BSA serum: $(Gal)_2$-TA-BSA, 25 ng/mL and 1:100,000 dilution of anti-$(Gal)_2$-TA-BSA serum; $(Gal)_3$-TA-BSA, 0.05 μg/mL and 1:25,000 dilution of anti-$(Gal)_3$-TA serum. Pre-immune sera exhibited no binding to the $(Gal)_n$-TA-BSA antigens at 1:2000 dilution. The specificities of the antisera and the relative inhibitory effectiveness of the conjugates were not dependent on the conditions of the assay system, as changes in the concentration of the coating antigens and the antisera dilutions did not change the results. In order to ascertain whether the β-D-galactosidase of the secondary antibody affected the assay results by hydrolysis of the Gal units of the $(Gal)_n$-TA-BSA, a peroxidase-conjugated, secondary antibody was also used. The results were identical, showing that the β-D-galactosidase-conjugated, anti-rabbit IgG had no effect on the competitive-binding experiments.

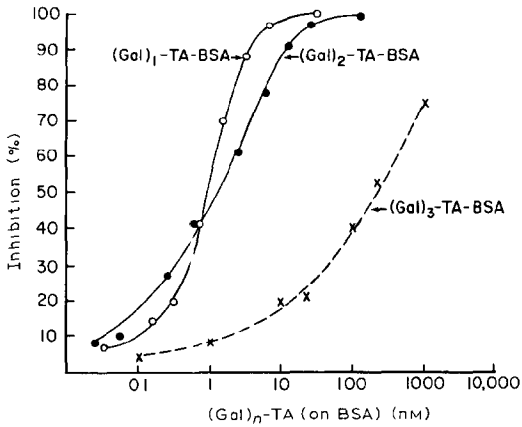

Fig. 1. Inhibition of binding of anti-$(Gal)_1$-TA-BSA antibodies to $(Gal)_1$-TA-BSA on the solid phase by $(Gal)_n$-TA-BSA conjugate antigens. {The details of the solid-phase ELISA, coating concentration of $(Gal)_1$-TA-BSA and antiserum dilution are described in the text. The $(Gal)_1$- and $(Gal)_2$-TA-BSA antigens gave 50% inhibitions at a concentration of about 1nM [calculated on the basis of the $(Gal)_n$-TA substitution of the conjugate]; $(Gal)_3$-TA-BSA was 180-fold less effective as an inhibitor.}

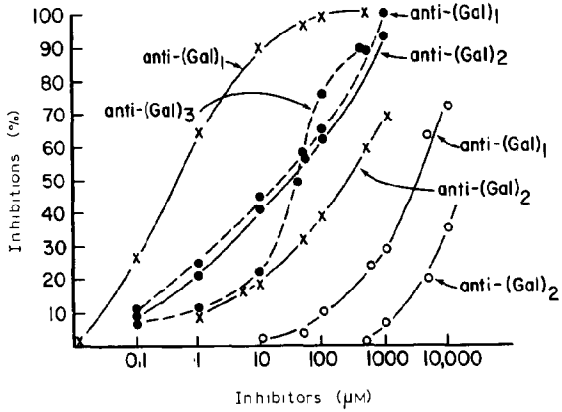

Fig. 2. Inhibitions of anti-$(Gal)_n$-TA-BSA antibodies to their homologous antigens on the solid phase by various haptens. [The inhibitors were pre-incubated with the diluted antisera prior to addition to the ELISA plate wells as described in the text. The haptens used were: $(Gal)_3$-TA with anti-$(Gal)_3$-TA-BSA (● - - ●), with anti-$(Gal)_2$-TA-BSA (●, solid line), and anti-$(Gal)_1$-TA-BSA (● - - ●); $(Gal)_1$-TA (× - ×); methyl β-D-Gal (○ - ○). The antisera are designated on the Figure. Other potential haptenic inhibitors, Lac, methyl α-D-Gal, Gal; the TA linkage group did not result in detectable inhibitions; $(Lac)_3$-TA exhibited much less relative inhibition at the concentrations listed in the text.]

Specificity of anti-$(Gal)_1$-TA-BSA. — Fig. 1 shows that the anti-$(Gal)_1$-TA-BSA antibodies were best inhibited with $(Gal)_1$-TA-BSA and $(Gal)_2$-TA-BSA, with 50% inhibitions obtained at 0.89 and 1.12nM, respectively [calculated on the basis of (Gal)*n*-TA substitutions]. With the haptens tested, $(Gal)_1$-TA gave 50% inhibition at 0.4μM, and $(Gal)_3$-TA at 22.4μM(see Fig. 2). Gal was a poor inhibitor, resulting in 30% inhibition at 100mM, and methyl β-D-Gal gave 50% inhibition at 2.5mM. Lac (100mM), TA (10mM), gentiobiose (10mM), melibiose (10mM), and methyl α-D-Gal (10mM) did not exhibit any detectable inhibitory activities, and $(Lac)_3$-TA gave 27% inhibition at 0.5mM.

Specificity of anti-$(Gal)_2$-TA-BSA. — Using the $(Gal)_n$-TA-BSA as inhibitors, anti-$(Gal)_2$-TA-BSA antibodies showed a relatively high degree of specificity for the homologous conjugate (see Fig. 3), giving 50% inhibition at 0.32nM. $(Gal)_1$-TA-BSA and $(Gal)_3$-TA-BSA were respectively 353- and 150-fold less effective than $(Gal)_2$-TA-BSA. The antibody–$(Gal)_2$-TA-BSA binding was inhibited 28% by 44μM $(Lac)_3$-TA-BSA. Using haptens (see Fig. 2), $(Gal)_3$-TA gave 50% inhibition at 282μM, and $(Gal)_1$-TA at 200μM, whereas $(Lac)_3$-TA yielded 35 and 60% inhibitions at 0.5 and 1mM, respectively. Methyl β-D-Gal caused 35% inhibition at 10mM, and no inhibition was seen with the other potential haptens at the concentrations used with anti-$(Gal)_1$-TA-BSA.

Specificity of anti-$(Gal)_3$-TA-BSA. — Fig. 4 shows that $(Gal)_3$-TA-BSA inhibited the homologous antiserum 4-fold better than $(Gal)_2$-TA-BSA, with 50% inhibitions obtained at 3.2 and 13.6nM, respectively. No inhibition was detected with $(Gal)_1$-TA-BSA at concentrations of up to 0.6mM, and $(Lac)_3$-TA-BSA inhibited to the extent of only 12% at 44μM. $(Gal)_3$-TA gave 50% inhibition at 45μM

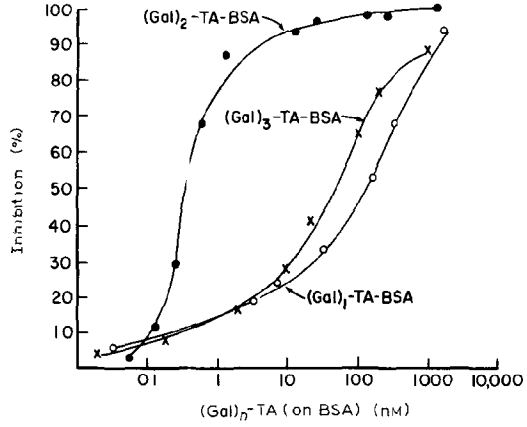

Fig. 3. Inhibition of binding of anti-$(Gal)_2$-TA-BSA antibodies to solid-phase $(Gal)_2$-TA-BSA by $(Gal)_n$-TA-BSA conjugates.

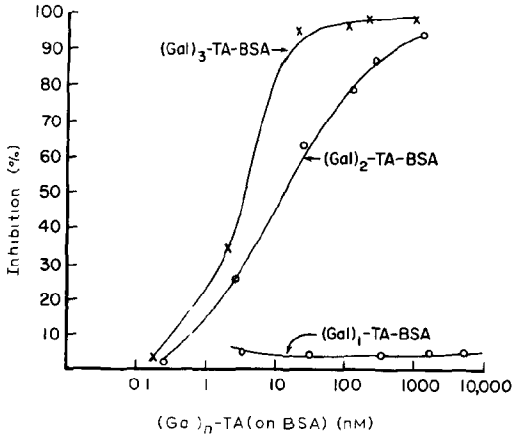

Fig. 4. Inhibition of binding of anti-$(Gal)_3$-TA-BSA antibodies to solid-phase $(Gal)_3$-TA-BSA by $(Gal)_n$-TA-BSA conjugates.

(see Fig. 2), and $(Lac)_3$-TA inhibited 9% at 0.5mM and 20% at mM; all other haptens tested did not result in any detectable inhibition at the concentrations used with anti-$(Gal)_1$- or anti-$(Gal)_2$-TA-BSA sera.

Cross-reactivities of the antisera were studied by allowing anti-$(Gal)_2$-TA-BSA to react with solid-phase $(Gal)_3$-TA-BSA, or anti-$(Gal)_3$-TA-BSA with solid-phase $(Gal)_2$-TA-BSA, and inhibitions, with the $(Gal)_2$- and $(Gal)_3$-TA-BSA (see Fig. 5). The patterns of inhibition were similar to those observed with each antiserum and its homologous antigen (see Figs. 3 and 4), except that the differential reactivities of the antisera were diminished.

When used at the same dilutions as in the $(Gal)_n$-TA-BSA antigen assays, the anti-$(Gal)_1$-, -$(Gal)_2$-, and -$(Gal)_3$-TA-BSA sera did not exhibit any detectable

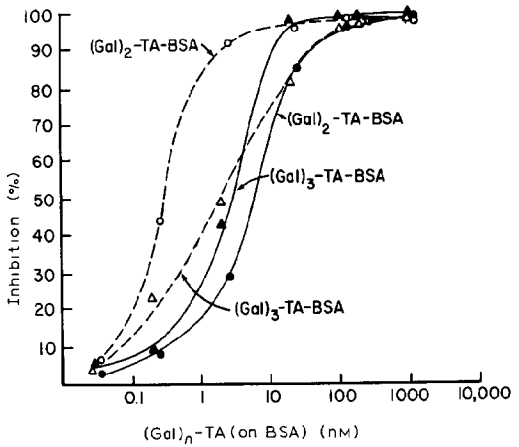

Fig. 5. Inhibition of binding of anti-$(Gal)_2$- and anti-$(Gal)_3$-TA-BSA antibodies to solid-phase $(Gal)_3$- and $(Gal)_2$-TA-BSA, respectively, by the $(Gal)_n$-TA-BSA conjugates.

antibody binding to desialized, ovine submaxillary mucin or ovomucoid, to hog mucin or to the first and second Smith-degradation products of hog mucin and desialized ovomucoid, or to $(Lac)_3$-TA-BSA. Detectable antibody binding was observed only for anti-$(Gal)_1$- and anti-$(Gal)_2$-TA-BSA sera at 1:500 dilution with the hog mucin adsorbed to the wells of the ELISA plates, but not to the other glycoproteins at the same dilution. Also, all three antisera exhibited significant antibody binding to $(Lac)_3$-TA-BSA at 1:2000 dilution.

DISCUSSION

The results show that there can be produced antisera to closely spaced carbohydrate units, and that the antibody populations of each antiserum exhibited a relative specificity for the number of Gal residues. For example, the anti-$(Gal)_2$-TA-BSA serum showed at least a 150-fold greater reactivity for the two Gal residues of $(Gal)_2$-TA-BSA, compared to the $(Gal)_1$- and $(Gal)_3$-TA-BSA antigens; the anti-$(Gal)_3$-TA-BSA antibodies exhibited a 4-fold greater inhibition with the homologous antigen than with $(Gal)_2$-TA-BSA, and no reactivity with $(Gal)_1$-TA-BSA. Although anti-$(Gal)_1$-TA-BSA reacted almost equally well with $(Gal)_1$- or $(Gal)_2$-TA-BSA antigens, much less inhibition was obtained with the $(Gal)_3$-cluster ligand–BSA conjugate.

The specificities observed with the $(Gal)_n$-TA hapten inhibitions was similar to that seen with the $(Gal)_n$-TA-BSA antigens. The anti-$(Gal)_1$-TA-BSA antibody binding to its homologous antigen was inhibited with a 10^3-fold smaller concentration of $(Gal)_1$-TA than the anti-$(Gal)_2$-TA-BSA binding to $(Gal)_2$-TA-BSA antigen. Also, at the highest concentration tested, $(Gal)_1$-TA did not inhibit the anti-$(Gal)_3$-TA-BSA serum. With the $(Gal)_3$-TA hapten, similar concentrations inhibited all three anti-$(Gal)_n$-TA-BSA sera, although the curvature of the sigmoi-

dal, inhibition curve for the anti-$(Gal)_3$-TA-BSA assay-system was greater than those of the other two antisera. The lesser curvature seen with anti-$(Gal)_1$- and anti-$(Gal)_2$-TA-BSA sera inhibited by $(Gal)_3$-TA is a result of the greater inhibitions obtained at low concentrations with the $(Gal)_3$-TA hapten. In the case of the anti-$(Gal)_1$- and -$(Gal)_2$-TA-BSA sera, the $(Gal)_3$-TA hapten contains more than one identical antigenic group. Thus, at low concentrations of the inhibitor, the antibodies can cross-link the $(Gal)_3$-TA molecules, to form circular complexes, resulting in a higher and a functional affinity, and accounting for the greater inhibitory activities with the anti-$(Gal)_2$ compared to the anti-$(Gal)_3$ sera. The $(Gal)_2$-TA hapten was not available for testing as an inhibitor.

For each of the anti-$(Gal)_n$-TA-BSA sera, the $(Gal)_n$-TA-BSA antigens exhibited inhibitions in the nM range of concentration, whereas the $(Gal)_1$- or $(Gal)_3$-TA haptens were effective inhibitors in μM concentrations. Thus, the functional-affinity factor of the multiple $(Gal)_n$-cluster ligands of the antigens was of the order of 10^3 to 10^4.

The inhibition data also suggested that the antibody combining-sites of each antiserum primarily interact with the Gal units, as the TA linking-group exhibited no inhibition at the highest concentration tested (10mM). This was an unpredicted result for the anti-$(Gal)_1$-TA-BSA, where the combining-site interaction would have been expected to include a portion of the TA linking-group. The poorer inhibitions obtained with methyl β-D-Gal and $(Gal)_1$-TA haptens of anti-$(Gal)_2$- compared to anti-$(Gal)_1$-TA-BSA sera indicated that the combining sites of anti-$(Gal)_2$-TA-BSA interact with the two Gal units. Also, the greater inhibition of anti-$(Gal)_3$-TA-BSA with its homologous antigen indicated antibody combining-site interactions mainly with two Gal units and with at least a portion of the third Gal residue.

The $(Gal)_3$-TA cluster-ligand consists of each Gal residue joined, *via* an $-OH_2C-$ group, to the same carbon atom which is adjacent to the amide nitrogen atom of the TA linking-arm. The Gal residues may be readily rotated about their glycosidic bonds to the common carbon atom, giving three distinct orientations with regard to the pentanamido portion of the TA linking arm (*i.e.*, changing the ψ torsion-angle of the glycosidic bond). The ϕ torsion-angle was kept at 50°, in accord with the exo-anomeric effect as discussed by Lemieux[12,13]. One of these orientations is shown in Fig. 6, with the rings of the Gal residues and the axial hydrogen atoms perpendicular to the pentanamido group. Two additional, and opposite, orientations can be achieved by changing the ψ torsion-angles, resulting in (*a*) the Gal rings and the axial hydrogen atoms being on the same side as the pentanamido group, or (*b*) each Gal ring oriented approximately parallel to the pentanamido group, with either all axial hydrogen atoms of the rings pointing in the same direction, or with one of the rings oppositely oriented. Lemieux[13] and Kabat *et al.*[14] showed that the binding of carbohydrate antigenic-determinant sequences to antibodies and lectins may involve hydrophobic interactions and be facilitated by intra-residue hydrogen-bonding of the hydroxyl groups. For example, β-D-Gal-(1→4)-β-

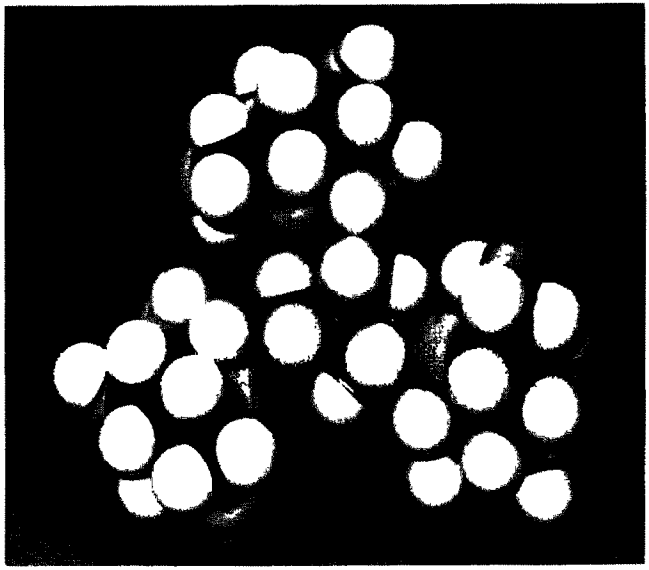

Fig. 6. Photograph of a molecular model of $(Gal)_3$-TA. [The 3 Gal residues were positioned by changing the ψ torsion angle of the glycosidic bond such that the axial hydrogen atoms on C-3 and C-5 were approximately perpendicular to the pentanamido group (which projects to the back of the photograph and is not visible). The ϕ torsion angles of the glycosidic bond are 50°. The 6 hydrogen atoms of the 3 $-OCH_2-$ groups are in the middle of the structure as photographed. The hydrophobic regions of the $(Gal)_3$-TA hapten that may interact with the antibody combining-sites are readily apparent.]

D-GlcNAc-(1→6)-R and related haptenic derivatives were found to bind to the monoclonal anti-I Ma antibody in a conformation that presents a hydrophobic region of the carbohydrate structure to the antibody binding site. The conformation for $(Gal)_3$-TA as shown in Fig. 6, with all of the axial hydrogen atoms projecting away from the linking arm, provides a readily visualized, hydrophobic surface that could interact with the antibody combining-sites as proposed by Lemieux[13] and by Kabat *et al.*[14].

Antibody combining-sites have been postulated to consist of longitudinal grooves or cavities, based upon X-ray crystallographic data and hapten-inhibition experiments[15–18]. The sizes and dimensions of the combining sites can be quite different, depending upon the particular antibody or myeloma protein studied. Considering the structure of $(Gal)_3$-TA, it is more probable that the antibody combining-site would be a pocket, rather than an elongated groove. The circle that subscribes the triangle formed by the three Gal residues of $(Gal)_3$-TA has an area of 2.27 nm^2, whereas the pocket type of antibody combining-site described had[16,17] circular openings of up to 1.76 nm^2. If anti-$(Gal)_3$-TA-BSA antibodies have a pocket with dimensions close to those calculated for the myeloma proteins, only 2 Gal residues could enter the pocket, and the third Gal residue could interact to some extent with a portion of the combining site adjacent to, or outside, the pocket. Such reasoning could explain why anti-$(Gal)_3$-TA-BSA was inhibited only 4-fold better with $(Gal)_3$-TA-BSA than by $(Gal)_2$-TA-BSA.

The inhibition data suggest that antibodies can be produced with a relative specificity to the number of branch points, where single residues extend from the point of branching. For example, the $(Gal)_2$-TA-BSA structure is equivalent to a disubstituted oligosaccharide having two residues linked to O-2 and O-4 of a third D-galactopyranose, and thus, the structures used in this study are similar to 2- or 3-substituted branch-points of oligosaccharides. The hapten-inhibition results with the anti-$(Gal)_2$- and anti-$(Gal)_3$-sera are similar to those obtained by using antibodies to certain blood-group-active oligosaccharide sequences. For example, the A and B active pentasaccharides contain either GalNAc or Gal, respectively, in α-D-(1→3)-glycosidic linkage to the trisaccharide sequence α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GlcNAc. Both the GalNAc (or Gal) and Fuc residues were shown to contribute to the interaction with anti-A or anti-B antibody combining-sites, as oligosaccharides lacking the Fuc residues exhibited much lower inhibitory activities[19]. Thus, the anti-A and -B antibodies are relatively specific for a disubstituted Gal residue, similar to the observed specificity of the anti-$(Gal)_2$-TA-BSA serum for the two closely spaced Gal residues on the TA group.

Interestingly, although methyl β-D-Gal exhibited weak inhibitory activities with anti-$(Gal)_1$-TA-BSA serum (50% inhibition at 5mM) and with anti-$(Gal)_2$-TA-BSA (35% inhibition at 10mM), Gal and Lac [β-D-Gal(1→4)-D-Glc] at 10mM showed no inhibitory activity. The antibodies may, in part, interact with the $-OH_2C-$ group linked to C-1 of the Gal residues, and the Glc residue of Lac apparently does not allow interaction in the antibody combining-sites comparable to that observed with methyl β-D-Gal.

$(Lac)_3$-TA (**4**) inhibited the anti-$(Gal)_n$-TA-BSA antibodies, but only at high concentrations of the hapten. Also, each of the anti-$(Gal)_n$-TA-BSA sera demonstrated binding to $(Lac)_3$-TA-BSA only at high concentrations of antibody (1:2000 dilutions), showing that the spatial relationships of the Gal residues are of obvious importance for binding with the combining-site regions of the antibodies. In addition, only the anti-$(Gal)_1$- and anti-$(Gal)_2$-TA-BSA sera (at 1:500 dilutions) reacted with hog mucin adsorbed to the polystyrene wells of the ELISA plates, and not to the other glycoproteins tested. Thus, only a small portion of the anti-$(Gal)_n$ antibodies of the antisera were cross-reactive with either $(Lac)_3$-TA or the hog mucin; perhaps those antibodies have sufficient binding-affinity for a single Gal residue or portion of a Gal residue. Lee *et al.*[20] estimated the affinity of the synthetic $(Gal)_3$-TA ligands to be 10^3-fold less than that of the naturally derived, Gal_3 cluster, asialo-triantennary glycopeptide, for binding to the hepatic Gal lectin. The difference was attributed to the distances of the Gal residues from the branching point, thus indicating that the position of branching is important for relative binding-affinities. For similar reasons, it was not unexpected that the anti-$(Gal)_n$-TA-BSA sera did not react with glycoproteins containing di- and tri-antennary oligosaccharides, for which the Gal residues may be one carbohydrate unit removed from the branching points.

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