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A Monoclonal Antibody Specific for Non-reducing Terminal Galα1-4Gal Residues

S MÅRTENSSON[1,2], T BRODIN[3], A-S CARLSTRÖM[4], J DAHMEN[4], T FREJD[4], A GUNNARSSON[5], U JANSSON[1], G MAGNUSSON[4] and A LUNDBLAD[1]

[1]*Department of Clinical Chemistry and* [2]*Department of Urology, University Hospital, S-221 85 Lund, Sweden*
[3]*Department of Tumour Immunology, The Wallenberg Laboratory, S-220 07 Lund, Sweden*
[4]*Swedish Sugar Co. Ltd., Research and Development, Box 6, S-232 00 Arlöv, Sweden*
[5]*Department of Carbohydrate Chemistry, Chemical Center, S-221 00 Lund, Sweden*

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A mouse monoclonal antibody (87.5) against Galα1-4Gal has been obtained after immunization with the disaccharide glycosidically coupled to a protein. The specificity was determined by studying its binding to a number of glycoconjugates and oligosaccharides.

The antibody which was found to be highly specific for terminal Galα1-4Gal residues is a powerful tool for the detection of this structure in glycoproteins and glycolipids by immunochemical *in vitro* methods. It is also useful for *in vitro* quantification of the free disaccharide.

A thin layer chromatographic overlay assay using glycolipids and an immunoperoxidase technique is also described. The antibody 87.5 is used in this assay to identify human uroepithelium glycolipids with terminal Galα1-4Gal residues.

Glycolipids containing the Galα1-4Gal structure have been identified as receptors for pyelonephritogenic *Escherichia coli* [1-4]. This structure is a common feature of the various antigens in the blood group P system. *E. coli* strains that bind to the P antigens have been denoted "P-fimbriated". In a study using a thin layer plate assay these strains of *E. coli* bind to glycolipids containing both terminal and internal Galα1-4Gal residues [5] and Galα1-4Gal coupled to latex particles has been proven to be a valuable tool in diagnosis of these microorganisms [6]. The free oligosaccharide has also been tested as a therapeutic agent in animal models of urinary tract infections [7, 8].

There is a clinical interest in obtaining information about possible correlation between

Abbreviations: Lactosylceramide, Galβ1-4GlcCer; globotriaosylceramide, $GbOse_3$-ceramide, Galα1-4Galβ 1-4GlcCer; globotetraosylceramide, globoside, $GbOse_4$-ceramide, GalNAcβ1-3Galα1-4Galβ1-4GlcCer.

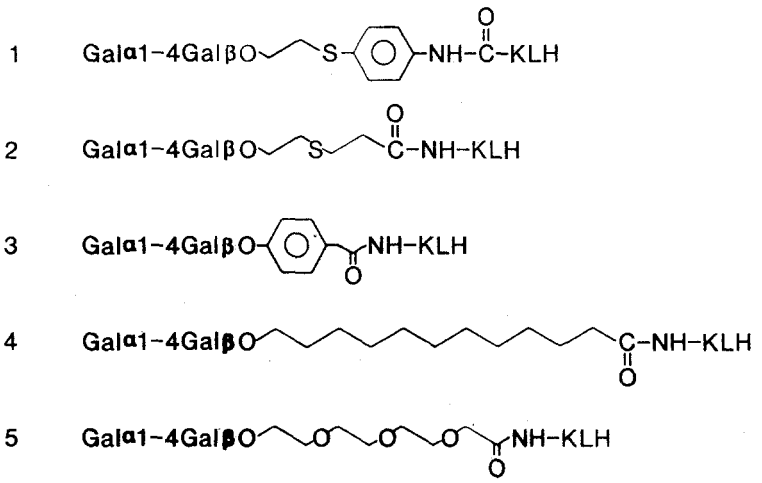


Figure 1. Structures of the oligosaccharides and linking arms used for coupling to proteins.

receptor density [9] and susceptibility for urinary tract infections. A monoclonal antibody against such a receptor would allow immunochemical quantification of the cell-surface receptor, and of the free receptor analogue in therapeutic situations.

Cell surface carbohydrates display changes in several tumour tissues [10], exemplified by the loss of ABH haptens in a variety of human cancers and the accumulation of globotriose in Burkitt lymphoma cells, the latter indicating a blocked biosynthesis of glycolipids in the globo series. Thus, a Galα1-4Gal specific antibody might reveal tumour associated disturbances in the blood group P system.

This paper describes the production of a mouse monoclonal antibody against Galα1-4Gal obtained after immunization with the disaccharide glycosidically coupled to a protein *via* a linking arm. The antibody is highly specific for terminal Galα1-4Gal residues and is useful in immunochemical quantification of the disaccharide. It was also applied in a TLC overlay assay of terminal Galα1-4Gal residues in glycolipids extracted from urinary bladder epithelium. The identity of antibody-reacting components was confirmed by GLC and MS.

Experimental Procedure

Mice

Female mice of the strain Balb/c A Bom (Gl Bomholtgaard Ltd, Ry, Denmark) were used for immunization and ascites production.

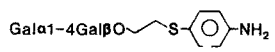
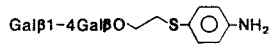
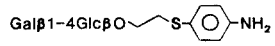
	Trivial name	Structure
1		Galα1-4Gal
2	Lactose	Galβ1-4Glc
3	Melibiose	Galα1-6Glc
4	Cellobiose	Glcβ1-4Glc
5	Maltose	Glcα1-6Glc
6	Globo-N-tetraose	GalNAcβ1-3Galα1-4Galβ1-4Glc
7	Globotriose	Galα1-4Galβ1-4Glc
8		
9		
10		Galα1-4Galα-O-CH_3
11		Galα1-4Galβ-O-C_2H_5
12		

Figure 2. Oligosaccharides and their derivatives used in this study.

Antigens

Galα1-4Gal was coupled *via* five different linking arms (Fig. 1) to keyhole limpet hemocyanin (KLH; Schwartz-Mann, Orangeburg, NY, USA) and bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) to give the corresponding neoglycoproteins for use as antigens in the immunizations. Antigens 1 and 2 were prepared by coupling [11, 12] of the corresponding glycosides [13] to KLH. The starting glycosides for the preparation of antigens 3-5 were prepared (unpublished procedure) by standard glycosidations of acetobromogalabiose [14]. Coupling to KLH was performed as for antigen 2 [12]. The BSA conjugates were prepared in the same way as the KLH antigens.

Oligosaccharides

Oligosacharides and their derivatives used are listed in Fig. 2. The syntheses of compounds 1 and 8-12 have been reported [13, 14]. Compounds 6 and 7 were obtained from BioCarb Chemicals, Lund, Sweden, and compounds 2-5 were purchased from Sigma. 6-^{3}H-Galα1-4GalβOEt was prepared by oxidation of Galα1-4GalβOEt with galactose oxidase [13] followed by reduction with NaB^3H_4.

Table 1. Immunization and fusion schedule.

Day	Antigen (μg)	Adjuvant[a]	Administration[b]
1	50	CFA	s.c
21	50	IFA	s.c
75	50	IFA	s.c
290	50	—	i.p
291	400	—	i.p
292	400	—	i.p
293	400	—	i.p
294	Fusion	—	—

[a] CFA, Complete Freund's adjuvant; IFA, Incomplete Freund's adjuvant.
[b] S.c. subcutaneous injection; i.p., intraperitoneal injection.

Glycolipids

Lactosylceramide, globotriaosylceramide and globotetraosylceramide were obtained from BioCarb Chemicals.

Immunization

The antigens were dissolved in 0.2 M Tris-HCl buffer, pH 7.2, (0.2 mg/ml) and emulsified with equal volumes of CFA or IFA. (Complete Freund's adjuvant, CFA, and incomplete Freund's adjuvant, IFA, were purchased from Difco Laboratories, Detroit, MI, USA). The first three injections were done subcutaneously (s.c.) and the remaining injections intraperitoneally (i.p.). The immunization schedule is given in Table 1.

Determination of Humoral Immune Response

The determination of the antibody titre in serum after immunizations was done by solid phase ELISA [15], using antigen 1-5 coupled to BSA as target antigens bound to microtitre plates (Dynatech Immulon, Plochingen, W.Germany). Mouse antibodies were detected by rabbit anti-mouse immunoglobulin-horseradish peroxidase, obtained from Dakopatts, Copenhagen, Denmark.

Hybridomas

Hybridomas were produced according to the method of Kennett [16] using 50% polyethyleneglycol (PEG 1500, Sigma). Spleen cells from immunized mice were fused with SP2/0 cells at a ratio 1.4/1. Supernatants were tested for antibody activity using the solid phase ELISA [15]. Cloning was performed as described by Nowinski *et al.* [17]. The antibody isotype was determined by double immunodiffusion [18] using rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM antisera, obtained from Miles, Elkhart, IN, USA.

Determination of Antibody Specificity

The specificity of the antibody was evaluated in a nitrocellulose filter assay by comparing various oligosaccharides as inhibitors of binding of 6-^{3}H-Galα1-4GalβOEt [19]. A solid phase ELISA technique using different glycolipids as target antigens in microtitre plates was also employed [20].

Preparation of Neutral Glycolipids

Normal uroepithelium was obtained at autopsy, within 24 h *post mortem*. Malignant uroepithelial tissue was obtained on transurethral surgery for bladder cancer. The tissue specimens were stored frozen at -30°C. After thawing, the specimens were homogenized in choroform/methanol, 1/2 by vol, and extracted by shaking in a water bath at 37°C for 30 min followed by chloroform/methanol, 2/1 by vol, for 30 min. After centrifugation the supernatants were mixed and concentrated to dryness by rotary evaporation. The extracts were desalted on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (10 × 100 mm) equilibrated with chloroform/methanol/water, 60/30/4.5 by vol. Neutral glycolipids were obtained by passing the material through a DEAE-Sephadex A-25 column (10 × 50 mm; Pharmacia) equilibrated with chloroform/methanol/water, 30/60/8 by vol. The neutral glycolipid fraction was acetylated [21] and further purified by chromatography on a Florisil column, 60-100 mesh (Kebo, Stockholm, Sweden) according to a procedure previously described [21]. The glycolipids were finally de-*O*-acetylated overnight in methanol/25% aqueous ammonia, 4/1 by vol, and evaporated to dryness by rotary evaporation.

Binding of the Antibody to Glycolipids Separated by Thin Layer Chromatography

TLC was carried out on Silica gel 60 HPTLC plates (E. Merck, Darmstadt, W. Germany). The chromatograms were developed with chloroform/methanol/water, 60/35/8 by vol. Binding of the monoclonal antibody was carried out using a procedure modified from Magnani *et al.* [22]. The dried chromatograms were dipped in petroleum ether containing 0.05% polyisobutylmethacrylate (Polyscience, Warrington, PA, USA) for 1 min. After drying, the plates were sprayed with phosphate buffered saline (PBS; 0.01 M sodium phosphate, pH 7.2 in 0.15 M NaCl) and overlayed with PBS containing 1% bovine serum albumin (PBS-BSA) for 30 min and then with the monoclonal antibody diluted 1/50 in PBS-BSA, followed by incubation for 1 h at room temperature. The chromatograms were washed by dipping in four successive changes of PBS and then covered with a biotinylated horse anti-mouse IgG antibody (Vector Laboratory, Burlingame, CA, USA) diluted 1/500 in PBS-BSA, for 1 h. The plates were washed as before and covered for 30 min with an avidin-biotinylated horseradish peroxidase complex (Vector) diluted 1/250 in PBS-BSA. After washing again the plates were developed for 2 min in a solution consisting of 20 mg 3-amino-9-ethylcarbazole (Sigma), 2.5 ml dimethylformamide, 50 ml 0.15 M sodium acetate buffer, pH 5.5 and 25 μl H_2O_2.

Identification of Antibody-binding Glycolipids

To a 25 μg glycolipid mixture, prepared from the TLC di- and trihexosylceramide region respectively, 1 ml of trifluoroacetic acid/trifluoroacetic anhydride, 1/50 by vol, was added (Janssen Chemicals, Beerse, Belgium). The solution was heated to 100°C for 48 h in

a sealed thick-walled glass tube (Caution - highly corrosive mixture under pressure). The mixture was cooled to room temperature and concentrated to dryness by rotary evaporation. *O*-Trifluoroacetyl groups were removed by evaporation from methanol (3 × 1ml). The released oligosaccharides were reduced, permethylated and analyzed by GC-MS [23].

Structural Analyses

Monosaccharides were analyzed as alditol acetates by GLC [24] and MS [25] after hydrolysis with 4 M trifluoroacetic acid at 100°C for 4 h. Methylation analyses were performed as previously described [26, 27]. GLC was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector. Separations were performed on (a) an SE-30 W.C.O.T. vitreous silica capillary column (25 m × 0.2 mm) at 170-220°C for partially methylated alditol acetates and at 180-320°C for permethylated oligosaccharide alditols, (b) a Silar 10 glass capillary column (4m × 0.25 mm) at 170-230°C for alditol acetates. MS was performed on a VG Masslab 12-250 quadrapole instrument fitted with the appropriate column. The spectra were recorded at 70 eV with an ionisation current of 100 μA and an ion-source temperature of 250°C. The spectra were processed on an on-line computer system (PDP-11/23, Digital Equipment Corporation).

Results

Immune Response

Five different antigens (Fig. 1) were studied in a total of 30 mice with 6 mice in each group. The immunization schedule is given in Table 1. Mice were bled before primary injection and two weeks after each of the first three immunizations. The antibody response was determined and expressed as average serum dilution at 50% of maximal binding to Galα1-4Gal coupled to BSA *via* the same linking arm as in the immunogen used for each group of mice. Group 1 and 4 gave the highest response and were hyperimmunized after the third immunization. The other three groups gave lower responses. Antibody titres in sera from group 1 and 4 mice were measured using all five derivatives (Fig. 1) coupled to BSA. Sera from group 1 mice gave higher titres with all target antigens than sera from group 4 mice (data not shown) indicating a higher content of antibodies specific for the Galα1-4Gal structure in sera from the group 1 mice. From these results it was decided to use the group 1 mice for production of monoclonal antibodies.

Yield of Monoclonal Antibodies

Spleen cells from the two mice in group 1 showing the highest titres were selected for cell fusion. From 864 wells plated, a total of 130 hybridomas producing antibodies against antigen 1 (BSA) (Fig. 1) were detected. Twenty-two of these also bound to antigen 2 (BSA). In further binding tests with globotriaosylceramide six of the 22 monoclonal antibodies bound to this target antigen (one IgG_{2a}, four IgG_{2b} and one IgG_3). Only one of these (denoted 87.5, and with isotype IgG_{2a}) bound the free disaccharide in the nitrocellulose filter assay. Antibody 87.5 was therefore selected for further studies.

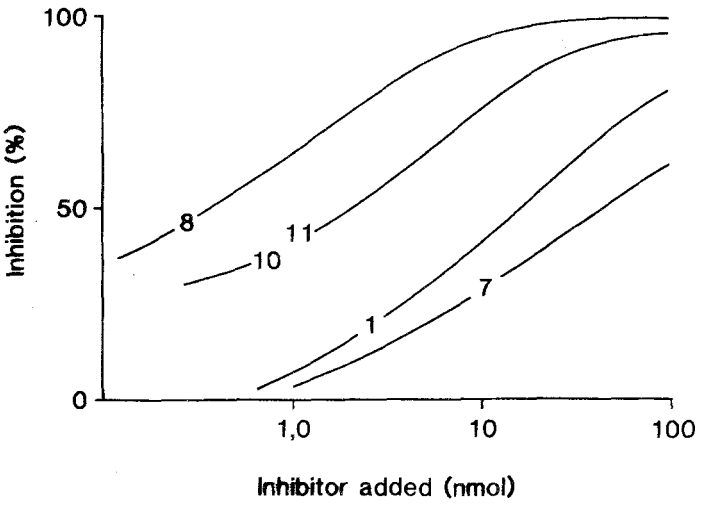


Figure 3. Inhibition of binding of 6-^{3}H-Galα1-4GalβOEt to hybridoma antibody 87.5 by oligosaccharides. See Fig. 2 for oligosaccharide structures.

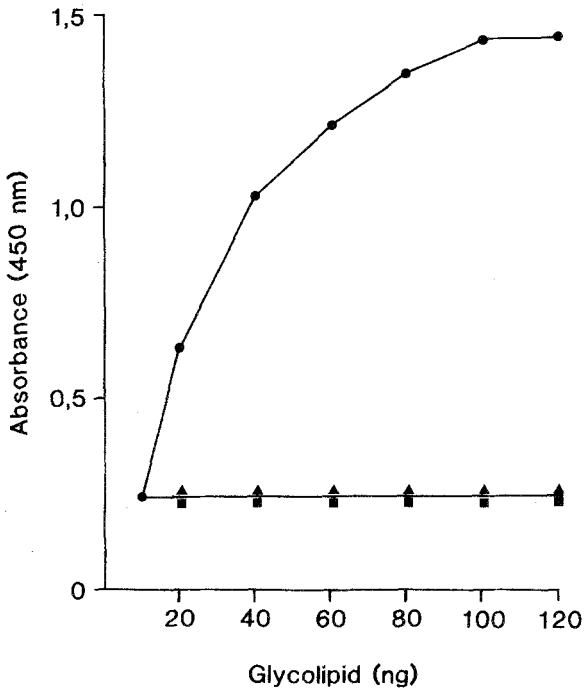


Figure 4. Binding of antibody 87.5 to microtitre plates coated with lactosylceramide (▲), $GbOse_3Cer$ (●) and $GbOse_4Cer$ (■) measured using the ELISA technique.

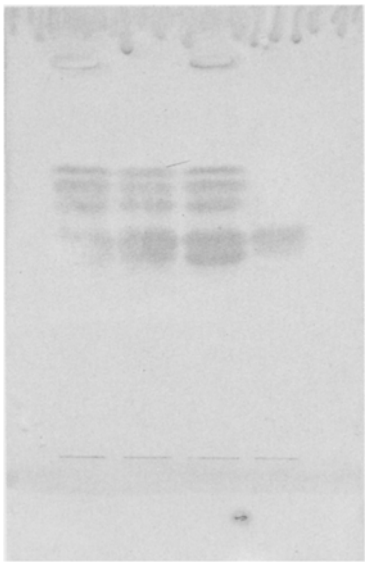


Figure 5. Thin layer chromatogram of neutral glycosphinglipids: (A) normal uroepithelium, (B, C) malignant uroepithelium, (D) reference glycosphingolipid $GbOse_3Cer$. The chromatogram was developed with chloroform/methanol/water, 60/35/8 by vol. Detection using antibody 87.5 as primary antibody.

Specificity of Hybridoma Antibody 87.5

The activities of various oligosaccharides and oligosaccharide derivatives as inhibitors of binding of 6-^{3}H-Galα1-4GalβOEt to hybridoma antibody 87.5 in a nitrocellulose filter radioimmunoassay are shown in Fig. 3. Substance 8 (Fig. 2), consisting of both the disaccharide and the linking arm of antigen 1 was the most potent inhibitor. The free disaccharide, Galα1-4Gal, displayed about 5% of this activity. The α-methyl and the β-ethyl glycosides of Galα1-4Gal were considerably more active than the free disaccharide. Globotriose had an activity slightly less than the free Galα1-4Gal whereas all other compounds tested, including globotetraose, which has the internal Galα1-4Gal structure, were totally inactive. The specificity was also tested in solid phase ELISA with lactosyl ceramide, globotriaosylceramide and globotetraosylceramide as target antigens (Fig. 4). Antibody 87.5 bound only to globotriaosylceramide in this assay.

Detection of Terminal Galα1-4Gal Residues in Normal and Malignant Uroepithelium

The neutral glycolipid fraction was prepared from urinary bladder mucosa obtained at autopsy of 17 individuals and from tumour biopsies of 14 patients with urinary bladder cancer. The tumours were of different grade and stage. All individuals were typed for their P-blood group. Eleven of the normals and 13 of the patients were of blood group P_1 whereas the remaining individuals were of blood-group P_2.

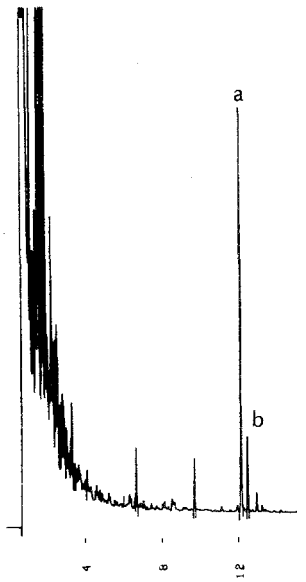


Figure 6. Gas-liquid chromatogram of permethylated oligosaccharide alditols from glycolipid fraction 1, liberated by trifluoroacetolysis. a) Galβ1-4GlcOL b) Galα1-4GalOL.

Thin layer chromatography of the neutral glycolipid fractions from the 31 individuals was carried out and the plates were overlayed with antibody 87.5. The antibody bound to glycolipids in the di- and tri-hexosylceramide region (Fig. 5). No significant differences in the binding pattern was observed between normals and tumour patients or between individuals with different blood group P phenotype.

Glycolipids in the di- and tri-hexosylceramide region from a normal individual were separated by preparative TLC, eluted from the plate and subjected to further characterization. Determination of the monosaccharide composition of the dihexosylceramide fraction (fraction 1) revealed galactose and glucose in the molar proportion 1.3 : 1.0. The trihexosylceramide fraction (fraction 2) contained galactose and glucose in the ratio 2.2 : 1.0. Fractions 1 and 2 were subjected to methylation analysis and purification on a silica gel column [27]. Non-reducing terminal galactose, 4-*O*-substituted galactose and 4-*O*-substituted glucose could be detected in fraction 1 and 2. The oligosaccharides released by trifluoroacetolysis were analysed by GC-MS as permethylated alditols. In glycolipid fraction 1 two disaccharides were observed. The disaccharides were identified by their mass spectra [28] and by comparison with authentic standards of Galα1-4GlcOL and Galα1-4GalOL (Fig. 6). According to the sugar analysis and trifluoroacetolysis of glycolipid fraction 1, Galα1-4Gal corresponds to about 15% of the dihexosylceramide content. Using the same techniques, a single trisaccharide identified as Galα1-4Galβ1-4Glc was detected in fraction 2.

Discussion

To obtain a monoclonal antibody highly specific for the nonreducing disaccharide terminal Galα1-4Gal residue the immunization and screening procedure has to be optimized for this purpose. This was achieved in two ways. Firstly, antigens with five different linking arms were tested in the immunizations. Mice, whose sera displayed the highest degree of cross reactivity when tested for binding to Galα1-4Gal coupled to BSA *via* the five different linking arms, were chosen for the fusion experiments. Secondly, the hybridomas were screened so as to select clones producing antibodies reacting with Galα1-4Gal coupled to BSA *via* two different linking arms. The antibodies were selected also to react with globotriaosylceramide, which contains Galα1-4Gal as the non-reducing terminal residue. The specificity studies clearly indicate that antibody 87.5 requires terminal Galα1-4Gal for its binding. The linking arm, however, is to some extent included in the antibody combining site as demonstrated by the increased inhibitory activity of the disaccharide when it carries a linking arm. The relatively high activity of the α-methyl and β-ethyl glycosides is probably due to their higher hydrophobicity as compared to the free disaccharide. No binding of 87.5 to globoside, which contains an internal Galα1-4Gal residue, could be detected. This is in contrast to what has been shown for P-fimbriated uropathogenic *E. coli* which binds to both terminal and internal Galα1-4Gal residues [5].

Since antibody 87.5 could bind *in vitro* to all the reference compounds containing terminal Galα1-4Gal residues it was found useful as a reagent to detect this structural element in glycolipids extracted from tissues and separated on thin-layer plates. Urinary bladder epithelium was chosen as a model and binding to the di- and tri-hexosylceramide region was demonstrated. Galα1-4Gal-ceramide was shown by GC-MS to constitute approximately 15% of the dihexosylceramide fraction in this material which most likely accounted for the antibody binding. In the trihexosylceramide region, globotriaosylceramide was the predominant compound which also was confirmed by GC-MS. No binding to larger glycosphingolipids with terminal Galα1-4Gal was observed.

The P_1 antigen (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) which has been isolated from red cells of individuals with blood group P_1-phenotype [29] is a glycolipid that might be expected to be present also in the urinary tract epithelium and partly responsible for the binding of uropathogenic *E. coli*. Antibody 87.5 which binds *in vitro* to Gal α1-4Galβ1-4GlcNAc coupled to BSA [30] (data not shown), should therefore also bind to the P_1 glycolipid. It can therefore be assumed that this compound is not present to any significant extent in urinary bladder epithelium. A study of the glycosphingolipid composition of human urethral epithelium cells also failed to detect the P_1 glycolipid [31].

Our studies comparing normal and malignant uroepithelial tissue did not reveal any differences in the distribution of terminal Galα1-4Gal structures. Since antibody 87.5 failed to bind to uroepithelial cells (data not shown), its usefulness is restricted to *in vitro* quantification of the free disaccharide Galα1-4Gal in various fluids, including serum and urine, which will be of great importance in experimental and possible clinical therapeutic situations where Galα1-4Gal glycosides would be used to inhibit bacterial infections *in vitro*. The antibody is also a powerful tool for the detection of terminal Galα1-4Gal residues of glycoproteins or glycolipids in immunochemical *in vitro* methods.

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