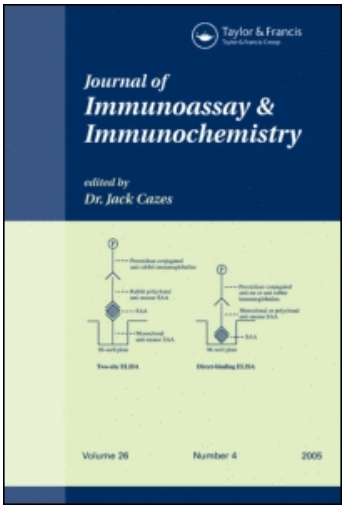

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

The Isolation and Characterization of Human Natural αGal-Specific IgG Antibodies Applicable to the Detection of αGal-Glycosphingolipids

E. P. Smorodin[a]; O. A. Kurtenkov[a]; I. N. Shevchuk[b]; R. H. Tanner[c]
[a] Department of Oncology & Immunology, National Institute for Health Development, Tallinn, Estonia [b] Department of Chemistry, Tallinn University of Technology, Tallinn, Estonia [c] National Institute of Chemical Physics & Biophysics, Tallinn, Estonia

To cite this Article Smorodin, E. P. , Kurtenkov, O. A. , Shevchuk, I. N. and Tanner, R. H.(2005) 'The Isolation and Characterization of Human Natural αGal-Specific IgG Antibodies Applicable to the Detection of αGal-Glycosphingolipids', Journal of Immunoassay and Immunochemistry, 26: 2, 145 — 156
To link to this Article: DOI: 10.1081/IAS-200051999
URL: http://dx.doi.org/10.1081/IAS-200051999

Journal of Immunoassay & Immunochemistry, 26: 145–156, 2005

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1081/IAS-200051999

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E. P. Smorodin and O. A. Kurtenkov
Department of Oncology & Immunology, National Institute for Health Development, Tallinn, Estonia

I. N. Shevchuk
Tallinn University of Technology, Department of Chemistry, Tallinn, Estonia

R. H. Tanner
National Institute of Chemical Physics & Biophysics, Tallinn, Estonia

Abstract: The Galα1-3Galβ (αGal) hapten is xenogeneic for humans; natural anti-αGal antibodies are present in human serum. To study the possible abnormal expression of the αGal in humans and the pathophysiological role of antibodies, the method of affinity purification of human anti-αGal IgG was developed. The specificity of antibodies was evaluated using polyacrylamide (PAA)-based glycoconjugates in direct and competitive enzyme-linked immunosorbent assays (ELISA). The purified antibodies exhibited αGal-restricted specificity. The IC_{50} value for αGal-PAA was equal to 4×10^{-8} M. In a competitive assay, the Galα1-3(Fucα1–2)Galβ-PAA (trisaccharide of blood group B) was found to be one hundred times less active inhibitor than αGal-PAA. The multivalent αGal-PAA was 1100 times more potent an inhibitor than the monovalent spacered αGal-saccharide. The antibodies did not show any reactivity to the negatively charged antigens (DNA, human tumor-derived mucins). At a concentration of $2\,\mu g/mL$, the antibodies agglutinated rabbit

Address correspondence to Dr. E. P. Smorodin, Department of Oncology & Immunology, National Institute for Health Development, Hiiu 42, 11619, Tallinn, Estonia. E-mail: evgeni.smorodin@tai.ee

erythrocytes but not hare erythrocytes. The high reactivity of antibodies to the αGal-glycosphingolipids of rabbit erythrocytes and the pig kidney was shown by a modified sensitive method of thin-layer chromatography with immunodetection.

Keywords: αGal, Natural antibodies, PAA-glycoconjugates, Glycosphingolipids, HPTLC

INTRODUCTION

The terminated saccharide Galα1-3Galβ (αGal) is expressed on red blood cells and nucleated cells of nonprimate mammals and New World monkeys in both glycolipids and glycoproteins.[1] The gene for α1,3galactosyltransferase in human is inactivated; therefore the αGal (cryptic afuco B epitope) may be only aberrantly expressed on human cells.[2] It was detected on human cancer cells, in papilloma virus-infected cells, and in an invasive trophoblast.[3–6] The natural antibodies specific to the αGal epitope in humans seem to be produced as a result of stimulation by the αGal antigens of enterobacteria.[7] The possible role of abnormally expressed αGal and anti-αGal antibodies in autoimmune diseases is discussed.[2] The presence of αGal in animal organs is an obstacle to their xenotransplantation to humans.[8]

The study of the αGal-expression with the help of animal antibodies is, to some extent, hampered. The high titre antisera cannot be obtained from most conventional animals because of their immunotolerance to self-antigens. The mouse monoclonal antibodies Gal-13, which are specifically bound to the terminal Galα1-3Galβ1-4GlcNAc, were obtained from only one of the 700 clones studied.[9] Natural anti-αGal antibodies are abundant in human serum and could be applied to the study of the αGal-expression but the heterogeneity and inactivation of antibodies during purification was observed.[10,11] Earlier, synthetic homogenous polyacrylamide (PAA) glycoconjugates[12] and affinity sorbents were used for the characterization of antibody specificity and for the purification of specific anti-carbohydrate antibodies.[13] In the present study, active and specific anti-αGal IgG antibodies were purified from human serum and their applicability to the detection of animal αGal-glycolipids is shown.

EXPERIMENTAL

Glycoconjugates and Saccharides

The following soluble PAA-glycoconjugates (30 kDa, 20% mol. carbohydrate, Syntesome, Russia – Germany) were used: αGal or Bdi, Galα1-3Galβ; B blood group trisaccharide (Btri), Galα1-3(Fucα1-2)Galβ; A blood group trisaccharide (Atri), GalNAcα1-3(Fucα1-2)Galβ; Tαα, Galα1-3GalNAcα;

Forssman disaccharide (Fs), GalNAcα1-3GalNAcβ; Thomsen–Friedenreich disaccharide (TF), Galβ1-3GalNAcα; T$\beta\beta$, Galβ1-3GalNAcβ; Tn, GalNAcα. The spacered saccharide αGal-sp (Bdi-sp), Galα1-3Galβ-O$(CH_2)_3NH_2$ was also used. Tris-PAA, tris(hydroxymethyl)aminomethane-PAA was used as a negative control.

Purification of Human α-Gal Specific IgG Antibodies

The anti-αGal antibodies were isolated from four pooled sera of blood groups A and O, of patients with gastric cancer, by affinity chromatography using Bdi-PAA covalently bound to the macroporous glass matrix (Syntesome). The unspecific binding sites of the sorbent (10 mL) were blocked with 8.5 mL of 1% human serum albumin (HSA) in 0.05 M Tris HCl/0.2 M NaCl/0.02% sodium azide, pH 7.5 (TBS). The solution was put through the column by circulation overnight at room temperature. Then the column was washed with TBS/5 mM EDTA. The serum (8 mL) was treated to remove the complement[14] and delipidated with n-heptane. The serum was put through the column in a closed cycle (3 h, room temperature). The column was washed with TBS/5 mM EDTA. The elution of antibodies was performed by 8 M urea/0.1 M glycine/0.05 M Tris HCl/10 mM EDTA pH 6.5 at 4–8°C in the opposite direction to the flow of the antibody adsorption—upwards. It provides a sharp gradient with efficient desorption because a more dense solution of the urea did not mix with a lower-density buffer. Dialysis of the product was performed immediately during elution.

Antibody Specificity Evaluation

The ELISA was performed as described in reference [14]. The inhibition assay (competitive ELISA) was run as follows. The diluted antibodies or serum were mixed with the solution of PAA-conjugate or other antigens (mucin, DNA) and incubated for 2 h at 26°C. The mixture was added to the wells of the Nunc-Immuno plate precoated with PAA-conjugate (5 μg/mL) and the plate was incubated for 2 h at 26°C. The goat antihuman IgG-alkaline phosphatase conjugate was added. After incubation (1.5 h, 26°C), the absorbance (A) at 405 nm of the reaction with p-nitrophenylphosphate disodium salt was measured. The specific IgM antibodies were determined using PAA-conjugates as described in reference [15]. The variation coefficient was 4%.

Isolation of Lipids

The lyophilized erythrocytes or small pieces of tissues (0.1–0.5 g) were rubbed in 1–2 mL of methanol and 25 μL of the solution of ionol

(2,6-di-tert-butyl-4-methylphenol, Merck) in methanol (20 mg/mL) was added. The mixture was incubated for 2 h at 37°C. The extraction of lipids was repeated three times with chloroform/methanol (1:2, v/v) with stirring for 1 h at 37°C. The combined extracts were evaporated under vacuum and dissolved in 1–1.5 mL of 0.3 M NaOH in methanol and incubated for 1 h at 37°C. After neutralization with 0.3 M acetic acid in methanol the sample was evaporated, diluted with 0.8 mL of deionized water and dialysed against deionized water for 48 h. The sample was concentrated by ultrafiltration; the residue was evaporated under vacuum almost to dryness and dissolved in methanol (lipid extract). Alternatively, the isolation of neutral lipids was performed. The sample was further dissolved in 1 mL of chloroform/methanol/water (30:60:8, v/v, Solvent A) and was then applied to a DEAE-Toyopearl 650 (M) column (Tosoh, Tokyo), (acetate form, equilibrated with Solvent A, 3 mL bed volume). The neutral lipid fraction was eluted with 15 mL of solvent A and then with 6 mL of methanol. The fraction was evaporated and redissolved in methanol.

High-Performance Thin-Layer Chromatography (HPTLC)

The lipid extract or the fraction of neutral lipids were examined by HPTLC (Silica gel 60 F_{254}, aluminium sheets, Merck) with a solvent system chloroform/methanol/water (60:35:8, v/v). The lipids were visualized by Coomassie Brilliant Blue staining.[16] Immunostaining with anti-αGal IgG was performed as follows. After chromatography, the sheet was dried in vacuum (1 h, 50°C) and impregnated in hexane by dipping on one side, then soaked for 1 minute with 0.1% isopropyl myristate (IPM), (Merck) in hexane. The alternative treatment of the TLH-sheet with 0.02, 0.05, or 0.2% polyisobutylmethacrylate (PIBM), (GlycoTech) was performed. The dried sheet was sprayed with TBS, soaked in degassed TBS/1% bovine serum albumin (BSA) for 1 h at room temperature. Then the sheet was incubated overnight at 4°C with anti-αGal IgG (1:400) in degassed TBS/0.2% BSA. After washing, the sheet was incubated (1.5 h, room temperature) with the rabbit-antihuman IgG-alkaline phosphatase conjugate (1:1000, DAKO) in TBS/0.1% BSA. The sheet was washed three times in TBS and once in 0.1 M Tris HCl/0.1 M NaCl/5 mM $MgCl_2$/0.02% sodium azide, pH 9.5, and soaked in the same buffer containing 5-bromo-4-chloro-3-indolylphosphate disodium salt (0.2 mg/mL) and nitroblue tetrazolium (0.4 mg/mL), (Sigma). The stained sheet was washed with distilled water and dried.

Isolation of Tumor-Derived Mucins

Mucins were isolated as described earlier from human malignant tumors:[13,14] colon (n = 2) and breast (n = 11) cancer were histologically verified.

Hemagglutination Assay

The agglutination of a 0.3% suspension of rabbit or hare erythrocytes in phosphate-buffered saline/0.01% BSA was performed as described in reference [17].

Statistics

The drawing of curves and calculations were performed using the Sigma Plot program.

RESULTS AND DISCUSSION

The Specificity of Anti-αGal IgG

The binding activity of serum IgG antibodies to the tested or structurally related saccharide was analysed by inhibition assay at a concentration of PAA-conjugates of 20 and/or 200 μg/mL. The inhibition of the antibody binding to the adsorbed PAA-conjugate by the same (tested) PAA-conjugate in the corresponding serum was higher than the inhibition by another (related) PAA-conjugate (Table 1). The inhibition of the IgG antibody binding with the adsorbed Btri-PAA by αGal-PAA and *vice versa* shows their cross-reactivity in relation to the antibodies of both sera with the phenotype of blood group O (Table 1, *VN, IV*). It is in accordance with earlier documented observations on cross-reactivity between natural anti-Gal and anti-B antibodies for individuals of blood groups A and O.[18] The anti-αGal IgG was isolated from pooled A- and O-blood group sera with a low IgG-binding activity to Btri-PAA (Table 2). The binding activity

Table 1. The specificity of the whole sera as tested by inhibition assay[a]

Adsorbed PAA-conjugate	PAA-conjugate as inhibitor, (concentration, μg/mL)	% of inhibition, serum index	
αGal	αGal (200)	90.4 *VN*	100.0 *IV*
αGal	αGal (20)	76.6 *VN*	87.8 *IV*
Btri	Btri (20)	86.0*VN*	87.1 *IV*
Btri	αGal (200)	82.8 *VN*	42.8 *IV*
αGal	Btri (200)	71.4 *VN*	92.1 *IV*
αGal	Tαα (200)	11.3 *FA*	31.9 *PM*

[a]The binding activity of antibodies was calculated as (Atest minus Acontrol). Atest is absorbance with PAA-conjugate; Acontrol is absorbance with Tris-PAA.

Table 2. The IgG-binding activity of pooled sera (dilution 1:50) used for purification of antibodies and purified anti-αGal IgG to PAA-conjugates. Direct ELISA

Adsorbed PAA-conjugate	αGal	Btri	Tαα	Atri	Fs	TF	Tn	Tββ
Binding activity of pooled sera, %[a]	100	6	0	ND	ND	1	9	ND
Binding activity of anti-αGal IgG, %[a]	100	2	0	0	0	0	0	0

[a]The activity of IgG antibodies (Atest minus Acontrol) to αGal ligand was taken as 100%.

ND: not determined.

to Btri-PAA was also found to be weak for the purified anti-αGal IgG; Btri-PAA was one hundred times less active inhibitor than αGal-PAA (Table 2, 3, Figure 1).

Although the Tαα epitope has terminated the Galα1-residue like αGal, at a dilution of 1:25 none of the fourteen αGal-positive sera investigated showed a clear IgG-binding activity to Tαα-PAA. The purified anti-αGal IgG did not bind Tαα-PAA either (Table 2). In a competitive ELISA, Tαα-PAA was a weak inhibitor as well (Table 1); at a concentration of 200 μg/mL it inhibited the binding of purified anti-αGal IgG to αGal-PAA by 12.3% only. The αGal (disaccharide Bdi) is a minimal ligand that exhibits a high affinity to anti-αGal IgG;[19] otherwise, the affinity may be too low for ELISA. The anti-αGal IgG bound none of the other saccharides to which antibody reactivity in human serum is usually present,[13] (Table 2).

The value of IC_{50} for αGal-PAA was also in the range of from 2 to 5×10^{-8} M (Table 3) as observed for the other purified natural anticarbohydrate IgG antibodies (anti-TF, -Tn, -Sialyl Tn IgG) with the corresponding PAA-conjugates, but αGal-sp was a comparatively less efficient inhibitor than the spacered Tn and Sialyl Tn saccharides.[13,14] The multivalent αGal-PAA was 1100 times more potent inhibitor than the monovalent αGal-spacer (Table 3, Figure 1).

Table 3. The IC_{50} values of inhibitors

Inhibitor	αGal-PAA	Btri-PAA	αGal-sp
IC_{50}[a]	41 nM (1.24 μg/mL)	4.4 μM	47 μM

[a]50% inhibition of the antibody binding with the adsorbed αGal-PAA by the corresponding inhibitors.

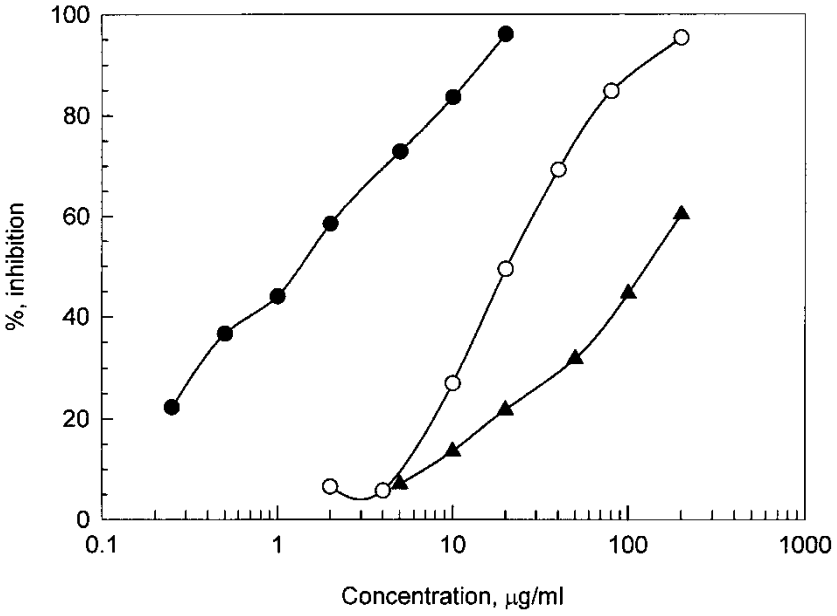

Figure 1. The inhibition of purified anti-αGal IgG binding to adsorbed αGal-PAA by soluble αGal-PAA (●), by free saccharide αGal-sp (○) and Btri-PAA (▼).

The purified anti-αGal IgG antibodies contained trace amounts of the corresponding specific IgM antibodies.

The Reactivity of Antibodies to Other Antigens

In the literature, there is information about the polyreactivity of human natural anti-αGal antibodies that was demonstrated by their binding to DNA and other antigens.[20] The polyreactivity of isolated antibodies should make them unsuitable for determination of a specific antigen. According to our observations, the anti-αGal IgG did not bind DNA (E. coli, plasmid) in either a direct (5 μg/mL of DNA for adsorption) or competitive ELISA (20 μg/mL). The antibodies did not bind the synthetic 60-mer MUC1-BSA conjugate (three tandem repeats of the MUC1 peptide core) in both direct (5 μg/mL) and competitive (100 μg/mL) assays either. Furthermore, the binding of anti-αGal IgG to human tumor-derived mucins was low: in a competitive ELISA at the concentration of mucins 100 μg/mL the maximal inhibition was 11%. Hence, the antibodies did not show any reactivity to the negatively charged antigens.

We support an opinion that the anti-αGal antibodies exhibit a restricted specificity and belong to a category different from polyreactive natural

antibodies which react with various antigens.[21] The interpretation of the results concerning polyreactivity appears to be debatable.[22] The reactivity of antibodies to other ligands may be an artefact due to an unsuitable treatment during purification. The exposure of the serum anti-αGal, -TF and -Tn antibodies to acid pH resulted in an unspecific binding to Tris-PAA and reduction of the specific binding to the corresponding ligands (unpublished observations). Natural anti-αGal IgG antibodies appeared to be tolerant to 8 M urea or basic pH (Tris buffer, pH 11) for a short time at a temperature of 4–8° C. The renovation of a native structure of the anticarbohydrate IgG by an efficient dialysis from urea or after neutralization of the basic buffer occurs apparently more favourably. If the anti-αGal antibody polyreactivity is still present in serum, then it is associated rather with the hidden activity.[23]

Thus, the affinity-purified anti-αGal IgG antibodies exhibited αGal-restricted specificity.

Reactivity of Antibodies to Glycolipids

The partial inactivation of human anti-αGal antibodies during affinity chromatography resulted in a poor analytical reagent for detection of αGal-glycosphingolipids.[11] New conditions for affinity chromatography afforded active and specific antibodies, which also showed a high reactivity to animal αGal-glycosphingolipids. The lipid extracts or neutral lipids of the pig kidney tissue and rabbit erythrocytes, where the αGal-terminated glycosylceramides are typically expressed,[9,24] were separated by HPTLC for immunodetection. The sensitivity of immunodetection depended on the treatment of TLC-sheets. The dipping of sheets in PIBM for fixation of silica gel as widely used results in a non-specifically stained diffuse band, which was detected without anti-αGal IgG as well (Figure 2, A, B; on the original chromatograms the specific bands were brown, the non-specific ones were lilac). This band interferes with immunodetection: although two lower specific bands for the extract of rabbit erythrocytes were scarcely visible, the αGal-glycosylceramide of the pig kidney remained invisible (Figure 2, B a, c). The intensity of immunostaining was higher and the non-specific staining was diminished when the sheets were treated with IPM instead of PIBM (Figure 2, C, D). The clear specific band with the mobility corresponding to the upper band for rabbit erythrocytes was demonstrated for the neutral lipids (as well as for crude lipid extracts, not shown) of the pig kidney (Figure 2, D a, c). Like monoclonal antibodies Gal-13,[9,24] anti-αGal IgG antibodies detected a similar pattern of two specific components for the glycolipids of rabbit erythrocytes and one in the samples of the pig kidney. Although αGal-glycosphingolipids with a longer saccharide chain were isolated from the pig kidney cortex, the pentaglycosylceramide is the most abundant.[11] Probably, the concentration

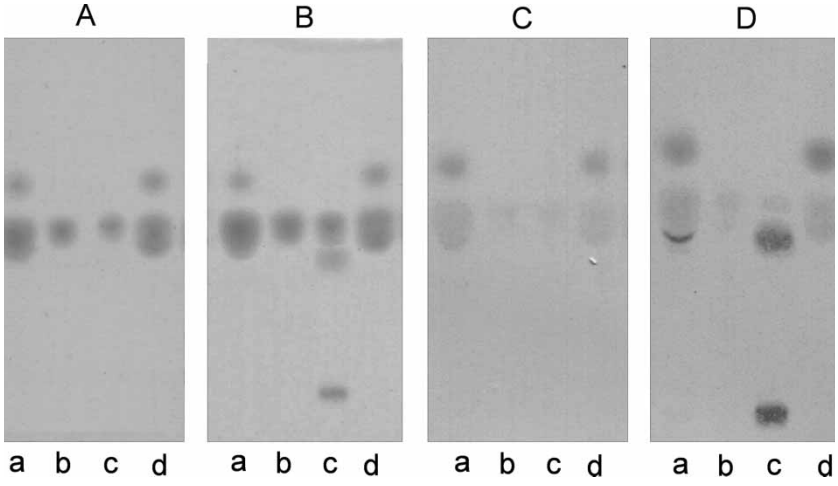

Figure 2. HPTLC of lipids in chloroform/methanol/water (60:35:8, v/v) and immunostaining with anti-αGal IgG antibodies. A, B—dipping of sheets in PIBM; A—control without anti-αGal IgG, B—test. C, D—dipping in IPM; C—control, D—test. Lane a—neutral lipids of pig kidney, b—lipid extract of hare erythrocytes, c—extract of rabbit erythrocytes, d—tumor extract of patient with gastric cancer.

of other components in the sample is too low to detect them without further purification. But taking into account the high activity of antibodies and the described modification of HPTLC immunostaining, the specific bands were visualized at a concentration of 0.5 μg/mL of IgG for 1 μL of crude extracts.

The effect of IPM on the immunodetection of αGal-glycosylceramides remains unclear. Probably, the treatment of silica gel with IPM makes the polar carbohydrate moiety of the glycolipid more accessible for antibody binding.

Unlike rabbit erythrocytes, the glycolipid extract of hare (Lepus europaeus) erythrocytes did not demonstrate any positive reaction with antibodies (Figure 2, B, D lane b). At a concentration of 2 μg/mL the anti-αGal IgG agglutinated rabbit erythrocytes, but at concentrations up to 80 μg/mL it did not agglutinate hare erythrocytes. This finding may be interpreted as specific differences in the αGal expression on red blood cells within the same animal family.

The top brown band of pig kidney sample was more visible than that of control (Figure 2, C, D lane a). A similar higher immunostaining was observed for the top band of the sample isolated from the tumor tissue of patient with gastric cancer (Figure 2, C, D lane d). Our preliminary investigations of human malignant tumor-derived extracts (gastric, $n = 4$; breast, $n = 7$) showed the variable reactivity of antibodies to this fraction.

Possibly, it is due to the cross-reactivity with spatial αGal-like saccharides.[25]

Thus, human natural affinity-purified anti-αGal IgG antibodies were shown to be an available reagent for the detection of αGal-glycosphingolipids in tissue extracts. The Bdi-PAA covalently bound to the glass matrix is an adequate immunosorbent for the purification of specific antibodies.

ABBREVIATIONS

ELISA	enzyme-linked immunosorbent assay
HPTLC	high-performance thin-layer chromatography
PAA	polyacrylamide
IPM	isopropyl myristate
PIBM	polyisobutylmethacrylate
sp	spacer arm
HSA	human serum albumin
BSA	bovine serum albumin
TBS	Tris-buffered saline
A	absorbance

ACKNOWLEDGMENTS

The authors are grateful to Dr. N. Bovin for consultations and providing us with synthetic glycoconjugates, to Dr. B. Sergeyev for design and calculations, to R. Syld, N. Shuvalova and A. Kull for technical assistance. This investigation was supported the Estonian Science Foundation, grants No. 4741 and No. 5436.

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Received August 24, 2004
Accepted September 30, 2004
Manuscript 3148