Glycoconjugate Journal 16, 481–485 (1999)

SHORT COMMUNICATION

The sugar moiety of Tamm-Horsfall protein is affected by the carbohydrate-deficient glycoprotein type I syndrome. A case study.

Teresa Olczak*, Mariusz Olczak and Aleksandra Kubicz

Institute of Biochemistry and Molecular Biology, University of Wroclaw, Tamka 2, 50-137 Wroclaw, Poland

As the sugar moiety of Tamm-Horsfall protein (THP) is affected by many pathological conditions, the aim of this study was to examine the influence of carbohydrate-deficient glycoprotein syndrome (CDG) on THP glycans. THP was isolated from urine of one patient with CDG type I and N-glycan profiling, analysis of monosaccharide content, determination of THP reactivity with specific lectins and with anti-THP antibodies were performed. THP of the CDG patient showed markedly lower amounts of all monosaccharides. Diminished amounts of lactosamine-type chains, galactose and α2,3 linked sialic acid were expressed in lower reactivity with PHA-L, DSA and MAA, respectively. These modifications were reflected in altered proportions of tetrasialylated and disialylated oligosaccharide chains. THP of the CDG patient reacted slightly more with anti-THP antibodies. Our results indicate that the CDG type I affects the THP sugar moiety and slightly enhances the THP immunoreactivity.

Keywords: **Tamm-Horsfall protein, carbohydrate-deficient glycoprotein syndrome, glycans, glycoproteins**

Abbreviations: **CBB, Coomassie Brilliant Blue R-250; CDG, carbohydrate-deficient glycoprotein syndrome; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; GU, glucose units; PBS, phosphate buffered saline, pH 7.4; PMM, phosphomannomutase; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; THP, Tamm-Horsfall protein; TPBS, 0.1% Tween 20 in PBS; MAA, *Maackia amurensis* lectin; SNA, *Sambucus nigra* lectin; DSA, *Datura stramonium* lectin; AAA, *Aleuria aurantia* lectin; ConA, *Canavalia ensiformis* lectin; PHA-L, *Phaseolus vulgaris* lectin; GNA, *Galanthus nivalis* lectin**

Introduction

Carbohydrate-deficient glycoprotein syndrome (CDG) constitutes a group of disorders with multisystemic involvement and developmental defects [1]. On the basis of different isoelectric focusing patterns of serum transferrin [2] at least four types of these disorders have been recognized. CDG type I is the most frequent form. The primary defect has been reported as a deficiency in phosphomannomutase (PMM) [3]. This enzyme converts mannose-6-phosphate to mannose-1-phosphate, which is a substrate for the synthesis of GDP-mannose. This nucleotide sugar is then used in the synthesis of dolichol-phosphate-mannose, which is essential for N-linked glycosylation and thus for the secretion of several glycoproteins as well as for the synthesis of glycosylphosphatidylinositol anchored proteins [4]. Patients with CDG type I underglycosylate glycoproteins by failing to add the entire N-linked oligosaccharide chains to the protein moiety and produce abnormal N-linked oligosaccharides [5]. As a consequence, truncated oligosaccharides are transferred to newly synthesized glycoproteins.

The disease shows an autosomal recessive mode of inheritance. Two expressed PMM genes, PMM1 and PMM2, are located on chromosome bands 22q13 and 16p13, respectively [6,7]. Several missense mutations in PMM2 are the cause of CDG type I whereas no disorder has been associated with defects in PMM1 [3,8].

We are currently studying the influence of pathological conditions on the carbohydrate part of Tamm-Horsfall protein (THP), the most abundant protein in normal human urine produced by kidney cells. N-linked glycans account

*To whom correspondence should be addressed. Teresa Olczak, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Tamka 2, 50-137 Wroclaw, Poland. Tel.: +48 71 3402-304; Fax: + 48 71 3402-608; E-mail: grabska@bf.uni.wroc.pl

for about 30% of molecular mass of THP. The majority of oligosaccharides have a polybranched structure varying from diantennary to tetraantennary sialylated compounds, containing N-acetylgalactosamine in the Sda+ immunodeterminant structure [9]. A minor component of high-mannose glycans was also found [10,11].

The pathophysiological function claimed for THP is in part related to its sugar moiety [12]. Structural and functional alterations of THP were reported for disorders of calcium metabolism [13] and diabetes mellitus [14]. THP oligosaccharides are also affected in some kidney diseases e.g. Bartter's syndrome [15] and also in malignancies of lymphoid cells [16]. The aim of our study was to examine whether the carbohydrate part of THP is influenced by CDG type I syndrome.

Patients and methods

Patients

Studies were carried out in one patient with CDG type I. Diagnosis was based on clinical investigations and biochemical findings as reported by Midro *et al.* [17]. The type of CDG syndrome has been recognized on the basis of isoelectric focusing patterns of serum transferrin. The control group consisted of five healthy children.

THP preparation

THP was prepared from 24-hour urine pool by adsorption on diatomaceous earth according to Serafini-Cessi *et al.* [18]. The purity, relative molecular weight and the identification of the isolated proteins were analyzed by SDS-PAGE, Western blotting and Ouchterlony double immunodiffusion as described previously [19].

Determination of THP immunoreactivity

Reactivity of THP with anti-human uromucoid/anti-THP antibodies (Serotec, UK) was determined using an enzyme-linked immunosorbent assay (ELISA). For this purpose, polystyrene plates (Nunc, Denmark) were coated with various amounts (0.5–300 ng) of patient and control THP in 100 µl per well of phosphate buffered saline, pH 7.4 (PBS). Then, 100 µl of sheep anti-human uromucoid/anti-THP antibodies (diluted 1:3000), followed by 100 µl of donkey anti-sheep IgG antibodies conjugated with POD (diluted 1:5000, Boehringer Mannheim, Germany) were added. The peroxidase reaction was carried out with 100 µl per well of 0.05% o-phenylenediamine in 50 mmol/l citrate/phosphate buffer, pH 5.0, containing 0.01 % H_2O_2. The colour was developed within 30 min, then the reaction was stopped with 25 µl of 12.5 % H_2SO_4 and the optical density was determined colorimetrically on Spectra Max 340 microplate reader (Molecular Devices, USA) at 492 nm. Washing, blocking and antibodies dilution were done with 0.1 % Tween 20 in PBS (TPBS). Blocking was carried out overnight at 4°C and other steps in the procedure for 1 h at 37°C. The determination of THP immunoreactivity was performed in five independent experiments.

Analysis of monosaccharide content

Monosaccharide analysis was carried out by high-performance liquid chromatography (HPLC) on a Knauer apparatus (Germany) with Shimadzu RF-551 fluorescence detector (Japan). The neutral and amino monosaccharides were obtained by acid hydrolysis with 3.0 mol/l trifluoroacetic acid for 5 h at 105°C and then labelled with 2-aminobenzoic acid (2-AA) [20] using 2-AA Labelling Kit (Oxford GlycoSciences, UK). The 2-AA derivatives were analyzed on a reverse-phase C-18 column (GlycoSep R, 4.6 × 150 mm, Oxford GlycoSciences) with fluorescence detection (λ_{max} excitation = 315 nm and λ_{max} emission = 400 nm). Gradient conditions were used as follows: buffer A - 0.25 % buthylamine:0.5 % phosphoric acid:1.0 % tetrahydrofurane in water and buffer B - 50 % methanol in water (0–10 min 25 % buffer B and 10–30 min 25–40 % buffer B). The total run time was 30 min at a flow rate 0.7 ml/min.

Reactivity with specific lectins

Reactivity with lectins was performed using the ELISA test as reported by Olczak *et al.* [16]. For this purpose the following DIG-conjugated lectins were used (Boehringer Mannheim): *Maackia amurensis* lectin (MAA) specific for NeuNAc linked α2,3 to Gal, *Sambucus nigra* lectin (SNA) for NeuNAc linked α2,6 to Gal, *Datura stramonium* lectin (DSA) for terminal Gal linked β1,4 to GlcNAc, *Aleuria aurantia* lectin (AAA) for α1,6 linked Fuc to GlcNAc, *Canavalia ensiformis* lectin (ConA) for diantennary and high-mannose type glycans, *Phaseolus vulgaris* lectin (PHA-L) for β1,6 linked lactosamin branch of complex N-glycans and *Galanthus nivalis* lectin (GNA) for terminal Man residues.

N-glycan profiling

N-glycan profiling was carried out as reported by Olczak *et al.* [16]. The sugar moieties were released from THP by hydrazinolysis [21]. The reaction was carried out at 95°C for 6 hours. The reducing ends of the oligosaccharides were fluorescently labelled with 2-aminobenzamide (2-AB, Oxford GlycoSciences) [20]. Oligosaccharide analysis was performed by HPLC on a normal-phase amide column (GlycoSep N, 4.6 × 250 mm, Oxford GlycoSciences) [22] and on an ion exchange column (GlycoSep C, 3 × 100 mm, Oxford GlycoSciences) [23] with fluorescence detection (λ_{max} excitation = 330 nm and λ_{max} emission = 420 nm).

Results

Purity of THP

The patient and control THP preparations appeared on SDS-PAGE as a single band of 93 and 96 kDa, respectively (Fig. 1). The identity of this band as THP was confirmed by immunoblotting, in which one band reacting with sheep anti-human uromucoid/anti-THP antibodies was visualized. Ouchterlony double immunodiffusion performed with anti-human uromucoid/anti-THP, anti-human albumin (Sigma, USA) and anti-human whole serum antibodies (Sigma) showed one precipitation band only with antibodies against human uromucoid/THP. Only preparations of the state of purity described above were used for further experiments.

Monosaccharide content of THP

The monosaccharide content of THP isolated from urine of the CDG patient and healthy controls is shown in Table 1. It demonstrates that the patient THP shows markedly lower amounts of all monosaccharides studied. THP isolated from urine of CDG patient contains 16.3% of total neutral and amino monosaccharides amount compared to 28.6 ± 6.4% of control THP preparations.

Characterization of THP carbohydrate chains

To characterize the type of carbohydrate chains we examined the reactivity of THP with specific lectins. As shown in Table 2 patient THP reacted weaker with PHA-L and DSA, indicating decreased amounts of lactosamine-type chains and terminal galactose, respectively. Lower amounts of α2,3 linked sialic acid were observed in patient THP as judged from its diminished reactivity with MAA. We were not able to detect terminal mannose residues as there was no reactivity with GNA in healthy individuals as well as in the examined patient. We found previously [11] that all THP preparations, also THP isolated from urine of the patient with CDG syndrome, contain high-mannose type glycans. This was examined after releasing of oligosaccharides by hydrazinolysis using N-glycan profiling and exoglycosidase sequencing.

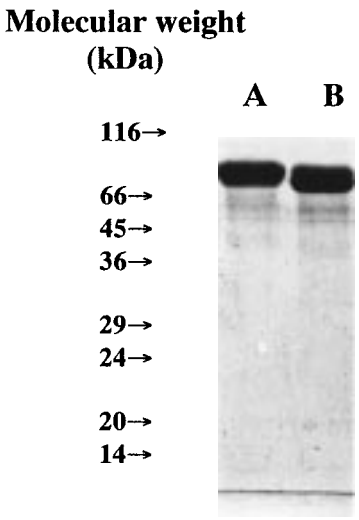

Figure 1. SDS-PAGE analysis of THP isolated from urine of control subject (A) and patient with CDG syndrome (B). 20 μg of THP was used for this experiment; gel was stained with CBB.

Table 1. Monosaccharide content of THP

Monosaccharide	*Controls (n = 5)*	*CDG syndrome (n = 1)*
Fuc	5.7 ± 1.2	4.2
Man	26.1 ± 3.6	13.1
Gal	34.2 ± 4.9	20.4
GlcNAc	61.5 ± 6.3	36.9
GalNAc	11.1 ± 2.8	4.2

The values are expressed as molecule of carbohydrate per molecule of protein; data are given as mean values ± SD.
Fuc = fucose, Man = mannose, GlcNAc = N-acetylglucosamine, GalNAc = N-acetylgalactosamine, NeuNAc = sialic acid

N-glycan profiles

Determination of monosaccharide content and reactivity with specific lectins showed that the carbohydrate part of THP is affected by CDG conditions. Thus, we performed more-thorough analysis of the THP oligosaccharide chains in this patient. For this purpose we carried out HPLC separations of the total glycan pools released from THP after hydrazinolysis. Separation on Glyco Sep N column (Fig. 2) resulted in various oligosaccharide structures differing

Table 2. Reactivity of THP with specific lectins

Lectin	*Controls (n = 5)*	*CDG syndrome (n = 1)*
SNA	1.21 ± 0.32	1.28
MAA	1.31 ± 0.42	0.79
DSA	1.72 ± 0.36	1.22
AAA	0.36 ± 0.23	0.37
ConA	0.47 ± 0.16	0.41
PHA-L	0.56 ± 0.22	0.31
GNA	not detected	not detected

Data are given as mean values of absorbance at 492 nm ± SD.
DSA = *Datura stramonium* lectin (specific for terminal Gal linked β1,4 to GlcNAc), AAA = *Aleuria aurantia* lectin (specific for α1,6 linked Fuc to GlcNAc), MAA = *Maackia amurensis* lectin (specific for NeuNAc linked α2,3 to Gal), SNA = *Sambucus nigra* lectin (specific for NeuNAc linked α2,6 to Gal), Con A = *Canavalia ensiformis* lectin (specific for diantennary and high-mannose type glycans), PHA-L = *Phaseolus vulgaris* lectin (specific for β1,6 linked lactosamine branch of complex N-glycans), GNA = *Galanthus nivalis* lectin (specific for terminal Man residues)

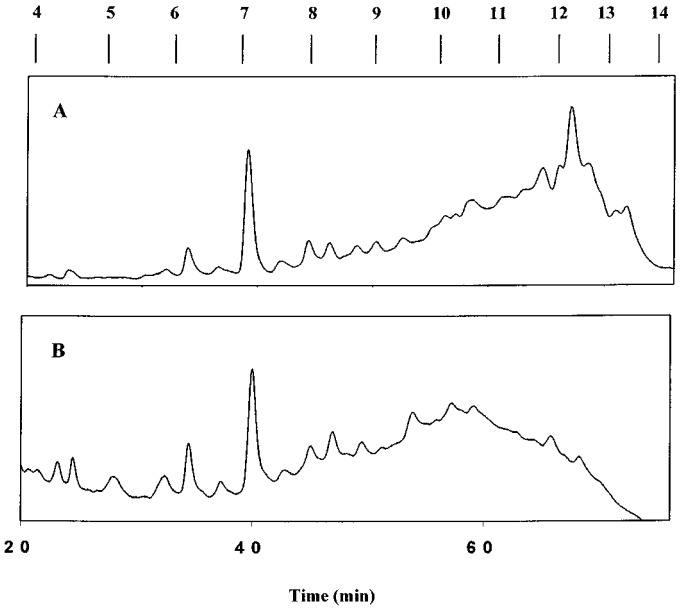

Figure 2. GlycoSep N separations of total THP glycans of control subject (A) and patient with CDG syndrome (B). Glycans were released from THP by hydrazinolysis and fluorescently labelled with 2-AB. The separation on the normal-phase amide column was performed as reported in Patients and methods. The assignment of peaks was made using GU values by comparison with a standard dextran ladder, the elution positions of which are shown at the top of the figure.

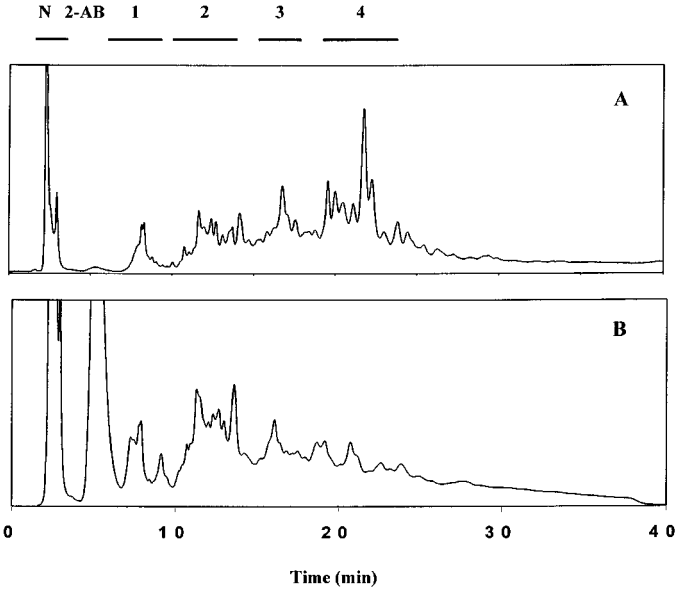

Figure 3. GlycoSep C separations of total THP glycans of control subject (A) and patient with CDG syndrome (B). Glycans were released from THP by hydrazinolysis and fluorescently labelled with 2-AB which is eluted in the second peak. The separation on the ion exchange column was performed at gradient conditions as reported in Patients and methods. Elution positions of neutral sugars (N) and glycans with charged groups (from 1 to 4 charged groups) are shown at the top of the figure.

mainly in glycans molecular size. Our results indicate that CDG is associated with changes in the proportions of the particular oligosaccharide chains. We observed namely diminished amounts of glycans with the highest GU values (11,0–13,5 GU), i.e. sialylated tetraantennary oligosaccharide chains.

Retention on the ion exchange column (GlycoSep C) is based on the anionic charge, related with the degree of oligosaccharides sialylation and sulfation. The profile obtained after separation on this column (Fig. 3) showed peaks differing in anionic charge. In THP of patient with CDG syndrome diminished amounts of tetrasialylated glycans and increased amounts of disialylated glycans were noticed.

Immunoreactivity of THP

To examine whether the changes of the carbohydrate moiety influence some biological properties we studied the immunoreactivity of THP. We examined the reactivity of the glycoprotein isolated from urine of the patient with CDG syndrome and control subjects with anti-human uromucoid/anti-THP antibodies using ELISA test. As shown in Fig. 4 THP isolated from urine of patient with CDG showed slightly higher immunoreactivity.

Discussion

A deficiency of asparagine-N-linked oligosaccharide transfer caused by CDG syndrome occurs in many glycoproteins. CDG patients have fewer oligosaccharides attached to serum glycoproteins (e.g. α_1-acid glycoprotein, transferrin, antithrombin III, thyroxine-binding globulin) [24,25], to B lymphocyte surface-expressed glycoproteins [26] and to

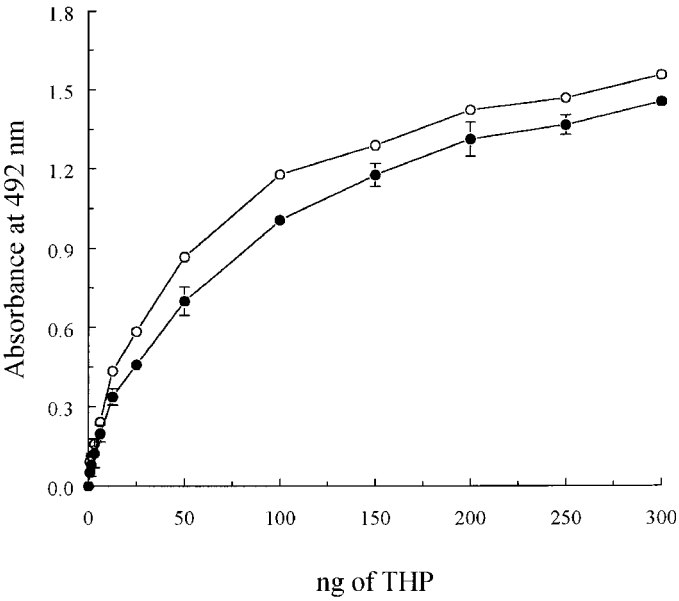

Figure 4. Reactivity of CDG (○) and control (●) THP preparations with anti-human uromucoid/anti-THP antibodies. 0.5–300 ng of THP was used in the ELISA test. Details in Patients and methods.

other proteins [27]. In this paper, we described an altered glycosylation of Tamm-Horsfall protein in a patient with CDG type I. We were able to study only one patient with this syndrome, because this disease is extremely rare and in Poland there is only one well documented case with this disorder. Thus, our results should be treated as a preliminary report.

Modifications of the oligosaccharide chains of THP isolated from CDG were reflected in lower amounts of all monosaccharides studied and in altered reactivity with lectins. Further support for differences of the sugar moiety came from experiments of glycan profiling. Patient THP showed lower amounts of tetrasialylated glycans and higher amounts of disialylated glycans. This indicates that the most evident alteration is caused by lower content of all monosaccharides and diminished amounts of tri- and tetrasialylated glycans. Midro *et al.* [17] found that also transferrin isolated from the same patient has diminished amounts of higher sialylated forms. We found previously [11] that THP of the same patient with CDG and THP of healthy individuals showed similar content of neutral oligosaccharides. This indicates that the observed changes in the degree of sialylation are not due to increased neuraminidase activity. The undersialylation of THP in CDG type I may result either from the absence of one or more N-linked oligosaccharide chains or from truncated oligosaccharide chains, as it was found in other glycoproteins [28,29]. Yuasa et al. [24] reported that α_1-acid glycoprotein, transferrin and antithrombin III in CDG syndrome have diminished molecular weight as compared with normal glycoprotein preparations. We found that the lower amount of total monosaccharides in THP of CDG patient is also reflected in slightly decreased molecular mass of this glycoprotein.

This paper shows that alterations of THP sugar moiety in the CDG patient affect the immunoreactivity of this glycoprotein. The increased reactivity of THP with anti-THP antibodies may result from insufficient glycosylation. We found previously [19] that partial desialylation and deglycosylation of THP enhances its immunoreactivity. It is possible that insufficient glycosylation may expose peptide epitopes that are normally restricted by oligosaccharides.

In conclusion, we have shown that CDG type I affects markedly the sugar moiety of THP. This was reflected in lower amounts of the monosaccharides and in altered proportions of the particular glycans, and also in slightly higher THP immunoreactivity. The present study give further evidence that CDG conditions affect glycosylation processes of various glycoproteins. It seems, that the alteration of glycosylation is a general effect of this syndrome.

Acknowledgments

The authors wish to express their sincere thanks to Prof. A. Midro and Prof. K. Zwierz from Medical Academy of Bialystok, who kindly supplied us with biological material and whoes contribution enabled us to perform this study.

References

1 Jaeken J, Carchon H, Stibler H (1993) *Glycobiology* **3:** 423–28.
2 Stibler H, Jaeken J, Kristiansson B (1991) *Acta Paediatr Scand Suppl* **375:** 21–31.
3 Van Schaftingen E, Jaeken J (1995) *FEBS Lett* **377:** 318–20.
4 Krasnewich DM, Holt GD, Brantly M, Skovby F, Redwine J, Gahl WA (1995) *Glycobiology* **5:** 503–10.
5 Powell LD, Paneerselvam K, Vij R, Diaz S, Manzi A, Buist N, Freeze H, Varki A (1994) *J Clin Invest* **94:** 1901–09.
6 Wada Y, Sakamoto M (1997) *Genomics* **39:** 416–17.
7 Martinsson T, Bjursell C, Stibler H, Kristianssen T, Skovby F, Jaeken J, Blennow G, Stromme P, Hanefeld F, Wahlstrom J (1994) *Hum Mol Genet* **3:** 2037–42.
8 Matthijs G, Schollen E, Pardon E, Veiga-Da-Cunha M, Jaeken J, Cassiman JJ, Van Schaftingen E (1997) *Nature Genetics* **16:** 88–92.
9 Hard K, Van Zadelhoff G, Moonen P, Kamerling JP, Vliegenthart JFG (1992) *Eur J Biochem* **209:** 895–915.
10 vanRooijen JJM, Voskamp AF, Kamerling JP, Vliegenthart JFG (1999) *Glycobiology* **9:** 21–30.
11 Olczak M, Olczak T (1999) *Clin Chim Acta* **282:** 35–44.
12 Kumar S, Muchmore AV (1990) *Kidney Int* **37:** 1395–1401.
13 Schnierle P, Hering F, Seiler H (1996) *Urol Res* **24:** 79–82.
14 Rambausek M, Dulawa J, Jann K, Ritz E (1998) *Eur J Clin Invest* **18:** 237–42.
15 Olczak T, Olczak M, Kubicz A, Dulawa J, Kokot F (1999) *Int J Clin Lab Res* **29:** 68–74.
16 Olczak T, Olczak M, Dereniowska M, Strzelczyk R, Kubicz A (1999) *Electrophoresis* **20:** 1382–89.
17 Midro AT, Hanefeld F, Zadrozna-Tolwinska B, Stibler H, Olchowik B, Stasiewicz-Jarocka B (1996) *Pediatr Pol* **LXXI:** 621–28.
18 Serafini-Cessi F, Bellabarba G, Malagolini N, Dall'Olio F (1989) *J Immunol Methods* **120:** 185–89.
19 Grabska T, Baginski T, Kubicz A, Kokot M, Kokot F, Dulawa J (1996) *Arch Immunol Therap Exp* **44:** 241–48.
20 Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB (1995) *Anal Biochem* **230:** 229–38.
21 Patel TP, Bruce JA, Merry SA, Bigge C, Wormald M, Jaques A, Parekh RB (1993) Biochemistry **32:** 679–93.
22 Guile GR, Rudd PM, Wing DR, Prime SB, Dwek RA (1996) *Anal Biochem* **240:** 210–26.
23 Guile GR, Wong SY, Dwek RA (1994) *Anal Biochem* **222:** 231.
24 Yuasa I, Ohno K, Hashimoto K, Iijima K, Yamashita K, Takeshita K (1995) *Brain Dev* **17:** 13–19.
25 Stibler H, Holzbach U, Kristiansson B (1998) *Scand J Clin Lab Invest* **58:** 55–61.
26 Bergmann M, Gross HJ, Abdelatty F, Moller P, Jaeken J, SchwartzAlbiez R (1998) *Glycobiology* **8:** 963–72.
27 Pohl S, Hoffman A, Rudiger A, Nimtz M, Jaeken J, Conradt HS (1997) *Glycobiology* **7:** 1077–84.
28 Yamashita K, Ideo H, Ohkura T, Fukushima K, Yuasa I, Ohno K, Takeshita K (1993) *J Biol Chem* **268:** 5783–89.
29 Wada Y, Gu J, Okamoto N, Inui (1994) *Biol Mass Spectrom* **23:** 108–09

Received 12 July 1999, revised and accepted 5 October 1999