Physiological significance of the marked increased branching of the glycans of human serotransferrin during pregnancy

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Human serotransferrin (Tf) presents a microheterogeneity based on the existence of biantennary and triantennary glycans of the *N*-acetyl-lactosaminic type. By affinity chromatography on a concanavalin A–Sephacrose column in well-defined conditions, human serotransferrin isolated from healthy donors was resolved into three carbohydrate molecular variants: Tf-I (less than 1%), Tf-II ($17 \pm 2\%$) and Tf-III ($82 \pm 3\%$) containing two triantennary glycans, one triantennary and one biantennary glycans and two biantennary glycans respectively. In addition, two 'isomers' of the triantennary glycans containing the third antenna β -1,4-linked to the α -1,3-mannose residue or β -1,6-linked to the α -1,6-mannose residue were characterized by methylation analysis in the ratio 1:1 in both Tf-I and Tf-II variants. On concanavalin A crossed immuno-affinity electrophoresis, the patterns exhibited by each of the three purified variants or by a mixture of these variants were compared with the patterns given by transferrin present in sera from non-pregnant and pregnant women. The results suggest that the relative proportions of transferrin carbohydrate variants was unchanged when the concentration of transferrin was increased in serum from normal donors, whereas in the serum of pregnant women, especially in the last 3 months of pregnancy, when the serum concentration of transferrin reached 4.5–5 g/l, the relative proportions of the carbohydrate variants Tf-I and Tf-II increased from 1 to $6 \pm 1\%$ and from 17 ± 2 to $26 \pm 3\%$ respectively while that of Tf-III decreased from 82 ± 3 to $67 \pm 3\%$. The binding of the three transferrin carbohydrate variants to the receptor of the syncytiotrophoblast plasma membranes was determined by using Scatchard-plot analysis. The number of binding sites remained constant with an increase in the number of triantennary glycans whereas a decrease up to 6-fold in the affinity constant was observed. Detection of the transferrin–receptor complex by immunoblotting in the presence of non-dissociating detergents revealed the existence of only one type of receptor or of a receptor possessing similar properties involved in the binding of each of the three serotransferrin carbohydrate variants.

INTRODUCTION

The plasma iron concentration in humans is subject to large modifications under various pathological and physiological conditions (Huebers & Finch, 1987). In particular, during pregnancy the high requirement of iron for development of the foetus (Fletcher & Sutter, 1969) induces an increase in plasma iron-binding capacity that is due to a rise in the serum transferrin (or serotransferrin) concentration (Laurell *et al.*, 1968). In order to assess to what extent the increase in the blood serotransferrin concentration during pregnancy is associated with a modification of the structure of the transferrin molecule and, in particular, to a variation of the glycan structure, we have analysed the serum of normal donors and of pregnant women by ConA crossed immuno-affinity electrophoresis. The three serotransferrin carbohydrate variants isolated by affinity chromatography on a ConA–Sephacrose column were used as standards in the ConA crossed immuno-affinity electrophoresis experiments.

Moreover, as serotransferrin is involved in the process of iron transfer to the foetus by a placental receptor-mediated mechanism (Wada *et al.*, 1979; Booth & Wilson, 1981; Pearse, 1982), the three carbohydrate

variants of serotransferrin were used in order to define their binding parameters to human syncytiotrophoblast microvillar membranes.

The detection of the placental receptor–transferrin complex by using an electrophoretic method and the dissociation of this complex by each of the three serotransferrin carbohydrate variants were performed in order to prove the existence of only one type of membrane transferrin receptor.

EXPERIMENTAL

Materials

Samples of venous-blood serum from 34 healthy gestating women at different terms through pregnancy were kindly obtained from a local Maternity Hospital and classified into the three following groups: group A, 1–3 months; group B, 4–6 months; group C, 7–9 months. Samples of venous blood serum from non-pregnant women, who were not receiving oral contraceptive treatment, were collected at the local Blood Transfusion Centre. These sera were used as controls and were classified into three groups (A, B and C) according to

Abbreviations used: Tf, serotransferrin; ConA, concanavalin A.

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their transferrin concentration. The different sera were stored at 4 °C, until used, in presence of 0.2% NaN₃.

Normal mature human placentae were obtained from a local Maternity Hospital within 30 min of vaginal delivery and placed immediately in ice-cold Hanks balanced salt solution (Flow Laboratories S.A., Puteaux, France).

Human serotransferrin was purchased from Behringwerke (Marburg am Lahn, West Germany). Antibodies to human serotransferrin were raised in rabbits according to the method of Vaitukaitis *et al.* (1971). Horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody was purchased from Institut Pasteur (Paris, France). Agarose gel type II, medium EEO, bovine serum albumin, immobilized neuraminidase (from *Clostridium perfringens*, type VI-A), phenylmethanesulphonyl fluoride, aprotinin, Trasylol, dithiothreitol and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ConA was supplied by Industrie Biologique Française (Clichy, France). ConA-Sepharose containing 10 mg of ConA/ml of settled gel and Sephadex G-25 prepacked columns (PD-10) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril) was provided by Pierce Eurochimie (Beijerland, The Netherlands), Na¹²⁵I (carrier-free, 15.6 mCi/mg) by Amersham International (Amersham, Bucks., U.K.), *O*-methyl α -D-glucopyranoside by Koch-Light Laboratories (Colnbrook, Bucks., U.K.) and fat-free powdered milk by Gloria S.A. (Paris, France). Nitrocellulose membranes BA 85 were from Schleicher und Schüll (Dassel, West Germany). Airfuge polycarbonate tubes were from Beckman Instrument Co. (Fullerton, CA, U.S.A.). All other reagents were of analytical grade.

Preparation of the carbohydrate molecular variants of human serotransferrin

A 0.6 g portion of human iron-saturated serotransferrin isolated from healthy donors' sera, dialysed against solution A (5 mM-sodium acetate buffer, pH 6.9, containing 1 M-NaCl, 1 mM-MgCl₂, 1 mM-CaCl₂ and 1 mM-MnCl₂), was loaded on a ConA-Sepharose column (3.5 cm $\times$ 60 cm) equilibrated with solution A at room temperature. Elution was first carried out with the equilibrating solution at a flow rate of 40 ml/h (volume of collected fractions: 4 ml) until the absorbance monitored at 280 nm had decreased to the baseline. The bound serotransferrin was eluted by solution A containing 15 mM-*O*-methyl α -D-glucoside. After dialysis against distilled water, the different fractions were freeze-dried. Desialylated carbohydrate molecular variants of transferrin were prepared by the method of Cornfield *et al.* (1978) with the use of immobilized neuraminidase in 0.1 M-sodium citrate buffer, pH 5.1.

Determination of the structure of the glycans isolated from the serotransferrin carbohydrate molecular variants

The molar proportions of the monosaccharides were determined on the mixture of glycopeptides isolated from the Pronase digest (Spik & Montreuil, 1969) of each of the three serotransferrin variants by methanolysis (Zanetta *et al.*, 1972) and g.l.c. of the trimethylsilylated methylglycosides (Kamerling *et al.*, 1975). Permethyl-ation of glycopeptides was carried out by the procedure of Finne *et al.* (1980) and identification of the methylated methyl glycosides was performed by g.l.c. in combination with m.s. (Fournet *et al.*, 1981).

ConA crossed immuno-affinity electrophoresis of the serotransferrin carbohydrate molecular variants

The total amount of serotransferrin in normal and pregnancy sera was determined by single radial immuno-diffusion in agarose against rabbit anti-(human serotransferrin) antibodies (Mancini *et al.*, 1965). The proportion of each serotransferrin carbohydrate molecular variant in the serum samples was estimated by ConA crossed immuno-affinity electrophoresis in agarose in accordance with Salier *et al.* (1980) and Nicollet *et al.* (1981). Briefly, 3 μ l samples of serum were run for 3 h in the presence of 2 mg of ConA/ml of gel in the first dimension. The second dimension was developed overnight in the presence of 8–10 μ l of rabbit anti-(human serotransferrin) serum (titre: 2 mg/ml) and 10 mg of *O*-methyl α -D-glucoside/ml of gel. Then the electrophoresis plates were washed, dried and stained with Amido Black and the proportions of the molecular variants were determined from the areas of the peaks.

Labelling of serotransferrin carbohydrate molecular variants

Serotransferrin was iodinated by using the Iodogen reagent and Na¹²⁵I in a quantity just sufficient to incorporate statistically one iodine atom per molecule of serotransferrin. The reaction was performed in accordance with Fraker & Speck (1978) for 10 min in ice, and excess of reagent was removed by gel filtration on a Sephadex G-25 column equilibrated with the incubation medium.

Preparation of the syncytiotrophoblast microvilli

The syncytiotrophoblast microvilli were isolated from placenta by using the method of Smith *et al.* (1977) as modified by Booth *et al.* (1980). The proteinase inhibitors (1 mMeach) aprotinin, Trasylol, phenylmethanesulphonyl fluoride and dithiothreitol and MgCl₂ were added to the suspension before homogenization in 10 mM-mannitol/2 mM-Tris/HCl buffer, pH 7.1, and centrifugation at 15000 *g* for 30 min. The final pellet was resuspended in Hanks balanced salt solution supplemented with 10 mM-mannitol to give a final concentration of 2 mg of protein/ml. The protein concentration of microvillar membranes was determined by the method of Peterson (1977). The purity of the microvillar preparation was checked by measurements of two microvillar marker enzyme activities: alkaline phosphatase (Beaufay *et al.*, 1974) and 5'-nucleotidase (Aronson & Touster, 1974). The syncytiotrophoblast microvillar suspension was used for binding studies within 4 h or divided into small portions immediately after isolation and stored at -70 °C.

Characterization of the receptor-transferrin complex and displacement of the endogenous transferrin by the different carbohydrate molecular variants of serotransferrin

Polyacrylamide-gel electrophoresis was carried out with a 4–15% acrylamide gradient in a discontinuous buffer of Laemmli (1970) in which SDS was replaced by a Triton X-100/sodium deoxycholate mixture (3:1, w/w) as recommended by Dewald *et al.* (1974). All gels contained the mixture of detergents at a final concentration of 0.1%. Approx. 20–40 μ g of solubilized membrane proteins was loaded on 1.5 mm-thick gel slabs in a vertical gel apparatus (Bio-Rad Laboratories).

Samples were prepared in the following conditions: the membranes (50–100 µg of protein), untreated or pre-incubated with unlabelled or ^{125}I -labelled serotransferrin molecular variants, were solubilized in 100 µl of sample buffer containing 0.75% Triton X-100 and 0.25% sodium deoxycholate. The samples were heated at 37 °C for 30 min and centrifuged at 110000 *g* for 5 min in a Beckman Airfuge. *M_r* markers and commercial human serotransferrin were solubilized in the same conditions. Pre-run was performed at 70 V until the front reached the separation gel and at 150 V overnight under refrigeration. Then the gel was stained for proteins with Amido Black or submitted to autoradiography for 3 days.

Placental membrane proteins separated by Triton X-100/sodium deoxycholate/polyacrylamide-gel electrophoresis were transferred from the polyacrylamide gel to nitrocellulose by Western blotting and detected with specific human serotransferrin antibodies and horseradish-peroxidase-conjugated second antibodies raised against rabbit IgG in accordance with Towbin *et al.* (1979) and Burnette (1981). For immunostaining the nitrocellulose sheets were incubated overnight at 4 °C with rabbit anti-(human serotransferrin) serum diluted 1/5000 in a solution containing 5% (w/v) powdered milk in Tris-buffer saline (50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl) to prevent non-specific adsorption. The nitrocellulose sheets were washed for 10 min successively with Tris-buffered saline, Tris-buffered saline containing 0.05% Tween 20 and Tris-buffered saline, then incubated at 4 °C for 4 h with horseradish-peroxidase-conjugated anti-(rabbit IgG) antibody diluted 1/5000 in Tris-buffered saline containing 5% powdered milk. After the same washing procedure, the peroxidase activity bound to nitrocellulose was developed in 0.04% diaminobenzidine in Tris-buffered saline containing 0.01 M- H_2O_2 .

Standard binding assays

The standard binding assays were carried out in 0.25 ml Airfuge polycarbonate tubes. The incubation mixture was constituted of 50 µl of placental brush-border-membrane protein suspension (1 mg/ml) in Hanks balanced salt solution containing 10 mM-mannitol and 100 µl of ^{125}I -serotransferrin variants (10 µg/ml) in the same medium supplemented with 1% (w/v) bovine serum albumin, pH 7.4. The mixture was incubated at 0 °C for 30 min and centrifuged in a Beckman Airfuge at 110000 *g* for 3 min. The packed membranes were washed twice with 150 µl of ice-cold Hanks balanced salt solution, then transferred with 3 × 150 µl of Hanks balanced salt solution into plastic tubes (13 mm × 75 mm) and residual radioactivity was determined with an LKB Multigamma counter.

The apparent association constant K_a and the number of specific receptors per µg of membrane protein were determined with ^{125}I -serotransferrin molecular variants at concentrations ranging from 80 to 2500 ng/0.15 ml. Non-specific binding was determined in the presence of a 100-fold molar excess of unlabelled serotransferrin carbohydrate molecular variant.

RESULTS

Isolation of the human carbohydrate molecular variants of serotransferrin

Preliminary assays have shown that the binding sites of 1 ml of ConA-Sephacrose were saturated by 5 mg of

pure serotransferrin variant Tf-III containing two biantennary glycans. A large-scale affinity chromatography was performed in these original conditions, in which the binding capacity of the ConA-Sephacrose column was completely saturated by the Tf-III variant present in the analysed sample of serotransferrin. With this procedure, a complete separation of Tf-III from Tf-II was achieved in a single step (Fig. 1). The homogeneity of each fraction was checked by ConA crossed immunoelectrophoresis, and no cross-contamination was observed (Figs. 2c, 2d and 2e). The relative proportions of the three well-resolved fractions were calculated to be 0.7:17:82 for Tf-I, Tf-II and Tf-III respectively.

Determination of the glycan structure present in the three molecular variants of transferrin

The glycan structure was determined on glycopeptides isolated from the Pronase digest of each serotransferrin variant. The glycopeptides from Tf-II were previously separated by affinity chromatography on a ConA-Sephacrose column into non-retained and retained glycopeptides. The carbohydrate molar proportions and the

Fig. 1. Affinity chromatography of human serotransferrin on a ConA-Sephacrose column

Tf-I and Tf-II were eluted in 5 mM-sodium acetate buffer, pH 6.9, containing 1 mM- CaCl_2 , 1 mM- MgCl_2 and 1 mM- MnCl_2 . Tf-III was eluted in the same buffer containing 15 mM-*O*-methyl α -D-glucopyranoside (MeGlc).

Table 1. Molar carbohydrate composition of glycopeptides isolated from the three carbohydrate molecular variants of human serotransferrin

The compositions are calculated on the basis of 3 mol of mannose residues/mol of glycopeptide

Variant from which glycopeptides were isolated	Carbohydrate composition (mol of residue/mol of glycopeptide)			
	NeuAc	Gal	Man	GlcNAc
Tf-I	2.9	4.0	3	4.9
Tf-II	2.4	2.6	3	4.4
Tf-III	1.8	2.1	3	3.8

Table 2. Molar proportions of monosaccharide methyl ethers present in the methanolysates of permethylated glycopeptides isolated from the three carbohydrate molecular variants of human serotransferrin

The molar proportions are calculated on the basis of 1 residue of 2,4-di-*O*-methylmannoside. The theoretical values are given in parentheses.

Methyl ether	Molar proportions		
	Glyco-peptides from Tf-I	Glyco-peptides from Tf-II	Glyco-peptides from Tf-III
4,7,8,9-Mer ₄ NeuAc	2.4 (3)	2.0 (2.5)	1.6 (2)
2,3,4,6-Me ₄ Gal	0.3 (0)	0.1 (0)	0.1 (0)
2,3,4-Me ₃ Gal	1.8 (2)	1.9 (2)	1.9 (2)
2,4,6-Me ₃ Gal	0.8 (1)	0.4 (0.5)	0
3,4,6-Me ₃ Man	0.9 (1)	1.4 (1.5)	1.9 (2)
3,6-Me ₂ Man	0.4 (0.5)	0.2 (0.25)	0
3,4-Me ₂ Man	0.5 (0.5)	0.2 (0.25)	0
2,4-Me ₂ Man	1	1	1
3,6-Me ₂ GlcNAc	3.6 (5)	3.0 (4.5)	3.2 (4)

methyl derivatives characterized in the different glycopeptides are listed in Tables 1 and 2. It may be noted that the yields of 3,6-di-*O*-methyl-*N*-acetylglucosamine in the three glycopeptides were lower than theoretical owing to the fact that methanolysis does not quantitatively split

the asparaginyl-glucosamine linkage (Fournet *et al.*, 1981).

From the results obtained and particularly from the molar proportions of the tri-*O*-methyl derivative of mannose and of the di-*O*-methyl derivatives of mannose, it was concluded that (i) Tf-I contains two sialylated 'isomeric' triantennary glycans in relative proportions of about 1:1, (ii) Tf-II contains one sialylated biantennary glycan and the two sialylated 'isomeric' triantennary glycans in relative proportions of 1:0.5:0.5 and (iii) Tf-III contains only sialylated biantennary glycans, the structure of which were previously determined (Spik *et al.*, 1975; Dorland *et al.*, 1977). Concerning the 'isomeric' triantennary glycans, from the results previously obtained by 500 MHz ¹H-n.m.r. spectroscopy (Spik *et al.*, 1985), the third antenna was found to be linked either by a β -1,4-linkage to the α -1,3-mannose residue or by a β -1,6-linkage to the α -1,6-mannose residue.

Analysis of the ConA crossed immuno-affinity electrophoresis patterns

ConA crossed immuno-affinity electrophoresis was performed in a first step on purified serotransferrin variants, which were used as standards. The electrophoretic migration of these variants was consistent with their behaviour on ConA-Sepharose column chromatography and with the affinity of the glycan structures towards ConA (Fig. 2). A comparative analysis of the standard patterns with the patterns exhibited by normal and pregnancy sera allows semi-quantitative determination of the changes in

Fig. 2. Crossed immuno-affinity electrophoresis in the presence of ConA in the first-dimension gel (1) and rabbit anti-(human serotransferrin) IgG in the second-dimension gel (2)

(a) Serum from normal donor; (b) serum from pregnant woman; (c), (d) and (e) serotransferrin variants Tf-I, Tf-II and Tf-III isolated on a ConA-Sepharose column. Coding system of glycan structure characterized in each of the serotransferrin variants is as follows: ●, GlcNAc; ◆, Man; ■, Gal; △, α -2,3-linked NeuAc; ○, α -2,6-linked NeuAc.

the proportions of the three transferrin variants and therefore modifications of the glycosylation. The results summarized in Table 3 indicate a 1.6-fold increase of the total amount of serotransferrin between the groups A and C of non-pregnant and pregnant women. However, although the increase in the serotransferrin concentration in apparently healthy non-pregnant women may be explained by an increase in the concentrations of the three serotransferrin variants, the increase in the serotransferrin concentration in pregnant women may be explained essentially by an increase in the concentration of serotransferrin variants containing triantennary glycans (Tf-I and Tf-II). It may be suggested, therefore, that some of the physiological factors that occur during the pregnancy are able to induce an increase in the biosynthesis of triantennary glycans.

Analysis of the binding of the three transferrin carbohydrate variants to the placental membrane receptors

The preparations of syncytiotrophoblast membrane vesicles were characterized routinely by assessment of the increase in alkaline phosphatase and 5'-nucleotidase activities. The final vesicle suspension ($n = 3$) contained $0.25 \pm 0.1\%$ of the homogenate proteins (mean $\pm$ S.E.M.) and the enzyme activities corresponded to an enrichment relative to the whole homogenate of 25-fold.

The purified syncytiotrophoblast membrane proteins were solubilized in a Triton X-100/sodium deoxycholate mixture, and the position of the receptor-bound transferrin was located after Triton X-100/sodium deoxycholate/polyacrylamide-gradient-gel electrophoresis, followed by electrotransfer to nitrocellulose and immunological staining of the transferrin.

The separation profile shown in Fig. 3(b) (lane 4)

indicates that in these conditions the receptor-bound transferrin was detected as a single band with an apparent M_r of 340 000. As no free serotransferrin (M_r 80 000) was visible, it may be suggested that most of the placental serotransferrin receptors were saturated with endogenous transferrin and that this complex was not dissociated in presence of the Triton X-100/sodium deoxycholate mixture.

In order to check if each of the three carbohydrate molecular variants of serotransferrin was able to displace the receptor-bound endogenous transferrin, the Triton X-100/sodium deoxycholate-solubilized membrane proteins were incubated with an excess of each of the ^{125}I -labelled serotransferrins and the ^{125}I radioactivity was detected by autoradiography after Triton X-100/sodium deoxycholate-polyacrylamide-gel electrophoresis. As shown in Fig. 3(c) (lanes 5–7), the ^{125}I radioactivity of labelled serotransferrin was not associated with material corresponding to an apparent M_r of 80 000 but with a single band possessing an apparent M_r of 340 000. These results suggest that each of the serotransferrin variants was able to displace the endogenous transferrin and to form a complex with a solubilized placental transferrin receptor. As these complexes possess an identical electrophoretic migration, it may be hypothesized that each of the three serotransferrin variants binds to a similar placental membrane receptor.

The binding parameters of the three serotransferrin variants to the placental membrane proteins were determined and, as shown in Fig. 4, saturable binding of each of the serotransferrin variants to the membranes was observed at transferrin ranging from 100 to 150 ng. As in a preliminary experiment the equilibrium was reached after 20 min, the binding assays were performed after 30 min incubation at 37 °C and the mean values of the

Table 3. Modifications of the percentage and of the amount of the carbohydrate molecular variants of transferrin from sera from pregnant women; comparison with sera from non-pregnant women containing similar transferrin concentration

The serum concentrations of total transferrin were estimated by single radial immunodiffusion. The serum concentrations of Tf-I, Tf-II and Tf-III were estimated from the percentages of total serotransferrin (given in parentheses) estimated from the area of the peak given by each molecular variant by ConA crossed immuno-affinity electrophoresis and the serum concentration of total transferrin. Abbreviation: N.D., not determined.

Origin of serum	Serum concentration (g/l)			
	Total serotransferrin	Tf-III	Tf-II	Tf-I
Group A				
Pregnant women: 0–3 months ($n = 7$)	2.76 ± 0.4	2.2 ± 0.35 (79.0 $\pm$ 2.80 %)	0.5 ± 0.06 (19.4 $\pm$ 2.80 %)	0.04 ± 0.02 (1.7 $\pm$ 0.90 %)
Non-pregnant women ($n = 25$)	2.80 ± 0.3	2.25 ± 0.26 (80.4 $\pm$ 2.5 %)	0.54 ± 0.09 (19.2 $\pm$ 3.1 %)	0.01 ± 0.01 (0.4 $\pm$ 0.9 %)
Group B				
Pregnant women: 3–6 months ($n = 12$)	3.89 ± 0.6	2.93 ± 0.46 (72.4 $\pm$ 3.5 %)	0.99 ± 0.15 (24.6 $\pm$ 2.1 %)	0.15 ± 0.07 (3.4 $\pm$ 1.6 %)
Non-pregnant women ($n = 9$)	3.86 ± 0.4	3.16 ± 0.35 (81.8 $\pm$ 2.7 %)	0.71 ± 0.1 (18.2 $\pm$ 1.9 %)	0.009 ± 0.01 (0.25 $\pm$ 0.5 %)
Group C				
Pregnant women: 6–9 months ($n = 15$)	4.41 ± 0.6	2.99 ± 0.46 (67.6 $\pm$ 2.5 %)	1.16 ± 0.15 (26.5 $\pm$ 2.3 %)	0.27 ± 0.06 (6.1 $\pm$ 1.3 %)
Non-pregnant women ($n = 5$)	4.40 ± 0.4	3.64 ± 0.37 (82.7 $\pm$ 2.2 %)	0.76 ± 0.04 (17.3 $\pm$ 1.6 %)	N.D. N.D.

Fig. 3. Detection of the serotransferrin-receptor complex of the placental microvillar membrane by polyacrylamide-gradient-gel electrophoresis in the presence of Triton X-100/sodium deoxycholate

(a) Amido Black staining. Lane 1, M_r standards (ferritin, catalase, lactate dehydrogenase and bovine serum albumin); lane 2, human serotransferrin; lane 3, placental membrane proteins (50 μ g). (b) Immunological staining of human serotransferrin after electrophoretic transfer on nitrocellulose. Lane 4, placental membrane proteins (50 μ g). (c) Autoradiography after incubation of placental membrane proteins (50 μ g) with each of the three 125 I-labelled human serotransferrin variants. Lane 5, Tf-I; lane 6, Tf-II; lane 7, Tf-III.

apparent affinity constants (K_a) determined from five series of experiments were $0.75 \times 10^7 \pm 0.15 \times 10^7$, $1.7 \times 10^7 \pm 0.25 \times 10^7$ and $4.5 \times 10^7 \pm 0.4 \times 10^7$ M^{-1} . The total numbers of specific binding sites calculated from the analysis of the Scatchard plots were $3.2 \times 10^{10} \pm 0.3 \times 10^{10}$, $2.8 \times 10^{10} \pm 0.4 \times 10^{10}$ and $2.0 \times 10^{10} \pm 0.2 \times 10^{10}$ per μ g of placental membrane proteins for Tf-I, Tf-II and Tf-III respectively. These results suggest that the receptors represent a homogeneous population of high-affinity binding sites with K_a values decreasing more than 2-fold as a function of increasing amount of triantennary glycans.

Similar values of K_a and number of receptors were found when the experiments were performed with the completely desialylated carbohydrate variants of serotransferrin, indicating that the sialic acid residues and probably also the sugar moieties are not involved in the interaction of the serotransferrin variants with the placental membrane receptors.

DISCUSSION

Affinity chromatography on ConA-Sepharose column allows the complete resolution of human serotransferrin into three carbohydrate variants. These variants, the existence of which was suggested by Spik *et al.* (1974) and Montreuil & Spik (1975), were previously identified by ConA affinity chromatography (Wong *et al.*, 1978; Spik, 1982; Spik *et al.*, 1983, 1985). However, in the

conditions that were described, the retarded serotransferrin variant (Tf-II) was not well separated from the retained serotransferrin variant (Tf-III) and a second affinity chromatography was necessary. In the present work the method was improved by loading quantities of total serotransferrin such that the variant Tf-III saturates the ConA-Sepharose column. Therefore during the chromatography variant Tf-II slightly retained by the ConA was constantly removed by variant Tf-III firmly bound to the lectin. This modification allows the preparation in a single step of large amounts of the three carbohydrate variants of the serotransferrin.

The structure of the glycans linked to the polypeptide chain of the three variants was analysed by methylation. Identification and quantification of the di- and tri-methyl derivatives of mannose confirm the existence of Tf-II of one biantennary and one triantennary glycan in equimolar proportions, and in addition allows demonstration of the presence of two 'isomeric' triantennary glycans in approximately equimolar proportions. Therefore these results are inconsistent with the hypothesis proposed by Bayard & Kerckaert (1980) and Kerckaert & Bayard (1982) concerning the uniformity of the glycan structures of glycoproteins, particularly of serotransferrin variants.

A comparative analysis of the electrophoretic behaviour of the three isolated serotransferrin variants used as standards and of the transferrin present in the sera of non-pregnant and pregnant women leads to the conclusion that pregnancy induces the biosynthesis of serotransferrin variants Tf-I and Tf-II containing triantennary glycans. Similar results were previously obtained from human α_1 -acid glycoprotein by Wells *et al.* (1981), who have shown that relatively less ConA-binding material was found in association with and after an increase in female sex hormone concentration. Moreover, Strel'chyonok *et al.* (1982, 1984) have shown that transcortin from serum of normal healthy donors contains two triantennary and three biantennary glycans whereas transcortin from the serum of pregnant women contains five triantennary glycans of the same type. From these results it may be suggested that the glycans of most of the serum glycoproteins, the concentration of which is increased during late pregnancy (Stimson, 1972), should be modified. In the present paper evidence has been given that the glycan structure modification is not due to a general increase of the glycoprotein biosynthesis by the liver, since in the case of non-pregnant women an increase of serum transferrin concentration is correlated to an increase of the concentration of the three variants.

The selective and significant increase in the Tf-I and Tf-II variants is not limited to the pregnancy, since in the serum of cirrhotic patients, where the concentration of serum transferrin is dramatically decreased, an increase of these two variants has been previously observed (Spik *et al.*, 1983; Debruyne *et al.*, 1984). A shift of biantennary glycans towards tri- and tetra-antennary glycans was also observed in α_1 -acid glycoprotein isolated from ascitic fluid of cirrhotic patients (Biou *et al.*, 1987). These results raise the question of the biological function of transferrin variants containing triantennary glycans, particularly in iron transport. As in the cirrhotic human liver, an accumulation of iron was described (Murray & Stein, 1966; Chapman *et al.*, 1983), and as the iron requirements of the foetus are important, mostly at the end of the pregnancy (Fletcher & Sutter, 1969), it may be suggested

Fig. 4. Binding of variants Tf-III (a), Tf-III (b) and Tf-I (c) to the human placental microvillar membranes

Reaction mixtures contained 50 μg of microvillar membrane proteins and various amounts of ^{125}I -labelled serotransferrin. ○, Total binding; □, non-specific binding measured by adding 100-fold molar excess of unlabelled transferrin to the assay mixtures; ----, specific binding. (d), (e) and (f) are the Scatchard analyses deduced from five different assays of respectively (a), (b) and (c).

that increase of Tf-I and Tf-II variants is correlated to an increase of iron transport.

The use of solubilized syncytiotrophoblast microvillar membranes does not allow the determination of placental iron uptake from the three serotransferrin variants. However, it may be noted that, in previous experiments performed by Dekker *et al.* (1985), the iron uptake from carbohydrate variants of human serotransferrin by rat reticulocytes was found to be identical, suggesting no role of the serotransferrin carbohydrate moiety in the mechanism of recognition and iron uptake.

As previously shown by Brown & Johnson (1981), the presence of a Triton X-100/sodium deoxycholate mixture does not dissociate the receptor-endogenous transferrin complex. Characterization in our experiments of a band of apparent M_r 340 000 corresponding to a complex formed by a receptor associated with two transferrin molecules is in good accordance with the M_r value of 364 000 calculated by Enns & Sussman (1981). The small difference in the M_r values may be explained by the fact that in the non-dissociating detergent Triton X-100 the mobility of the membrane proteins is raised (Dewald *et al.*, 1974). No additional bands were found when the solubilized placental membranes were incubated with each of the labelled serotransferrin variants, suggesting that only one type of specific receptor or of a receptor of the same M_r is involved in the interaction with the three serotransferrin variants.

Binding-site homogeneity and a 6-fold decrease in the association constants raise the question of the significance of the increase in serotransferrin triantennary glycans. Further studies concerning the nature and the role of the factors that affect the biosynthesis of serotransferrin glycans in physiological and pathological conditions are therefore necessary in order to assess whether the selective increase of the branching has any biological significance.

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