Glycobiology vol. 8 no. 11 pp. 1053–1064, 1998

Expression of N-linked sialyl Le^x determinants and O-glycans in the carbohydrate moiety of human amniotic fluid transferrin during pregnancy

Johannes J.M.van Rooijen, Udo Jeschke[1], Johannis P.Kamerling and Johannes F.G.Vliegenthart[2]

Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80075, NL-3508 TB Utrecht, The Netherlands and [1]Universität Rostock, Frauenklinik, P.O. Box 18055, Rostock, Germany

Received on January 16, 1998; revised on March 27, 1998; accepted on April 11, 1998

[2]To whom correspondence should be addressed

Transferrin, a glycoprotein involved in iron transport in body fluids, was isolated from amniotic fluid of a hydramnios patient by sequential anion-exchange chromatography and gel filtration. The N-glycans of human amniotic fluid transferrin (hAFT) were enzymatically liberated by PNGase-F digestion, isolated by gel filtration and fractionated by (high-pH) anion-exchange chromatography. After alkaline borohydride treatment of native hAFT, the released O-glycans were isolated by gel filtration and fractionated by anion-exchange chromatography. Structure elucidation of 14 N- and 2 O-glycans was performed by 500 or 600 MHz ^{1}H-NMR spectroscopy. Besides conventional N-glycans established earlier for human serum transferrin (hST), new (α1–3)-fucosylated N-glycans were found, representing sialyl Le^x elements. Furthermore, as compared to hST, a higher degree of (α1–6)-fucosylation and an increase in branching from di- to triantennary compounds has been detected. The presence of O-glycans is demonstrated for the first time in transferrin.

Key words: amniotic fluid/NMR/carbohydrate/pregnancy/sialyl Le^x

Introduction

Transferrins are glycoproteins involved in iron transport in body fluids. They consist of a single polypeptide chain with a molecular mass of approximately 80 kDa and can bind two Fe(III) atoms per mol tightly but reversibly (Crichton, 1990). Two major classes of transferrins in humans can be distinguished, namely, serotransferrin, mainly found in serum, and lactotransferrin, found in abundance in milk but also in specific granules of polymorphonuclear leukocytes.

The carbohydrate structures and glycosylation sites of these various transferrins have been characterized. Human serum transferrin (hST) contains two potential N-glycosylation sites, namely, Asn413 and Asn611 both located in the C-lobe (Mac Gillivray *et al*., 1983). The N-glycans exhibit microheterogeneity and consist for ~85% of sialylated *N*-acetyllactosamine-type diantennary oligosaccharides and for the remaining 15% of sialylated triantennary oligosaccharides (Spik *et al*., 1975, 1985; Dorland *et al*., 1977). Human lactotransferrin (hLT) contains also two occupied N-glycosylation sites, namely, Asn138 and Asn479, although a hLT glycovariant, derived from a kidney 293(S) cell line, was shown to contain additional glycosylation at Asn624 (Rey *et al*., 1990; Van Berkel *et al*., 1996). The glycans of hLT are (α2-6)-sialylated *N*-acetyllactosamine-type diantennary structures, mainly (α1-6)-fucosylated, containing the Le^x-epitope in a minor amount (Spik *et al*., 1982). The distribution of the carbohydrate chains is symmetric over the N- and C-lobes in native hLT (Baker *et al*., 1987).

During pregnancy, the plasma iron-binding affinity in women is increased significantly by a rise in hST concentration to meet the high requirement of iron for the development of the fetus (Laurel *et al*., 1968; Fletcher and Sutter, 1969). Furthermore, structural changes were reported in the glycans of hST during pregnancy (Leger *et al*., 1989), especially in the last three months an increase in branching from diantennary to triantennary glycans was detected.

Here, we describe the isolation of transferrin from the amniotic fluid of a pregnant woman with hydramnion. In addition, the structures of the N- and O-linked carbohydrate chains of human amniotic fluid transferrin (hAFT) are reported, and discussed in relation to the glycans of hST and pregnancy.

Results

hAFT was isolated from amniotic fluid in a two-step procedure using anion-exchange chromatography on DEAE-Sepharose CL-6B (Figure 1a) followed by gel filtration on Superdex 75 (Figure 1b). The reddish-colored protein behaved similar to hST as a single band at 78 kDa on SDS–PAGE under reducing conditions. Sequence analysis of the first six N-terminal amino acids of hAFT revealed Val-Pro-Asp-Lys-Thr-Val, identical with the N-terminal amino acid sequence in hST. The amino acid composition of hAFT showed to be in good agreement with that of hST, although differences are observed (Table I). Monosaccharide analysis of the purified protein revealed a carbohydrate content of 9.4% (w/w) with Man, GlcNAc, GalNAc, Gal, Neu5Ac, and Fuc in the molar ratio of 3.0:5.6:1.0:5.0:2.4:1.3. The value of GlcNAc has been corrected for the relatively stable GlcNAc-Asn linkage (Kamerling and Vliegenthart, 1989).

The N-linked carbohydrate chains of hAFT were split off enzymatically with PNGase-F and the released oligosaccharides were separated from the protein by gel filtration on Superdex 75. Monosaccharide analysis of PNGase-F–treated hAFT showed the presence of 1.9% (w/w) carbohydrate, with a molar ratio of 0.8:1.0: 1.0:3.2:1.2:0.3 for Man, GlcNAc, GalNAc, Gal, Neu5Ac, and Fuc, demonstrating increased amounts of Gal, GalNAc, and Neu5Ac. The released N-glycans did not contain GalNAc, indicating that GalNAc-containing structures are still attached to the protein.

The possible location of GalNAc in the carbohydrate chains of hAFT was also investigated via binding studies with *Dolichos biflorus* and *Arachis hypogaea* lectin. After desialylation, no interaction was observed with *Dolichos biflorus*, indicating that terminal GalNAc was not present in the carbohydrate moiety of hAFT. In contrast, *Arachis hypogaea* lectin did bind to hAFT, identifying the presence of the core-1 unit, Gal(β1–3)GalNAc, of O-linked carbohydrate chains (Sueyoshi *et al*., 1988).

© 1998 Oxford University Press

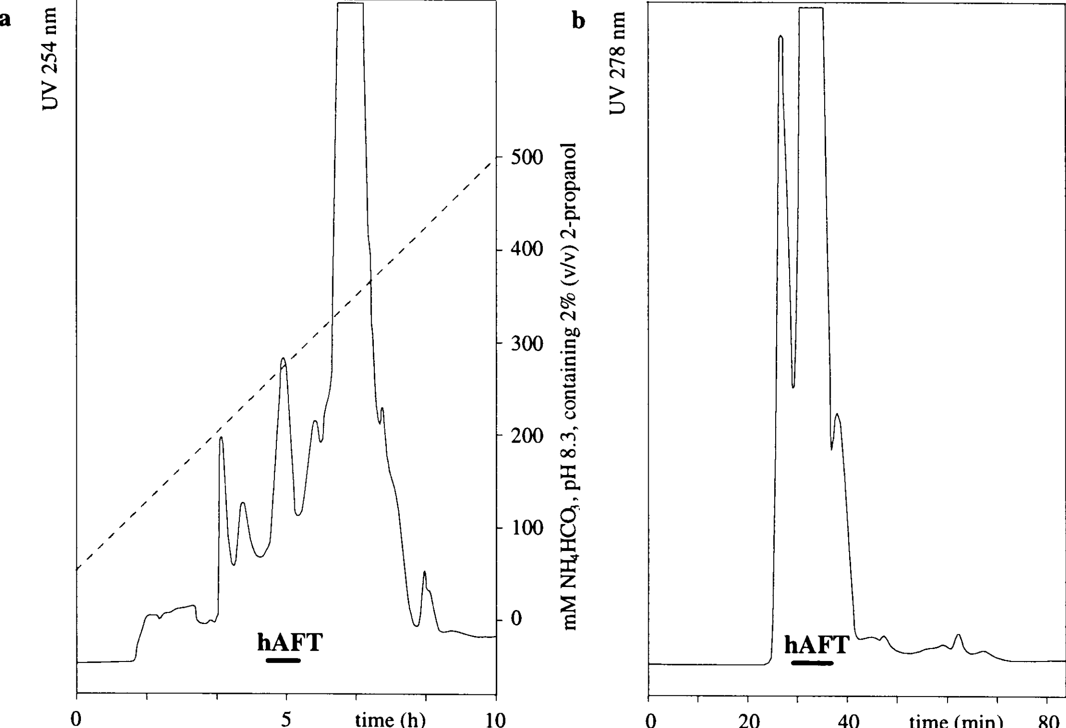


Fig. 1. Isolation of human amniotic fluid transferrin from amniotic fluid by sequential DEAE-Sepharose CL-6B and Superdex 75 column chromatography. (**a**) Elution profile of amniotic fluid on a DEAE-Sepharose CL-6B column (5.0 × 15 cm), eluted with a linear concentration gradient of 50–500 mM NH_4HCO_3, pH 8.3, containing 2% (v/v) 2-propanol at a flow rate of 60 ml/h. The transferrin-positive fraction, indicated as hAFT, was isolated, lyophilized, desalted, and lyophilized again. (**b**) Elution profile of the transferrin-containing DEAE-Sepharose fraction on a Superdex 75 column (2.6 × 60 cm), eluted with 100 mM NH_4HCO_3, pH 7.0, at a flow rate of 4.0 ml/min. The transferrin-positive fraction, indicated as hAFT, was isolated, lyophilized, desalted, and lyophilized again.

Table I. Amino acid composition of human amniotic fluid transferrin (hAFT) as compared with human serum transferrin (hST)

Amino acid	Relative amount (mole %)	
	hAFT	hST
Asx[a]	11.7	12.7
Thr	5.4	4.8
Ser	9.7	6.6
Glx[b]	10.2	9.5
Pro	3.1	5.1
Gly	10.8	8.0
Ala	10.3	9.1
Val	6.3	7.1
Cys	nd[c]	nd
Met	nd	nd
Ile	2.2	2.4
Leu	9.2	9.4
Tyr	3.3	4.1
Phe	3.8	4.4
Lys	7.4	9.3
His	2.4	3.1
Arg	3.9	4.2
Trp	nd	nd

[a]Asx is Asn and Asp taken together.
[b]Glx is Gln and Glu taken together.
[c]nd, Not determined.

N-Linked carbohydrate chains

The desalted carbohydrate-containing material obtained after PNGase-F digestion, was fractionated by anion-exchange chromatography on Resource Q. Three carbohydrate-positive fractions were obtained denoted **Q1**, **Q2**, and **Q3** having elution positions corresponding to those of mono-, di-, and trisialylated *N*-acetyllactosamine-type N-linked oligosaccharides, respectively (Figure 2). The neutral fraction did not contain sufficient amounts of carbohydrate for further analysis. Fractionation by HPAEC on CarboPac PA-1 yielded for fraction **Q1** five main subfractions, denoted **Q1.1–Q1.5** (Figure 3a), for **Q2** six subfractions **Q2.1–Q2.6** (Figure 3b), and for **Q3** nine subfractions **Q3.1–Q3.9** (Figure 3c). The HPAEC-fractions were investigated by 500 or 600 MHz ^{1}H-NMR spectroscopy, and relevant ^{1}H chemical shifts of the elucidated oligosaccharide structures are compiled in Table II and III. The numbering of the monosaccharide residues is exemplified in the structure of compound **Q3.2**. The structures of the identified oligosaccharides are summarized in Table IV, together with their relative amounts. Fractions not discussed below were mixtures too heterogeneous for structure determination, or did not contain enough material for structure determination by ^{1}H-NMR spectroscopy.

The N-linked oligosaccharides identified have either an (α1–6)-fucosylated or a nonfucosylated *N*,*N*′-diacetylchitobiose element at their reducing ends. These glycans can be distinguished by the anomeric signals of αGlcNAc-**1** and GlcNAc-**2**, together with the *N*-acetyl methyl signals of GlcNAc-**1** and GlcNAc-**2** in combination with the presence or absence of the H-1 and CH_3 signals of Fuc (Hård *et al*., 1990; de Waard *et al*., 1991). The branching patterns of the elucidated N-glycans are deduced from the sets of chemical shifts of the H-1 signals of Man-**4** and

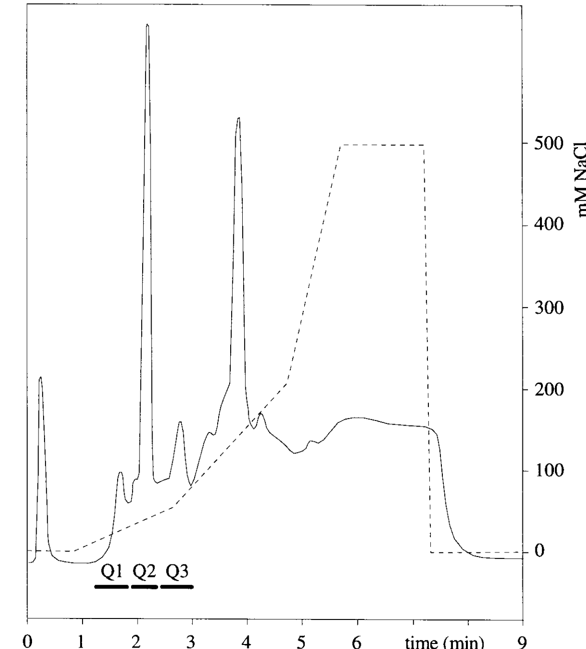


Fig. 2. Elution profile of the oligosaccharide fraction derived from PNGase-F-treated hAFT on Resource Q. The column (1 ml) was eluted with a NaCl concentration gradient as indicated in the figure at a flow rate of 4 ml/min. Fractions **Q1** to **Q3** were isolated, lyophilized, desalted, and lyophilized again.

Man-**4′**, together with those of the H-2 signals of Man-**3**, Man-**4**, and Man-**4′** (Vliegenthart *et al*., 1983; Hård *et al*., 1990). Furthermore, sialic acids occur in (α2–3)- or (α2–6)-linkage to Gal(β1–4)GlcNAc, as deduced from the H-3e, H-3a, and NAc signals of Neu5Ac and the H-3 signal of Gal to which the sialic acid residue is linked (Vliegenthart *et al*., 1983).

The ^{1}H-NMR spectrum of the main fraction of **Q1**, **Q1.3**, indicated the presence of a nonfucosylated (α2–6)-monosialylated diantennary compound with a terminal β-Gal residue on the Man(α1–6) branch (cf. compound **N1.4**; Hård *et al*., 1992). Fraction **Q1.2** is the (α1–6)-fucosylated analog of compound **Q1.3** (cf. compound **N1.5**; Hård *et al*., 1992).

The main component in FPLC-fraction **Q2** is fraction **Q2.4**. This fraction contains the nonfucosylated (α2–6)-disialylated diantennary oligosaccharide, also found in other transferrins as the main constituent (Dorland *et al*., 1977; van Pelt *et al*., 1987; Fu and van Halbeek, 1992; D'Andrea *et al*., 1994). The (α1–6)-fucosylated analog is present as the major compound in fraction **Q2.2** (cf. compound **2–2**; de Waard *et al*., 1991). The major compound in fraction **Q2.5** is the nonfucosylated disialylated diantennary carbohydrate chain, bearing (α2–3)- and (α2–6)-linked Neu5Ac in the Man(α1–6) and Man(α1–3) branch, respectively (cf. compound **N2.1.1**; Damm *et al*., 1989a). The (α1–6)-fucosylated analog is present in the minor fraction **Q2.3** (cf. compound **2–1**; de Waard *et al*., 1991).

The ^{1}H-NMR spectrum of fraction **Q2.1** (Figure 4a) indicates the presence of the sialyl Le^x determinant, confirmed by the chemical shifts of Fuc H-1 at δ 5.130, H-5 at δ 4.80 (320 K), and CH_3 at δ 1.171, and Neu5Ac H-3a at δ 1.796, H-3e at δ 2.765, and NAc at δ 2.030 (Kamerling and Vliegenthart, 1992). The Man(α1–3) branch of the diantennary structure is terminated with an (α2–6)-linked Neu5Ac residue, and the sialyl Le^x determinant is located at the Man(α1–6) branch, as indicated by the characteristic chemical shifts of Man-**4** H-1 at δ 5.132 and H-2 at δ 4.196, Man-**4′** H-1 at δ 4.917 and H-2 at δ 4.107, and Man-**3** H-2 at δ 4.256 (Stanley and Atkinson, 1988).

```
                    Fuc(α1-3)\
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q2.1
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)/
```

The main subfraction in FPLC-fraction **Q3** is **Q3.6**, which contains a nonfucosylated trisialylated triantennary structure with (α2–6)-linked Neu5Ac at Gal-**6′** and Gal-**6** and (α2–3)-linked Neu5Ac at Gal-**8**. The structural-reporter-group signals of the branching pattern and Neu5Ac are similar to those reported earlier for the corresponding oligosaccharide alditol, glycopeptide, and oligosaccharide alditol missing GlcNAc-**1** (van Pelt *et al*., 1987, 1988). This structure is also found in hST as the main trisialylated triantennary structure present (Spik *et al*., 1985; van Pelt *et al*., 1987; Fu and van Halbeek, 1992). The (α1–6)-fucosylated analog is found in fraction **Q3.4** (cf. compound **N3.10.3**; Hård *et al*., 1992). Fraction **Q3.8** consists of a nonfucosylated trisialo tri′-antennary oligosaccharide with (α2–6)-linked Neu5Ac at Gal-**6′** and Gal-**6**, and (α2–3)-linked Neu5Ac at Gal-**8′**. The branching pattern as well as the position of the (α2–6)-linked Neu5Ac and the (α2–3)-linked Neu5Ac are inferred from the typical structural-reporter-group signals of Man-**4** H-1 at δ 5.137 and H-2 at δ 4.201, Man-**4′** H-1 at δ 4.870 and H-2 at δ 4.10, and Man-**3** H-2 at δ 4.256 (cf. compound **hST3b** and **E**; van Pelt *et al*., 1987). Fraction **Q3.7** contains an isomer of compound **Q3.6**, having (α2–6)-linked Neu5Ac at Gal-**6** and (α2–3)-linked Neu5Ac at Gal-**6′** and Gal-**8** (cf. compound **343**, van Pelt *et al.* (1988); cf. compound **N3.8**, Hård *et al.* (1992)). No (α1–6)-fucosylated analogs were found for compounds **Q3.7** and **Q3.8**. The main compound of fraction **Q3.5** is a nonfucosylated trisialo triantennary compound containing three (α2–6)-linked Neu5Ac residues (cf. compound **34**; Vliegenthart *et al*., 1983).

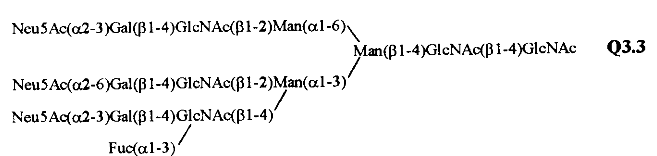
Fraction **Q3.2** is an analog of compound **Q3.6**, containing the sialyl Le^x determinant at Man-**4** O-4 as indicated by ^{1}H-NMR analysis (Figure 4b). Structural-reporter-group signals for (α1–3)-linked Fuc at GlcNAc-**7** are Fuc H-1 at δ 5.104, H-5 at δ 4.822 (320 K), and CH_3 at δ 1.169 (Kamerling and Vliegenthart, 1992), together with GlcNAc-**7** H-1 at δ 4.561 and NAc at δ 2.065, and Gal-**8** H-1 at δ 4.513 and H-3 at δ 4.09.

```
                  6'                5'                4'
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)\
                                           Man(β1-4)GlcNAc(β1-4)GlcNAc      Q3.2
                  6                 5                4      /3        2           1
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)/
                  8                 7         /
Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4)/
                            /
                  Fuc(α1-3)/
                  Fuc
```

Fraction **Q3.3** is an (α1–3)-fucosylated analog of compound **Q3.7**. ^{1}H-NMR analysis revealed the sialyl Le^x determinant at Man-**4** O-4 (Figure 4c) as indicated by the structural-reporter-group signals for (α1–3)-linked Fuc at GlcNAc-**7** of Fuc H-1 at δ 5.103, H-5 at δ 4.820 (320 K), and CH_3 at δ 1.169 (Kamerling and Vliegenthart, 1992), together with GlcNAc-**7** H-1 at δ 4.562 and NAc at δ 2.065, and Gal-**8** H-1 at δ 4.513. As compared to the structure of **Q3.2**, the (α2–6)-linked Neu5Ac residue at Gal-**6′** in

Fig. 3. Elution profile of the Resource Q N-glycan fractions on CarboPac PA-1. The column (0.9 × 25 cm) was eluted with 0.1 M NaOH using NaOAc concentration gradients as indicated in the figures, at a flow rate of 4.0 ml/min. (**a**) **Q1**, (**b**) **Q2**, (**c**) **Q3**.

Q3.2 has been replaced by an (α2–3)-linked Neu5Ac residue in **Q3.3**.



O-Linked carbohydrate chains

Gel filtration on Bio-Gel P-4 of the mixture of O-linked oligosaccharide alditols obtained from intact hAFT by alkaline borohydride treatment gave rise to two carbohydrate-containing fractions, denoted **O1** and **O2** (Figure 5a). These fractions were subfractionated by FPLC on Resource Q (Figure 5b), yielding fractions **O1.1** and **O2.1**, respectively. The ^{1}H-NMR spectrum of fraction **O2.1** indicated the presence of the neutral core-1 unit Gal(β1–3)GalNAc-ol (cf. compound **2**; Kamerling and Vliegenthart, 1992). **O1.1** showed to be a mixture of monosialylated O-linked glycans wherein the monosialylated linear trisaccharide

Table II. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of diantennary N-linked oligosaccharides, derived from human amniotic fluid transferrin

Reporter group	Residue	Chemical shift (δ, ppm) in						
		Q1.2	**Q1.3**	**Q2.1**	**Q2.2**	**Q2.3**	**Q2.4**	**Q2.5**
H-1	GlcNAc-**1**α	5.180	5.188	5.190	5.181	5.182	5.190	5.189
	GlcNAc-**2**	4.66[a]	4.61	4.62	4.67	4.66	4.61	4.61
	Man-**3**	nd[b]	nd	4.768[e]	4.787	nd	nd	nd
	Man-**4**	5.135	5.136	5.132	5.133	5.135	5.133	5.134
	Man-**4'**	4.930	4.930	4.917	4.941	4.924	4.947	4.926
	GlcNAc-**5**	4.605	4.606	4.606	4.605	4.606	4.605	4.605
	GlcNAc-**5'**	4.583	4.583	4.577	4.605	4.572	4.605	4.573
	Gal-**6**	4.447	4.447	4.443	4.445	4.444	4.443	4.444
	Gal-**6'**	4.472	4.473	4.517	4.446	4.551	4.447	4.550
	Fuc	-	-	5.130	-	-	-	-
	Fuc$_\alpha$	4.886	-	-	4.889	4.891	-	-
	Fuc$_\beta$	4.894	-	-	4.896	4.899	-	-
H-2	Man-**3**	4.253	4.254	4.256	4.257	4.255	4.256	4.254
	Man-**4**	4.197	4.193	4.196	4.196	4.196	4.194	4.198
	Man-**4'**	4.11	4.111	4.107	4.115	4.11	4.114	4.12
H-3a[c]	Neu5Ac	1.716	1.717	1.719	1.718	1.719	1.718	1.718
	Neu5Ac'	-	-	1.796	1.718	1.801	1.718	1.801
H-3e[c]	Neu5Ac	2.669	2.668	2.667	2.669	2.667	2.668	2.668
	Neu5Ac'	-	-	2.765	2.673	2.759	2.676	2.759
H-5	Fuc	nd	-	4.80[e]	nd	nd	-	-
NAc	GlcNAc-**1**	2.039	2.039	2.037	2.039	2.039	2.038	2.04
	GlcNAc-**2**$_\alpha$	2.095	2.081	2.084	2.098	2.096	2.084	2.082
	GlcNAc-**5**	2.068	2.069	2.068	2.068	2.068	2.069	2.068
	GlcNAc-**5'**	2.048	2.048	2.037	2.068	2.044	2.066	2.04
	Neu5Ac	2.030	2.030	2.030[d]	2.030[d]	2.030[d]	2.030[d]	2.030[d]
CH$_3$	Fuc	-	-	1.171	-	-	-	-
	Fuc$_\alpha$	1.209	-	-	1.210	1.210	-	-
	Fuc$_\beta$	1.220	-	-	1.221	1.221	-	-

Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225). Compounds are represented by short–hand symbol notation: open triangles, Neu5Ac (α2–3); open circles, Neu5Ac (α2—**6**); solid circles, GlcNAc; solid diamonds, Man; solid squares, Gal; open squares, Fuc. For numbering of the monosaccharide residues, see text. α and β stand for the anomeric configuration of GlcNAc-**1**.
[a]Some values are given only with two decimals because of spectral overlap.
[b]nd, Not determined.
[c]Neu5Ac is linked to Gal-**6**, Neu5Ac′ is linked to Gal-**6′**.
[d]Signal stemming from two NAc groups (Neu5Ac + Neu5Ac′).
[e]Measured at 320 K.

alditol Neu5Ac(α2–3)Gal(β1–3)GalNAc-ol was the most abundant component (cf. compound **78**; Kamerling and Vliegenthart, 1992). Evidence for an extra GlcNAc residue attached to GalNAc-ol was found in the reporter signal at δ 2.066 but the relative intensities of the rest of other structural-reporter-group signals were too low for detailed analysis.

Discussion

In early pregnancy, the amniotic fluid represents a dialysate of solutes in maternal circulation, whereas the composition at term of the amniotic fluid is the net result of several sites of exchange with fetal regulation. Between 10 weeks of pregnancy and term, the major proportion of the soluble proteins in amniotic fluid is thought to be of maternal origin which enters the fluid by diffusion processes through the amnion and chorion (Sutcliffe, 1975). A relation exists between the concentration of transferrin in the maternal serum and in the amniotic fluid as well as for other serum proteins depending on the molecular mass of the protein. Therefore, it is likely that hAFT has a maternal origin, although a fetal contribution is possible. Fetal urine, nasopharyngeal secretions and the skin of the fetus before and during the keratinization process, transfer components to the amniotic fluid, but no relation exists between the concentration of protein in

Table III. ^{1}H-Chemical shifts of structural–reporter-group protons of the constituent monosaccharides of tri- and tri′-antennary N-linked oligosaccharides, derived from human amniotic fluid transferrin

Reporter group	Residue	Chemical shift (δ, ppm) in						
		Q3.2	**Q3.3**	**Q3.4**	**Q3.5**	**Q3.6**	**Q3.7**	**Q3.8**
H-1	GlcNAc-**1**α	5.191	5.189	5.181	5.190	5.187	5.191	5.194
	GlcNAc-**2**	4.61[a]	4.61	4.67	4.61	4.61	4.61	4.61
	Man-**3**	4.758[e]	4.756[e]	nd[b]	nd	nd	nd	nd
	Man-**4**	5.127	5.128	5.129	5.134	5.130	5.131	5.137
	Man-**4'**	4.936	4.908	4.933	4.936	4.937	4.909	4.870
	GlcNAc-**5**	4.593	4.592	4.592	4.596	4.591	4.595	4.60
	GlcNAc-**5'**	4.600	4.574	4.600	4.606	4.598	4.570	4.60
	Gal-**6**	4.446	4.444	4.440	4.440	4.447	4.446	4.443
	Gal-**6'**	4.446	4.547	4.446	4.447	4.447	4.547	4.443
	GlcNAc-**7**	4.561	4.562	4.545	4.570	4.546	4.547	-
	GlcNAc-**7'**	-	-	-	-	-	-	4.56
	Gal-**8**	4.513	4.513	4.545	4.440	4.546	4.547	-
	Gal-**8'**	-	-	-	-	-	-	4.56
	Fuc	5.104	5.103	-	-	-	-	-
	Fuc_{α}	-	-	4.885	-	-	-	-
	Fuc_{β}	-	-	4.899	-	-	-	-
H-2	Man-**3**	4.214	4.213	4.217	4.220	4.216	4.213	4.256
	Man-**4**	4.214	4.213	4.217	4.220	4.216	4.213	4.201
	Man-**4'**	4.113	4.108	4.11	4.113	4.111	4.11	4.10
H-3a[c]	Neu5Ac	1.718	1.719	1.717	1.717	1.717	1.716	1.717
	Neu5Ac'	1.718	1.800	1.717	1.717	1.717	1.802	1.717
	Neu5Ac*	1.797	1.800	1.800	1.717	1.802	1.802	-
	Neu5Ac^	-	-	-	-	-	-	1.801
H-3e[c]	Neu5Ac	2.672	2.669	2.670	2.669	2.672	2.671	2.670
	Neu5Ac'	2.672	2.759	2.670	2.669	2.672	2.758	2.670
	Neu5Ac*	2.759	2.759	2.759	2.669	2.757	2.758	-
	Neu5Ac^	-	-	-	-	-	-	2.758
H-5	Fuc	4.822[e]	4.820[e]	nd	-	-	-	-
NAc	GlcNAc-**1**	2.037	2.038	2.038	2.037	2.038	2.038	2.040
	GlcNAc-$\mathbf{2}_{\alpha}$	2.082	2.082	2.097	2.082	2.083	2.081	2.081
	GlcNAc-**5**	2.065	2.065	2.066	2.068	2.066	2.065	2.066
	GlcNAc-**5'**	2.065	2.044	2.066	2.065	2.066	2.043	2.066
	GlcNAc-**7**	2.065	2.065	2.074	2.100	2.074	2.073	-
	GlcNAc-**7'**	-	-	-	-	-	-	2.043
	Neu5Ac	2.030[d]	2.031[d]	2.031[d]	2.030[d]	2.031[d]	2.031[d]	2.031[d]
CH_3	Fuc	1.169	1.169	-	-	-	-	-
	Fuc_{α}	-	-	1.210	-	-	-	-
	Fuc_{β}	-	-	1.221	-	-	-	-

Chemical shifts are given at 300 K and were measured in $^{2}H_2O$ relative to internal acetone (δ 2.225). Compounds are represented by short-hand symbol notation: open triangles, Neu5Ac (α2–3); open circles, Neu5Ac (α2—**6**); solid circles, GlcNAc; solid diamonds, Man; solid squares, Gal; open squares, Fuc. for numbering of the monosaccharide residues, see text. α and β stand for the anomeric configuration of GlcNAc-**1**.

[a]Some values are given only with two decimals because of spectral overlap.

[b]nd, Not determined.

[c]Neu5Ac is linked to Gal-**6**, Neu5Ac′ is linked to Gal-**6′**, Neu5Ac* is linked to Gal-**8**, and Neu5Ac^ is linked to Gal-**8′**.

[d]Signal stemming from three NAc groups (Neu5Ac + Neu5Ac′ + Neu5Ac*/Neu5Ac^).

[e]Measured at 320 K.

Table IV. N-Linked carbohydrate chains, including relative amounts, obtained from human amniotic fluid transferrin

Code	Relative amount (mol-%)	Structure
Q1.2	3	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Fuc(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /
Q1.3	7	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /
Q2.1	5	Fuc(α1-3) \ Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /
Q2.2 **Q2.3**	7 3	**Neu5Ac(α2-6)** **Neu5Ac(α2-3)**Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Fuc(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /
Q2.4 **Q2.5**	23 13	**Neu5Ac(α2-6)** **Neu5Ac(α2-3)**Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /
Q3.2 **Q3.3**	4 1	**Neu5Ac(α2-6)** **Neu5Ac(α2-3)**Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4) / Fuc(α1-3) /
Q3.4	2	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Fuc(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4) /
Q3.5 **Q3.6**	<1 6	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / **Neu5Ac(α2-6)**Gal(β1-4)GlcNAc(β1-4) / **Neu5Ac(α2-3)**
Q3.7	2	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-4) /
Q3.8	2	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6) \ Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /

For a given pair of compounds, the bold part indicates the variable part of the structure. The molar ratio of oligosaccharides present in the FPLC fractions was determined on the basis of the C=O groups (absorbance at 214 nm) being known after structural identification (Damm *et al.*, 1989b). The molar ratio of constituent oligosaccharides within each FPLC fraction was determined on the basis of the peak height in the HPAEC profiles. Peak heights were not corrected for response factors.

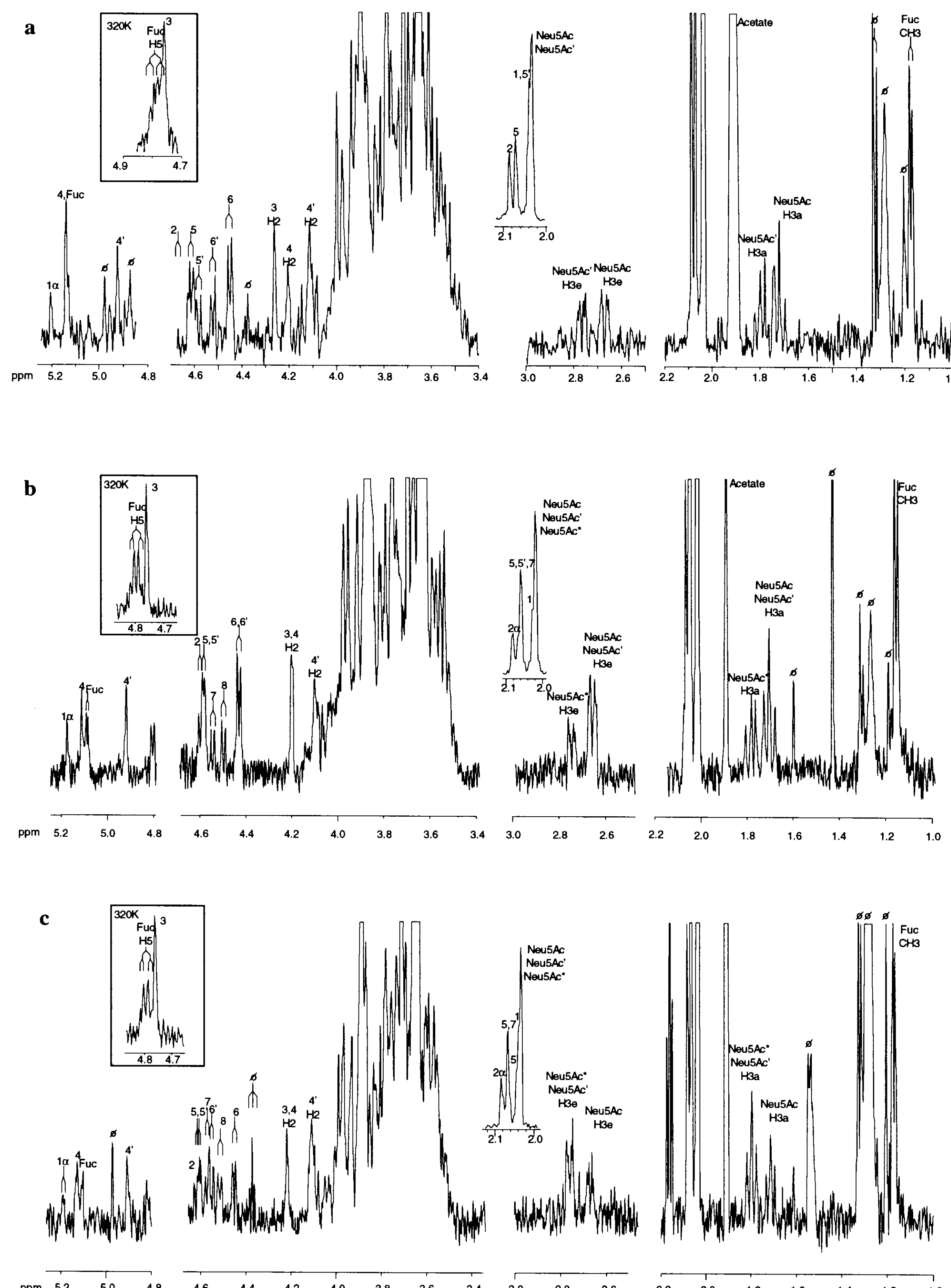


Fig. 4. 600 MHz ^{1}H-NMR spectra of fractions **Q2.1** (**a**), **Q3.2** (**b**), and **Q3.3** (**c**). The denotation of the protons refers to the corresponding residues in the structure. Spectra were recorded at 300 K. For the elucidation of the Fuc H-5 and the Man-**3** H-1 signals, spectra were recorded at 320 K. ∅ means noncarbohydrate contaminant.

amniotic fluid and in fetal serum. During hydramnios, the protein concentration in amniotic fluid appears to be within the expected range in comparison with normal pregnancy. Only in congenital malformation, which causes 19.8% of the total occurrence of hydramnios, increasing levels of serum α-fetoprotein (AFP) have been reported (Harris *et al.*, 1974). No data about increased

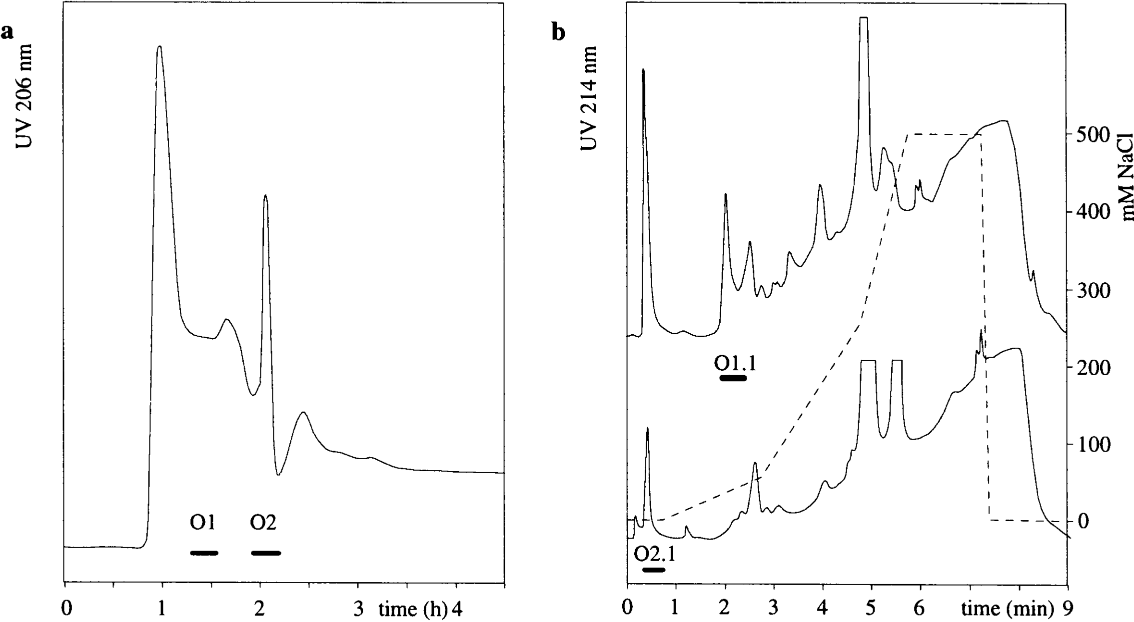


Fig. 5. (**a**) Elution profile on Bio-Gel P-4 of alkaline borohydride-treated hAFT. The column (50 × 1.0 cm) was eluted with 10 mM NH_4HCO_3, pH 7.0, at a flow rate of 15 ml/h. Fractions **O1** and **O2** were isolated and lyophilized. (**b**) Elution profile of **O1** and **O2** on Resource Q. The column (1 ml) was eluted with a NaCl concentration gradient as indicated in the figure at a flow rate of 4 ml/min. Fractions **O1.1** and **O2.1** were isolated, lyophilized, desalted, and lyophilized again.

secretion of serum proteins by the fetus during hydramnios has been reported.

In this report, the structures of 14 N- and 2 O-linked carbohydrate chains of hAFT have been determined. With respect to the N-glycosylation, mono-, di-, and tri-sialylated structures, including three sialyl Le^x-containing carbohydrates were found. As indicated in Table IV, sialylated diantennary carbohydrate chains constitute the major part of the liberated N-glycans but in a much lower amount (55%) as reported for hST (Spik *et al.*, 1975; Dorland *et al.*, 1977). The relative molar amount of triantennary compounds is increased to 32%, which is in agreement with the reported increased branching of hST during pregnancy (Leger *et al.*, 1989). An increase in branching could be correlated with an increase in iron transport. For cirrhotic patients, it is known that both iron accumulation in the human liver, and increased branching of the N-glycans of hST occurs (Yamashita and Kobata, 1996). However, a 6-fold decrease in the affinity constant of a molecular transferrin variant, containing only triantennary carbohydrate structures, suggests otherwise (Leger *et al.*, 1989). Increased branching is also observed for other glycoproteins during pregnancy, such as α_1-acid glycoprotein (AGP), transcortin, human chorionic gonadotropin α (αhCG), and fibronectin (Wells *et al.*, 1981; Strel'chyonok *et al.*, 1982; Zhu *et al.*, 1984; Blithe and Iles, 1995). Although the biological function is mostly unclear increased branching can affect binding properties, for example, increased branching in αhCG prevents the molecule from binding to available free β-subunits in order to stimulate uterine decidua cell PRL secretion. Furthermore, it can play a role in protective properties, for example, increased branching in fibronectin renders the protein to be more resistant to proteolysis while not affecting its other functional properties.

Interestingly, 40% of the diantennary N-glycans show the presence of (α2–3)-linked Neu5Ac, a linkage type which is only present in the triantennary glycans of hST (Spik *et al.*, 1985). This feature is also displayed when comparing human serum- and amniotic fluid fibronectin. Amniotic fluid-derived fibronectin has primarily sialic acid linked (α2–3) to galactose, whereas plasma fibronectin has primarily sialic acid linked (α2–6) to galactose (Krusius *et al.*, 1984). Fucosylation of the innermost *N*-acetylglucosamine is present in the N-glycans of hAFT but not in those of hST.

One of the novel features of hAFT is the presence of the sialyl Le^x determinant incorporated into three N-glycans, indicating α-1,3-fucosyltransferase activity in amniotic fluid. Indeed, an α-1,3-fucosyltransferase was identified in amniotic fluid, which accepted substrates of the general structure Neu5Ac-(α2–3)Gal(β1–4)GlcNAc(β-) in order to synthesize sialyl Le^x (Mitsakos and Hanisch, 1989). It is tempting to assume that the biosynthesis of the sialyl Le^x structure takes place in the amniotic fluid. However, so far GDP-Fuc has not been demonstrated to occur in amniotic fluid. This is subject of further investigation. Increased expression of sialyl Le^x may abrogate the maternal immune/inflammatory response by blocking the primary adhesive interactions required for the expression of such activities (Clark *et al.*, 1996). This hypothesis is supported by the increase of α-1,3-fucosyltransferase activity resulting in an increased expression of sialyl Le^x determinants on the glycans of AGP during acute inflammation (Hakomori, 1985; Wieruszeski *et al.*, 1988; de Graaf *et al.*, 1993). It should be noted that both native hLT (Matsumoto *et al.*, 1982; Spik *et al.*, 1982) and hST present in some patients with hepatocellular carcinoma (Campion *et al.*, 1989; Yamashita *et al.*, 1989) are reported to contain the Le^x-epitope. With respect to hST, the presence of the Le^x-epitope is probably due to an increase of α-1,3-fucosyltransferase activity (Yamamoto *et al.*, 1984) as reported earlier for malignant cells and cancer sera (Kim *et al.*, 1980; Aoyagi *et al.*, 1996).

Another novel feature of hAFT is the presence of O-linked glycans. HAFT contained in addition to Gal(β1–3)GalNAc, also Neu5Ac(α2–3)Gal(β1–3)GalNAc. Although in the present report no disialo compound Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]-GalNAc was found, hAFT shows remarkable homology with the O-glycans of amniotic fluid fibronectin (Krusius *et al.*, 1984).

Interestingly, the O-glycosylation sites increase in amniotic fluid fibronectin as compared to serum fibronectin, although the O-glycan structures do not change. It is known that O-linked glycans can play a role in protecting sensitive domains of a glycoprotein against proteolytic attack (Varki, 1993). It is possible that in the environment of the amniotic fluid, where active growth and tissue remodeling of the fetus may be associated with increased protease activities, O-glycosylation protects the protease-sensitive domains on amniotic fluid fibronectin and amniotic fluid transferrin.

Material and methods

Isolation of human amniotic fluid transferrin (hAFT)

HAFT was isolated from the amniotic fluid of a pregnant woman, who suffered from hydramnion causing an overproduction of amniotic fluid, generated in the amnion gap.

Two portions of amniotic fluid (each 1.5 l) were collected in the 22th and 26th week of the pregnancy, respectively, and pooled. The solution was dialyzed twice against distilled water and then once against 50 mM NH_4HCO_3, pH 8.3, containing 2% (v/v) 2-propanol. Portions of dialyzed amniotic fluid (200 ml) were fractionated on a DEAE-Sepharose CL-6B column (5.0 × 15 cm; Pharmacia), equilibrated with 50 mM NH_4HCO_3, pH 8.3, containing 2% (v/v) 2-propanol. The elution was performed with a linear concentration gradient of 50–500 mM NH_4HCO_3, pH 8.3, containing 2% (v/v) 2-propanol at a flow rate of 60 ml/h for 10 h and monitored at 254 nm. Fractions, containing hAFT, were pooled and lyophilized. Purity was checked on 12.5% slabgels by SDS–PAGE with Coomassie brilliant blue staining (Laemmli, 1970).

Subsequently, hAFT-containing material was applied to a Superdex 75 column (2.6 × 60 cm, Pharmacia), equilibrated with 100 mM NH_4HCO_3, pH 7.0. The elution was performed isocratically at a flow rate of 4.0 ml/min and monitored at 278 nm. The main fraction, containing hAFT, was lyophilized, desalted by HiTrap (Pharmacia FPLC system; four columns connected, 4 × 5 ml; eluent, 5 mM NH_4HCO_3; flow rate, 3 ml/min; detection, 214 nm), and lyophilized again. The purity was checked by SDS–PAGE, amino acid composition (de Baaij *et al.*, 1986) and N-terminal amino acid analysis (Tarentino, 1985). A monosaccharide analysis was performed as described (Kamerling *et al.*, 1975).

Liberation and isolation of N-glycans

The N-linked carbohydrate chains were released enzymatically from the glycoprotein with PNGase-F (Boehringer Mannheim). HAFT (70 mg) was dissolved in 7 ml 50 mM Tris–HCl, pH 7.0, containing 50 mM EDTA. After addition of 1.4 ml 10% (w/v) SDS and 0.7 ml 10% (v/v) 2-mercaptoethanol, the solution was boiled for 5 min and subsequently cooled down to room temperature. Then, 2.8 ml 10% (v/v) Nonidet P-40 was added, and the mixture was incubated with 20 U PNGase-F at ambient temperature. After 24 h, the mixture was boiled for 3 min, cooled down to room temperature, and incubated again with 20 U PNGase-F for 24 h. SDS–PAGE on a 12.5% slabgel and Coomassie brilliant blue staining was used to check the degree of deglycosylation. The released carbohydrate chains were isolated by gel filtration chromatography on a Superdex 75 column (2.5 × 60 cm, Pharmacia) eluted with 100 mM NH_4HCO_3, pH 7.0, at a flow rate of 4.0 ml/min and monitored at 206 nm. Carbohydrate-positive fractions were identified by spraying aliquots on a TLC plate with orcinol/H_2SO_4. These fractions were pooled, lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

Table V. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of O-linked carbohydrate chains of human amniotic fluid transferrin

Residue	Reporter group	Chemical shift (δ, ppm) in	
		O1.1	O2.1
GalNAc-ol	H-2	4.387	4.399
	H-3	4.078	4..064
	H-4	3.496	3.505
	H-5	4.188	4.196
	NAc	2.045	2.049
Gal	H-1	4.546	4.475
	H-3	4.125	nd[a]
	H-4	3.930	nd
Neu5Ac	H-3a	1.801	-
	H-3e	2.772	-
	NAc	2.033	-

Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ 2.225). Compounds are represented by short-hand symbol notation: open triangles, Neu5Ac (α2–3); solid squares, Gal; open triangles, GalNAc.
[a]nd, Not determined.

The carbohydrate-containing fraction, generated by PNGase-F digestion, was subfractionated according to charge on a Resource Q anion-exchange column (1 ml, Pharmacia). The column was eluted with 2.8 ml H_2O, followed by linear concentration gradients of 0–50 mM NaCl in 8 ml H_2O, 50–200 mM NaCl in 8 ml H_2O and finally by 200–500 mM NaCl in 4 ml H_2O at a flow rate of 4 ml/min. The fractionation was monitored at 214 nm and the carbohydrate-positive fractions (orcinol/H_2SO_4) were lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

The carbohydrate-containing Resource Q fractions were subfractionated on a CarboPac PA-1 pellicular anion-exchange column (0.9 × 25 cm, Dionex) attached to a Dionex LC system. Elutions were carried out with a concentration gradient of NaOAc in 0.1 M NaOH as indicated in the figures at a flow rate of 4.0 ml/min. Detection was performed with a gold electrode and triple-pulse amperometry, comprising the following pulse potentials and durations: E_1 = 0.05 V (300 ms); E_2 = 0.65 V (60 ms); E_3 = –0.95 V (180 ms). Collected fractions were immediately neutralized by addition of 5 M HOAc, lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

Lectin assay

HAFT was desialylated by sialidase (*Vibrio cholerae*, Boehringer Mannheim) as described by the manufacturer. An aliquot of a desialylated hAFT solution was transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad) and dried by air. The remaining active sites on the blot were blocked by incubating with blocking solution (Boehringer Mannheim) for 30 min. After removing the blocking solution, the blot was soaked into 10 ml 50 mM Tris–HCl/200 mM NaCl, pH 7.4, containing 170 mU peroxidase-labeled lectin from *Dolichos biflorus* or 340 mU peroxidase-labeled lectin from *Arachis hypogaea*, and shaken gently for 30 min. Next, the blot was washed five times with 10 ml 50 mM Tris–HCl/

200 mM NaCl, pH 7.4, for 5 min. Color development was performed without shaking in 10 ml staining buffer (10 mg 3,3′,5,5′-tetramethylbenzidine and 10 mg dioctylsulfosuccinate in 2.5 ml ethanol adjusted to 10 ml with a citrate-phosphate buffer (7.6 mM citric acid and 27 mM Na_2HPO_4) and activated by adding 10 ml H_2O_2.

Liberation and isolation of O-glycans

The O-linked carbohydrate chains were released by alkaline borohydride treatment. HAFT (10 mg) was dissolved in 5 ml 0.1 M NaOH containing 1 M $NaBH_4$ (Nakajima and Ballou, 1974), and the solution was stirred at 37°C under N_2 for 48 h, then acidified to pH 5.0 with 4 M HOAc at 0°C, and subsequently applied to a Dowex 50W-X8, H^+ column (15 × 0.5 cm, 100–200 mesh, Fluka). The column was eluted with 75 ml 0.01 M formic acid, and the eluate was lyophilized. Boric acid was removed by repeated coevaporation with methanol under reduced pressure. Finally, the material was resuspended in water and fractionated on a Bio-Gel P-4 column (50 × 1.0 cm, 200–400 mesh, Bio-Rad) using 10 mM NH_4HCO_3, pH 7.0, as eluent at a flow rate of 15 ml/h. The eluate was monitored at 206 nm and the collected carbohydrate-positive fractions (orcinol/H_2SO_4) were lyophilized and subsequently fractionated according to charge by FPLC on a Resource Q anion-exchange column as described above. Carbohydrate-containing fractions were lyophilized, desalted by HiTrap (Pharmacia), and lyophilized again.

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, samples were treated twice with 99.9% ^2H_2O with intermediate lyophilization. Finally, samples were dissolved in 99.96% ^2H_2O (MSD-Isotopes). ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or on a Bruker AMX-600 spectrometer (SON-hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen). The probe temperature was 27°C, unless indicated otherwise. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 in ^2H_2O at 27°C; Vliegenthart *et al*., 1983). Typically, one-dimensional (1D) spectra were recorded with a spectral width of 5 kHz, collecting 128–3072 free induction decays (FIDs) of 8k or 16k complex data points. Suppression of the residual water signal was achieved by applying the WEFT pulse sequence (Water Eliminated Fourier Transform; Hård *et al*., 1992). The resolution of the 1D spectra was enhanced by a Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a fourth order polynomial function.

Acknowledgments

We thank Dr. Gerrit Gerwig for performing the monosaccharide analysis. This investigation was supported by the Netherlands Foundation for Chemical Research (NWO/SON) and by the Deutsche Forschungsgemeinschaft (DFG, Je 181/2–1 and 2–2).

References

Aoyagi,Y., Isemura,M., Yosizawa,Z., Suzuki,Y., Sekine,C., Ono,T. and Ichida,F. (1996) Fucosylation of serum α-fetoprotein in patients with primary hepatocellular carcinoma. *Biochim. Biophys. Acta*, **830,** 217–223.

Baker,E.N., Rumball,S.V. and Anderson,B.F. (1987) Transferrins: insight into structure and function from studies on lactotransferrin. *Trends Biochem. Sci*., **12,** 350–353.

Blithe,D.L. and Iles,R.K. (1995) The role of glycosylation in regulating the glycoprotein hormone free α-subunit and free β-subunit combination in the extraembryonic coelomic fluid of early pregnancy. *Endocrinology*, **136,** 903–910.

Campion,B., Leger,D., Wieruszeski,J.M., Montreuil,J. and Spik,G. (1989) Presence of fucosylated triantennary, tetraantennary and pentaantennary glycans in transferrin synthesized by the human hepatocarcinoma cell line Hep G2. *Eur. J. Biochem*., **184,** 405–413.

Clark,G.F., Oehninger,S., Patankar,M.S., Koistinen,R., Dell,A., Morris,H.R., Koistinen,H. and Seppala,M. (1996) A role for glycoconjugates in human development: the human feto-embryonic defence system hypothesis. *Hum. Reprod*., **11,** 467–473.

Crichton,R.R. (1990) Proteins of iron storage and transport. *Adv. Protein Chem*., **40,** 315–332.

Damm,J.B.L., Bergwerff,A.A., Hård,K., Kamerling,J.P. and Vliegenthart,J.F.G. (1989a) Sialic acid patterns in N-linked carbohydrate chains. Structural analysis of the *N*-acetyl-/*N*-glycolyl-neuraminic-acid-containing N-linked carbohydrate chains of bovine fibrinogen. *Recl. Trav. Chim. Pays-Bas*, **108,** 351–359.

Damm,J.B.L., Voshol,H., Hård,K., Kamerling,J.P. and Vliegenthart,J.F.G. (1989b) Analysis of *N*-acetyl-4-*O*-acetylneuraminic-acid-containing N-linked carbohydrate chains released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F. Application to the structure determination of the carbohydrate chains of equine fibrinogen. *Eur. J. Biochem*., **180,** 101–110.

D'Andrea,G., D'Alessandro,A.M., Salucci,M.L. and Oratore,A. (1994) Primary structure of the major glycan from human seminal transferrin. *J. Prot. Chem*., **13,** 31–36.

de Baaij,J.A., Jansen,F.W. and Voortman,G. (1986) The use of an amino acid analyzer in food quality control. *Science Tools*, **33,** 17–32.

de Graaf,T.W., van der Stelt,M.E., Anbergen,M.G. and van Dijk,W. (1993) Inflammation induced expression of sialyl Lewis^x-containing glycan structures on α_1-acid glycoprotein (orosomucoid) in human sera. *J. Exp. Med*., **177,** 657–666.

de Waard,P., Koorevaar,A., Kamerling,J.P. and Vliegenthart,J.F.G. (1991) Structure determination by ^{1}H NMR spectroscopy of (sulfated) sialylated N-linked carbohydrate chains released from porcine thyroglobulin by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F. *J. Biol. Chem*., **266,** 4237–4243.

Dorland,L., Haverkamp,J., Schut,B.L., Vliegenthart,J.F.G., Spik,G., Strecker,G., Fournet,B. and Montreuil,J. (1977) The structure of the asialo-carbohydrate units of human serotransferrin as proven by 360 MHz proton magnetic resonance spectroscopy. *FEBS Lett*., **77,** 15–20.

Fletcher,J. and Sutter,P.E.N. (1969) The transport of iron by the human placenta. *Clin. Sci*., **36,** 209–220.

Fu,D. and van Halbeek,H. (1992) N-Glycosylation site mapping of human serotransferrin by serial lectin affinity chromatography, fast atom bombardment-mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy. *Anal. Biochem*., **206,** 53–63.

Hakomori,S.I. (1985) Aberrant glycosylation in cancer cell membranes as focussed on glycolipids: overview and perspectives. *Cancer Res*., **45,** 2405–2414.

Hård,K., Mekking,A., Damm,J.B.L., Kamerling,J.P., de Boer,J.P., Wijnands,R.A. and Vliegenthart,J.F.G. (1990) Isolation and structure determination of the intact sialylated N-linked carbohydrate chains of recombinant human follitropin expressed in Chinese hamster ovary cells. *Eur. J. Biochem*., **193,** 263–271.

Hård,K., van Zadelhoff,G., Moonen,P., Kamerling,J.P. and Vliegenthart,J.F.G. (1992) The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel *N*-acetylgalactosamine-containing N-linked carbohydrate chains. *Eur. J. Biochem*., **209,** 895–915.

Harris,R., Jennison,R.F., Barson,A.G., Laurence,K.M., Ruoslahti,E. and Seppala,M. (1974) Comparison of amniotic-fluid and maternal serum α-fetoprotein levels in the early antenatal diagnosis of spina bifida and anencephaly. *Lancet*, **1,** 428–429.

Kamerling,J.P. and Vliegenthart,J.F.G. (1989), Carbohydrates. In Lawson,A.M. (ed.), *Clinical Biochemistry; Principles, Methods, Applications. Mass Spectrometry.* Walter de Gruyter, Berlin, pp. 175–263.

Kamerling,J.P. and Vliegenthart,J.F.G. (1992) High resolution ^{1}H-nuclear magnetic resonance spectroscopy of oligosaccharide-alditols released from mucin-type O-glycoproteins. *Biol. Magn. Reson*., **10**, 1–194.

Kamerling,J.P., Gerwig,G.J., Vliegenthart,J.F.G. and Clamp,J.R. (1975) Characterization by gas-liquid chromatography-mass spectrometry and proton magnetic resonance spectroscopy of pertrimethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. *Biochem. J*., **151,** 491–495.

Kim,Y.S., Kim,Y.W., Siddiqui,B. and Tsao,D. (1980) Membrane-associated, fucose-containing glycoproteins and glycolipids of cultured epithelial cells from human colonic adenocarcinoma and fetal intestine. *Eur. J. Cancer Clin. Oncol*., **18,** 1329–1336.

Krusius,T., Fukuda,M., Dell,A. and Ruoslahti,E. (1984) Structure of the carbohydrate units of human amniotic fluid fibronectin. *J. Biol. Chem*., **260,** 4110–4116.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680–685.

Laurel,C.B., Kullander,S. and Thorell,J. (1968) Effect of administration of a combined estrogen-proestin contraceptive on the level of individual plasma proteins. *Scand. J. Clin. Lab. Invest*., **21,** 337–343.

Leger,D., Campion,B., Decottignies,J.P., Montreuil,J. and Spik,G. (1989) Physiological significance of the marked increased branching of the glycans of human serotransferrin during pregnancy. *Biochem. J*., **257,** 231–238.

Mac Gillivray,R.T.A., Mendez,E., Shewale,J.G., Sinha,S.K., Linebach-Zins,J. and Brew,K. (1983) The primary structure of human serum transferrin. The structures of seven cyanogen bromide fragments and the assembly of the complete structure. *J. Biol. Chem*., **258,** 3543–3553.

Matsumoto,A., Yoshima,H., Takasaki,S. and Kobata,A. (1982) Structural study of the sugar chains of human lactoferrin: findings of four novel complex-type asparagine-linked sugar chains. *J. Biochem*., **91,** 143–155.

Mitsakos,A. and Hanisch,F.G. (1989) One-step purification of an α(1–3)-L-fucosyltransferase from human amniotic fluid by fetuin-agarose affinity chromatography. *Biol. Chem. Hoppe Seyler*, **370,** 239–243.

Nakajima,T. and Ballou,C.E. (1974) Characterization of the carbohydrate fragments obtained from *Saccharomyces cerevisiae* mannan by alkaline degradation. *J. Biol. Chem*., **249,** 7679–7684.

Rey,M.W., Woloshuk,S.L., de Boer,H.A. and Pieper,F.R. (1990) Complete nucleotide sequence of human mammary gland lactoferrin. *Nucleic Acids Res.,* **18,** 5288.

Spik,G., Bayard,B., Fournet,B., Strecker,G., Bouquelet,S. and Montreuil,J. (1975) Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett*., **50,** 296–299.

Spik,G., Debruyne,V., Montreuil,J., van Halbeek,H. and Vliegenthart,J.F.G. (1985) Primary structure of two sialylated triantennary glycans from human serotransferrin. *FEBS Lett*., **183,** 65–69.

Spik,G., Strecker,G., Fournet,B., Bouquelet,S., Montreuil,J., Dorland,L., van Halbeek,H. and Vliegenthart,J.F.G. (1982) Primary structure of the glycans from human lactotransferrin. *Eur. J. Biochem*., **121,** 413–419.

Stanley,P. and Atkinson,P.H. (1988) The LEC11 chinese hamster ovary mutant synthesizes *N*-linked carbohydrates containing sialylated, fucosylated lactosamine units. *J. Biol. Chem*., **263,** 11374–11379.

Strel'chyonok,O.A., Avvakumov,G.V., Matveentseva,I.V., Akhrem,L.V. and Akhrem,A.A. (1982) Isolation and characterization of glycopeptides of human transcortin. *Biochim. Biophys. Acta*, **705,** 167–173.

Sueyoshi,S., Tsuji,T. and Osawa,T. (1988) Carbohydrate-binding specificities of five lectins that bind to *O*-glycosyl-linked carbohydrate chains. Quantitative analysis by frontal-affinity chromatography. *Carbohydr. Res*., **178,** 213–224.

Sutcliffe,R.G. (1975) The nature and origin of the soluble protein in human amniotic fluid. *Biol. Rev*., **50,** 1–33.

Tarentino,A.L. (1985) Deglycosylation of asparagine-linked glycans by peptide N-glycosidase F. *Biochemistry*, **24,** 4665–4671.

van Berkel,P.H.C., van Veen,H.A., Geerts,M.E.J., de Boer,H.A. and Nuijens,J.H. (1996) Heterogeneity in utilization of N-glycosylation sites Asn^{624} and Asn^{138} in human lactoferrin: a study with glycosylation-site mutants. *Biochem. J.,* **319,** 117–122.

van Pelt,J., Damm,J.B.L., Kamerling,J.P. and Vliegenthart,J.F.G. (1987) Separation of sialyl-oligosaccharides by medium pressure anion-exchange chromatography on Mono-Q. *Carbohydr. Res*., **169,** 43–51.

van Pelt,J., Hård,K., Kamerling,J.P., Vliegenthart,J.F.G., van Diggelen,O.P. and Galjaard,H. (1988) Storage of sialic acid-containing carbohydrates in the placenta of a human galactosialidosis fetus. Isolation and structural characterization of 16 sialyloligosaccharides. *Eur. J. Biochem*., **177,** 327–338.

Varki,A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, **3,** 97–130.

Vliegenthart,J.F.G., Dorland,L. and van Halbeek,H. (1983) High-resolution, ^{1}H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem*., **41,** 209–374.

Wells,C., Bøg-Hansen,T.C., Cooper,E.H. and Glass,M.R. (1981) The use of Concanavalin A-crossed immuno-affinoelectrophoresis to detect variations of α_1-acid glycoprotein. *Clin. Chim. Acta*, **109,** 59–67.

Wieruszeski,J.M., Fournet,B., Konan,D., Biou,D. and Durand,G. (1988) 400-MHz ^{1}H-NMR spectroscopy of fucosylated tetrasialyl oligosaccharides isolated from normal and cirrhotic α_1-acid glycoprotein. *Eur. J. Cancer Clin. Oncol*., **238,** 390–394.

Yamamoto,Y., Tsuji,T., Tarutani,O. and Osawa,T. (1984) Structural changes of carbohydrate chains of human thyroglobulin accompanying malignant transformations of thyroid glands. *Eur. J. Biochem*., **143,** 133–144.

Yamashita,K. and Kobata,A. (1996), Cancer cells and metastasis. In Montreuil,J., Vliegenthart,J.F.G. and Schachter,H. (eds.), *Glycoproteins and Disease*. Elsevier Science, Amsterdam, pp. 197–210.

Yamashita,K., Koide,N., Endo,T., Iwaki,Y. and Kobata,A. (1989) Altered glycosylation of serum transferrin of patients with hepatocellular carcinoma. *J. Biol. Chem*., **264,** 2415–2423.

Zhu,B.C., Fisher,S.F., Pande,H., Calaycay,J., Shively,J.E. and Laine,R.A. (1984) Human placental (fetal) fibronectin: increased glycosylation and higher protease resistance than human fibronectin. Presence of polylactosamine glycopeptides and properties of a 44-kilodalton chymotryptic collagen-binding domain: difference from human plasma fibronectin. *J. Biol. Chem*., **259,** 3962–3970.